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The evolutionary biology of ageing: demography, death, and diet

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memento mori

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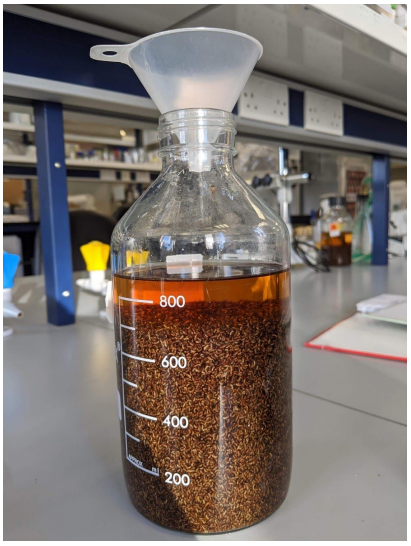

Declaration of intellectual contribution

I, the author, confirm that the Thesis is my own work. I am aware of the University's Guidance on the Use of Unfair Means. This work has not been previously presented for an award at this, or any other, university. Each chapter contains a list of author contributions.

The following are publications arising from this PhD:

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Abstract

The emergence and maintenance of the phenomenon of ageing remains only partially understood within an evolutionary framework. All organisms are born, reproduce, and die - but the variability with which these life-history traits exhibit between, and within, species is also yet to be fully elucidated. In this thesis, I investigate the biology of ageing using inbred fruit flies (*Drosophila melanogaster*). Utilising a demographic-based approach, where the lifespan of large numbers of genetically identical individuals are assayed, my focus is to better understand the mechanisms which underpin the apparent paradox of dietary restriction (DR), plasticity of lifespan, and the genetic regulation of ageing. In chapter one, I investigate the genotype-specific nature of dietary-driven lifespan plasticity, and find significant genetic variance in the relationship between diet and lifespan. I emphasise the need for care in DR experiments when considering diet and experimental confounds, and argue a full reaction norm is imperative prior to concluding a null response to DR. In chapter two, I test an explicit and key prediction of the consensus model of DR, reporting that in two circumstances of returning to resource abundance, individuals are not better equipped to survive or reproduce - in direct contrast to the model predictions. I propose a novel framework with which to interpret DR, based on both costs of DR, and rich-feeding. In chapter three, I estimate the heritability of ageing. When partitioning variance by considering ageing a population-based emergent trait, I find the explanatory power of total genetic variance to be far higher than has previously been recorded, offering renewed optimism for gene therapy interventions. I argue that previous estimates of the heritability of lifespan are compressed by the inherent stochastic nature of the trait. Overall, this thesis details novel insights into the evolutionary basis of DR, and the putative higher-order regulation of ageing.

Introduction

Background

Ageing is characterised by a progressive functional decline, concomitant with an age-dependent mortality risk increase (and almost invariably, a decrease in fecundity) post-maturation. Ageing and its inevitable outcome – death – present a challenge to evolutionary theory: a hypothetical Darwinian Demon with infinite lifespan and fecundity would increase its fitness merely by its continued survival and reproduction. Hence, the reduction of lifespan by an increase in mortality risk cannot be considered adaptive, since it reduces an organism's potential to maximise reproductive output. So why did such a maladaptive mechanism evolve, and why does it persist? As pointed out by Williams, maintaining functional integrity ought to be straightforward, when juxtaposed with the more complex task of development (Williams, 1957). Functional explanations of senescence posit the gradual, irreversible accumulation of cellular damage to be inevitable. However, despite high conservation of both cellular components acting in concert to repair, renew and replicate DNA (Morita *et al.*, 2010), and of ageing-controlling pathways like insulin-signalling (Kenyon, 2010), taxa vary widely in both median lifespan and age-specific mortality rates (Jones *et al.*, 2014). Should ageing be merely the accumulation of damage, and this damage be repairable (it is), then ageing ought not to occur, except in instances of prolonged energy deficit. Indeed, the partitioning of germline and soma, and high fidelity of DNA replication and repair in the germline (Sabour and Schöler, 2012) - which results in an offspring devoid of any age-related maladies of its parents - is directly indicative of an under-utilised capacity to repair the soma.

But ageing, whilst not necessarily ubiquitous, has a broad taxonomic distribution and is a fundamental facet of life. A lack of consensus remains over whether some branches within the tree of life are susceptible to organismal-level senescence, or if the negligible senescence observed in some species (most notably in *Hydra* (Schaible *et al.*, 2015) and *Heterocephalus glaber* (Ruby *et al.*, 2018b)) is merely a result of a deficient framework to delineate what constitutes a singular organism, or experimental artefact. Ageing is, nonetheless, a characteristic prevalent amongst all birds and mammals (Jones *et al.*, 2014), with lifespan having a modest level of heritability in humans (Herskind *et al.*, 1996; Mitchell *et al.*, 2001) and primates (Martin *et al.*, 2002). Genes which determine patterns of ageing, directly or indirectly, are thought to have existed prior to the evolution of eukaryotes, and orthologous ageing genes have been identified in many model organisms (Guarente and Kenyon, 2000; Smith *et al.*, 2008). In light of this, senescence might be considered an evolved trait with common multifactorial origins; common elements identified between and within species' ageing processes may then hold the key to deciphering the intricacies of senescence, and identify possible medical interventions.

But while an appreciation of the cellular hallmarks of senescence is flourishing (López-Otín *et al.*, 2013; López-Otín and Kroemer, 2021), a mechanism describing the inextricable link

with physiological ageing remains unresolved, although senolytics provide robust evidence of its existence (Xu *et al.*, 2018). In addition, no universal molecular marker of physiological ageing exists, nor 'master switch' identified, despite advances in quantifying chronological age via methylation clocks (Horvath, 2013). Likewise, the relationship between chronological and physiological age is complex, with markers of functional decline sometimes observed to be a better predictor of mortality than chronological age (Rera, Clark and Walker, 2012; Tricoire and Rera, 2015; Dambroise *et al.*, 2016).

In light of this, it is understandable that a considerable effort has been invested in understanding *why* the process of ageing occurs, as a precursor to devising strategies to combat it. In the absence of a clear functional framework to delineate the process of senescence, functional biologists are restricted to narrow corridors of research. Where the evolutionary process is not the object of study, evolutionary biology attempts to provide a direction for functional research to focus attention on - in a manner akin to providing a torch and compass to a traveller lost in a forest. The evolutionary field of ageing aims to move us towards a more thorough understanding of the factors involved in organism-level senescence. An improved understanding will be especially crucial in medicine, where an ability to diminish the impact of age-related degeneration and disease would yield profound benefits for humankind both socially and economically.

The evolutionary biology of ageing

Ageing has proven such a fascinating intellectual quandary, that the first known attempt to understand it was Aristotle, who considered the process to be an exuding of moisture and heat (King, 2001). More recent historical attempts, derived from the thinking of Weismann (Weismann, 1889), considered ageing an adaptive causative agent in mortality: offspring must be afforded the space and resources necessary to survive, and only via altruistic parental death can this occur. A 'for the good of the species' explanation remained the consensus view until the 1950's, where evolutionary biologists like Bidder, Haldane, Charlesworth and Fisher, drove the field to re-examine the assumption that group selection was compatible with Darwinian natural selection.

Modern models of ageing have also rejected this line of thinking, and largely see the existence of ageing as a failure of natural selection to maintain the integrity of the individual, at ages that would rarely be attainable in nature (but see Longo, Mitteldorf and Skulachev, 2005). The first modern hypothesis of ageing - mutation accumulation - explains ageing as the natural consequence of extrinsic (environmental) causes of mortality (Haldane, 1941; Medawar, 1952; Hamilton, 1966). Even in the complete absence of physiological ageing, extrinsic causes of mortality would result in a selective pressure on genetic variants that impact fitness, which is in constant decline throughout the lifespan of

the organism, due to the accumulating chance to die by extrinsic factors. The corollary of this, is that mutations which precipitate ageing can freely accumulate within the germline with little or no selective pressure to be purged. Individuals who continued to escape extrinsically-caused death for long enough, would eventually encounter the impact of late-acting deleterious mutations.

That the evolutionary value of survival and somatic integrity declines with age - the 'selective shadow' - is a key insight upon which later theories lie. The model of antagonistic pleiotropy (Williams, 1957) is an extension of this, whereby pleiotropic genes with positive effects on fitness in early-life would still undergo positive selection, even if they carried more pronounced deleterious effects in late-life; the benefit accorded by selection is based not only on the magnitude of the effect, but also the probability of the individual living long enough to be affected by it. Ageing here, again, would be caused by the amalgamation of harmful effects occurring in late-life - and the classification 'late-life' determined by the magnitude of extrinsic forces of mortality.

Comparative analysis in ants (Keller and Genoud, 1997) and experimental evolution in the fly (Partridge, Prowse and Pignatelli, 1999; Stearns *et al.*, 2000) supports the idea of intrinsic ageing being modulated by extrinsic mortality. Likewise, empirical evidence has, at times, given credence to both mutation accumulation (MA) and antagonistic pleiotropy (AP). Genetic variance increases with age, as predicted by MA, but not AP (Charlesworth and Hughes, 1996; Hughes *et al.*, 2002, but see Promislow *et al.*, 1996). Relaxation of purifying selection has also been shown to increase genetic drift, exacerbate MA, and shape genome-wide distribution of deleterious alleles (Cui *et al.*, 2019; Willemsen *et al.*, 2020). The evidence for pleiotropic polymorphisms acting antagonistically is scarce in nature, however (Leroi *et al.*, 2005). Additionally, much of the evidence in support of AP, in naturalistic settings (Paaby *et al.*, 2014), and in selection experiments (Rose and Charlesworth, 1980; Zwaan, Bijlsma and Hoekstra, 1995), also implicates life-history trade-offs between lifespan and fecundity. Indeed, the trade-off between early-life fecundity and longevity has been observed to be, in some experiments, causal (Sgrò and Partridge, 1999), with delayed mortality attributable to reproduction. These costs of reproduction therefore hint that physiological trade-offs at an evolutionary scale ought to be considered more so than the above genetic theories of ageing would imply.

The constraint on lifespan given costs associated with reproduction is a key component of life-history theory, which attempts to explain how natural selection shapes organisms to best utilise resources to optimise survival and reproduction via co-evolved traits (Stearns, 1989; Edward and Chapman, 2011). The disposable soma hypothesis (DS; Kirkwood, 1977; Kirkwood and Holliday, 1979) is still predicated on the notion of the selective shadow, but pits the energy allocation of current reproduction against future reproductive capacity and

survival - an effect which, putatively, occurs at an evolutionary level due to pleiotropic genes (Flatt, 2011). DS pertains to an immediate physiological trade-off: investment in reproduction can only occur at the expense of repair and maintenance. Since repair is costly, energy is scarce, and extrinsic mortality places restrictions on lifespan, repair beyond what is necessary would result in an organism with high late-life condition, but limited investment in reproduction. In contrast to earlier genetic theories, which determine ageing as an unearthing of late-acting deleterious mutations, DS thus contends the process of investing into cellular repair (somatic maintenance) is optimised to maximise fitness - and optimisation will always lead to repair mechanisms operating at a less than full capacity. Here, like in the genetic theories of ageing, differential extrinsic pressures placed on populations will then necessitate disparate strategies to invest energy wisely, and would be expected to result in negatively correlated intrinsic rates of mortality (Cichoń, 1997; Drenos and Kirkwood, 2005).

An abundance of evidence links costs of reproduction (Höglund and Sheldon, 1998) with a shortening of lifespan (Partridge, Gems and Withers, 2005; Tatar, 2010; Flatt, 2011). However, the physiological trade-off between lifespan and reproduction is not absolute, and evidence for its uncoupling can be seen in a number of experiments on mutants (Clancy *et al.*, 2001; Dillin, Crawford and Kenyon, 2002; Hwangbo *et al.*, 2004) or experimentally sterilised organisms which sometimes show no increase in lifespan (Kenyon *et al.*, 1993; Arantes-Oliveira *et al.*, 2002). Comparatively, correlations of lifespan and fecundity are usually negative, however they can also show a positive relationship (Dick, Ross and Yampolsky, 2011). It is possible that a laboratory environment may represent an uncharacteristically favourable one, where *ad libitum* feeding mitigates the need for strong trade-offs, however life-history predicts these trade-offs even in nutrient-rich environments (Stearns, 1989). Moreover, evidence of this decoupling also exists in naturalistic settings, in eusocial insects (Kramer *et al.*, 2015; Schrempf *et al.*, 2017) and in birds (Apanius and Nisbet, 2006). However, costs may exist, and simply not be apparent, since costs of reproduction exist not only in egg formation, but also in a less direct manner, like in lactation (Simons *et al.*, 2011) or parental provisioning (Sanz and Tinbergen, 1999), for example. Alternatively, costs may only be apparent under particular environmental conditions (Jenkins, McColl and Lithgow, 2004).

While the classic evolutionary models above have provided insight into the biology of ageing and have a fair degree of explanatory power, there remains no overarching model to explain the existence, and continuation of, ageing. Indeed, some of the most robust predictions derived from these models, like the direct link between extrinsic and intrinsic mortality, are not without caveat. Higher extrinsic mortality can in some circumstances select for longer lifespans - for example, if applied in a condition-dependent manner (Chen and Maklakov, 2012), if there are age-specific costs (Abrams, 1993), if the cost of mating is

extreme (Shokhirev and Johnson, 2014), if females choose older, higher quality males (Beck *et al.*, 2002), or if fecundity increases with age (Reznick *et al.*, 2004).

Likewise, further advances in the field have cast doubt on the the prerequisites of an ageing organism. Williams (Williams, 1957) predicted a strict delineation between germline and soma, but asexual bacteria display reproductive senescence (Ackermann, Stearns and Jenal, 2003). More likely necessary is the delineation between parent and offspring (Partridge and Barton, 1993), but even *E. coli*, which divides in a ostensibly symmetric manner has been shown to be both functionally asymmetric, and capable of ageing (Stewart *et al.*, 2005). Given the available evidence, it is possible to broadly conclude that ageing is likely maintained as a byproduct of selection being imposed on organisms for lifetime reproductive success and an unregulated, non-programmed (Kowald and Kirkwood, 2016) outcome of the declining force of selection at old-age.

Measuring ageing via mortality demography

Assessments of senescence in both wild and captive populations are hampered by the impracticability of determining ageing, or predicting time-to-death. Assaying of prognostic biomarkers like DNA methylation (Horvath, 2013) or telomere length (Zhao, Li and Liu, 2017; Wilbourn *et al.*, 2018) is possible, but lacks relevance to all individuals. Individuals die of a wide variety of proximate causes; ageing itself is a polygenic trait (Tesi *et al.*, 2020) with hugely-multifaceted intrinsic deterioration (Grotewiel *et al.*, 2005). It is in all instances, however, a process which culminates in death. Given this, time-to-death represents the most objective, generalisable - and perhaps most biologically relevant - measurement of ageing.

While individual age at death (in the form of maximum lifespan recorded) has been used as an indirect index of ageing rate, it remains an exceptionally poor surrogate measure of lifespan (Speakman, 2005), not least because lifespan is a highly stochastic trait (Caswell, 2009). Deriving useful information therefore requires a population-based approach. Population averages can be used, but more information can be extracted via a demographic approach. Demographic ageing within a heterogenous population is, however, confounded by noise related to such genetic heterogeneity (Vaupel, Manton and Stallard, 1979; Vaupel and Zhang, 2010) and underlying individual variation in robustness is a likely cause of the phenomenon of late-life mortality deceleration (Chen, Zajitschek and Maklakov, 2013). Given this, studies on model organisms which can establish inbred populations, are uniquely well-suited to inferring mechanisms of ageing, via precise genotype-phenotype association.

Demographic ageing - the increase in mortality rate, or risk, with age - a parameterisation of the decline of physiological function with age, was first described by actuary Benjamin Gompertz (Gompertz, 1825), and remains one of the most commonly used models. Several derivations of this model exist (Makeham, 1860; Tjørve and Tjørve, 2017), and other unrelated models exist to parameterise mortality (Wilson, 1994), but the simple two parameter Gompertz function (Greenwood, 1928) is a suitable fit for all-cause mortality (Juckett and Rosenberg, 1993).

The hazard function is described as:

$$u(x) = a \,.\, e^{\beta x} \qquad \textbf{Eqn. 1}$$

where $u(x)$ represents mortality rate (or risk) at age x, a denotes the level of mortality at the initial age (or initial mortality), and β is the mortality increase by age. Given this, the Gompertz describes a mortality risk which increases exponentially with age, and consequently, a log-linear increase.

These two parameters reveal two key assumptions inherent within the model, with which to discriminate demographic senescence: frailty and actuarial ageing rate. The ageing rate (β, the age-dependent increase in mortality risk) suggests an accumulation of damage accrued over an individual's lifespan, intensifying risk to causes of mortality; the magnitude of this increase indicates how accelerated mortality risk becomes with age. Frailty, a corresponds to the age-independent initial mortality. Differences in the frailty parameter between individuals are ordinarily interpreted as the immediate susceptibility to die from intrinsic ageing, given differential basal vulnerability to disease (Kirkwood, 2015), or number of defects present in an individual at birth (Gavrilov and Gavrilova, 2001).

Most biological interpretations of these parameters suggest the Gompertz explicitly describes only intrinsic, physiological, ageing (but see Ricklefs and Scheuerlein, 2002). It will, however, also provide an appropriate fit for field data, which will capture other elements of mortality, like age-dependent extrinsic mortality (Simons *et al.*, 2019). An additional, additive, Makeham parameter, γ, can be incorporated into the Gompertz to account for age-independent extrinsic causes of mortality - predation or starvation, for example (Makeham, 1860). However, in laboratory-based studies in controlled environments, this is rarely necessary. Notably, unlike in the similar Weibull function (Weibull, 1951), the relationship between frailty and ageing rate is multiplicative, rather than additive. The biological rationale of these models are therefore clearly distinct, although both have a high goodness of fit for mortality data (Ricklefs and Scheuerlein, 2002).

The existence of two demographic parameters with different biological interpretations illustrates a key point - that lifespan is a composite of frailty, ageing rate, and the

imposition of extrinsic causes of mortality. Lifespan should, thus, not be directly conflated with ageing, and a demographic approach is necessary to tease apart biological implications - this consideration is especially true for heterogeneous populations (Kowald, 2002).

But both ageing rate and frailty are useful comparative metrics in the field of ageing when they can be appropriately quantified; variation in each potentially implies different processes acting as causative agents. Both parameters also show significant heritability (Tatar and Carey, 1994; Promislow *et al.*, 1996), suggesting significant genetic variation upon which natural selection could act. Furthermore, the biological relevance of Gompertz parameters may be more significant than other population-based metrics of longevity, like median lifespan, since one median lifespan estimate can incorporate a range of Gompertz parameters (Simons, Koch and Verhulst, 2013; Garratt, Nakagawa and Simons, 2016).

Dietary restriction is an example of robust lifespan plasticity

One source of considerable variation within populations is phenotypic plasticity - the capacity of an individual genotype to respond differentially to varying environmental conditions. This is to be expected: organisms do not live in static environments, and are expected to have evolved life-histories which allow for a range of responses to maximise fitness under different environmental conditions (Stearns, 1992). In quantitative genetics, phenotypic variance of a trait can be partitioned into the sum of genetic and environmental factors which impact its expression, in addition to an element of unexplained stochastic error ($V_P = V_G + V_E + V_S$; Kilfoil, Lasko and Abouheif, 2009; Flatt, 2014). In this sense, phenotypic plasticity can be considered the response of one individual, or genotype, over a continuous environmental context (Dingemanse *et al.*, 2010). Alternatively, V_P can refer to qualitative differences in the same environmental factor. Importantly, the range of responses to a particular environmental variable - or reaction norm (Flatt, 2014) - is not always linear (Tatar, 2011; Metaxakis and Partridge, 2013). Additionally, environmental factors can interact with genetics (GxE effects), and/or affect correlated traits, which can elicit phenotypes which can only be understood when considering this interplay. As such, phenotypic plasticity can, in many cases, present an important experimental confound, or lead to erroneous conclusions if the full nature of the response curve is unknown.

Plasticity of lifespan in particular is observable across a range of variable environmental conditions, such as temperature (Hosono *et al.*, 1982; Conti *et al.*, 2006). Some of the most prominent examples are the specialised life-histories of invertebrate diapause (Tatar and Yin, 2001) and dauer state in *C. elegans* (Braendle, Milloz and Félix, 2008), or of eusocial insects (Keller and Jemielity, 2006). However, the restriction of calories or protein above the

level of malnutrition - dietary restriction (DR) - is perhaps one of the best examples of robust lifespan plasticity, a phenomenon present across taxa, and in nearly all species where it has been studied (Min *et al.*, 2007; Solon-Biet *et al.*, 2014; Fontana and Partridge, 2015; Mattison *et al.*, 2017). DR's importance to the field of ageing is two-fold: First, as a dietary perturbation it produces a counterintuitive extension of lifespan, allowing for an insight into the mechanisms of ageing. Second, given its high degree of conservation, it is commonly assumed to operate via shared evolutionary mechanisms - something which is suggestive of a translatable potential (Fontana and Partridge, 2015).

Several highly conserved genes - particularly those interconnected with insulin, or insulin-like growth factor (IGF-1) signalling - have, in the past, been inferred to be responsible for the longevity-promoting effects of DR across species, implying a level of conservation of the DR response may indeed exist at the genetic level. Insulin and IGF-1 show a correlated response with DR (Breese, Ingram and Sonntag, 1991; Argentino *et al.*, 2005), and a downstream effector of insulin signalling, the nutrient sensor mechanistic target of rapamycin (mTOR), was previously implicated in directly mediating extension of lifespan of DR (Kapahi *et al.*, 2004; Kaeberlein *et al.*, 2005; Hansen *et al.*, 2007). Autophagy has also been determined to be a requirement for the lifespan extending effect of DR in *C. elegans* (Jia and Levine, 2007; Bagherniya *et al.*, 2018), potentially mediated via upregulation of the insulin signalling transcription factor family FOXO (Hansen *et al.*, 2008; Webb and Brunet, 2014). Similarly, senescent cell removal is upregulated under DR (Fontana *et al.*, 2018; Fontana, Nehme and Demaria, 2018), but synergy of these interventions has not yet been tested.

Nonetheless, the complete molecular mechanisms of DR are far from elucidated; more recently studies have found mTOR acts in a non-overlapping fashion with DR, suggesting mTOR is not sufficient to induce the full DR-mediated longevity response (Bjedov *et al.*, 2010; Garratt, Nakagawa and Simons, 2016; Birkisdóttir *et al.*, 2021). Additionally, while DR mimetics are currently under investigation, progress will likely be hampered by the potential for species- or genotype-specific mechanisms to exist. Indeed, the most compelling evidence of this problem, is the mechanisms by which physiological benefits are accrued appear to differ between species. For example, both DR and mTOR knockdowns extend lifespan in demographically discrete manners (Mair *et al.*, 2003; Simons, Koch and Verhulst, 2013; Garratt, Nakagawa and Simons, 2016). Likewise, the magnitude of the DR longevity effect is genetically heterogenous (Dick, Ross and Yampolsky, 2011; Metaxakis and Partridge, 2013) and dietary optima vary between sex (Maklakov *et al.*, 2008; Jensen *et al.*, 2015; Camus *et al.*, 2017). Overall, no single genetic manipulation, or medical intervention has been shown to be effective in inhibiting the effects of DR in multiple models.

As such, there remains considerable scope for attention to be directed towards the evolutionary biology of DR, both to direct mechanistic research, and to interrogate the assumed conservation of the physiology of the response. The consensus model of the evolutionary biology of DR (Shanley and Kirkwood, 2000; Kirkwood and Shanley, 2005) is predicated upon DS. Ostensibly however, this seems to be a paradox: DS purports physiological trade-offs occur due to resource scarcity; further scarcity should not elongate lifespan. Shanley and Kirkwood posit an addendum to Kirkwood's DS hypothesis to reconcile these ideas. In a manner similar to diapause, they suggest a potentially adaptive explanation for the DR longevity effect, where harsh conditions necessitate an optimal 'wait for the good times' approach. Under certain ecological conditions, like where juvenile survivorship reduces in accordance with resources, or when a minimum reproductive overhead exists, Shanley and Kirkwood suggest energy allocated to reproduction should be preferentially re-allocated to somatic maintenance. The DR state, therefore, represents an attempt by the organism to ensure somatic integrity for when conditions improve, and to make good use of energy which otherwise would fail to yield a fitness return. There exists some tangential evidence for this model - DR often reduces fecundity concomitant with lifespan extension (Lee *et al.*, 2008; Skorupa *et al.*, 2008; Grandison *et al.*, 2009; Moatt *et al.*, 2016).

However, evidence is accruing which is difficult to reconcile with the consensus evolutionary model of DR. This evidence can be separated into discrete categories: First, fecundity and lifespan can be decoupled, and this is also apparent under DR, and DR mimetic, conditions (Mair *et al.*, 2004; Grandison, Piper and Partridge, 2009; Bjedov *et al.*, 2010; Drewry, Williams and Hatle, 2011) suggesting the reproductive response is merely correlated to that of lifespan. Note, however, investment towards reproduction is not solely represented by fecundity. Second, while DR precipitates lifespan extension in flies, it does so by reducing frailty - not ageing rate (Good and Tatar, 2001; Mair *et al.*, 2003). This is indicative of a reduction in the vulnerability to die from ageing-related damage accumulation, rather than a reduction of damage accumulation itself. Third, the model assumes a very high initial allocation towards reproduction, and only explains the relationship between diet and longevity over a narrow range of caloric intake (Mitteldorf, 2001). Fourth, flies fed on a rich diet actually invest more into somatic maintenance than those under DR (O'Brien *et al.*, 2008). It has also been argued that more increasing degrees of restriction ought to increasingly attenuate the longevity effect (Speakman, 2020). These criticisms are, however, contingent upon an interpretation of the modelled, absolute (as opposed to relative) reallocation of resources towards somatic maintenance (Shanley and Kirkwood, 2000). Lastly, studies utilising a geometric framework approach have detailed the protein:carbohydrate ratio in flies, rather than calories, to be the primary response axis of longevity in flies (Lee *et al.*, 2008; Maklakov *et al.*, 2008; Jensen *et al.*, 2015). More recently, results have indicated the partitioning of dietary cholesterol may be causal in the

nature of this relationship (Zanco *et al.*, 2021). These data indicate a more nuanced role of nutrition in longevity, than the simple energy reallocation implied by the consensus model.

Accordingly, there have been attempts to reconcile these data with a more parsimonious explanation of DR. A nutrient-centered, constraint-based approach - the lethal protein hypothesis (Lee *et al.*, 2008; Fanson *et al.*, 2009; Mautz *et al.*, 2019; Moatt *et al.*, 2020) - inverts the typical assumption of DR: that a DR diet confers a protective, pro-longevity effect on the soma. Instead it posits dietary protein as possessing direct physiological costs on lifespan, but that organisms may consume it in excess due to its requirement for reproductive output. An alternative evolutionary hypothesis which may underpin the lethal protein hypothesis has also been proposed: nutrient recycling (Adler and Bonduriansky, 2014; Moatt *et al.*, 2020; Speakman, 2020). Adler and Bonduriansky suggest the longevity effect of DR originates from the upregulation of autophagy and apoptosis in the nutrient-deprived state (Longo and Fontana, 2010) - processes which are, however, inhibited by growth pathways, which are in turn required to maximise reproductive output. As such, key somatic repair mechanisms are inhibited under conditions of nutrient abundance. The DR effect is so highly conserved, they suggest, not only because apoptosis and autophagy will free up valuable energetic resources at a time of scarcity, but also curtail total daily energy expenditure, thereby maximising immediate reproductive output (Mitchell *et al.*, 2017). Both ideas contain a reasonably sound theoretical basis, but currently lack empirical examination of key predictions (but see Fanson, Fanson and Taylor, 2012). Further investigation will be necessary to fully elucidate the mechanisms which underlie the evolution of the lifespan extending capacity of DR.

The fly as a model organism

The fruit fly, *Drosophila melanogaster*, has, for over a century, been used as a model organism in research spanning from early genetics of heritable traits, to physiology, and has been the organism of study in Nobel prize winning research - most recently for its role in the elucidation of circadian rhythms (Liu *et al.*, 1992; Price *et al.*, 1998). The fly has many general properties which make it an attractive option for its use in research, including: a simple, compact genome; a broad genetic homology with humans, including disease-causing genes (Yamamoto *et al.*, 2014); homology with organ systems (Choma *et al.*, 2011), and ease of use in genetic crossing schemes.

Pathways which regulate ageing are highly-conserved (Guarente and Kenyon, 2000; Smith *et al.*, 2008), and this extends to ageing-related phenomena like DR (Wuttke *et al.*, 2012). Given this, ageing is widely considered to possess common evolutionary origins, and thus its study does not preclude the use of model organisms.

A demographic approach to the question of ageing requires very large sample sizes to accurately associate mortality demography to the relevant life-history traits: lifespan and reproductive output. The fly is the ideal candidate for such a large-scale project given its short generation time (roughly ten days from egg to eclosion), short lifespan (median of around thirty days), very high fecundity, and fractional upkeep costs. Huge sample sizes suitable for fine detailing of mortality curvature can be acquired in a few generations, and require minimal care. This approach has previously proven highly effective in furthering understanding of late-life mortality deceleration (Miyo and Charlesworth, 2004) and quantifying heritability of ageing parameters (Promislow *et al.*, 1996). DR has also been extensively studied utilising a demographic approach in the fly, with mortality being strongly modulated simply by alterations in yeast concentrations of food. Seminal studies in this area have revealed a counterintuitive immediate - but reversible - reduction in the risk to die (frailty) when protein intake is restricted (Good and Tatar, 2001; Mair *et al.*, 2003).

Heterogeneity within assayed populations can cloud genotypic effects on the ageing process (Chen, Zajitschek and Maklakov, 2013), and will nullify any attempts to associate mortality with genotype, given the confounds of additional environmental interactions with genetics. But the fly also provides an excellent opportunity to efficiently, and stably, experiment with inbred lines. The Drosophila Genetic Reference Panel (DGRP) is an isofemale panel of 200 lines, with high-quality, publicly available sequencing data, generated from a population in North Carolina, USA (Mackay *et al.*, 2012). Utilising this panel allows for the delineation of genotype-specific mortality, and lines have already been shown to be highly variable (Durham *et al.*, 2014; Ivanov *et al.*, 2015) - while still maintaining a degree of standing genetic variation expected in the wild. One potential caveat to this approach, however, is the magnitude of inbreeding depression prevalent within the panel (Schou *et al.*, 2018).

Concluding remarks

Over the course of my PhD, I have sought to better understand the evolutionary principles which underpin the phenomena of dietary restriction, and ageing as a whole. The work detailed in this thesis constitutes a synthesis of the above concepts. In chapter one, I investigate the importance of genetic variance in the lifespan reaction norms to diet and desiccation. In chapter two, I exploit the huge sample size permitted by the fly to test the consensus evolutionary model of DR. In chapter three, I aim to establish estimates of the heritability of ageing, employing in the process, our relatively novel means of assaying mortality, to partition the variance of population-based metrics of lifespan.

Chapter One

The relationship between longevity and diet is genotype dependent and sensitive to desiccation in *Drosophila melanogaster*

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McCracken, A. W., Buckle, E., & Simons, M. (2020).

Abstract

Dietary restriction (DR) is a key focus in ageing research. Specific conditions and genotypes were recently found to negate lifespan extension by DR, questioning its universal relevance. However, the concept of dietary reaction norms explains why DR's effects might be obscured in some situations. We tested the importance of dietary reaction norms by measuring longevity and fecundity on five diets in five genotypes, with and without water supplementation in female *Drosophila melanogaster* (N>25,000). We found substantial genetic variation in the response of lifespan to diet. Flies supplemented with water rescued putative desiccation stress at the richest diets, suggesting water availability can be an experimental confound. Fecundity declined at these richest diets, but was unaffected by water, and this reduction is thus most likely caused by nutritional toxicity. Our results demonstrate empirically that a range of diets need to be considered to conclude an absence of the DR longevity effect.

Introduction

Dietary restriction (DR), the limitation of food intake but avoiding malnutrition, extends lifespan. The generality of the DR response has been questioned, however, by reports that DR does not extend lifespan under certain experimental conditions (Ja *et al.*, 2009; Piper *et al.*, 2010; Dick, Ross and Yampolsky, 2011; Austad, 2012) or in a considerable proportion of the genotypes tested (Liao *et al.*, 2010; Rikke *et al.*, 2010; Dick, Ross and Yampolsky, 2011; Swindell, 2012; Mitchell *et al.*, 2016; Wilson *et al.*, 2020; Jin *et al.*, 2020). These conclusions are routinely based upon experiments using two diets (dietary dyad) alone, whereas it is recognised that a change in the continuous relationship between diet and lifespan (reaction norm) can obscure lifespan extension by DR (Tatar, 2011; Flatt, 2014). The bell-shaped nature of the dietary reaction norm dictates that one particular diet concentration, in one genotype or environment, will result in the longest lifespan; lower or higher diet concentrations will induce a shortened lifespan due to malnutrition or overfeeding, respectively. Where a particular dietary dyad falls on this reaction norm will determine the magnitude of the DR effect and can even lead to the erroneous conclusion that DR shortens lifespan (Fig.1).

Few studies have examined dietary reaction norms in more detail by titrating the supply of protein or calories across multiple genotypes or environments, and none have tested both genetic and environmental effects on dietary reaction norms simultaneously. Of these studies, a fraction employed transgenic or lab strains (Clancy *et al.*, 2002; Min *et al.*, 2008; Skorupa *et al.*, 2008; Grandison *et al.*, 2009; Wang *et al.*, 2009; Tatar, 2011) and demonstrated varying degrees of genetic variance in the plastic response to diet. Across these studies, shifts in dietary reaction norms on the x- or y-plane are more apparent than

changes in the overall shape of the relationship between diet and longevity (Tatar, 2011; Flatt, 2014). Whether genetic variation in transgenic and lab strain experiments is representative of standing genetic variation of natural populations is, however, unclear. A naturalistic appreciation of the genetic variation of the DR response becomes particularly important when null responses are interpreted to question the universal properties of DR important in translating its benefits to our own species. One previous study did measure detailed reaction norms using wild-derived outbred populations and found a degree of genetic variance for the relationship between diet and lifespan (Metaxakis and Partridge, 2013). However, the estimate of genetic variance of a population level trait, such as lifespan, when estimated from between outbred stains (Whitlock and Fowler, 1999) will be affected by mortality heterogeneity (Chen, Zajitschek and Maklakov, 2013), which can bias the estimated level of genetic variance upwards or downwards.

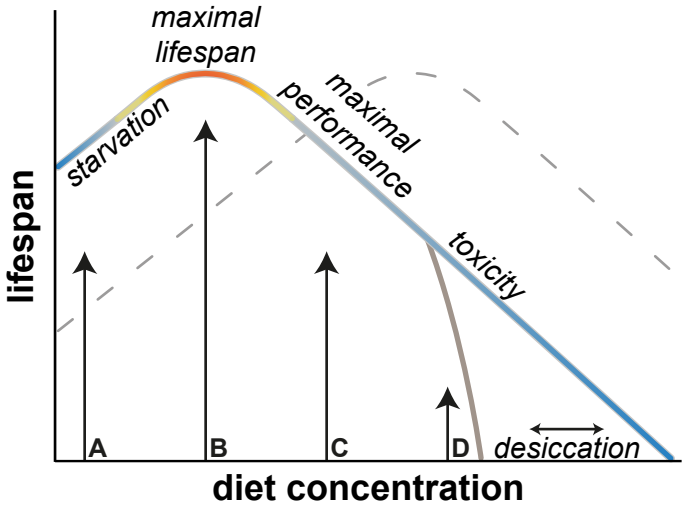

Figure 1. Schematic of multiple thresholds in the lifespan reaction norm to diet. Diet concentration has a bell-shaped relationship with lifespan, ranging from malnutrition (**A**), DR (**B**), maximal performance - or highest Darwinian fitness - at a relatively rich diet (**C**), to overfeeding, leading to nutritional toxicity (**D**). As a detailed reaction norm is rarely known, a dietary dyad (although often used) can lead to misleading conclusions. A dietary dyad (**A** and **C**) can show no response at all owing to the symmetry in the shape of the reaction norm. Furthermore, genetic or environmental effects can alter the shape or shift the reaction norm (dashed line), or lead to effects at only specific parts of the reaction norm (solid gray, e.g. desiccation). For example, diets **B** and **C** result in a DR response on the focal curve, but malnutrition on the dashed curve.

When specific environmental effects interact or interfere with the DR reaction norm, the use of dietary dyads - or the neglect of environmental confounds, like desiccation - could similarly lead to misleading conclusions. For flies specifically, water supplementation has been suggested to diminish the effect of DR on lifespan (Ja *et al.*, 2009; Dick, Ross and Yampolsky, 2011). The conclusion that water completely explains DR has been discredited (Piper *et al.*, 2010), but flies nonetheless value water as a resource and consume 1-2µl per day, with higher consumption at higher dietary yeast (Fanson, Yap and Taylor, 2012) and sugar concentrations (van Dam *et al.*, 2020). Hence, erroneous conclusions could be drawn from diet responses if desiccation presents a genotype- or diet-specific hazard.

Here, we present DR reaction norms for fecundity and longevity across five genotypes in female flies (*Drosophila melanogaster*) with and without water supplementation using high sample sizes. We show empirically across five wild-derived, inbred lines that there are strong genetic and environmental elements to dietary reaction norms, and therefore the thorough appreciation of reaction norms is critical when interpreting diet effects across genotypes and environments.

Materials and methods

Fly husbandry, experimental protocol and dietary regimes

For lifespan experiments adult *Drosophila melanogaster* were provided with either 0.5%, 2%, 5%, 8% or 14% autolysed yeast media. All other media components (13% table sugar, 6% cornmeal, 1% agar and 0.225% [w/v] nipagin) remained the same, given the dietary protein axis is the main lifespan determinant in flies (Lee *et al.*, 2008; Jensen *et al.*, 2015). Note, cornmeal concentration was halved in 14% yeast media to allow dispensing of this media. Halving cornmeal concentration in all diets would have impacted viscosity of media at lower yeast concentrations, possibly resulting in yeast granules settling at the bottom of vials, and would have made our diets less comparable to our own previous work (McCracken et al., 2020). Full cornmeal concentration 14% diets, we speculate, would have intensified, rather than have relieved, desiccative stress at this yeast concentration. Statistical analyses and figures consider our diets to be nominal (categorical) measurements, and do not imply a fixed degree of difference attributable to yeast concentration. Purpose-built demography cages included two openings, one for the supplementation of food, and one for water-agar (2% agar) or empty vial. Cages contained between 70-125 females each (mode of ~ 100 females), with 5 cages per treatment, per genotype (N = 50 cages per genotype). For one genotype, DGRP-195, sample size was even higher: an additional two cages of water-supplemented, and control cages at 2% media. All experimental flies were reared and mated on 8% media for 48 hours, and kept in cages on 8% media until age 3-4 days, when experimental dietary treatments started. Flies

were scored every 48 hours, where dead flies were removed and counted, and food vials were replaced.

To establish dietary reaction responses, flies were exposed to continuous diets with the addition, or absence of water-agar supplementation. To test the effect of water supplementation on longevity, we provided an additional vial of water-agar ('water supplementation'), or an empty vial ('control'), to each cage. Separation of food and water sources allowed flies to choose their source of nourishment, and eliminated the need for hydration to be coupled with caloric intake. Dietary treatments were balanced for age, and date of eclosion. All flies presented were grown within one batch. The experiment was carried out on a small collection of DGRP lines (Mackay *et al.*, 2012; DGRP-195; 217; 239; 362; 853), which were generated through full-sib mating of wild-type females in 2003. These lines were a subset of the lines we used in McCracken *et al.*, 2020, where we observed different responses comparing 2% and 8% yeast diets. Previously observed responses of the five lines to 2% yeast diets – either typical, or starvation, were replicated in the results presented here.

Fecundity

Feeding vials were imaged and analysed using image analysis software QuantiFly (Waithe *et al.*, 2015) to determine the relative quantity of egg laying. Egg counts based on image recognition do not necessarily provide an absolute count, as with manual egg-counting, but are suitable for comparative estimates. The combined estimate achieved using image analysis has the advantage of using egg laying from many females in the same vial, averaging out biological variation between females. Vials were removed, during normal scoring periods, from all cages containing eggs from flies aged 11 or 12 days.

Data analysis

For survival analysis, mixed Cox-proportional hazard models were used that included 'cage' as random term to correct for uncertainty of pseudo-replicated effects within demography cages (Ripatti and Palmgren, 2000; Therneau, Grambsch and Pankratz, 2003). Additional specific tests of coefficients are provided that combine the single and interaction term (in a z-test, using the maximum s.e. of the factors compared) to test how survival was changing in water-treated flies, compared to respective control treatments. Note, formal tests for proportionality of hazards are not available for mixed effects Cox regressions. For survival data comparisons, we report the full model, and models fitted within each genotype separately (see Table S1-12). By splitting the analysis between genotypes, bias introduced by deviations in proportionality of hazards between genotypes is avoided. Qualitative conclusions remain similar, irrespective of how these models are fitted. Interpretations from the Cox mixed effects model are based on a full model including the 3-way interaction between diet, water supplementation and genotype. Coefficients are

reported as logged hazards with significance based on Z-tests. Right-censoring was included, and dietary treatments were considered categorical factors.

Egg laying was analysed using linear models of log-transformed fecundity count data. Flies only differed by one day in age, and age was equally distributed across treatment and measured in a balanced design. BIC with backward elimination of terms was used for model comparisons and selection, and resulted in a model that contained the terms, and interaction between genotype and diet. Water was added to our models to directly test for any effect on fecundity, but this proved negligible (Table S13,14).

For hazard ratio figures, ratios are plotted as coefficients derived from within-line Cox mixed-effect models, with error bars representing 95% confidence intervals.

Results and Discussion

The genotypes tested showed a classical bell-shaped response to diet. Longer lifespans were observed at intermediate dietary yeast concentrations, consistent with DR (Fig.2A,S4; Table S1-6; P<0.001). All genotypes also exhibited a reduction in survival at very lowest yeast concentrations (starvation), and at the very highest (maximal performance or nutritional toxicity). We detected considerable genetic variation in the response to diet (genotype * diet; χ^2=162, df=16, P< 0.001) with the diet of maximum longevity, and the magnitude of the diet response, differing between genotypes (Fig.2). This result held even upon the exclusion of our highest yeast concentration diet (χ^2=217, df=12, P< 0.001).

To test the effect of desiccation, we compared longevity under control conditions to water-supplemented. Supplemental water reduced mortality particularly at higher yeast concentrations, and we found genetic variance for this environmental effect (genotype * diet * water; χ^2=160, df=16, P<0.001; Fig.2B; Table S2-6). At the highest yeast concentrations, this amounted to a 1.5- to 50-fold reduction in hazard rate. This result also held when excluding our highest yeast concentration diet (χ^2=75, df=12, P< 0.001). Given this, particular caution should be afforded when considering the effect of desiccation, especially in organisms without *ad libitum* access to water and when fed a concentrated diet. To assess statistically whether water supplementation abolished DR-induced life extension (Ja *et al.*, 2009; Piper *et al.*, 2010) we ran our statistical models within the water treatment only, but found no evidence for this suggestion (Fig.S1, Table S7-12). The observed mortality can thus be partitioned into nutrition- or hydration-based causes. We therefore conclude desiccation can play an experimentally confounding role in DR, but is not causal in the link between nutrition and longevity, since the removal of desiccation as a variable does not eliminate the longevity response to diet.

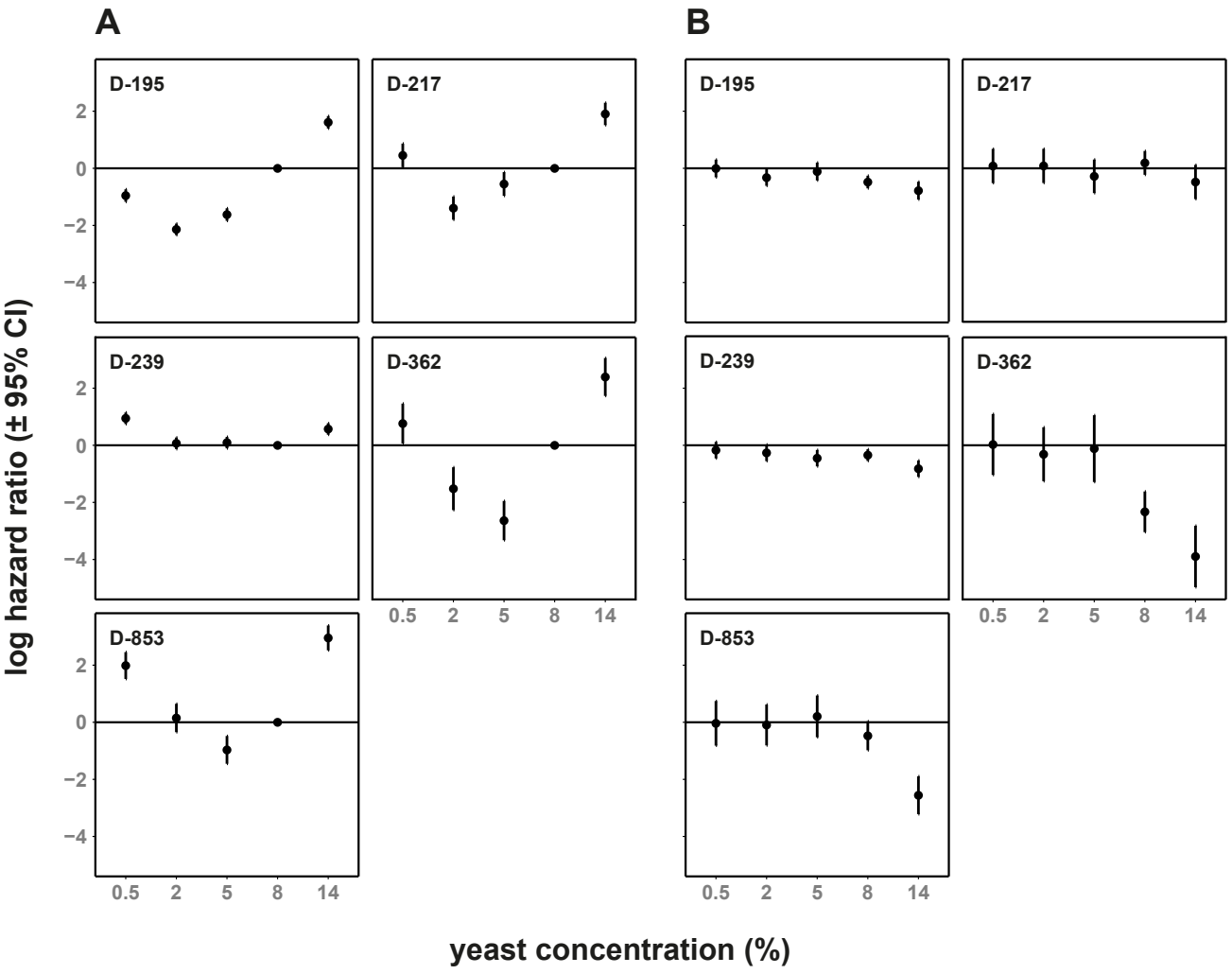

Figure 2. Log hazard ratios of diet and water supplementation in a panel of DGRP genotypes. **A** - Dietary reaction norms vary in a genotype-specific manner. **B** - Water-supplementation, relative to control treatment, rescues desiccation in a diet and genotype-dependent manner. Hazard ratios represent risk to die, therefore higher values indicate shorter lifespans and are relative. Ratios are plotted as coefficients derived from within-line Cox mixed-effect models, with error bars representing 95% confidence intervals (CIs). For **A**, 8% yeast treatment was treated as a reference and as such, no CIs are available. Rates here are relative to 8% yeast diet, and lines represent this standard. N = 12,782 females total; 2,404-2,635 per genotype. For **B**, hazard rates are relative to the corresponding control for each diet. Horizontal lines represent a water effect size of 0. N = 25,519 females total; 4,800-5,282 per genotype.

DR is known to reduce reproductive output and is commonly interpreted as a response to decreased energy availability (Moatt *et al.*, 2016). The effect of overfeeding on reproduction, although appreciated in humans (Broughton and Moley, 2017), has received little attention (McCracken *et al.*, 2020). These two responses were evident in egg laying: an increase with yeast concentration, and a stabilisation, or decline at the highest yeast

concentrations (Fig.S2,3; Table S13,14). As with mortality, genetic lines also differed in the reproductive response to diet ($F=6.3$, $df=16$, $P< 0.001$). Reduced egg laying together with a reduction in survival, lowered predicted lifetime reproductive at the richest diet (Fig.S3). Egg laying was not affected by water supplementation (Fig.S2; Table S13,14; $P>0.15$). Notably, even when water rescued mortality caused by desiccation at the high yeast concentrations, egg laying was unaffected (Fig.2,S2). Given this, we infer the decline in reproductive output at the highest yeast concentration was not due to desiccation stress, but nutritional toxicity. By contrast, the rescue of mortality at high yeast concentrations by water supplementation is therefore likely to be separate and driven by desiccation. However, since the reduction in fecundity was only observed in our highest yeast concentration diet, and this was the only diet in which cornmeal concentration was halved (see Materials and Methods), it is possible the reduction in cornmeal acts as a nutritional limiter of reproductive output, and this will require further testing.

In conclusion, we observe significant genetic, and environmentally induced, variation in the lifespan and fecundity responses to diet. Our data uses females only, but similar effects in males could explain observations of sexual dimorphism in the response to diet and likewise requires investigation (Jensen *et al.*, 2015; Camus *et al.*, 2017; Regan *et al.*, 2016). These data now directly demonstrate that specific care is needed when interpreting effects of DR across genotypes, experimental conditions or environments. We acknowledge that carrying out full reaction norms in all DR experiments would be laborious, especially in mammalian models (reviewed in Selman and Swindell, 2018). Still, it is increasingly acknowledged that personalising the degree of DR to genotype or environment will be key to translating the benefits of DR to humans (Perez-Matos and Mair 2020). When genetic variance in DR is the object of study, we suggest selecting dietary dyads that differ only minimally when genetic variance in DR is the object of study. Such a strategy reduces the chance that tested diets diverge considerably from maximal lifespans, leading to starvation or nutritional toxicity (Fig.1). Furthermore, we suggest when environmental conditions, such as water (Ja *et al.*, 2009), sex (Regan *et al.*, 2016) and microbiome (Wong, Dobson and Douglas, 2014) are presumed to negate the DR response, that a post-hoc reaction norm is performed. Similar considerations hold for mechanistic research. Should, for example, a genetic manipulation remove the DR response, only a full dietary reaction norm can demonstrate how such an effect arises: by either a shift in, or compression of, the reaction norm (Tatar, 2011; Flatt, 2014). The importance of reaction norms when studying DR has been stressed before, but this is the first high sample size data across multiple wild-type inbred genotypes and diets, including an environmental confound, that demonstrates this empirically.

Acknowledgements

We thank the Deplancke laboratory for supplying DGRP lines and members of Simons' lab for their valuable assistance. We would also like to thank the two anonymous reviewers for their valuable comments.

Author Contributions

Conceptualization: A.W.M., M.J.P.S.; Methodology: A.W.M., E.B., M.J.P.S.; Formal analysis: A.W.M., M.J.P.S.; Investigation: A.W.M., E.B., M.J.P.S.; Resources: M.J.P.S.; Data curation: A.W.M.; Writing - original draft: A.W.M.; Writing - review & editing: A.W.M., E.B., M.J.P.S.; Visualization: A.W.M.; Supervision: M.J.P.S.; Project administration: M.J.P.S.; Funding acquisition: A.W.M., M.J.P.S.

Supplement

Figures

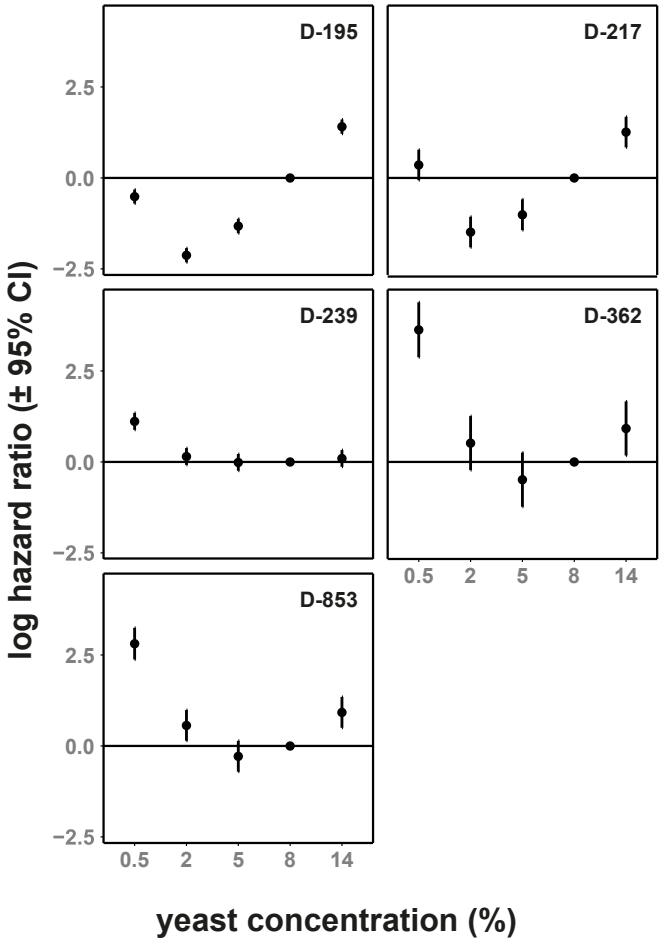

Fig. S1. Log hazard ratios of diet within water-treated cages in a panel of DGRP genotypes. Reaction norms to diet still differ in water-treated circumstances. Hazard ratios represent the inverse of typical survival reaction norms to diet. 8% yeast treatment was treated as a reference and as such, no CIs are available. Rates here are relative to 8% yeast diet, and lines represent this standard. N = 12,737 females total; 2,396-2629 per genotype. Hazard ratios have the benefit over median lifespan in that they are directly related to the appropriate statistics used for time-to-event data. In addition, they are directly comparable in a quantitative fashion across genotypes of different lifespans, as they express a relative risk.

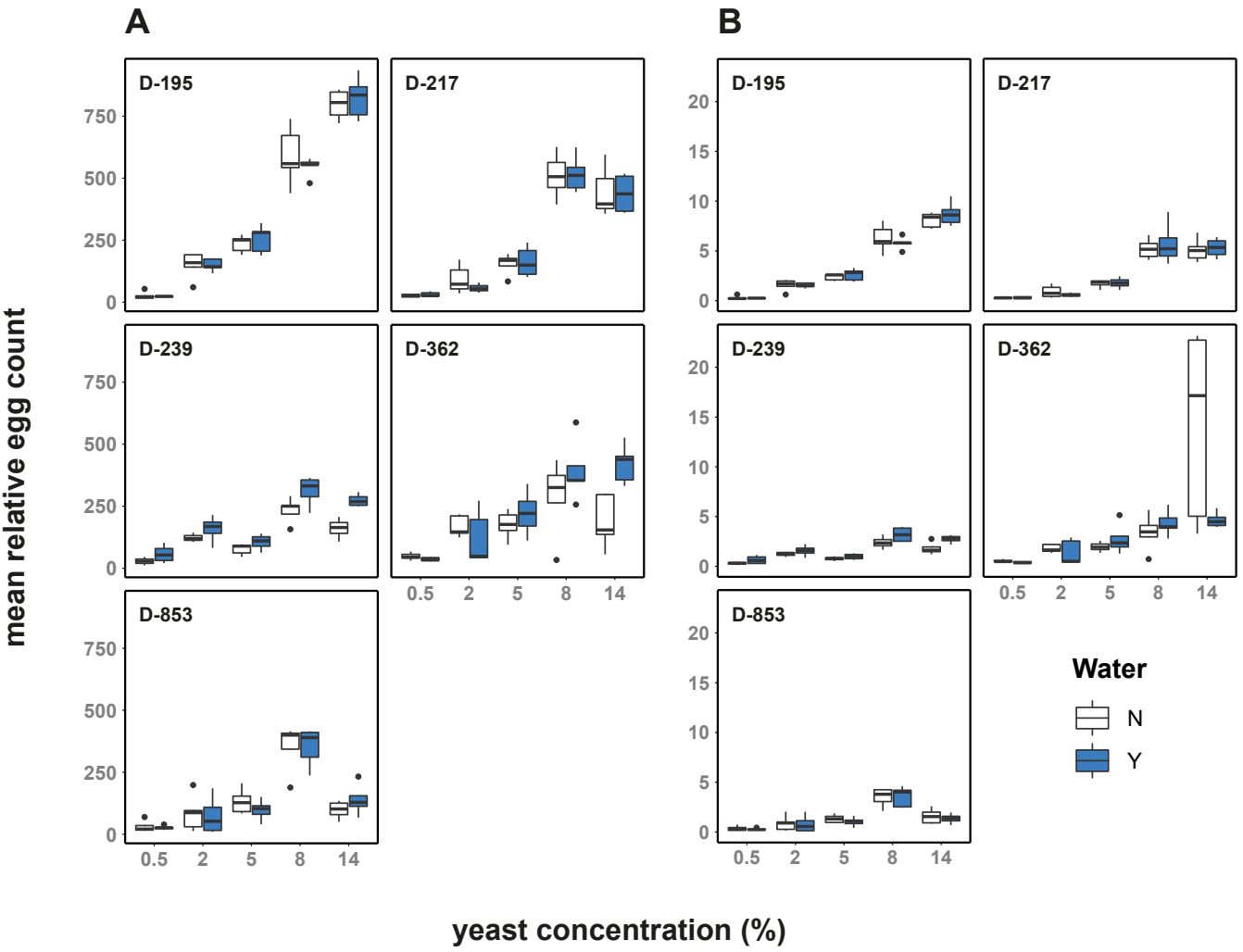

Fig. S2. Fecundity analysis of panel under all conditions. Fecundity has a positive relationship with dietary yeast concentration, except at the highest yeast concentration assayed (14%) for most genotypes. **A** - raw egg counts. **B** - mortality-corrected counts. Counts generated using QuantiFly software. Counts are relative, but directly comparable. Flies assayed at age 11-12 days, with boxplots aggregating totals (median, with the box depicting a quartile each way, and whiskers showing the range; outliers plotted as dots). Each cage was assayed on 1 scoring day at this age. Mortality corrected counts (**B**) generated by dividing raw counts, by N flies remaining in cage at the time of assaying. N = 25,519 females total; 4,800-5,282 per genotype. Note that DGRP-362 experienced significant mortality at this age under 14% yeast dietary treatment. This is the cause of the discrepancy in significance between raw, and age-adjusted fecundity counts. Note, egg-laying was not assessed throughout life and in natural circumstances lifespan of the fly is truncated by extrinsic factors, e.g. predation. We nonetheless, tentatively conclude that the enhanced mortality and reduced egg laying on very rich diets is caused by nutritional toxicity.

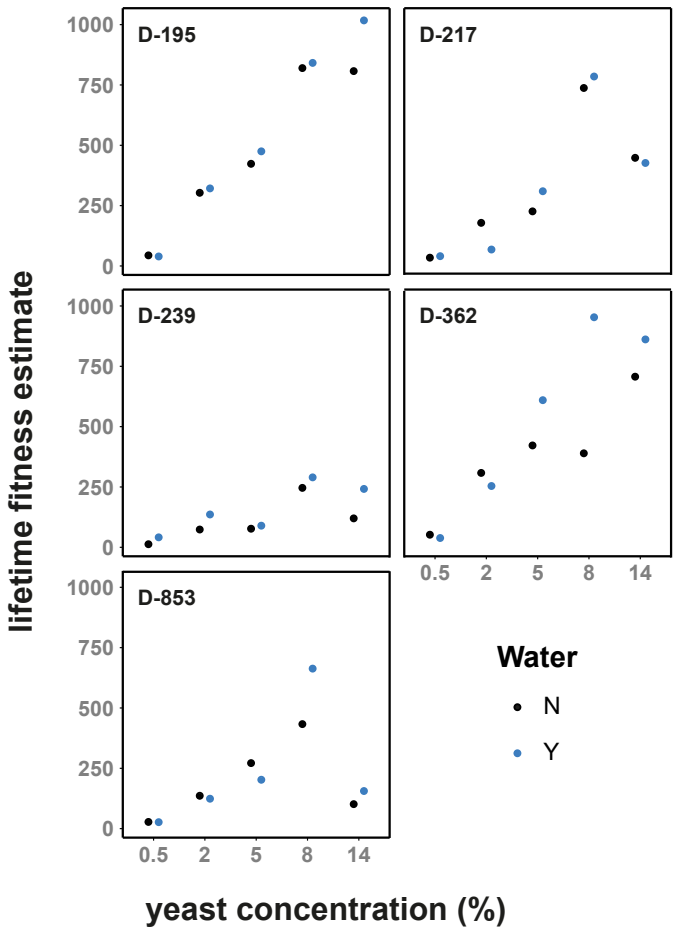

Fig. S3. Lifetime reproductive fitness estimates of panel under all conditions. Lifetime fitness has a positive relationship with dietary yeast concentration, except at the highest yeast concentration assayed (14%) for most genotypes. Mortality-adjusted egg counts from Fig. S2 were multiplied by the area under the relevant survival curve (restricted mean) to generate lifetime estimates. N = 25,519 females total; 4,800-5,282 per genotype.

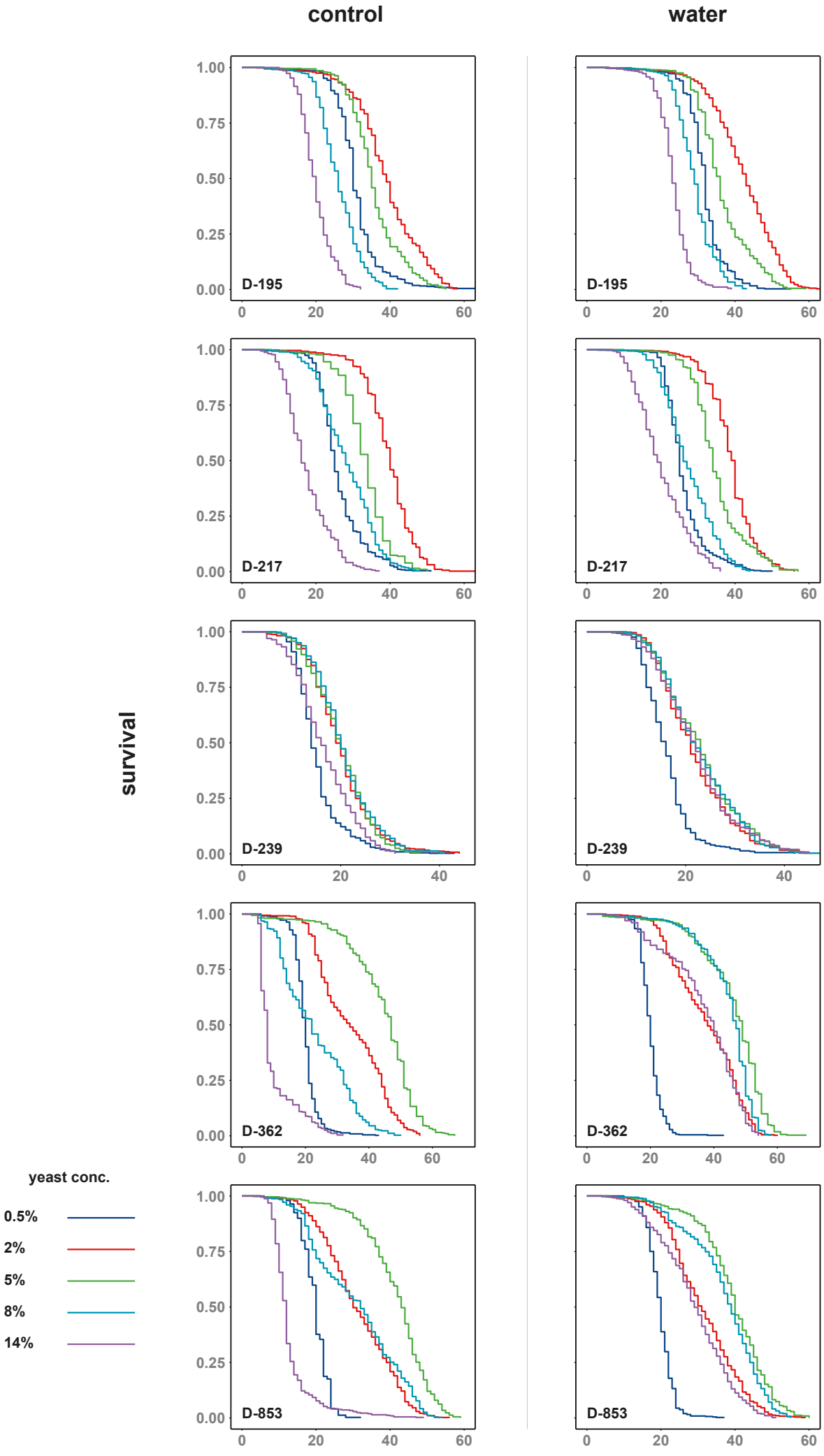
control
water
survival
age (days)
D-195
D-217
D-239
D-362
D-853
yeast conc.
0.5%
2%
5%
8%
14%

Fig. S4. Survival curves of panel in response to diet. Dietary reaction norms vary in a genotype-specific manner. Survival curves are separated by genotype, and water-supplementation status. N = 25,519 females total; 4,800-5,282 per genotype.

Tables

Table S1. Effect of diet and water supplementation on mortality across 5 DGRP lines (DGRP-195 is reference).

	Full Model			
coefficient	estimate	exp	s.e.	p
water	-0.390	0.677	0.228	0.086
0.5% yeast	-0.796	0.451	0.228	<0.001
2% yeast	-1.856	0.156	0.206	<0.001
5% yeast	-1.296	0.274	0.225	<0.001
14% yeast	1.043	2.837	0.226	<0.001
217	-0.444	0.641	0.226	0.05
239	0.763	2.145	0.227	0.001
362	0.104	1.110	0.227	0.645
853	-1.090	0.336	0.223	<0.001
0.5% yeast * water	0.470	1.601	0.322	0.144
2% yeast * water	0.030	1.031	0.300	0.92
5% yeast * water	0.277	1.319	0.322	0.39
14% yeast * water	-0.156	0.856	0.322	0.629
217 * 0.5% yeast	1.195	3.302	0.320	<0.001
217 * 2% yeast	0.632	1.881	0.310	0.042
217 * 5% yeast	0.786	2.195	0.321	0.014
217 * 14% yeast	0.643	1.903	0.322	0.046
239 * 0.5% yeast	1.793	6.008	0.321	<0.001
239 * 2% yeast	1.887	6.599	0.308	<0.001
239 * 5% yeast	1.429	4.174	0.321	<0.001
239 * 14% yeast	-0.382	0.683	0.324	0.238
362 * 0.5% yeast	1.754	5.775	0.319	<0.001
362 * 2% yeast	0.262	1.300	0.311	0.399
362 * 5% yeast	-1.322	0.267	0.315	<0.001
362 * 14% yeast	1.880	6.551	0.316	<0.001
853 * 0.5% yeast	3.087	21.901	0.318	<0.001
853 * 2% yeast	2.029	7.604	0.307	<0.001
853 * 5% yeast	0.287	1.332	0.320	0.37
853 * 14% yeast	2.100	8.168	0.320	<0.001
217 * water	0.551	1.736	0.322	0.087
239 * water	-0.101	0.904	0.322	0.754
362 * water	-2.015	0.133	0.319	<0.001
853 * water	-0.101	0.904	0.323	0.753
217 * 0.5% yeast * water	-0.568	0.567	0.455	0.212
217 * 2% yeast * water	-0.114	0.893	0.441	0.797
217 * 5% yeast * water	-0.702	0.496	0.456	0.123
217 * 14% yeast * water	-0.448	0.639	0.456	0.327
239 * 0.5% yeast * water	-0.145	0.865	0.457	0.751
239 * 2% yeast * water	0.126	1.134	0.440	0.775
239 * 5% yeast * water	-0.427	0.652	0.455	0.348
239 * 14% yeast * water	-0.383	0.682	0.457	0.402
362 * 0.5% yeast * water	1.990	7.319	0.456	<0.001
362 * 2% yeast * water	2.068	7.912	0.439	<0.001
362 * 5% yeast * water	2.007	7.444	0.457	<0.001
362 * 14% yeast * water	-1.942	0.143	0.451	<0.001
853 * 0.5% yeast * water	-0.027	0.974	0.458	0.954
853 * 2% yeast * water	0.351	1.420	0.441	0.427
853 * 5% yeast * water	0.433	1.541	0.457	0.344
853 * 14% yeast * water	-2.056	0.128	0.455	<0.001

Table S2. Effect of diet and water supplementation on mortality within DGRP-195.

	Estimates from individual model				Effect of water, compared to no water		
coefficient	estimate	exp	s.e.	p	estimate	exp	p
water supplementation	-0.481	0.618	0.113	<0.001			
0.5% yeast	-0.954	0.385	0.112	<0.001			
2% yeast	-2.139	0.118	0.103	<0.001			
5% yeast	-1.620	0.198	0.112	<0.001			
14% yeast	1.612	5.012	0.112	<0.001			
0.5% yeast * water	0.475	1.608	0.158	0.003	-0.006	0.994	0.971
2% yeast * water	0.156	1.169	0.147	0.289	-0.324	0.723	0.028
5% yeast * water	0.369	1.446	0.160	0.021	-0.112	0.894	0.483
14% yeast * water	-0.301	0.740	0.159	0.059	-0.781	0.458	<0.001

Table S3. Effect of diet and water supplementation on mortality within DGRP-217.

	Estimates from individual model				Effect of water, compared to no water		
coefficient	estimate	exp	s.e.	p	estimate	exp	p
water supplementation	0.190	1.209	0.213	0.373			
0.5% yeast	0.454	1.574	0.211	0.032			
2% yeast	-1.395	0.248	0.208	<0.001			
5% yeast	-0.549	0.578	0.212	0.01			
14% yeast	1.905	6.716	0.200	<0.001			
0.5% yeast * water	-0.108	0.898	0.306	0.724	0.082	1.08	0.789
2% yeast * water	-0.105	0.901	0.306	0.732	0.085	1.08	0.781
5% yeast * water	-0.470	0.625	0.300	0.117	-0.280	0.75	0.350
14% yeast * water	-0.668	0.513	0.306	0.029	-0.479	0.62	0.118

Table S4. Effect of diet and water supplementation on mortality within DGRP-239.

	Estimates from individual model				Effect of water, compared to no water		
coefficient	estimate	exp	s.e.	p	estimate	exp	p
water supplementation	-0.343	0.710	0.105	0.001			
0.5% yeast	0.946	2.575	0.104	<0.001			
2% yeast	0.070	1.072	0.104	0.502			
5% yeast	0.094	1.098	0.103	0.363			
14% yeast	0.572	1.771	0.103	<0.001			
0.5% yeast * water	0.173	1.188	0.148	0.243	-0.170	0.843	0.249
2% yeast * water	0.079	1.082	0.148	0.593	-0.264	0.768	0.075
5% yeast * water	-0.108	0.898	0.147	0.464	-0.451	0.637	0.002
14% yeast * water	-0.476	0.621	0.147	0.001	-0.819	0.441	<0.001

Table S5. Effect of diet and water supplementation on mortality within DGRP-362.

	Estimates from individual model				Effect of water, compared to no water		
coefficient	estimate	exp	s.e.	p	estimate	exp	p
water supplementation	-2.330	0.097	0.363	<0.001			
0.5% yeast	0.763	2.144	0.356	0.032			
2% yeast	-1.520	0.219	0.383	<0.001			
5% yeast	-2.638	0.072	0.351	<0.001			
14% yeast	2.393	10.950	0.339	<0.001			
0.5% yeast * water	2.358	10.571	0.545	<0.001	0.028	1.029	0.958
2% yeast * water	2.016	7.509	0.482	<0.001	-0.314	0.731	0.515
5% yeast * water	2.213	9.145	0.597	<0.001	-0.116	0.890	0.845
14% yeast * water	-1.564	0.209	0.549	0.004	-3.894	0.020	<0.001

Table S6. Effect of diet and water supplementation on mortality within DGRP-853.

	Estimates from individual model				Effect of water, compared to no water		
coefficient	estimate	exp	s.e.	p	estimate	exp	p
water supplementation	-0.473	0.623	0.259	0.067			
0.5% yeast	1.987	7.293	0.239	<0.001			
2% yeast	0.150	1.162	0.254	0.554			
5% yeast	-0.967	0.380	0.248	<0.001			
14% yeast	2.955	19.204	0.223	<0.001			
0.5% yeast * water	0.436	1.547	0.399	0.274	-0.037	0.964	0.926
2% yeast * water	0.379	1.461	0.365	0.299	-0.094	0.910	0.797
5% yeast * water	0.680	1.973	0.373	0.068	0.206	1.229	0.58
14% yeast * water	-2.083	0.125	0.339	<0.001	-2.556	0.078	<0.001

Table S7. Effect of diet on mortality across 5 water-supplemented DGRP lines (DGRP-195 is reference).

	Full Model			
coefficient	estimate	exp	s.e.	p
0.5% yeast	-0.345	0.709	0.228	0.131
2% yeast	-1.921	0.146	0.202	<0.001
5% yeast	-1.060	0.347	0.224	<0.001
14% yeast	0.930	2.535	0.225	<0.001
217	0.107	1.113	0.228	0.638
239	0.683	1.980	0.228	0.003
362	-1.991	0.137	0.218	<0.001
853	-1.242	0.289	0.221	<0.001
217 * 0.5% yeast	0.658	1.931	0.320	0.04
217 * 2% yeast	0.567	1.764	0.311	0.068
217 * 5% yeast	0.096	1.101	0.321	0.764
217 * 14% yeast	0.206	1.229	0.324	0.524
239 * 0.5% yeast	1.771	5.877	0.318	<0.001
239 * 2% yeast	2.119	8.326	0.305	<0.001
239 * 5% yeast	1.042	2.834	0.321	0.001
239 * 14% yeast	-0.799	0.450	0.322	0.013
362 * 0.5% yeast	3.937	51.246	0.315	<0.001
362 * 2% yeast	2.434	11.404	0.307	<0.001
362 * 5% yeast	0.691	1.995	0.318	0.03
362 * 14% yeast	-0.071	0.932	0.323	0.826
853 * 0.5% yeast	3.228	25.234	0.312	<0.001
853 * 2% yeast	2.482	11.960	0.303	<0.001
853 * 5% yeast	0.739	2.094	0.322	0.022
853 * 14% yeast	0.033	1.033	0.319	0.918

Table S8. Effect of diet on mortality within water-supplemented DGRP-195.

	Estimates from individual model			
coefficient	estimate	exp	s.e.	p
0.5% yeast	-0.512	0.599	0.098	<0.001
2% yeast	-2.124	0.120	0.100	<0.001
5% yeast	-1.322	0.267	0.102	<0.001
14% yeast	1.410	4.095	0.100	<0.001

Table S9. Effect of diet on mortality within water-supplemented DGRP-217.

	Estimates from individual model			
coefficient	estimate	exp	s.e.	p
0.5% yeast	0.357	1.429	0.214	0.095
2% yeast	-1.487	0.226	0.215	<0.001
5% yeast	-1.011	0.364	0.216	<0.001
14% yeast	1.260	3.527	0.215	<0.001

Table S10. Effect of diet on mortality within water-supplemented DGRP-239.

	Estimates from individual model			
coefficient	estimate	exp	s.e.	p
0.5% yeast	1.114	3.046	0.119	<0.001
2% yeast	0.150	1.162	0.118	0.204
5% yeast	-0.012	0.988	0.117	0.919
14% yeast	0.096	1.101	0.118	0.413

Table S11. Effect of diet on mortality within water-supplemented DGRP-362.

	Estimates from individual model			
coefficient	estimate	exp	s.e.	p
0.5% yeast	3.631	37.764	0.386	<0.001
2% yeast	0.518	1.678	0.379	0.172
5% yeast	-0.487	0.614	0.380	0.2
14% yeast	0.920	2.509	0.379	0.015

Table S12. Effect of diet on mortality within water-supplemented DGRP-853.

	Estimates from individual model			
coefficient	estimate	exp	s.e.	p
0.5% yeast	2.811	16.623	0.222	<0.001
2% yeast	0.564	1.759	0.216	0.009
5% yeast	-0.284	0.753	0.216	0.19
14% yeast	0.923	2.518	0.216	<0.001

Table S13. Effect of diet and water supplementation on fecundity across 5 DGRP lines, derived from linear model estimates oflog-transformed raw fecundity counts (DGRP-195 is reference). Counts generated using QuantiFly software. Counts are relative, but directly comparable.

	Full Model			Compared to 8%	
coefficient	estimate	s.e.	p	estimate	p
intercept	2.731	0.067	<0.001		
water	0.039	0.027	0.15		
0.5% yeast	-1.374	0.092	<0.001		
2% yeast	-0.595	0.092	<0.001		
5% yeast	-0.367	0.092	<0.001		
14% yeast	0.157	0.092	0.09	2.888	<0.001
217	-0.044	0.092	0.634		
239	-0.331	0.095	0.001		
362	-0.294	0.092	0.002		
853	-0.221	0.095	0.021		
217 * 0.5% yeast	0.113	0.130	0.387		
217 * 2% yeast	-0.268	0.134	0.048		
217 * 5% yeast	-0.164	0.132	0.216		
217 * 14% yeast	-0.224	0.132	0.092	-0.110	0.404
239 * 0.5% yeast	0.509	0.139	<0.001		
239 * 2% yeast	0.310	0.139	0.027		
239 * 5% yeast	-0.119	0.134	0.373		
239 * 14% yeast	-0.262	0.136	0.056	-0.436	0.001
362 * 0.5% yeast	0.524	0.130	<0.001		
362 * 2% yeast	0.212	0.130	0.106		
362 * 5% yeast	0.180	0.130	0.168		
362 * 14% yeast	-0.202	0.130	0.122	-0.339	0.009
853 * 0.5% yeast	0.249	0.134	0.065		
853 * 2% yeast	-0.233	0.132	0.079		
853 * 5% yeast	-0.137	0.132	0.3		
853 * 14% yeast	-0.657	0.134	<0.001	-0.721	<0.001

Table S14. Effect of diet and water supplementation on fecundity across 5 DGRP lines, derived from linear model estimates oflog-transformed mortality-adjusted fecundity counts (DGRP-195 is reference). Counts generated using QuantiFly software. Counts are relative, but directly comparable.

	Full Model			Compared to 8%	
coefficient	estimate	s.e.	p	estimate	p
intercept	0.776	0.067	<0.001		
water	-0.004	0.027	0.873		
0.5% yeast	-1.379	0.093	<0.001		
2% yeast	-0.602	0.093	<0.001		
5% yeast	-0.384	0.093	<0.001		
14% yeast	0.148	0.093	0.114	0.925	<0.001
217	-0.050	0.093	0.594		
239	-0.350	0.096	<0.001		
362	-0.239	0.093	0.011		
853	-0.241	0.096	0.013		
217 * 0.5% yeast	0.101	0.132	0.444		
217 * 2% yeast	-0.301	0.136	0.028		
217 * 5% yeast	-0.113	0.134	0.398		
217 * 14% yeast	-0.165	0.134	0.218	-0.067	0.617
239 * 0.5% yeast	0.542	0.141	<0.001		
239 * 2% yeast	0.311	0.141	0.028		
239 * 5% yeast	-0.120	0.136	0.378		
239 * 14% yeast	-0.237	0.138	0.087	-0.439	0.001
362 * 0.5% yeast	0.485	0.132	<0.001		
362 * 2% yeast	0.177	0.132	0.182		
362 * 5% yeast	0.186	0.132	0.16		
362 * 14% yeast	0.166	0.132	0.209	0.075	0.568
853 * 0.5% yeast	0.284	0.136	0.038		
853 * 2% yeast	-0.227	0.134	0.091		
853 * 5% yeast	-0.111	0.134	0.406		
853 * 14% yeast	-0.550	0.136	<0.001	-0.643	<0.001

Chapter Two

The hidden costs of dietary restriction: Implications for its evolutionary and mechanistic origins

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Abstract

Dietary restriction (DR) extends life span across taxa. Despite considerable research, universal mechanisms of DR have not been identified, limiting its translational potential. Guided by the conviction that DR evolved as an adaptive, pro-longevity physiological response to food scarcity, biomedical science has interpreted DR as an activator of pro-longevity molecular pathways. Current evolutionary theory predicts that organisms invest in their soma during DR, and thus when resource availability improves, should outcompete rich-fed controls in survival and/or reproduction. Testing this prediction in *Drosophila melanogaster* (N > 66,000 across 11 genotypes), our experiments revealed substantial, unexpected mortality costs when flies returned to a rich diet following DR. The physiological effects of DR should therefore not be interpreted as intrinsically pro-longevity, acting via somatic maintenance. We suggest DR could alternatively be considered an escape from costs incurred under nutrient-rich conditions, in addition to costs associated with DR.

Introduction

Ageing has attracted extensive scientific interest, both from a fundamental and biomedical perspective. Dietary restriction (DR) extends health- and lifespan across taxa, from baker's yeast to mice, with very few exceptions (Nakagawa *et al.*, 2012; Fontana and Partridge, 2015). The reduction of total calories - or restriction of macronutrients, such as protein - extends lifespan reliably (Lee *et al.*, 2008; Solon-Biet *et al.*, 2014; Jensen *et al.*, 2015). Although the precise universal mechanisms that connect DR to ageing remain elusive, translation of DR's health benefits to human medicine is deemed possible. The widespread assumption of DR's translational potential originates from the notion that DR's beneficial effects are facilitated by shared evolutionary conserved mechanisms, as beneficial effects of DR are observed across taxa. Experiments on our close evolutionary relatives, rhesus monkeys (*Macaca mulatta*) have demonstrated that DR could be translational (Mattison *et al.*, 2017). Still, the mechanisms by which these benefits are accrued physiologically may differ between species, as no single genetic or pharmaceutical manipulation mimicking the benefits of DR across model organisms exists (Selman, 2014). In addition, genetic heterogeneity within species presents an additional layer of complexity, since efficacy of DR-driven longevity extension can differ between genotypes (Dick, Ross and Yampolsky, 2011; Tatar, 2011). Mechanistic insight will be key, since DR as a human lifestyle intervention has limited scope, given the degree of self-restraint required. It is therefore warranted to direct scrutiny towards the evolutionary theory of DR, since it underpins the assumed universality of physiological mechanisms by which DR confers health benefits.

Shared universal mechanisms can only be inferred from the ubiquity of the DR longevity response in the animal kingdom, when the selection pressures responsible for such evolutionary conservation are understood. The DR response itself may have evolved once, and mechanisms might be conserved. Alternatively, DR could have undergone convergent evolution, either using similar mechanisms - or by adopting alternative ones (Mair and Dillin, 2008). These evolutionary scenarios provide distinct predictions as to how informative mechanistic research in other animals will prove for human medicine. Only if the DR response is rooted in ancient physiology (i.e. evolved once or through convergent evolution) can possible translation of mechanistic research on model organisms be confidently inferred. The DR effect itself is interpreted as an evolved, adaptive, pro-longevity physiological response to limiting food availability (Holliday, 1989). Life-history theory (Stearns, 1989) - a central tenet of evolutionary biology - states resources are limited, and thus predicts trade-offs between reproduction and survival, even in nutrient-rich environments. As such, DR presents an enigma: why do organisms live longer on a constrained energy budget?

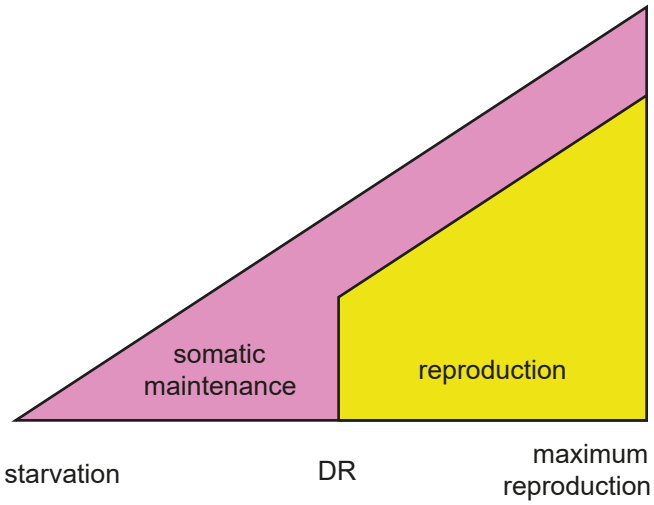

Figure 1. Schematic of the evolutionary model of DR. Resource availability is varied from left to right, from very low (where starvation would occur) to very high (where maximum reproduction would occur). The theoretical optimal allocation to somatic maintenance (**pink**) versus reproduction (**yellow**) is depicted at a given resource availability. When resource availability decreases, investment in both somatic maintenance and reproduction is reduced, until a threshold is met. Below this point resources are so scarce that investment in reproduction does not yield a fitness return. This could occur when offspring produced cannot recruit into the population due to the harsh resource environment, or because the capital (start-up) costs of breeding cannot be met. Here, investment in reproduction is lost and is wholly allocated to somatic maintenance. It is this evolved resource allocation decision to invest into somatic maintenance under DR conditions, that is thought to underlie lifespan extension under DR.

The currently accepted evolutionary model for DR (Shanley and Kirkwood, 2000; Kirkwood and Shanley, 2005) uses a life-history perspective on ageing to explain this enigma. The model proposes that below a certain resource threshold, organisms will reallocate energy almost exclusively towards somatic maintenance (Fig. 1). In certain ecological situations (e.g. severely reduced juvenile survival, or when the energy budget is lower than the initial costs, or the cost of one unit of reproduction) investment into reproduction will cease to yield fitness. The optimal, fitness-maximising strategy under these harsh conditions would be to terminate investment into reproduction and utilise this energy to gain fitness when conditions improve. Crucially, this life-history strategy would favour an increase in resources devoted to maintenance and repair during DR - allowing organisms to survive bouts of famine with an intact or superior soma (Shanley and Kirkwood, 2000; Kirkwood and Shanley, 2005). This 'somatic maintenance response' has been presumed to be the primary causative agent in the pro-longevity DR response (Kirkwood and Austad, 2000; Speakman and Mitchell, 2011; Fontana and Partridge, 2015). There are few alternatives to the somatic maintenance response model that can explain the evolutionary biology of DR (but see Blagosklonny, 2006; Adler and Bonduriansky, 2014; Regan *et al.*, 2019) and its elemental phenotypic predictions have undergone minimal empirical examination (but see Zajitschek *et al.*, 2018).

This attractive evolutionary rationale has given credibility to the assumption that physiological changes in the DR animal are inherently pro-longevity, since it implies DR increases investment into somatic maintenance. For example, transcriptomic upregulation of what could be interpreted as maintenance and repair processes under DR, have lent credence to this hypothesis (Lee *et al.*, 1999; Kirkwood and Austad, 2000; Pletcher, Libert and Skorupa, 2005). Directionality of these associations is often ambiguous, however, as, for example, downregulation of DNA repair under DR could be interpreted as either a reduction in DNA damage generation, or reduced investment into repair (Lee *et al.*, 1999; Pletcher, Libert and Skorupa, 2005). In other words, a potentially simpler rationale is often neglected: the surge of 'maintenance and repair' gene expression as a mere stress response to metabolic disruption. The health benefits observed under DR might originate from a passive response - one not necessarily evolved as an adaptive regulatory response that increases somatic maintenance in response to DR. Under these circumstances, lifespan extension could be a simple correlated response to currently unknown, but strongly conserved, physiology. For example, the limitation of metabolic rate or reduction in specific metabolites as a direct consequence of DR could reduce conserved associated physiological dysfunction, and thereby extend lifespan. The negative physiological effects dietary restricted organisms suffer, e.g. compromised immune function (Kristan, 2008) and cold intolerance (Adler and Bonduriansky, 2014), could arise from a similar passive response, and are not necessarily the result of a regulated trade-off. DR is sometimes considered a hormetic response - mild stress, resulting in the stimulation of conserved cellular reactions

leading to beneficial health (Rattan, 2008) – which would be a similar example of a passive response. One example of such a hormetic response is the activation of heat shock proteins, which show only very transient expression, but long-lasting effects on life expectancy (Tatar, Khazaeli and Curtsinger, 1997).

The distinction between passive-correlated versus adaptive-programmed pro-longevity responses will be key to identifying the mechanisms of DR and developing translation to humans. The current, widely-accepted evolutionary model of DR (Shanley and Kirkwood, 2000; Kirkwood and Shanley, 2005) supports an adaptive phenotypic response and provides a key prediction: organisms should increase investment into their soma during periods of DR, and therefore, when their resource availability improves, should outcompete age-matched rich-fed controls in survival and/or reproduction. Here we provide an experimental phenotypic test of this prediction, utilising a large-scale demographic approach detailing mortality and fecundity in *Drosophila melanogaster* fed different dietary regimes. Our results revealed substantial mortality and fecundity costs when returning to a rich diet after a period of DR, falsifying the key prediction provided by the evolutionary biology of DR. These effects were independent of genotype, duration of DR, number of dietary fluctuations, and we excluded large confounding effects arising from access to water (Fanson, Yap and Taylor, 2012), the social environment (Chakraborty *et al.*, 2019), the microbiome (Wong, Dobson and Douglas, 2014) and sex (Regan *et al.*, 2016). Our results therefore suggest that the effects of DR are not necessarily intrinsically pro-longevity, i.e. by increasing investment into somatic maintenance, and could alternatively be considered an escape from costs incurred under nutrient-rich conditions and/or costs associated with DR. These insights question the relevance of the somatic maintenance explanation of DR in guiding biomedical research into its mechanisms. Our alternative paradigm - a passive, not necessarily directly adaptive response to DR - gives renewed credibility to a range of mechanistic hypotheses of DR: hormesis (Sinclair, 2005), a reduction in metabolism causing reduced oxidative damage generation (Mair and Dillin, 2008; Redman *et al.*, 2018) and improved mitochondrial functioning (Weir *et al.*, 2017), or a reduction of waste products from specific metabolic pathways (Hipkiss, 2006).

Materials and methods

Fly husbandry

Wild-type inbred isofemale flies from the *Drosophila melanogaster* Genetic Reference Panel (Mackay *et al.*, 2012) were acquired from the Bloomington Stock Centre and the lab of Bart Deplancke (EPFL). Flies were cultured on rich media (8% autolysed yeast, 13% table sugar, 6% cornmeal, 1% agar and nipagin 0.225% [w/v]) with bottles for growing and mating, containing an additional 0.4% [v/v] propanoic acid. For lifespan experiments, adult

flies were subsequently provided with either the same rich media, or a restricted media (2% autolysed yeast) in vials. These dietary concentrations are neither particularly rich, nor restricted, in comparison to published work (Mair *et al.*, 2003; Grandison, Piper and Partridge, 2009). Diets remain difficult to compare between studies as ingredients and fly media preparation differ between labs. Our rich and restricted diets induce consistent lifespan differentials. Moreover, recent work carried out using a wider range of diets suggests our diets are in the area of largest response for most genotypes. Restricted media retained the composition of all other media components, given the dietary protein axis is the main lifespan determinant in flies (Lee *et al.*, 2008; Jensen *et al.*, 2015). Cooked fly media was kept for a maximum of 2 weeks at 4-6 °C and was warmed to 25°C before use.

Experimental mortality protocol and demography cages

Flies were expanded in bottles (Drosophila PP Flask Square Bottom; Flystuff) on a rich diet. Experimental flies were grown in bottles (incubated at 25°C) sprinkled with granulated live yeast, in which 12 females and 2 males had been egg-laying for a period of ~60 hours. Bottles were sprinkled with water, daily, if media appeared dry, until pupation began. Upon eclosion, the adult F1 generation was transferred, daily to generate age-matched cohorts, to mating bottles for 48 hours before being sorted under light CO_2 anaesthesia (Flystuff Flowbuddy; < 5 litres / min) and transferred to purpose-built demography cages (Good and Tatar, 2001). Lifespan experiments were carried out in a climate-controlled room (12:12 LD, 25°C and 50-60% relative humidity). Cages contained between 100-125 females each; the number of cages was treatment-dependent. All flies were kept on rich media until age 3-6 days whereupon they were divided between the dietary treatments. Individual lifespan was determined from the time when the individual entered the experimental cage (at 2 days of age) until death or censoring. A census of flies was taken every other day: dead flies were counted and removed, and fresh media was provided at this time. Flies that were alive, but stuck to the side of the vial, escaped flies and individuals affixed to the food (~10.5% of deaths) were right-censored.

Fecundity

A subsection of fly feeding vials were imaged and analysed using QuantiFly (Waithe *et al.*, 2015) to determine relative amounts of egg laying.

Dietary regimes

Two main temporal dietary regimes were imposed on several genotypes of mainly female flies using two diets, restricted (DR, 2% yeast) and rich (8% yeast) with controls of continuous exposure to these diets.

1) To test whether a prolonged period of DR resulted in superior survival and reproduction when conditions improved, flies were exposed to a continuously restricted diet that was

switched to a rich diet at ~45-60% survival of the continuous rich diet group ('long-switch'). All flies of the same genotype were switched on the same day, irrespective of eclosion date.

2) We further tested whether short bouts of DR had similar effects, which also allowed us to test whether effects observed in the long-switch regime were exclusive to older flies. In these diets, flies were repeatedly switched between restricted and rich diets at 4-day intervals ('4-day switch'). By starting half of the experimental cohort on restricted or rich diets, current dietary treatments were mirrored and balanced across the cohort.

These experiments were performed on DGRP-195 at high sample size (N = 14,102). Subsequently, to test whether these effects were general, these experiments were expanded to a panel of DGRP lines (DGRP-105; 136; 195; 217; 239; 335; 362; 441; 705; 707; 853) in one large experiment of N = 37,897. Several other parts of the experiments (see below) were run separately (for specific grouping see Supplement). Dietary treatments were balanced for age. From this experiment, fecundity estimates were also taken from feeding vials on four consecutive scoring days (for 4-day switch, and continuous treatments) and one scoring day before, and after, the dietary switch (for long-switch, and continuous rich treatment).

Supplementary dietary regimes

We tested a range of other dietary regimes to test specific hypotheses, alongside the treatments listed above, using line DGRP-195. 1) We tested whether DR could instantly reduce mortality by imposing a short duration (4 days) of DR, in late-life, *sensu* Mair *et al.*, 2003, before returning to a rich diet ('short reverse-switch'). 2) We increased the frequency of the dietary switch to two days ('2-day switch') to investigate the length of DR necessary for the observed phenotypes, and 3) further changed the ratio of the time spent on either diet; two days of either rich or restricted diet, to four days of the reverse ('4-to-2-day switch').

Tests of specific hypotheses: microbiome, water balance, sex and social effects

We tested whether the dietary phenotypes observed were due to four potential previously suggested confounding factors: 1) the microbiome (Wong, Dobson and Douglas, 2014), 2) water balance (Fanson, Yap and Taylor, 2012), 3) social effects (Chakraborty *et al.*, 2019), 4) sex-differences in the DR response (Magwere, Chapman and Partridge, 2004; Regan *et al.*, 2016). These were confirmed not to interfere with the observed phenotype (see Results). DGRP-195 were used exclusively for these experiments, under the continuous restricted, continuous rich, and long-switch diets. As our original dietary switch genotype, we reasoned that exclusion of these potentially confounding variables in this one genetic line would preclude them from being principal causative agents. Note, however, that this does mean we cannot strictly exclude that in other genotypes these confounding effects

are more important. 1) We assessed whether disruption of the gut microbiome was responsible for the mortality phenotype observed by wholesale abating the microbiome. Flies were provided media upon which an array of broad-spectrum antibiotics (50 µl of a stock solution, comprised of 100 µg/ml ampicillin, 50 µg/ml vancomycin, 100 µg/ml neomycin, and 100 µg/ml metronidazole) were pipetted and left for 24hrs. We assumed dissolution incorporation in the top 1 ml of food. Antibiotic treatment began four days prior to dietary switch treatments, and concluded 8 days thereafter. Ablation of the microbiome was confirmed by whole-fly homogenisation (age 20 days; 8 days post-antibiotic treatment) and growth of solution on MRS agar plates (Oxoid; see Fig. S7). Individuals (6 control and 6 antibiotic treated) were removed from cages containing a continuous restricted diet, washed in ethanol, and rinsed in PBS (Gibco). Homogenisation took place in 500 µl of PBS, and solute was transferred to a 96-well plate for 1:10 serial dilutions. Dilutions were spotted on plates with, and without antibiotic (500 µl of stock solution) and incubated at 25°C for 72 hours. Plates were coated with parafilm to mimic anoxic conditions. 2) Flies were provided with ~1cm^3 portion of water-agar (2% [w/v]) accompanying media in vials, to eliminate desiccation as a proximal cause. Water-agar supplementation began at age 4 days and continued throughout the flies' full life course. 3) Social effects were excluded by housing flies individually in vials. These flies were taken from experimental cages and put on the experimental diets at the dietary switch 4) Males were assessed for mortality in the 4-day switch dietary regime.

Experimental batches

All demography experiments contained the relevant controls, grown and assayed for mortality at the same time. Where data are plotted in a single figure, this constitutes results gathered from a batch of flies at the same chronological time.

Data Analysis

Mixed Cox-proportional hazard models were used that included 'cage' as a random term to correct for uncertainty of pseudo-replicated effects within demography cages (Therneau, Grambsch and Pankratz, 2003). We used interval-based models that used time-dependent covariates to estimate the differential mortality risks associated with diet (and with time spent on a diet, after diets changed), as imposed in the different dietary regimes. These models allow a statistical association, within the Cox-proportional hazard risk, with the current state (e.g. diet) and mortality. Flies in the long-switch dietary regime were also analysed in a state-dependent manner, coding for long-switch only when this state change occurred. Repeated switching regimes were considered lifelong treatments and tested in interaction with the state variable diet. Each model used continuous rich food and DGRP-195 as the reference category, except if otherwise stated.

Interactions between dietary regime, diet and genotype were fitted to test for differential effects of diet on mortality depending on the regime it was provided. Additional specific tests of coefficients are provided that combine the single and interaction term (in a Z-test, using the maximum SE of the factor compared) to test how mortality risk was changing compared to specific reference categories of interest (e.g. compared to continuous DR). For comparisons between genotypes we report full models including all data and models fitted within each genotype separately. The latter corrects for deviations in proportionality of hazards between the genotypes. Qualitative conclusions remain similar, and formal tests for proportionality of hazards are not available for mixed effects Cox regressions. Models without a time-dependent covariate for diet were also run to compare overall longevity differences as a result of alternating exposure to DR (2-day switch, 4-day switch and their combination). These models therefore test the integrated effect on mortality disregarding any within dietary treatment diet effects. Coefficients are reported as logged hazards with significance based on Z-tests. Right-censoring was included, as indicated above.

Egg laying was analysed as a mixed generalized Poisson model using cage as random term, and fitting age as a non-continuous factor in the analysis. Estimates from models are presented (effects of dietary regime) as well as model comparisons using log-likelihood comparison with chi-square to test overall effects of genotype. Effects of different dietary regimes were estimated within the same model. Comparisons of genotypic effects were performed for each different dietary regime separately compared to continuous treatment, as not to conflate genetic variance across different categories with each other.

Results

Hidden costs of Dietary Restriction

The use of large populations of animals, possible in the fruit fly and other small organisms, allows the measurement of age-dependent mortality risk - the risk to die at a given age. Such a demographic approach can be useful to infer underlying biology (Simons, Koch and Verhulst, 2013; Dammann *et al.*, 2019) and can be used experimentally to investigate instantaneous effects of treatments on mortality (Good and Tatar, 2001; Mair *et al.*, 2003). We used an experimental demographic approach comprising 11,084 individual deaths (Table S1) to test the phenotypic predictions from the evolutionary theory of DR: increased investment in somatic maintenance under DR allows the animal to better perform when nutrient availability improves.

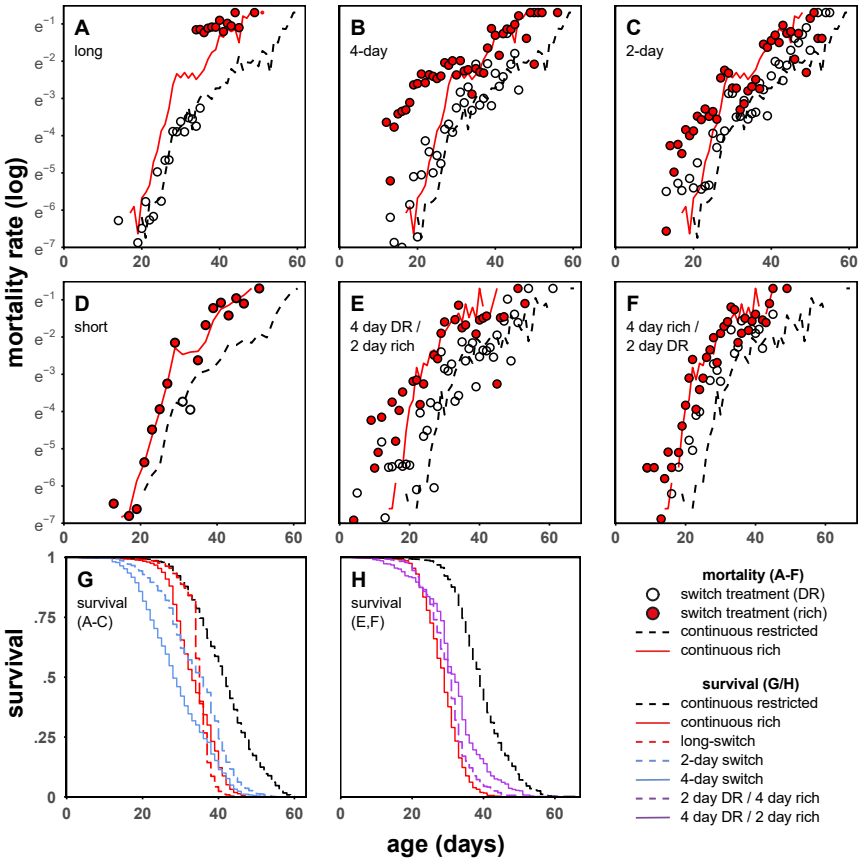

Figure 2. The effect of different dietary regimes on age-specific mortality risk in DGRP-195. Age-specific mortality risk (**A-F**) allows an investigation of instantaneous changes in mortality risk upon dietary switches (points) across the different dietary regimes used. Mortality risk at continuous rich (solid red) and restricted diets (dashed black) are plotted as lines. The exacerbation of mortality due to switch phenotypes is the difference between mortality at continuous rich diet (red line) and mortality of switch treatment when on a rich diet (red points). The open dots in the switch treatment, the DR condition, should overlay the continuous DR treatment (dash) if the dietary switch does not modulate the effect of the DR diet (or act as a pure control prior to a single switch, as in **A**. N = 19,086 females total; 995-3,769 per treatment. **A** – long-switch. When returning to a rich diet after a long period of DR, mortality is exacerbated compared to flies fed a rich diet continuously. **B** – 4-day switch. Switching from a DR to a rich diet repeatedly every four days, increases mortality on rich diets compared to continuously rich fed flies. Flies are still able to modulate their mortality in response to DR, even when diet fluctuates rapidly. **C** – 2-day switch. Mortality on rich diets is only mildly increased and flies still respond to DR even when it is only imposed for two days. **D** – short reverse-switch. After a long period on a rich diet, DR for 4 days returns flies to mortality of continuous DR. The x-axis of panel **D** is age-adjusted to correct for age differences (1-3 days) at the time of the diet switch for illustration purposes only. **E** – 4-day DR, 2-day rich switch (4-to-2 day switch). Flies respond to DR, but encounter a slightly blunted effect compared to continuous DR. **F** – 4-day rich, 2-day DR switch (4-to-2 day switch). The effect of DR is reduced when imposed for 2 days following 4 days on a rich diet. **G** – survival plot of panels **A-C** with associated continuous diet controls. Total survival of both the 4-day switching dietary regime and the long-switch is lowered compared to continuously rich diets, despite flies spending a considerable extent of their lives on

restricted diets. Flies on DR outlive all other categories. **H** – survival plot of panels **E** and **F** with associated continuous diet controls. Despite spending up to two-thirds of their lives on DR in these asymmetrical regimes, survival benefits are modest, compared to continuous DR. Dietary switch treatments contain daily time-points (dots) for the dietary switch treatments, as treatments were mirrored and balanced, with half of flies starting on DR, and half on rich diets.

DR imposed continuously throughout adult life resulted in a significant reduction in mortality rate (Fig. 2; Table S1 ,2; $P < 0.001$, 3 times lower hazard). In addition, switching flies to DR at older ages instantly reduced mortality levels to the levels of flies that had experienced continuous DR ('short reverse-switch'; Fig. 2D; Table S1). Such mortality amnesia - a complete absence of historic diet effects - has been reported previously in flies (Good and Tatar, 2001; Mair *et al.*, 2003).

Our expectation, based on the current evolutionary model of DR, was that if flies were returned to rich food conditions after a period of DR, they would have a superior soma compared to flies that experienced rich food continuously. Resources allocated to somatic maintenance should result in higher fitness (Fig. 1). In contrast, our 'long-switch' treatment resulted in a substantial increase in mortality risk compared to flies kept on a rich diet throughout life (Fig. 2A; Table S1; $P < 0.001$, 3.7 times higher hazard). Mortality peaked immediately (within 48h; 5.1 times higher hazard) after the switch from a restricted to a rich diet. The magnitude of this mortality difference decreased slowly thereafter, resulting in no difference between the continuous rich diet and the long-switch treatments after eight days (Fig. 2A; Table S3; $P < 0.001$).

Repeated diet switching

The long-switch dietary treatment could be dependent on several specific aspects of the imposed dietary regime, and this would not necessarily falsify the somatic maintenance hypothesis of DR. First, the effects of the long-switch treatment could be contingent upon the prior duration of DR. Indeed, it has been suggested that DR evolved in response to relatively short, intermittent bouts of famine (Shanley and Kirkwood, 2000; Kirkwood and Shanley, 2005). Second, it has been suggested that the longevity response to DR originated from selection pressures on relatively young individuals (Shanley and Kirkwood, 2000). Thus, younger flies might not show the heightened mortality we observed. Third, it could be that sudden changes in diet *per se* are harmful. To test these three potential confounds we used short recurring bouts of DR, alternating between a rich and a DR diet every four days ('4-day switch'). In this dietary regime, mortality on the rich diet compared to the continuous rich diet was similarly exacerbated (Fig. 2B; Table S1, 2; $P < 0.001$, 2.4 times higher hazard). This 4-day switch dietary regime also allowed us to examine whether flies were able to instantly and repeatedly modulate their mortality risk in response to diet,

similar to the short reverse-switch treatment (Fig. 2D). Flies indeed modulated their mortality in response to the diet they were currently fed, with a degree of surprising immediacy. Mortality risk on DR, within the 4-day switch regime, repeatedly decreased to levels similar to that of flies continuously exposed to a restricted diet (Fig. 2B; Table S1). Nonetheless, mortality risk during these periods of DR imposition was significantly higher than that of continuous DR-treated flies (Table S1; $P < 0.001$, 1.6 times higher hazard). We suggest this increase in mortality seen on DR in the 4-day switch treatment is due to either accrued physiological costs or more probable, a carry-over of deaths directly resulting from the rich diet, but recorded on the DR diet.

Mortality costs depend on the duration of Dietary Restriction

A closer examination of the timing of mortality within the 4-day switching paradigm showed that the mortality response was strongest in the second 48 hours after exposure to both DR and rich diets (Table S4; $P < 0.001$). This suggests a period of acclimation to both DR and rich diets is necessary before their physiological effects are fully realised. To test the importance of the duration of exposure to DR and rich diets for the mortality phenotypes observed, further dietary regimes were used. First, switching from DR to rich conditions was carried out at increased frequency - alternating every 2 days ('2-day switch'; Table S1, 2). This 2-day switch dietary regime confirmed that sustained exposure to the diets (longer than 2 days) was required to cause the full magnitude of the mortality phenotypes observed. On a rich diet, the 2-day switch regime showed slightly higher mortality compared to the continuous rich diet (Fig. 2C; hazard = 1.1, $P < 0.05$) and mortality on DR in the 2-day switch regime did not reduce to the levels seen in continuously dietary restricted flies (Fig. 2C; hazard = 1.3, $P < 0.001$). Together these diet-specific mortality effects resulted in an overall lifespan extension in the 2-day switch regime (Fig. 2G; Table S2; $P < 0.001$). As flies spend an equal amount of time on DR or rich diets in the 2-day switch regime, the reduction of mortality under DR can be considered to be relatively more rapid than the induction of exacerbated mortality on rich food (after a period of DR). We reasoned that the exacerbation of mortality on rich food requires an extended period on either restricted or rich food. To test this directly, asymmetrical dietary regimes were used.

In this additional set of experiments, we combined the 4-day and 2-day switching regimes: treatments were comprised of 4 days on either a DR or rich diet, followed by 2 days on the other ('4-to-2 day switch'). Similar to the 4-day switch, this dietary regime was repeated sequentially. These '4-to-2' regimes showed no marked increase in mortality on the rich diet compared to flies on a continuous rich diet (Fig. 2E, F; Table S5). Relative to a continuous DR treatment, the effect of DR within this paradigm was markedly reduced, especially when flies were restricted for 2 days only (Fig. 2F; Table S5). This reduction in

the mortality response to DR in the '4-to-2' regimes amounted to a marked reduction in the total longevity extension achieved when compared to continuous DR. When flies spend two-thirds of their lives on DR, lifespan was only extended by half (compared to continuous DR), and only a quarter when flies spend one-third of their lives on DR (Fig. 2E, F; Table S6). These experiments again suggest a period exceeding 2 days on either diet is required to induce marked mortality effects.

Note that within the long-switch treatment, the mortality exacerbation observable on rich food was strongest within the first 2-day interval (Fig. 2A; Table S3). Additionally, our short reverse-switch induced a full DR response - mortality amnesia - within 2 days (Fig. 2D; Table S1). Moreover, the ameliorated mortality exacerbation of our additional switch experiments (2-day, and 4-to-2 day switches) strongly suggest that the sudden dietary perturbations themselves are not the cause of premature mortality in our switching regimes. From these combined results we therefore conclude that the additional mortality costs observable on a rich diet are contingent upon the prior duration of DR. The increase in mortality when resource availability is reinstated, we report here, is in direct contrast to DR having evolved as a life-history strategy to invest into somatic maintenance to prepare for times when resources are plentiful again.

Genetic variance

The above set of diet experiments were conducted using the wild-type inbred lineage DGRP-195. To eliminate the possibility that the dietary responses described above were the result of rare genetic effects inherent to this specific genetic line, we performed the same dietary perturbations in a panel of randomly selected inbred genotypes (DGRP-105; 136; 195; 217; 239; 335; 362; 441; 705; 707; 853). Across our panel, we detected an increase in longevity under DR conditions (Fig. 3, 4; additive model, DR hazard = -0.21 ± 0.08, $P < 0.001$). There were considerable genetic effects in response to diet however (interaction model: $\chi^2 = 204.8$ (df=10), $P < 0.001$), with some genotypes showing elevated mortality under restricted-diet conditions, compared to continuously-fed rich diet flies (Fig. 3, 4). This degree of variation in response to DR can be explained by genetic variation in the reaction norm to diet and not necessarily as an absence of the longevity response to DR. Animals react to an increasing degree of food restriction of food by first reducing reproduction, then by increasing lifespan - the DR longevity response. Further food restriction, beyond the nutritional optimum for longevity, decreases lifespan through starvation. A particular combination of one restricted and one rich diet will therefore not always induce the same longevity response in a range of genotypes, when these genotypes differ in their reaction norm to diet (Dick, Ross and Yampolsky, 2011; Tatar, 2011).

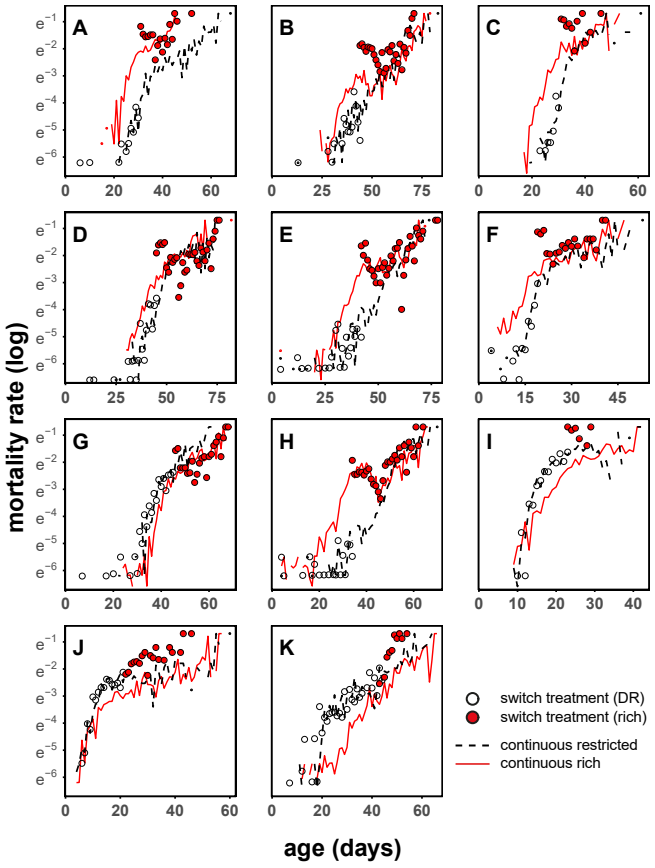

Figure 3. Long-switch treatment in a panel of 11 DGRP genotypes. A – 195; **B** – 105; **C** – 217; **D** – 441; **E** – 705; **F** – 707; **G** – 136; **H** – 362; **I** – 239; **J** – 335; **K** – 853. N = 29,702 females total; ~2,725 females per genotype; 13,375 for continuous rich treatments, and ~8,170 each for the two other treatments. The dietary switch for the long-switch treatment group occurred at 45- 65% of continuous rich treatment flies. All panels contain daily time-points as in Fig. 2. Exposure to a high nutrient diet after a period of DR resulted in marked increase in mortality compared to a continuous rich diet in all lines (9 out of 11 significant). There was genetic variation in this response with DGRP-136 (**G**) and DGRP-362 (**H**) showing the smallest effects. This marked overshoot was not contingent upon DR extending lifespan. Lines that showed 'starvation' on a DR diet still showed significant overshoots when they were switched to a rich diet, where recovery from starvation was expected, even when compared to continuous DR diets (**I**, **J**, **K**)

Across genotypes, exposure to the rich diet after a period of DR (long-switch) resulted in exacerbated mortality, exceeding that of flies fed a rich diet for their whole lives (Fig. 3; additive model, hazard = 0.997 ± 0.056, $P < 0.001$). There was significant genetic variance for this trait ($\chi 2 = 124$ (df=10), $P < 0.001$). Still, all genotypes showed a mortality overshoot, compared to a continuous rich diet, following a switch from DR to high nutrient conditions (Fig. 3; Table S7, 8; 9 of 11 significant; range: 1.12 to 5.21 times hazard). Genetic variation assessed in a larger amount of lines could be used to uncover the associated

mechanisms, but our objective here was to exclude the possibility of rare genetic effects inherent to a single line being responsible for the phenotypes we observed. Alternating the diet from DR to rich every 4 days decreased longevity compared to the continuous rich diet, across the genetic panel (additive non-interval based model, hazard = 0.24 ± 0.047, P < 0.001). Again, we found significant genetic variance for the response to this dietary regime (χ 2 = 117 (df=10), P < 0.001). Lines differed in their responses: 5 of the 11 showed marked decreases in survival; 1 showed an increase in survival, and the remaining 5 showed statistically non-significant effects (Table S8). Interval-based models showed that mortality rates increased at the rich diets following a period of DR, as in the long-switch, in all lines (significant in 7 of 11; Table S10,11). There was a modest positive genetic correlation in the increase in mortality induced by the long-switch and 4-day-switch dietary regimes (correlation of coefficients from Table S9, 11; r_s = 0.45, P = 0.17), suggesting these dietary phenotypes originate from similar physiology.

Hidden costs: independent of a pro-longevity DR response

Our restricted diet unexpectedly induced a putative starvation response - observable as an increased mortality rate - in four lines (DGRP-136, 239, 335, 853; Fig. 3, 4; Table S7, 9, 10, 11). These contrasting responses to DR serendipitously allowed us to see whether the dietary switching phenotypes were contingent on the direction of the DR response. Surprisingly, when lines that showed starvation were refed on a rich diet (long-switch), mortality did not decrease, but increased (Table S7, 8; 3 out of 4 showed a significant increase), even beyond the heightened mortality seen on DR (Fig. 3; Table S12). Similarly, within the 4-day switching regime, mortality risk was exacerbated at a rich diet. The pattern of mortality even reversed, compared to individuals fed diets continuously, with lines now showing a putative DR-longevity response within the 4-day switch dietary regime (Fig. S1; Table S10, 11, 13). These outcomes were particularly remarkable since exposure to a richer diet was expected to rescue the starvation response. In contrast to any recovery upon a return to a rich diet, individual mortality risk surged beyond that of flies fed rich diets continuously. These observations further fit with our interpretation that the dietary responses we report here are general in flies, and are not contingent on the phenotypic pro-longevity response to DR. In addition, the reversal of the mortality patterns in the 4-day switch regime suggests differences in the reaction norm to nutrient restriction, as discussed above, could be largely responsible for the genetic variance in the DR longevity response we observe. We find that short bouts of refeeding on this dietary regime present genetic lines normally experiencing starvation on DR, the opportunity to overcome malnourishment and extend lifespan. This indicates that these specific genetic lines are not refractory to the lifespan extension effects of DR, but are merely more susceptible to low-nutrient conditions. Given this, we predict that the starvation that these

lines exhibit under DR would, under slightly higher-nutrient conditions, result in a pro-longevity DR response.

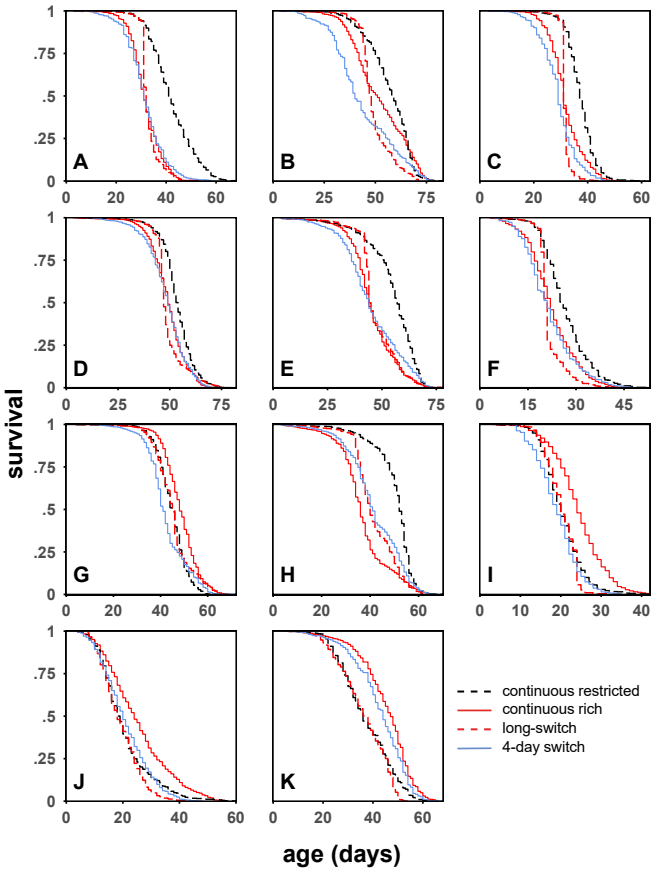

Figure 4. Survival curves of DGRP panel for both dietary regimes. A – 195; **B** – 105; **C** – 217; **D** – 441; **E** – 705; **F** – 707; **G** – 136; **H** – 362; **I** – 239; **J** – 335; **K** – 853. Total survival on the different dietary regimes across the genetic panel tested. Rich diets after a period of DR resulted in such an increase in mortality, that total survival of the cohort was lower (or equal to) those fed a continuous rich diet for their whole life (**A-F**). N = 37,897 females total; ~3,450 females per genotype; 13,375 for continuous rich treatments, and ~8,170 for all other treatments.

Cost of mortality not compensated for by fecundity increase

We recognised our results would not necessarily discredit the evolutionary model of DR should the observed costs in mortality be compensated fully, or partially, by an increase in fecundity. Egg production across the DGRP panel experiment was measured from vials in each dietary regime and expressed both as a total count (age-specific fitness of the population; Fig. S2, 3; Table S14, 16) or eggs per fly (age-specific reproductive output, corrected for mortality differences; Fig. S2, 3; Table S15, 17). All lines responded strongly to DR by reducing reproductive output. Within the 4-day switching paradigm, DR also

induced a rapid reduction in fecundity (Fig. S3; Table S16, 17). As with the mortality response, genetic lines also differed in fecundity response to the dietary treatments (long-switch: F=57 (df=2), P < 0.001 ; 4-day switch: χ 2 = 187 (df=9), P < 0.001). However, in both metrics, our switching diets underperformed in reproductive output compared to the continuous rich diet (Fig. S2, 3; Table S14-17), confirming our mortality phenotypes were not compensated by higher fecundity upon a return to nutrient rich conditions.

Mortality phenotypes were not contingent on condition of the microbiome, social housing, water or sex.

A switch to rich diets after a sustained period of DR (long-switch) still resulted in an increase of mortality when flies were treated with antibiotics (Table S18; P < 0.001), provided additional water (Table S19; P = 0.002), or when mortality was assessed in isolation (Table S20; P = 0.014). Males responded, similarly to females, by increasing mortality on rich diets if this was preceded by 4 days of DR (4-day switch, Table S21; P = 0.001, long-switch not tested).

Discussion

DR has been tested across multiple species and the resulting lifespan extension has consistently - with very few exceptions (Adler and Bonduriansky, 2014) - been interpreted as provoking anti-ageing, pro-longevity physiology. This interpretation is based on the widely-accepted evolutionary theory of DR (Shanley and Kirkwood, 2000; Kirkwood and Shanley, 2005) which predicts that during periods of DR, investment in somatic maintenance is actively increased, to await better times when fitness can be gained. In contrast, we find that periods of DR did not result in a superior soma, and instead resulted in large increases in mortality, and reductions in fecundity, when nutrient availability returned to plentiful. Our results question the current explanation of DR's evolutionary origins, and thereby its relevance in interpreting DR's mechanistic origins.

Other studies have raised similar concerns but have only very rarely measured the consequences of the relevant life-history event: a period of DR followed by a period of rich food conditions. Direct measurement of investment into the soma using stable isotopes showed no increased investment under DR (O'Brien *et al.*, 2008). Experimental evolution across fifty generations under DR, failed to support the current evolutionary theory of DR (Zajitschek *et al.*, 2018). Further lack of support, we suggest, originates from the remarkably immediate reduction in mortality – a reduction in frailty, rather than actuarial ageing rate (Good and Tatar, 2001; Mair *et al.*, 2003; Simons, Koch and Verhulst, 2013) or historic physiological effects of diet – seen when flies are dietary restricted. A limited number of previous studies with *Drosophila* have shown such a response (Good and Tatar,

2001; Mair *et al.*, 2003). We confirmed these results (Fig. 2D), but also show for the first time that flies are capable of reducing mortality repeatedly, in response to multiple switches in diet (Fig. S1). Since DR does not slow ageing demographically, but results in an instant lowering of mortality - without any accrued beneficial effects - this is in itself evidence against increased somatic investment under DR (Simons, Koch and Verhulst, 2013).

In the reverse scenario, when flies resumed rich diets after DR, their performance was markedly lower than that of flies that were fed rich diets for their entire lives. Notably, this effect held even when DR caused starvation - resulting in exacerbated mortality on the diet that should have provided an opportunity to refeed. Previous studies did not detect the same mortality costs in dietary regimes analogous to our long-switch (Mair *et al.*, 2003), although in the raw non-smoothed data, some exacerbation of mortality can be seen in some conditions. There are a number of potential variables which could explain these differences. First, the duration of DR prior to a rich diet appears to be integral to inducing exacerbated mortality on rich diets (Fig. 2). Second, the existence and intensity of both the long-switch and 4-day switch phenotype, are genotype-dependent (Fig. 3, S1). This matter is further complicated by the lack of complete synchronicity between both phenotypes, across genotypes (Fig. 3, S1). Last, the longevity response to both a restricted diet, and the re-introduction of a rich one, may be contingent on the macronutrient composition of both (Lee *et al.*, 2008; Jensen *et al.*, 2015). Earlier work diluted media reducing both carbohydrates and protein (Mair *et al.*, 2003), in contrast to our method of reducing yeast concentration alone.

Genotypes will differ in their longevity reaction norm to diet, rendering it impossible to know *a priori* whether a certain dietary composition constitutes the exact optimal longevity-directed diet (Tatar, 2011; Flatt, 2014). Genetic variation in the response to DR, reported in rodents (Mitchell *et al.*, 2016) and flies (Wilson *et al.*, 2018), might therefore not necessarily, or wholly, constitute variation in the physiological mechanisms that connect DR to ageing. We propose that our dietary phenotypes may also be contingent upon the direction and degree in which these diets deviate from the optimum, which may be one explanation for the dissimilarity of results observed in similar experiments. These considerations may also explain why the precise duration of DR is important, in line with the recent finding that the duration of starvation is critical in the lifespan extension generated via intermittent fasting (Catterson *et al.*, 2018). In addition, larval diet, timing and the order of how diets were fluctuated contributed to differential mortality observed when fluctuating diet (van den Heuvel *et al.*, 2014). 'Choice' experiments – where poor and rich diets are fed to flies in conjunction – result in heightened mortality, compared to continuous feeding (Ro *et al.*, 2016). These effects are dependent on serotonin signalling, suggesting

that the *perceived*, rather than *actual* composition of food ingested modulates ageing (Libert *et al.*, 2007).

In light of this, it is important to consider the renewed interest in intermittent fasting in both rodent and human studies (Fontana and Partridge, 2015; Mattson, Longo and Harvie, 2017). Studies in the previous century on rodents, already demonstrated that inducing intermittent fasting, by feeding animals every other day or by other means, extends lifespan in a similar manner to caloric restriction (reviewed in Anson, Jones and de Cabo, 2005). Two recent studies in mice suggest the same, although the effects are not as large as full caloric restriction (Mitchell *et al.*, 2019) and outcomes for systemic ageing have been questioned (Xie *et al.*, 2017). Human data on intermittent fasting is promising (Mattson, Longo and Harvie, 2017) and has potential application in specific diseases (Cignarella *et al.*, 2018), but conclusive evidence from clinical trials is currently lacking (Horne, Muhlestein and Anderson, 2015). Our work now suggests that intermittent DR, dependent on its duration, can have negative consequences. These observations fit with the 'refeeding syndrome' - a clinical condition that occurs at refeeding after a period of starvation (Mehanna, Moledina and Travis, 2008). It remains to be determined which duration of starvation or DR would instigate such harmful physiological effects upon refeeding to the extent that it offsets its physiological benefits in humans. The responses we observe however are clearly not expected under the somatic maintenance hypothesis of DR, as flies appear to become maladapted to rich nutrient conditions under DR. In this vein, we appreciate that it has been suggested that naturalistic dietary conditions required to investigate DR are not appropriately mimicked in the lab, and that DR itself is a lab-based artefact (Harper, Leathers and Austad, 2006). Note that such a suggestion would preclude any inference from DR to our own species based on evolutionary arguments. That animals in the lab experience an unnaturally heightened nutritional state, not often available in the wild, is an idea not well supported. Careful studies have shown that wild and domestic mice have similar mass-adjusted metabolic rate, even though they differ genetically and experience vastly different environments (Austad and Kristan, 2003).

At present, no mechanistic explanation is apparent which explains the exacerbated mortality when flies return to a rich diet after a period of DR. We have excluded water balance (Fanson, Yap and Taylor, 2012), social effects (Chakraborty *et al.*, 2019), the microbiome (Wong, Dobson and Douglas, 2014) and sex-specific effects (Regan *et al.*, 2016) being wholly responsible for our observations. We therefore conclude that in conjunction with physiological costs associated with a rich diet, there are hidden costs associated with DR. These costs appear only when a rich diet is resumed after DR. The difference in mortality rates between our switching treatments (Fig. 2B, C, E, F) demonstrate a minimum period of acclimation to a restricted diet is necessary to generate the detectable costs of it. This suggests a physiological change at DR that makes animals

more sensitive to rich diets, in direct contrast to predictions from evolutionary theory. Drawing from our observation of exacerbated mortality upon resumption of a rich diet - even when DR caused starvation - we suggest this exacerbation results from physiological adaptations that compensate for the lack of certain components within a restricted diet. Moreover, we observe these phenotypes across a range of genotypes with varying nutritional requirements - inferred from the existence of starvation in some lines on our experimental DR diet. This suggests that these effects will hold over a wide range of diet concentrations. Future experiments that gradually change diets over time, or titrate the difference in the diet required to recapitulate the observed phenotypes, could test this directly. We suggest that the physiological compensation that occurs at DR sensitises animals to the physiological costs associated either with the elevated intake, or metabolism of such a specific dietary component, leading to the exacerbation in mortality we observed. These effects could also originate more directly from compensation to nutrient restriction leading to an upregulation of nutrient intake and metabolic recycling pathways, that upon resumption of the high nutrient diet could lead to a detrimental influx of specific harmful dietary components, or a higher flux through metabolic pathways (e.g. the generation of toxic by-products). Intriguingly these same, otherwise hidden, mechanisms might also underlie why animals fed rich diets continuously are shorter lived than those on DR: as an escape from costs associated with the intake or metabolism of a (or several) dietary component(s) (Fig. 5). This paradigm also explains why flies can rapidly and repeatedly lower their mortality in response to DR.

A recent re-appreciation of the evolutionary biology of DR (and molecular nutrient-sensing pathways) suggest that phenotypic plasticity is at the core of the evolutionary explanation of DR (Regan *et al.*, 2019). Indeed, we find that flies are highly plastic in modulating their reproduction to dietary conditions. Death through depletion of bodily resources to use in reproduction would not be optimal if the animal expects resources to increase at some point in their lives. Such phenotypic plasticity does not however directly explain however why animals on DR live longer than their fully-fed counterparts, except if phenotypic plasticity itself or the act of reproduction carries specific costs. We know from careful experiments in model organisms that the effects of DR are largely independent of reproduction (Mair *et al.*, 2004; Grandison, Piper and Partridge, 2009; Drewry, Williams and Hatle, 2011; Tatar, 2011). This therefore suggests that the reduction of reproduction with DR is a correlated phenotypic response that is not causative in the DR longevity response. Such observations fit with recent elegant experiments showing that artificial selection for reproduction during DR does not affect the DR longevity response (Zajitschek *et al.*, 2018).

All current evidence to date suggests that uptake of the macronutrient protein is responsible for the effects of diet on longevity (Lee *et al.*, 2008; Solon-Biet *et al.*, 2014; Jensen *et al.*, 2015). We suggest that DR's effect on longevity is not via increased

investment in somatic maintenance, but the result from a forced escape from the intrinsically harmful effects of dietary protein. The reason why animals would still choose to eat or absorb intrinsically harmful components, such as protein from their diets, is most likely for its use in reproduction in both sexes (Lee *et al.*, 2008; Speakman and Mitchell, 2011; Jensen *et al.*, 2015). The specific physiological mechanisms that underlie these costs, lie at the heart of DR's lifespan extending capacities. Our identification of novel dietary phenotypes in the fly that expose these otherwise hidden costs could prove a powerful new experimental phenotype for the mechanistic study of DR. We suggest that the quest to identify the mechanisms of DR will be aided by acceptance that somatic maintenance is not necessarily responsible for the life-extension seen under DR.

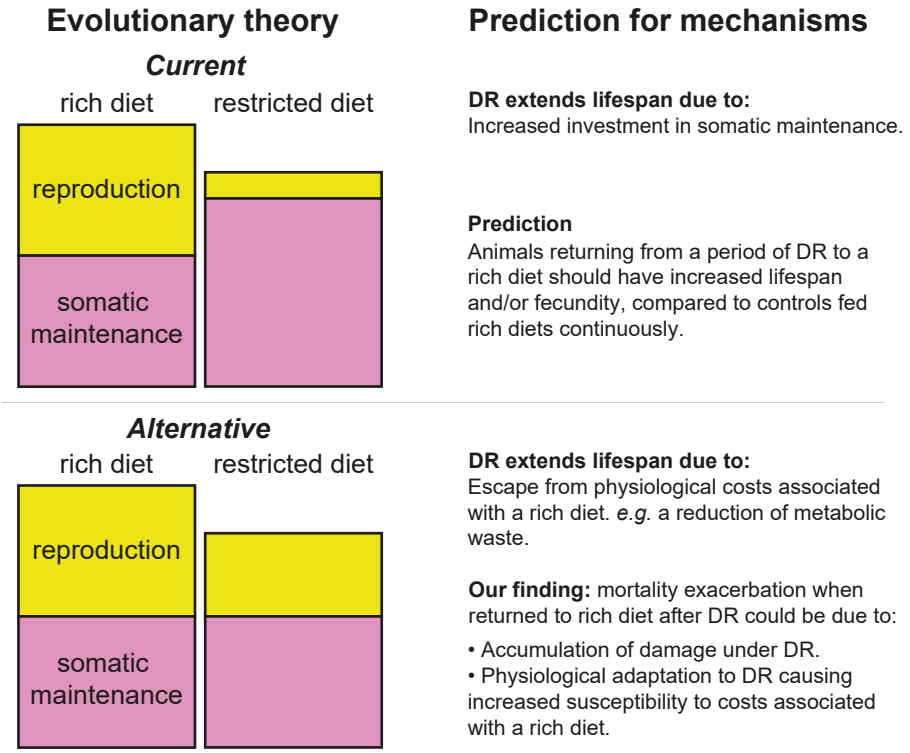

Fig 5. Schematic of the current, and alternative, hypotheses of DR. Reduced resource availability leading to increased investment towards somatic maintenance explains lifespan extension under DR (see Fig. 1), in the most commonly supported current evolutionary theory. This increased investment may be absolute, or relative to total resource availability. In our alternative model, based on the conclusions from the experiments we present here, the reduction of resource availability simply elicits a correlated reduction in available resources allocated towards reproductive output. The extension of lifespan observed under DR would then be a similarly passive response: an escape from unidentified costs incurred under a rich diet. These costs may be related to heightened metabolism, or arising from direct insults of excessive protein intake. In addition, we propose restricted diets promote the accumulation of unknown costs, which are only observable upon resumption of a rich diet (not

depicted here; see Discussion). These hidden costs of DR would be responsible for the exacerbation of mortality observed when a rich diet is resumed. We suggest these costs result from a period of physiological adaptation to a restricted diet, compensating for particular components of a rich diet. Such compensation on the DR diet, essentially maladapting the organisms to rich diet conditions, is directly contrary to current evolutionary theory that suggests investment in somatic maintenance occurs to survive to reap fitness benefits when resources are plentiful again.

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Author Contributions

A.W.M. and M.J.P.S. designed and interpreted the experiments with help from M.T. A.W.M. drafted the first version of the manuscript. A.W.M. and M.J.P.S. revised the manuscript with help from M.T., G.A., and L.H. A.W.M. led the data acquisition with help from M.J.P.S., G.A., and L.H. M.J.P.S. supervised the project.

Supplement

Figures

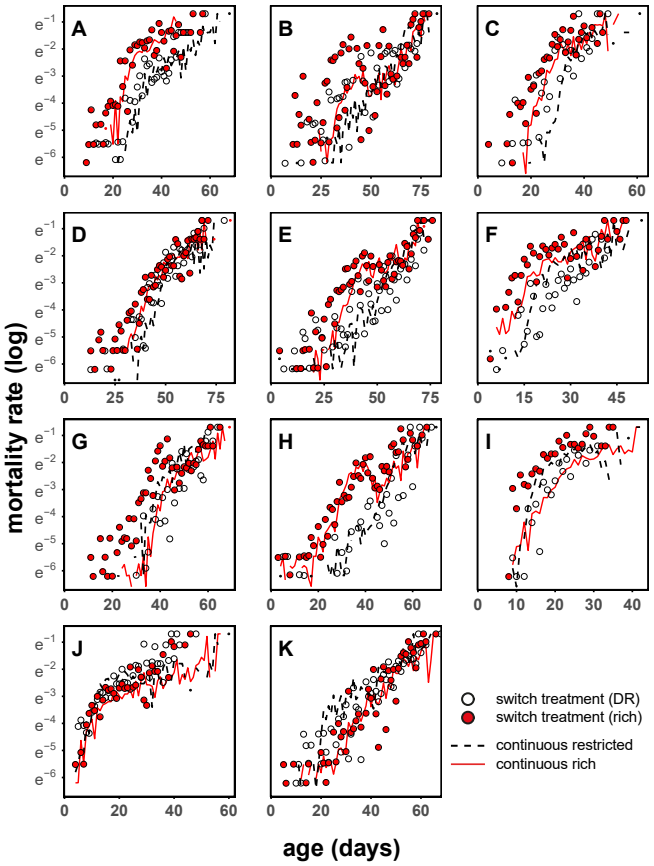


Figure S1. 4-day switch treatment in a panel of 11 DGRP genotypes. **A** – 195; **B** – 105; **C** – 217; **D** – 441; **E** – 705; **F** – 707; **G** – 136; **H** – 362; **I** – 239; **J** – 335; **K** – 853. Continuous rich, and restricted treatments plotted as lines (solid red and dashed black, respectively). Switch treatments plotted as points (white and red). The exacerbation of mortality due to switch phenotypes is observable as the difference between mortality at continuous rich diet (red line), and mortality of switch treatment when on a rich diet (red points). N = 29,740 total; ~2,725 per genotype; 13,375 for continuous rich treatments, and ~8,170 for continuous rich and 4-day switch treatments. Dietary switch for 4-day switch treatment group occurred every 4 days, and was mirrored at each time point. Continuous rich and restricted treatments are twinned with long switch treatment experiment (Fig. 2). All panels contain daily time-points, as in Fig.2.

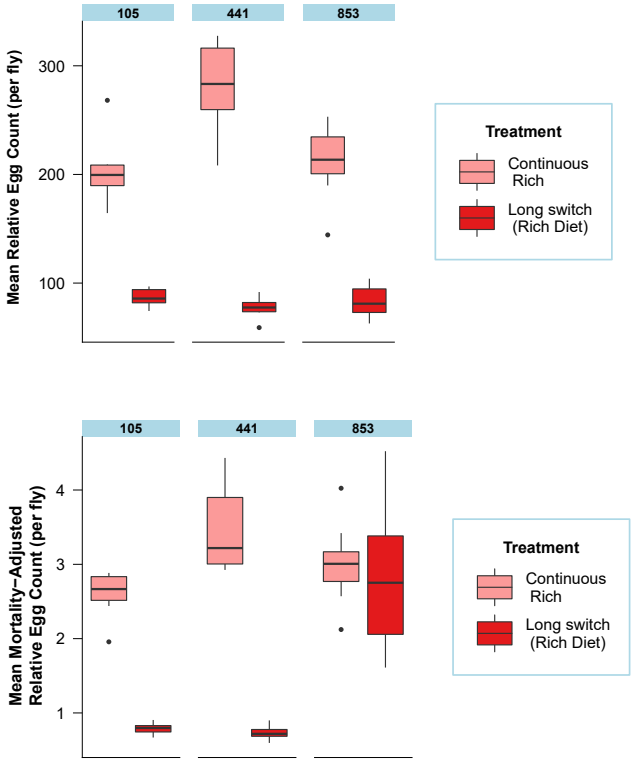
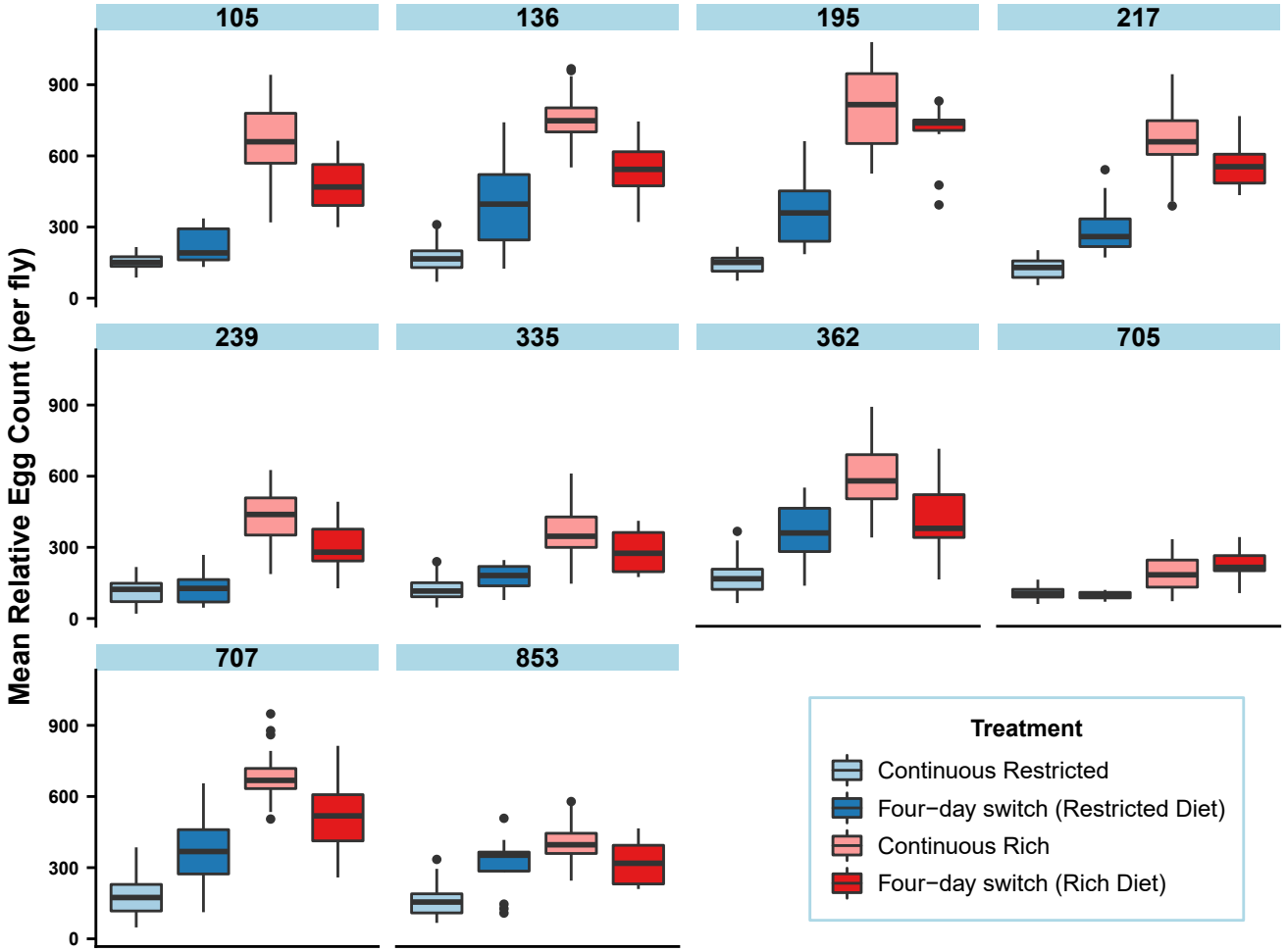


Figure S2. Fecundity analysis of long switch treatment from 3 DGRP genotypes. No compensation via fecundity for reduced lifespans in switch treatment. Raw (above) and mortality corrected (below) egg counts of DGRP-105; 441; 853 from long switch treatment experiment (Fig. 2). Counts generated using QuantiFly software. Counts are relative, but directly comparable. Flies assayed between age 44-47 days, with boxplots (median, with the box depicting a quartile each way, and whiskers showing the range; outliers plotted as dots) aggregating totals. Each cage was assayed once, on the first scoring day post dietary switch. Mortality corrected counts (below) generated by dividing raw counts, by N flies remaining in cage at the time of assaying. N = on average, 7 cages assayed, per treatment, per genotype.


105
136
195
217
239
335
362
705
707
853
Mean Relative Egg Count (per fly)
0
300
600
900
Treatment
Continuous Restricted
Four−day switch (Restricted Diet)
Continuous Rich
Four−day switch (Rich Diet)

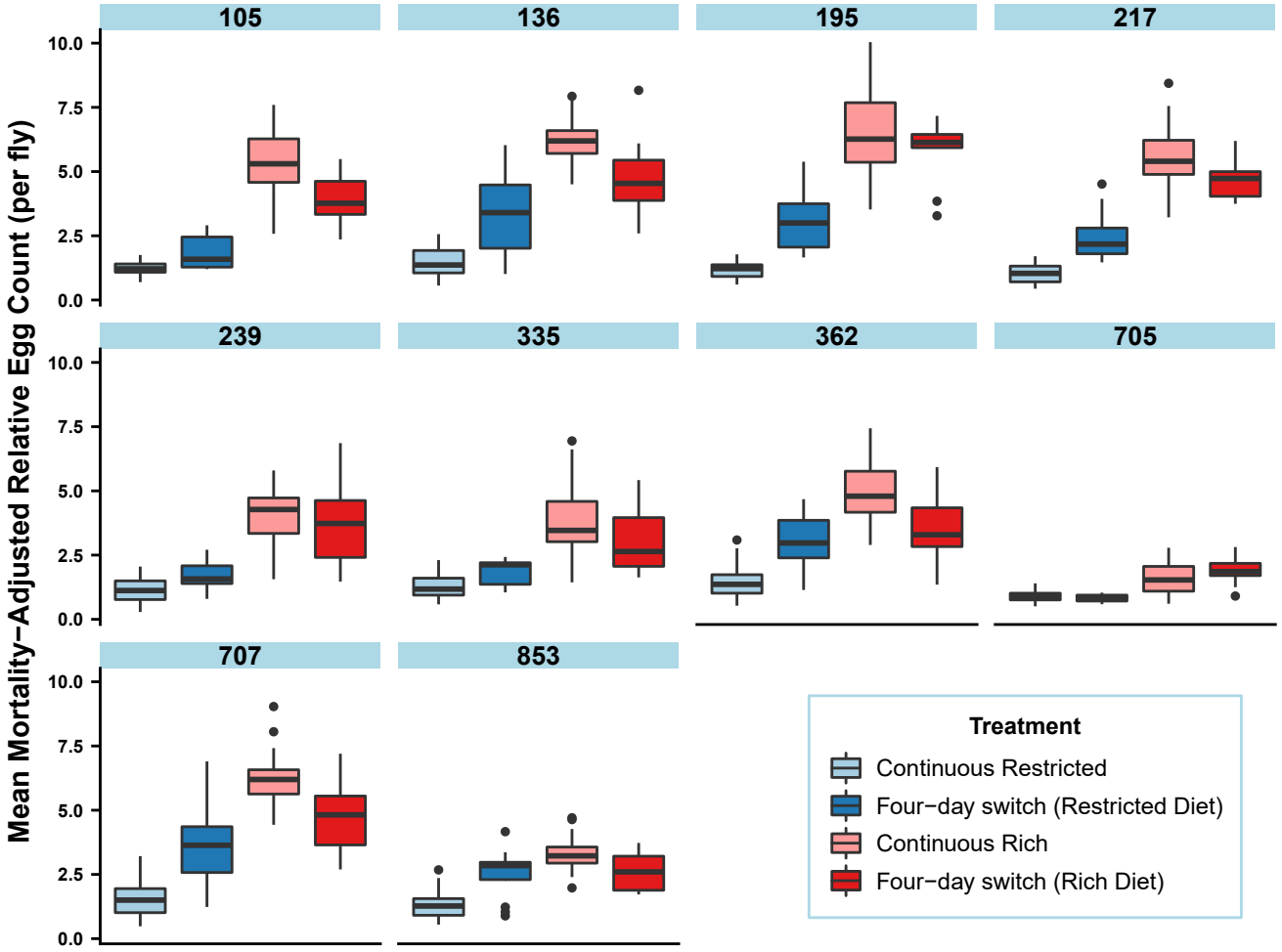

105
136
195
217
239
335
362
705
707
853
Mean Mortality−Adjusted Relative Egg Count (per fly)
0.0
2.5
5.0
7.5
10.0
Treatment
Continuous Restricted
Four−day switch (Restricted Diet)
Continuous Rich
Four−day switch (Rich Diet)

Figure S3. Fecundity analysis of 4-day switch treatment from 10 DGRP genotypes. No compensation via fecundity for reduced lifespans in switch treatment. Raw (**above**) and mortality corrected (**below**) egg counts of DGRP-105; 136; 195; 217; 239; 335; 362; 705; 707; 853 from long switch treatment experiment (Fig. 2). Counts generated using QuantiFly software. Counts are relative, but directly comparable. Flies assayed between age 8-21 days, with boxplots aggregating totals (median, with the box depicting a quartile each way, and whiskers showing the range; outliers plotted as dots). Each cage was assayed on 4 consecutive scoring days. Mortality corrected counts (below) generated by dividing raw counts, by N flies remaining in cage at the time of assaying. N = on average, 7 cages assayed, per treatment, per genotype.

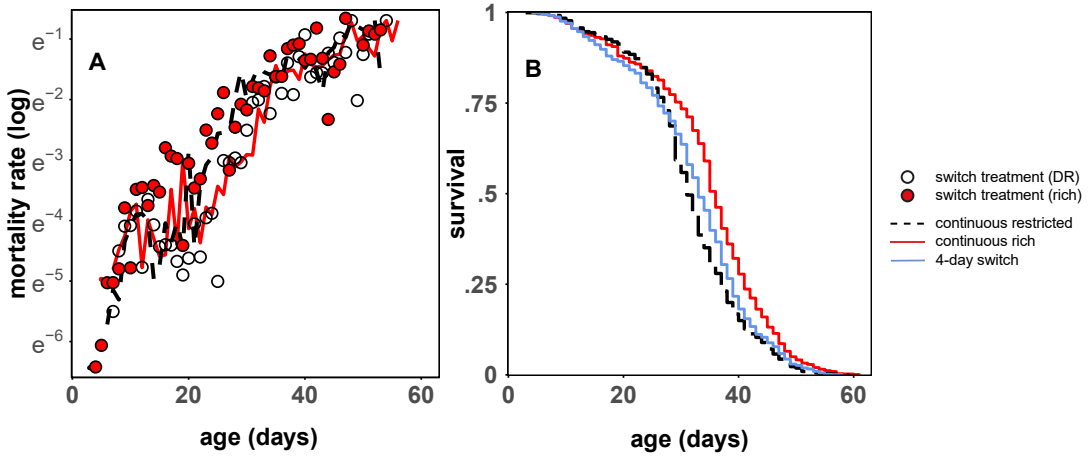


Figure S4. 4-day switch treatment of DGRP-195 males. A – 4-day switch mortality; **B** – 4-day switch survival. Muted response to 4-day switch treatment in males. Rich diet in the 4-day switch increased mortality compared to continuously rich fed flies. Continuous rich, and restricted treatments plotted as lines (solid red and dashed black, respectively). Switch treatment plotted as points (white and red). The exacerbation of mortality due to switch phenotypes is observable as the difference between mortality at continuous rich diet (red line), and mortality of switch treatment when on a rich diet (red points). N = 4,429 total; ~1,475 per treatment. Dietary switch for 4-day switch treatment group occurred every 4 days, and was mirrored at each time point. Both panels contain daily time-points, as in Fig.2.

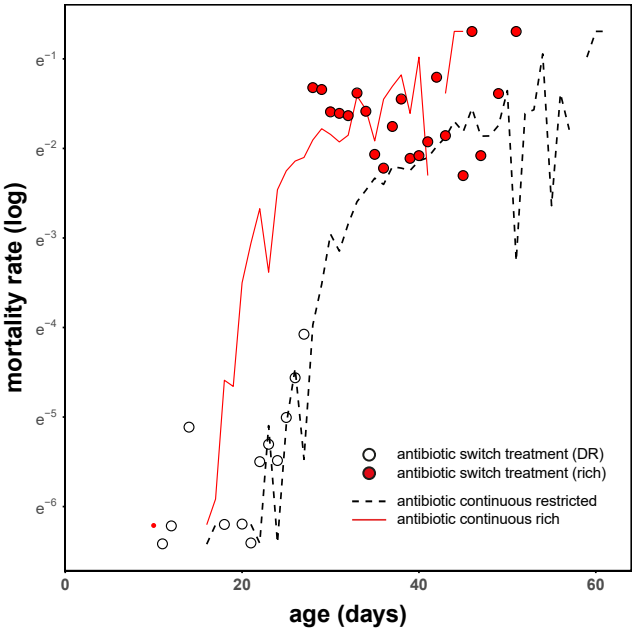


Figure S5. Antibiotic long switch treatment of DGRP-195. Long switch phenotype independent of antibiotic treatment. Antibiotic treatment took place in all treatments four days prior to dietary switch, and concluded eight days thereafter. Continuous rich, and restricted treatments plotted as lines (solid red and dashed black, respectively). Switch treatment plotted as points (white and red). The exacerbation of mortality due to switch phenotypes is observable as the difference between mortality at continuous rich diet (red line), and mortality of switch treatment when on a rich diet (red points). N = 2,605 total; ~870 per treatment. (See Fig. S7 for confirmation of ablation of microbiome). Figure contains daily time-points, as in Fig.2.

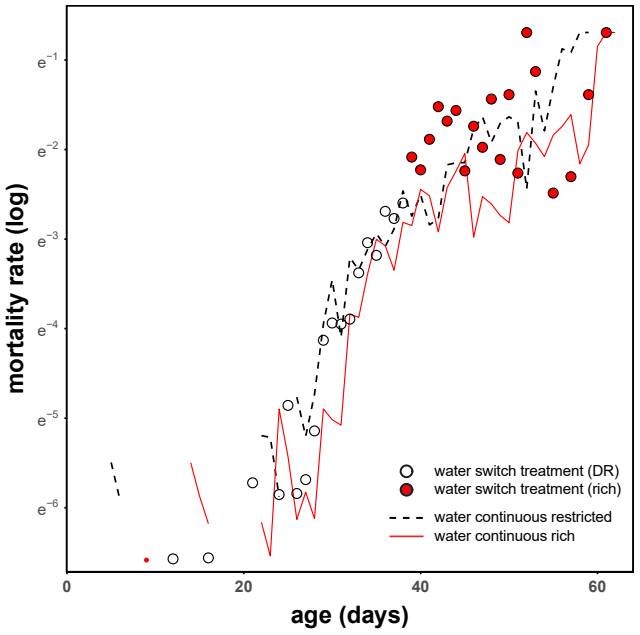
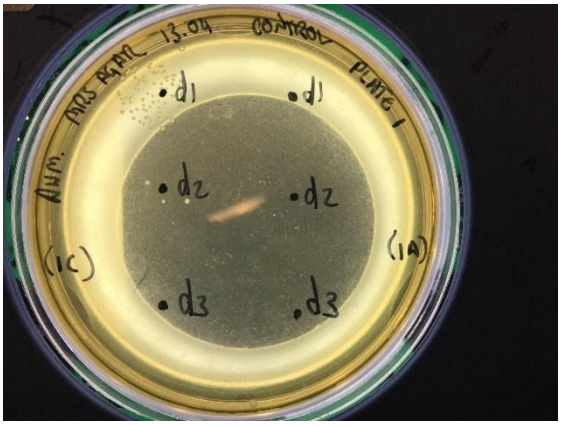
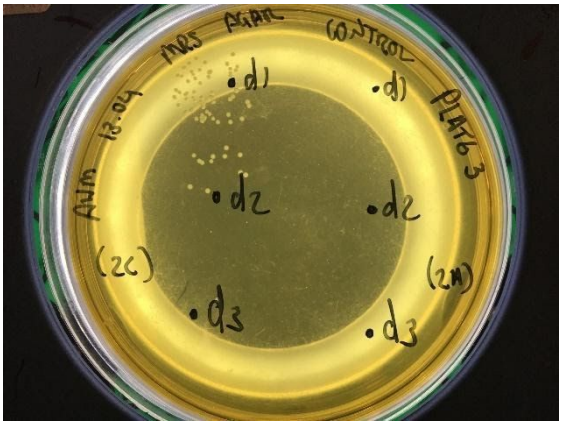
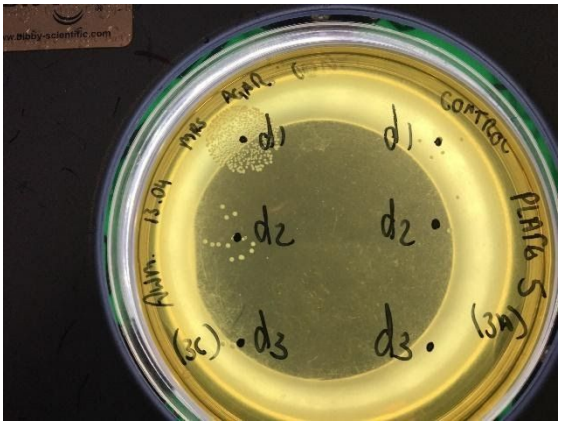
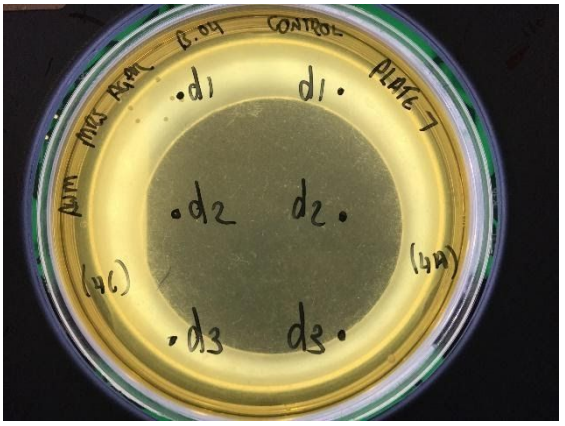


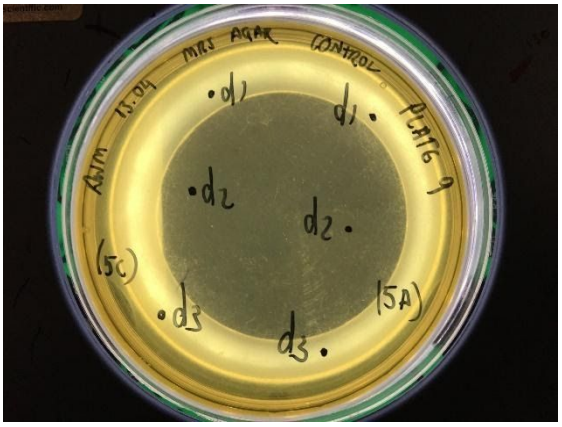
Figure S6. Water supplemented long switch treatment of DGRP-195. Long switch phenotype independent of water supplementation. Water supplementation took place in all treatments throughout life of the cage. Continuous rich, and restricted treatments plotted as lines (solid red and dashed black, respectively). Switch treatment plotted as points (white and red). The exacerbation of mortality due to switch phenotypes is observable as the difference between mortality at continuous rich diet (red line), and mortality of switch treatment when on a rich diet (red points). N = 2,562 total; ~850 per treatment. **NB** water supplementation did change the response to DR. This effect was followed up with an experiment containing five different genotypes across a range of diets, with only a shift in reaction norm detected (manuscript in preparation). DR is not explained by dehydration, as is sometimes suggested, nor is the long switch phenotype. Figure contains daily time-points, as in Fig.2.

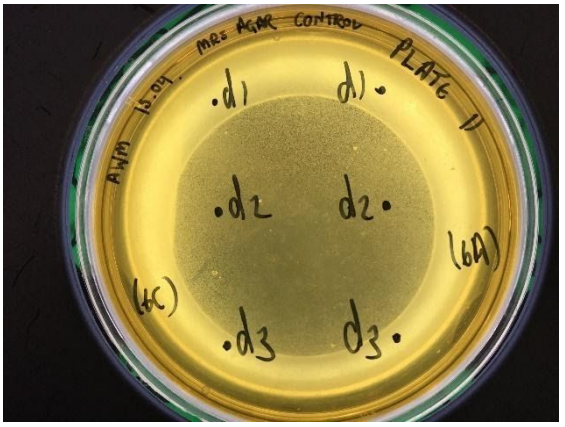

AWM. MRS AGAR 13.04 CONTROL PLATE 1
d1
d1
d2
d2
(1C)
(1A)
d3
d3

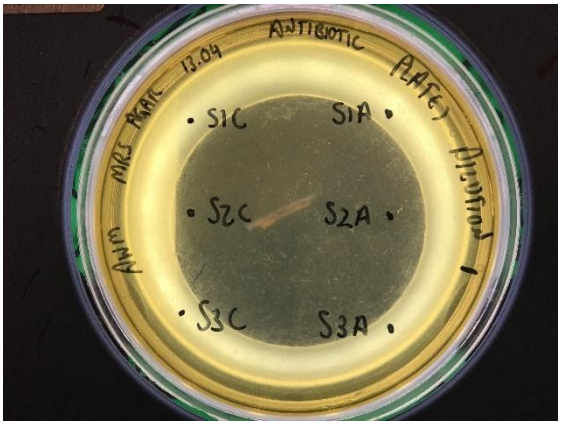

AWM 13.04 MRS AGAR CONTROL PLATE 3
d1
d1
d2
d2
(2C)
(2A)
d3
d3


AWM. 13.04 MRS AGAR CONTROL PLATE 5
d1
d1
d2
d2
(3C)
d3
d3
(3A)


AWM MRS AGAR 13.04 CONTROL PLATE 7
d1
d1
d2
d2
(4C)
(4A)
d3
d3


AWM 13.04 MRS AGAR CONTROL PLATE 9
d1
d1
d2
d2
(5C)
(5A)
d3
d3


AWM 13.04 MRS AGAR CONTROL PLATE 11
d1
d1
d2
d2
(6C)
(6A)
d3
d3


AWM MRS AGAR 13.04 ANTIBIOTIC PLATE 1 Ampicillin
S1C
S1A
S2C
S2A
S3C
S3A

Sample	Colonies at dilution 1	Colonies at dilution 2	Colonies at dilution 3	Colonies at antibiotic plate dilutions
1 (control)	75	3	0	0
2 (control)	50	12	1	0
3 (control)	250	12	2	0
4 (control)	8	0	0	NA
5 (control)	0	0	0	NA
6 (control)	1	0	0	NA
7 (antibiotic treated)	0	0	0	0
8 (antibiotic treated)	0	0	0	0
9 (antibiotic treated)	6	2	0	0
10 (antibiotic treated)	0	0	0	NA
11 (antibiotic treated)	0	0	0	NA
12 (antibiotic treated)	0	0	0	NA

Figure S7. Confirmation of ablation of microbiome. Images of bacterial colonies visible on MRS agar plates (above) and estimated colony count (below). Twelve samples ceded from control, or antibiotic-treated cages. Lysate was diluted post-homogenisation and grown on control, or antibiotic-treated plates. No growth visible under antibiotic treated plate conditions. 98.4% reduction of total microbiota observed at dilution 1. 92.5% reduction of total microbiota observed at dilution 2.

Table S1. Effect of dietary regimes on interval-based log hazard ratios of mortality in DGRP-195.

	Full Model				Versus continuous DR		
coefficient	estimate	exp (estimate)	s.e.	p	estimate	exp (estimate)	p
DR	-1.179	0.308	0.047	**<0.001**			
long switch	1.310	3.705	0.055	**<0.001**			
4-day switch	0.858	2.358	0.062	**<0.001**			
2-day switch	0.125	1.133	0.063	**0.047**			
short reverse-switch	0.272	1.312	0.051	**<0.001**			
4-day switch * DR	-0.409	0.665	0.085	**<0.001**	0.449	1.567	**<0.001**
2-day switch * DR	0.255	1.291	0.074	**0.001**	0.380	1.462	**<0.001**
short switch * DR	-0.466	0.627	0.116	**<0.001**	-0.195	0.823	0.092

Table S2. Effect of dietary regimes on longevity in DGRP-195.

	Full Model			
coefficient	estimate	exp (estimate)	s.e.	p
DR	-1.136	0.321	0.058	**<0.001**
2-day switch	-0.224	0.799	0.064	**<0.001**
4-day switch	0.386	1.471	0.064	**<0.001**

Table S3. Time-dependent effect of mortality increase induced by a long-switch from reduced to rich diets in DGRP-195

	Full Model			
coefficient	estimate	exp (estimate)	s.e.	p
day 2	1.629	5.101	0.085	**<0.001**
day 4	0.847	2.334	0.093	**<0.001**
day 6	0.452	1.572	0.121	**<0.001**
day 8	-0.045	0.956	0.216	0.84
day 10	-0.226	0.798	0.369	0.54
day 12	-0.077	0.926	0.601	0.9
> day 14	-1.038	0.354	1.066	0.33

Table S4. Time-dependent effect of mortality increase induced by a 4-day switch in DGRP-195

	Full Model			
coefficient	estimate	exp (estimate)	s.e.	p
DR	-1.303	0.272	0.094	**<0.001**
2nd interval	0.227	1.255	0.055	**<0.001**
2nd interval * DR	-0.715	0.489	0.143	**<0.001**

Table S5. Effect of asymmetrical dietary regimes on mortality in DGRP-195.

	Full Model				Versus continuous DR		
coefficient	estimate	exp (estimate)	s.e.	p	estimate	exp (estimate)	p
DR	-1.684	0.186	0.087	**<0.001**			
4d-DR|2d-Rich	-0.209	0.811	0.097	**0.032**			
2d-DR|4d-Rich	-0.157	0.854	0.095	0.097			
4d-DR|2d-Rich * DR	0.458	1.581	0.113	**<0.001**	0.249	1.283	**0.027**
2d-DR|4d-Rich * DR	0.929	2.531	0.124	**<0.001**	0.771	2.163	**<0.001**

Table S6. Effect of asymmetrical dietary regimes on longevity in DGRP-195.

	Full Model			
coefficient	estimate	exp (estimate)	s.e.	p
DR	-1.692	0.184	0.087	**<0.001**
4d-DR|2d-Rich	-0.805	0.447	0.095	**<0.001**
2d-DR|4d-Rich	-0.343	0.710	0.094	**<0.001**

Table S7. Mortality increases in response to a rich diet after a period of DR (long-switch) across a panel of 11 DGRP lines (195 is reference)

	Full Model				Effect of DR			Long switch versus rich diet		
coefficient	estimate	exp	s.e.	p	estimate	exp	p	estimate	exp	p
DR	-1.723	0.179	0.157	**<0.001**						
long switch	0.973	2.645	0.160	**<0.001**						
105	-2.681	0.068	0.146	**<0.001**						
136	-1.969	0.140	0.152	**<0.001**						
217	-0.080	0.923	0.159	0.61						
239	0.899	2.457	0.156	**<0.001**						
335	0.190	1.209	0.155	0.22						
362	-1.022	0.360	0.155	**<0.001**						
441	-2.107	0.122	0.152	**<0.001**						
705	-1.861	0.156	0.154	**<0.001**						
707	1.073	2.925	0.156	**<0.001**						
853	-1.803	0.165	0.152	**<0.001**						
105 * DR	1.296	3.655	0.217	**<0.001**	-0.427	0.653	**0.049**			
136 * DR	1.995	7.355	0.213	**<0.001**	0.273	1.314	0.199			
217 * DR	0.622	1.863	0.219	**0.004**	-1.100	0.333	**<0.001**			
239 * DR	2.427	11.319	0.211	**<0.001**	0.704	2.022	**0.001**			
335 * DR	2.947	19.052	0.208	**<0.001**	1.225	3.403	**<0.001**			
362 * DR	0.343	1.409	0.219	0.12	-1.379	0.252	**<0.001**			
441 * DR	1.002	2.724	0.215	**<0.001**	-0.720	0.487	**0.001**			
705 * DR	0.602	1.826	0.217	**0.006**	-1.120	0.326	**<0.001**			
707 * DR	0.886	2.424	0.215	**<0.001**	-0.837	0.433	**<0.001**			
853 * DR	2.549	12.796	0.210	**<0.001**	0.827	2.286	**<0.001**			
105 * long switch	0.168	1.183	0.220	0.44				1.141	3.130	**<0.001**
136 * long switch	-0.381	0.683	0.222	0.086				0.592	1.807	**0.008**
217 * long switch	1.152	3.165	0.226	**<0.001**				2.125	8.370	**<0.001**
239 * long switch	0.870	2.387	0.224	**<0.001**				1.843	6.313	**<0.001**
335 * long switch	-0.305	0.737	0.221	0.17				0.668	1.950	**0.002**
362 * long switch	-0.982	0.375	0.223	**<0.001**				-0.009	0.991	0.968
441 * long switch	-0.172	0.842	0.220	0.43				0.800	2.226	**<0.001**
705 * long switch	-0.605	0.546	0.222	**0.006**				0.368	1.445	0.097
707 * long switch	0.188	1.206	0.218	0.39				1.160	3.191	**<0.001**
853 * long switch	0.117	1.124	0.225	0.6				1.090	2.973	**<0.001**

Table S8. Models run within each genotype testing for increases in response to a rich diet after a period of DR (long-switch)

	Estimates from individual models			
coefficient	estimate	exp	s.e.	p
105 DR	-0.501	0.149	0.606	**0.001**
136 DR	0.647	0.120	1.910	**<0.001**
195 DR	-1.737	0.197	0.176	**<0.001**
217 DR	-1.102	0.178	0.332	**<0.001**
239 DR	0.775	0.071	2.170	**<0.001**
335 DR	0.570	0.115	1.768	**<0.001**
362 DR	-1.201	0.130	0.301	**<0.001**
441 DR	-0.503	0.101	0.605	**<0.001**
705 DR	-0.935	0.095	0.393	**<0.001**
707 DR	-0.646	0.076	0.524	**<0.001**
853 DR	0.828	0.093	2.288	**<0.001**
105 long switch	1.258	0.156	3.520	**<0.001**
136 long switch	0.113	0.132	1.120	0.39
195 long switch	0.453	0.203	1.573	**0.025**
217 long switch	1.650	0.187	5.205	**<0.001**
239 long switch	1.483	0.103	4.406	**<0.001**
335 long switch	0.881	0.136	2.413	**<0.001**
362 long switch	0.145	0.137	1.157	0.29
441 long switch	0.313	0.109	1.368	**0.004**
705 long switch	0.329	0.102	1.389	**0.001**
707 long switch	0.854	0.083	2.348	**<0.001**
853 long switch	1.373	0.122	3.949	**<0.001**

Table S9. Effect of alterating DR and rich diets every 4 days (4-day switch) on longevity across 11 DGRP lines (195 is reference)

	Full Model				Effect compared to rich diet		
coefficient	estimate	exp	s.e.	p	estimate	exp	p
4-day switch	-0.140	0.869	0.114	0.22			
105	-2.476	0.084	0.096	**<0.001**			
136	-1.858	0.156	0.098	**<0.001**			
217	-0.065	0.937	0.100	0.52			
239	0.918	2.505	0.099	**<0.001**			
335	0.226	1.254	0.100	**0.024**			
362	-0.988	0.373	0.099	**<0.001**			
441	-1.969	0.140	0.097	**<0.001**			
705	-1.769	0.171	0.098	**<0.001**			
707	1.054	2.869	0.100	**<0.001**			
853	-1.697	0.183	0.098	**<0.001**			
105 * 4-day switch	0.644	1.904	0.159	**<0.001**	0.504	1.656	**0.002**
136 * 4-day switch	0.632	1.880	0.157	**<0.001**	0.492	1.635	**0.002**
217 * 4-day switch	0.490	1.632	0.158	**0.002**	0.350	1.419	**0.027**
239 * 4-day switch	1.053	2.866	0.157	**<0.001**	0.913	2.491	**<0.001**
335 * 4-day switch	1.026	2.789	0.158	**<0.001**	0.886	2.425	**<0.001**
362 * 4-day switch	-0.343	0.710	0.157	**0.029**	-0.483	0.617	**0.002**
441 * 4-day switch	0.163	1.177	0.158	0.3	0.023	1.023	0.885
705 * 4-day switch	-0.032	0.968	0.157	0.84	-0.172	0.842	0.273
707 * 4-day switch	0.153	1.165	0.158	0.33	0.013	1.013	0.935
853 * 4-day switch	0.365	1.441	0.157	**0.02**	0.225	1.253	0.152

Table S10. Effect of alterating DR and rich diets every 4 days (4 day switch) on mortality at each diet, across 11 DGRP lines (195 is reference)

								Compared to continious diets					
	Full Model				Effect of DR			4 day switch at rich diet			4 day switch at DR		
coefficient	estimate	exp	s.e.	p	estimate	exp	p	estimate	exp	p	estimate	exp	p
DR	-1.378	0.252	0.129	**<0.001**									
4-day switch	0.305	1.357	0.132	**0.02**									
4-day switch * DR	-0.078	0.925	0.164	0.63									
105	-2.649	0.071	0.109	**<0.001**									
136	-1.907	0.148	0.111	**<0.001**									
217	-0.064	0.938	0.115	0.58									
239	0.907	2.476	0.113	**<0.001**									
335	0.227	1.255	0.114	**0.046**									
362	-1.024	0.359	0.113	**<0.001**									
441	-2.046	0.129	0.111	**<0.001**									
705	-1.846	0.158	0.112	**<0.001**									
707	1.051	2.859	0.113	**<0.001**									
853	-1.733	0.177	0.111	**<0.001**									
105 * DR	1.171	3.224	0.180	**<0.001**	-0.207	0.813	0.248						
136 * DR	1.804	6.071	0.178	**<0.001**	0.426	1.531	**0.017**						
217 * DR	0.822	2.275	0.178	**<0.001**	-0.556	0.574	**0.002**						
239 * DR	2.046	7.740	0.179	**<0.001**	0.668	1.951	**<0.001**						
335 * DR	2.104	8.197	0.178	**<0.001**	0.726	2.066	**<0.001**						
362 * DR	0.307	1.359	0.179	0.087	-1.071	0.343	**<0.001**						
441 * DR	0.994	2.701	0.179	**<0.001**	-0.384	0.681	**0.031**						
705 * DR	0.527	1.695	0.178	**0.003**	-0.851	0.427	**<0.001**						
707 * DR	0.797	2.218	0.179	**<0.001**	-0.581	0.559	**0.001**						
853 * DR	2.090	8.086	0.178	**<0.001**	0.712	2.038	**<0.001**						
105 * 4-day switch	0.530	1.699	0.184	**0.004**				0.835	2.305	**<0.001**			
136 * 4-day switch	0.648	1.912	0.182	**<0.001**				0.953	2.595	**<0.001**			
217 * 4-day switch	0.372	1.451	0.183	**0.042**				0.677	1.969	**<0.001**			
239 * 4-day switch	1.019	2.771	0.182	**<0.001**				1.324	3.759	**<0.001**			
335 * 4-day switch	0.451	1.569	0.186	**0.015**				0.756	2.129	**<0.001**			
362 * 4-day switch	-0.285	0.752	0.182	0.12				0.020	1.020	0.914			
441 * 4-day switch	-0.158	0.854	0.184	0.39				0.148	1.159	0.421			
705 * 4-day switch	-0.185	0.831	0.182	0.31				0.120	1.127	0.511			
707 * 4-day switch	0.167	1.182	0.183	0.36				0.472	1.603	**0.01**			
853 * 4-day switch	-0.140	0.869	0.185	0.45				0.165	1.179	0.372			
105 * 4-day switch * DR	-0.580	0.560	0.223	**0.009**							0.177	1.194	0.427
136 * 4-day switch * DR	-1.735	0.176	0.226	**<0.001**							-0.859	0.423	**<0.001**
217 * 4-day switch * DR	-0.272	0.762	0.223	0.22							0.327	1.387	0.142
239 * 4-day switch * DR	-1.846	0.158	0.225	**<0.001**							-0.600	0.549	**0.008**
335 * 4-day switch * DR	-0.400	0.670	0.219	0.068							0.277	1.319	0.207
362 * 4-day switch * DR	-0.838	0.433	0.238	**<0.001**							-0.896	0.408	**<0.001**
441 * 4-day switch * DR	0.179	1.196	0.219	0.41							0.249	1.282	0.256
705 * 4-day switch * DR	-0.050	0.951	0.222	0.82							-0.009	0.992	0.969
707 * 4-day switch * DR	-0.910	0.403	0.232	**<0.001**							-0.516	0.597	**0.026**
853 * 4-day switch * DR	-0.477	0.621	0.218	**0.028**							-0.390	0.677	0.073

Table S11. Interval models run within each genotype testing for differential effects of diet in the 4-day switch dietary regime

	Estimates from individual models			
coefficient	estimate	exp	s.e.	p
105 DR	-0.162	0.129	0.850	0.21
136 DR	0.576	0.134	1.780	**<0.001**
195 DR	-1.464	0.197	0.231	**<0.001**
217 DR	-0.695	0.132	0.499	**<0.001**
239 DR	0.697	0.075	2.008	**<0.001**
335 DR	0.516	0.128	1.675	**<0.001**
362 DR	-0.954	0.105	0.385	**<0.001**
441 DR	-0.466	0.140	0.628	**0.001**
705 DR	-0.741	0.078	0.476	**<0.001**
707 DR	-0.483	0.053	0.617	**<0.001**
853 DR	0.738	0.106	2.093	**<0.001**
105 4-day switch	0.789	0.132	2.201	**<0.001**
136 4-day switch	1.090	0.135	2.974	**<0.001**
195 4-day switch	0.265	0.195	1.303	0.18
217 4-day switch	0.756	0.134	2.131	**<0.001**
239 4-day switch	1.367	0.078	3.925	**<0.001**
335 4-day switch	0.351	0.137	1.421	**0.01**
362 4-day switch	0.065	0.106	1.068	0.54
441 4-day switch	0.139	0.143	1.149	0.33
705 4-day switch	0.171	0.081	1.186	**0.036**
707 4-day switch	0.543	0.054	1.720	**<0.001**
853 4-day switch	0.176	0.113	1.192	0.12
105 4-day switch * DR	-0.686	0.155	0.503	**<0.001**
136 4-day switch * DR	-1.965	0.165	0.140	**<0.001**
195 4-day switch * DR	0.018	0.221	1.018	0.94
217 4-day switch * DR	-0.166	0.158	0.847	0.29
239 4-day switch * DR	-1.956	0.119	0.141	**<0.001**
335 4-day switch * DR	-0.292	0.150	0.746	0.052
362 4-day switch * DR	-1.023	0.160	0.360	**<0.001**
441 4-day switch * DR	0.199	0.159	1.220	0.21
705 4-day switch * DR	-0.242	0.116	0.785	**0.037**
707 4-day switch * DR	-1.113	0.120	0.329	**<0.001**
853 4-day switch * DR	-0.579	0.129	0.560	**<0.001**

Table S12. Models run within each genotype testing for increases in response to a rich diet after a period of DR (long-switch) but within lines that showed starvation only, and with DR as reference category

	Estimates from individual models			
coefficient	estimate	exp	s.e.	p
136 rich diet	-0.647	0.524	0.120	**<0.001**
239 rich diet	-0.775	0.461	0.071	**<0.001**
335 rich diet	-0.570	0.566	0.115	**<0.001**
853 rich diet	-0.828	0.437	0.093	**<0.001**
136 long switch	0.113	1.120	0.132	0.39
239 long switch	1.483	4.406	0.103	**<0.001**
335 long switch	0.881	2.413	0.136	**<0.001**
853 long switch	1.373	3.949	0.122	**<0.001**

Table S13. Interval models run within each genotype testing for differential effects of diet in the 4-day switch dietary regime, but within lines that showed starvation only, and with DR as reference category.

	Estimates from individual models			
coefficient	estimate	exp	s.e.	p
136 rich diet	-0.576	0.562	0.134	**<0.001**
239 rich diet	-0.697	0.498	0.075	**<0.001**
335 rich diet	-0.516	0.597	0.128	**<0.001**
853 rich diet	-0.738	0.478	0.106	**<0.001**
136 4-day switch	-0.875	0.417	0.169	**<0.001**
239 4-day switch	-0.589	0.555	0.111	**<0.001**
335 4-day switch	0.059	1.061	0.148	0.69
853 4-day switch	-0.403	0.668	0.124	**0.001**
136 4-day switch * rich diet	1.965	7.135	0.165	**<0.001**
239 4-day switch * rich diet	1.956	7.071	0.119	**<0.001**
335 4-day switch * rich diet	0.292	1.340	0.150	0.052
853 4-day switch * rich diet	0.579	1.785	0.129	**<0.001**

Table S14. Linear model of estimates of (log-transformed) fecundity (from Quantifly) in the long-switch dietary treatment. A return to rich conditions from DR, resulted in reduced fecundity, rather than the predicted increase.

	Full model		
coefficient	estimate	s.e.	p
rich diet	2.281	0.038	**<0.001**
long switch	-0.367	0.037	**<0.001**
441	0.149	0.036	**<0.001**
853	0.010	0.035	0.768
age 45	0.040	0.041	0.339
age 46	0.028	0.042	0.512
age 47	-0.018	0.054	0.736
441 * long switch	-0.195	0.050	**<0.001**
853 * long switch	-0.043	0.050	0.4

Table S15. Linear model of estimates of (log-transformed) fecundity (from Quantifly), corrected for number of flies in the cage, in the long-switch dietary treatment. A return to rich conditions from DR, resulted in reduced fecundity, rather than the predicted increase. Note, this correction uses the census after egg-laying and thus overcorrects for mortality. Estimates compared are thus biased upwards, and provide the most sensitive test for an upregulation in response to dietary treatment.

	Full model		
coefficient	estimate	s.e.	p
Intercept	0.374	0.048	**<0.001**
long switch	-0.515	0.046	**<0.001**
441	0.079	0.046	0.092
853	0.041	0.044	0.355
age 45	0.049	0.052	0.349
age 46	0.060	0.053	0.262
age 47	0.132	0.067	0.058
441 * long switch	-0.158	0.063	**0.017**
853 * long switch	0.471	0.063	**<0.001**

Table S16. Mixed model (correcting for Cage) of estimates of (log-transformed) fecundity (from Quantifly) in the 4-day switching paradigm. Repeated short-term exposure to DR did not increase, but rather decreased fecundity, relative to a continuous rich diet.

	Full model		
coefficient	estimate	s.e.	p
Intercept	2.924	0.057	**<0.001**
DR	-0.642	0.030	**<0.001**
4-day switch	-0.131	0.047	**0.005**
136	0.028	0.032	0.39
195	0.092	0.033	**0.006**
217	0.018	0.032	0.569
239	-0.162	0.032	**<0.001**
335	-0.302	0.032	**<0.001**
362	-0.092	0.033	**0.005**
705	-0.570	0.032	**<0.001**
707	-0.021	0.032	0.513
853	-0.248	0.032	**<0.001**
Age 9	-0.046	0.059	0.444
Age 10	-0.073	0.054	0.171
Age 11	-0.040	0.055	0.474
Age 12	0.011	0.053	0.841
Age 13	-0.086	0.054	0.11
Age 14	-0.104	0.053	**0.049**
Age 15	-0.115	0.054	**0.032**
Age 16	-0.139	0.053	**0.009**
Age 17	-0.070	0.054	0.196
Age 18	-0.049	0.055	0.375
Age 19	-0.202	0.056	**<0.001**
Age 20	-0.212	0.056	**<0.001**
Age 21	-0.367	0.072	**<0.001**
4-day switch * DR	0.287	0.065	**<0.001**
136 * DR	-0.036	0.043	0.401
195 * DR	-0.114	0.044	**0.01**
217 * DR	-0.109	0.043	**0.011**
239 * DR	0.017	0.043	0.691
335 * DR	0.164	0.043	**<0.001**
362 * DR	0.072	0.043	0.09
705 * DR	0.422	0.043	**<0.001**
707 * DR	0.032	0.043	0.447
853 * DR	0.211	0.043	**<0.001**
136 * 4-day switch	-0.014	0.066	0.829
195 * 4-day switch	0.071	0.067	0.285
217 * 4-day switch	0.053	0.066	0.424
239 * 4-day switch	-0.029	0.066	0.656
335 * 4-day switch	0.015	0.066	0.818
362 * 4-day switch	-0.060	0.066	0.362
705 * 4-day switch	0.219	0.066	**0.001**
707 * 4-day switch	0.002	0.066	0.976
853 * 4-day switch	0.017	0.066	0.795
136 * 4-day switch * DR	0.215	0.092	**0.02**
195 * 4-day switch * DR	0.162	0.093	0.081
217 * 4-day switch * DR	0.166	0.092	0.071
239 * 4-day switch * DR	-0.048	0.092	0.601
335 * 4-day switch * DR	-0.023	0.092	0.805
362 * 4-day switch * DR	0.218	0.092	**0.018**
705 * 4-day switch * DR	-0.422	0.092	**<0.001**
707 * 4-day switch * DR	0.149	0.092	0.106
853 * 4-day switch * DR	0.112	0.092	0.225

Table S17. Mixed model (correcting for Cage) of estimates of (log-transformed) fecundity (from Quantifly), corrected for number of flies in the cage, in the 4-day switching paradigm. Repeated short-term exposure to DR did not increase, but rather decreased fecundity, relative to a continuous rich diet. Note, this correction uses the census after egg-laying and thus overcorrects for mortality. Estimates compared are thus biased upwards, and provide the most sensitive test for an upregulation in response to dietary treatment.

	Full model		
coefficient	estimate	s.e.	p
Intercept	0.812	0.056	**<0.001**
DR	-0.640	0.030	**<0.001**
4-day switch	-0.126	0.046	**0.006**
136	0.051	0.032	0.111
195	0.094	0.033	**0.004**
217	0.029	0.031	0.363
239	-0.124	0.031	**<0.001**
335	-0.180	0.032	**<0.001**
362	-0.057	0.032	0.08
705	-0.556	0.031	**<0.001**
707	0.048	0.032	0.133
853	-0.225	0.032	**<0.001**
Age 9	-0.053	0.059	0.37
Age 10	-0.089	0.053	0.094
Age 11	-0.059	0.055	0.283
Age 12	0.008	0.053	0.883
Age 13	-0.083	0.053	0.12
Age 14	-0.099	0.052	0.06
Age 15	-0.107	0.053	**0.045**
Age 16	-0.120	0.053	**0.022**
Age 17	-0.043	0.054	0.419
Age 18	-0.027	0.055	0.626
Age 19	-0.161	0.055	**0.003**
Age 20	-0.167	0.055	**0.003**
Age 21	-0.151	0.071	**0.034**
4-day switch * DR	0.297	0.064	**<0.001**
136 * DR	-0.027	0.042	0.517
195 * DR	-0.119	0.044	**0.007**
217 * DR	-0.114	0.042	**0.007**
239 * DR	0.074	0.042	0.081
335 * DR	0.170	0.042	**<0.001**
362 * DR	0.064	0.042	0.129
705 * DR	0.418	0.042	**<0.001**
707 * DR	0.000	0.042	0.992
853 * DR	0.213	0.042	**<0.001**
136 * 4-day switch	0.003	0.065	0.969
195 * 4-day switch	0.078	0.066	0.236
217 * 4-day switch	0.050	0.065	0.446
239 * 4-day switch	0.076	0.065	0.243
335 * 4-day switch	0.019	0.065	0.767
362 * 4-day switch	-0.062	0.065	0.342
705 * 4-day switch	0.217	0.065	**0.001**
707 * 4-day switch	-0.002	0.065	0.97
853 * 4-day switch	0.013	0.065	0.841
136 * 4-day switch * DR	0.178	0.091	0.051
195 * 4-day switch * DR	0.156	0.092	0.089
217 * 4-day switch * DR	0.164	0.091	0.072
239 * 4-day switch * DR	-0.060	0.091	0.511
335 * 4-day switch * DR	-0.030	0.091	0.738
362 * 4-day switch * DR	0.215	0.091	**0.018**
705 * 4-day switch * DR	-0.431	0.091	**<0.001**
707 * 4-day switch * DR	0.207	0.091	**0.023**
853 * 4-day switch * DR	0.098	0.091	0.283

Table S18. Effect of returning to a rich diet after a period of DR (long-switch) after ablation of the microbiome (Antibiotics on rich diet is reference)

	Full Model			
coefficient	estimate	exp (estimate)	s.e.	p
DR on Antibiotics	-0.894	0.409	0.181	**<0.001**
long switch on Antibiotics	1.313	3.717	0.142	**<0.001**
DR	-1.201	0.301	0.223	**<0.001**
Rich diet	-0.087	0.917	0.159	0.59

Table S19. Effect of returning to a rich diet after a period of DR (long-switch) with supplementation of water

	Full Model			
coefficient	estimate	exp (estimate)	s.e.	p
DR with water supplementation	0.648	1.911	0.138	**<0.001**
long switch with water supplementation	0.385	1.470	0.126	**0.002**

Table S20. Effect of returning to a rich diet after a period of DR (long-switch) with flies in isolation in vials. Test from chi-square tests on proportions by age.

	Dead				Mortality rate				P value from proportion test	
Age	DR	Rich to DR	Rich	DR to Rich (long switch)	DR	Rich to DR	Rich	DR to Rich (long switch)	Rich versus DR	Rich versus long switch
28	0	0	5	16	0.000	0	0.100	0.320	0.063	**0.014**
30	0	0	8	5	0.000	0	0.178	0.152	**0.006**	1
32	1	0	16	13	0.020	0	0.432	0.464	**<0.001**	0.997
34	1	0	8	4	0.020	0	0.381	0.267	**<0.001**	0.72
36	1	0	8	2	0.020	0	0.667	0.200	**<0.001**	0.079
38	4	0	2	2	0.087	0	0.500	0.250	0.102	0.829
40	3	0	0	3	0.071	0	0.000	0.500	1	1
42	2	0	0	3	0.051	0	0.000	1.000	1	0.505

Table S21. Effect of switching from DR to rich food every four days (4-day switch) in males

	Full Model			
coefficient	estimate	exp (estimate)	s.e.	p
DR	0.362	1.437	0.117	**0.002**
4-day switch	0.410	1.507	0.120	**0.001**
4-day switch * DR	-0.768	0.464	0.129	**<0.001**

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Chapter Three

Heritability of ageing in the fruit fly is high when accounting for stochastic variability in lifespan

Manuscript in preparation

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Summary

Ageing is often interpreted as having low heritability - a puzzling finding given both the multitude of genetic manipulations which impact longevity, and diversity of lifespan between species. Heritability of lifespan is almost exclusively derived from analysing individual ages of death. However, lifespan and ageing are not synonymous; lifespan is the ultimate consequence of a heterogeneous ageing process, where time to death is considered stochastic in both statistical and theoretical models. Lifespan will thus show variance due to stochasticity, even in the absence of genetically or environmentally induced variance, compressing estimates of the heritability of ageing. Here, we estimate genetic variance in ageing, accounting for stochasticity by estimating multiple independent population-level estimates of median lifespan, using inbred lines of *Drosophila melanogaster* (n = 146 genotypes; 222,542 total flies). We find heritability of ageing in both sexes to be substantially higher than previous estimates (H^2 = 0.914 - 0.924), and this estimate held across experiments conducted up to 22 months apart. When genetic determination of the ageing process is the object of study, we argue it is necessary to tease apart ageing's biological relevance from stochastic effects. Our stochasticity-corrected estimates of heritability hold mechanistic relevance by providing the upper bound of genetic determination of ageing. However, compared to conventional estimates of heritability they do not provide information on natural selection, since this will act on the stochastic phenotypic trait expressed. Our results indicate that ageing is strongly genetically determined, providing a renewed optimistic outlook for personalised gene therapy interventions to ameliorate ageing.

Introduction

The role of biology in the determination of ageing is self-evident - a human being can expect to live at least 400 times longer than a fruit fly (Tacutu *et al.*, 2018) - but the genetics underpinning differences in species' characteristic age, or indeed the broad within-species variation of lifespan, remains far from elucidated. *A priori*, ageing might be expected to have a large heritable component. In humans, twin studies and extensive pedigree data have provided ample opportunity for its study, yet narrow-sense heritability of lifespan has been estimated to be surprisingly low (~0.15 - 0.25; Herskind *et al.*, 1996; Kerber *et al.*, 2001; Mitchell *et al.*, 2001). Recent evidence suggests even this may be an overestimate (Kaplanis *et al.*, 2018; Ruby *et al.*, 2018a). These modest estimates are especially contentious given the robustness of species-level life-history correlations with lifespan (de Magalhães, Costa and Church, 2007), differential ageing rates of species (Jones *et al.*, 2014), and the wealth of known genetic manipulations which can impact longevity (Friedman and Johnson, 1988; Kenyon *et al.*, 1993; Clancy *et al.*, 2001; Kapahi *et al.*, 2004; Min *et al.*, 2008) - all of which imply strict genetic control of ageing.

As a metric, heritability provides an estimate of the relative importance of genetics in determining a trait. Broad-sense heritability (H^2) can be used to infer the degree of phenotypic variance attributable to total genetic variance ($H^2 = V_G / V_P$). Here, phenotypic variance can be considered the sum of genetic and environmental factors which impact expression of the trait, in addition to an element of unexplained, stochastic error ($V_P = V_G + V_E + V_S$; Kilfoil, Lasko and Abouheif, 2009; Flatt, 2014). H^2 in particular, is comprised of additive, dominance and epistatic genetic variance - and will additionally capture any maternal or paternal effects (Fitch *et al.*, 1998; Groothuis *et al.*, 2005), which contribute to phenotypic variance. It may also, depending on its estimation, unintentionally incorporate a degree of environmental variance. In contrast, narrow-sense heritability (h^2) consists solely of additive genetic variance. As a ratio of the response to selection, and the selection differential (Falconer and Mackay, 1996), it provides insight into the scope of change expected between generations for a particular trait.

Previous work carried out in both fly hemiclones, and the *D. melanogaster* Genetic Reference Panel (DGRP; Mackay *et al.*, 2012) has estimated heritability of lifespan, typically by using analysis of variance (ANOVA) on individual ages of death (Table 1; Lehtovaara *et al.*, 2013; Durham *et al.*, 2014; Ivanov *et al.*, 2015; Huang *et al.*, 2020). While the lower range of evaluated lifespan broad-sense heritability ($H^2 = 0.29$ - 0.42) generally exceeds studies of h^2 in other organisms - in humans (Herskind *et al.*, 1996; Kerber *et al.*, 2001; Mitchell *et al.*, 2001), in *C. elegans* (Johnson and Wood, 1982), and in primates (Martin *et al.*, 2002) - the degree of observed genetic variance which explains variability in lifespan remains relatively low.

Table 1. Broad-sense heritability of lifespan estimates in *Drosophila melanogaster* from previous publications, where each individual represents a data point. Sample size represents n per genotype, per sex. NB Lehtovaara *et al.* report an intermediate of broad- and narrow-scale.

publication	heritability	population	sample size	method
Durham et al., 2014	0.29	DGRP	22	ANCOVA
Ivanov et al., 2015	0.413	DGRP	25	ANOVA
Huang et al., 2020	0.36-0.42	DGRP	72	ANOVA
Lehtovaara et al., 2013	0.25-0.4	hemiclones	200	MCMC

One possible explanation for the contrast between low heritability of lifespan and the expected strong genetic control of ageing, lies in the extent to which stochasticity mediates the phenotypic variance of lifespan. When a trait has little stochastic variance and can be measured with high accuracy - and, where needed, repeatedly, from the same individual - genetic determination can be high. For example, quantified heritability of human height is large, despite known environmental effects which can alter the phenotype substantially

(Silventoinen *et al.*, 2003; Polderman *et al.*, 2015; Jelenkovic *et al.*, 2016; Wainschtein *et al.*, 2019). Measurement error will impact estimates of phenotypic variance, but can be controlled by generating multiple estimates of individuals. However, individual age at death - sometimes assumed a direct metric for ageing - can not only be measured once per individual, but also has a highly stochastic element. Such heterogeneity caused by stochasticity is illustrated by the wide range of lifespan, even in the absence of genetic or environmental variance, within genetically homogenous individuals under identical environmental conditions (Fig. 1; Gavrilov and Gavrilova, 2001; Caswell, 2009). Note, variability in age of death here is not due to measurement error, as lifespan measurements can be assumed to have negligible error when animals are observed relatively frequently. Rather, theoretical and parametric models of ageing parameratise probability distributions of death (Gavrilov and Gavrilova, 2001; Avraam, de Magalhaes and Vasiev, 2013; Kirkwood, 2015). Empirical evidence of this stochasticity also exists in model organisms (Herndon *et al.*, 2002). As a consequence of neglecting the impact of stochastic variance on lifespan, it routinely remains undetected, and will instead be allocated to environmental variance, artificially inflating phenotypic variance in the process. Crucially, phenotypic variance of lifespan in this instance would remain high even were longevity to be tightly regulated at a population level (like ageing rate, for example; Kirkwood, 2015).

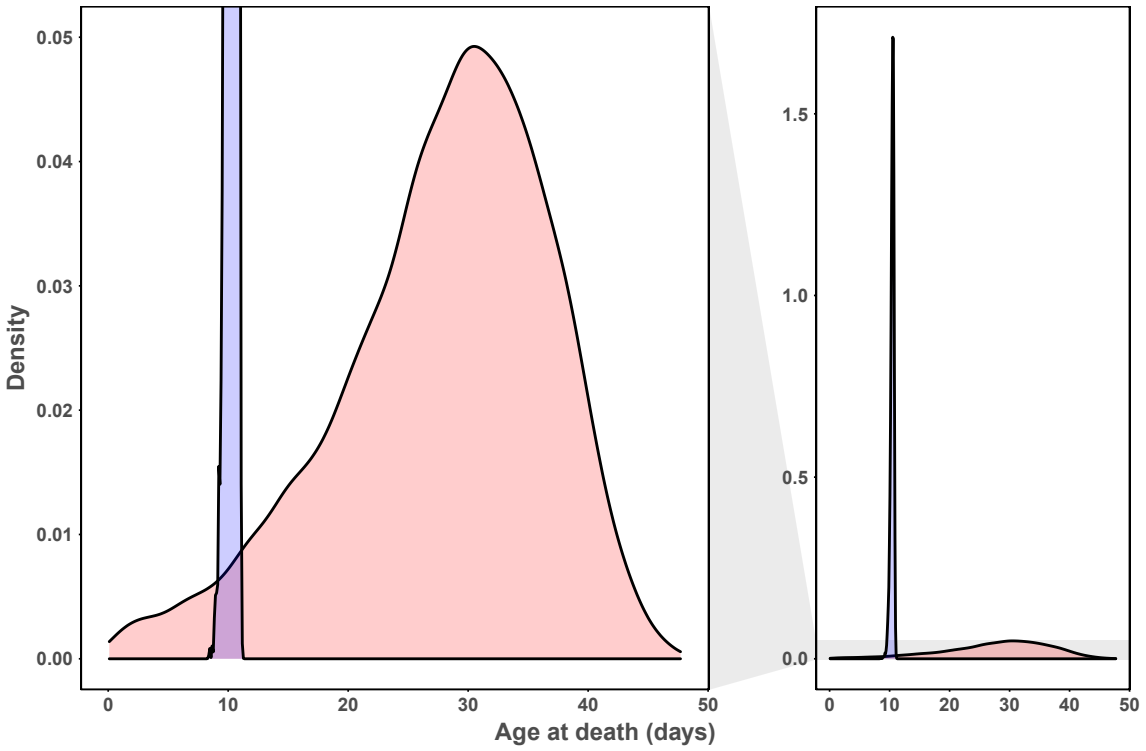

Figure 1. The Gompertz distribution is probability based, and predicts a wide lifespan distribution. Probability density function comparison of a likely Gompertz distribution, with one where stochasticity would be unidentifiable. The two parameter Gompertz model of ageing has a strong fit to lifespan data across species and situations (Juckett and Rosenberg, 1993; Gavrilova and Gavrilov, 2015). **Red distribution** - median Gompertz parameters modelled from across the full female dataset (shape = 0.13; rate = $2.069e^{-3}$). **Blue distribution** - an example of an extreme Gompertz distribution, where phenotypic variance at our resolution of 2 days would remain unobserved (shape = 4.5; rate = $1e^{-20}$). The average female fly in our dataset could record a time to death across a wide range (red distribution; {2. . 50}; $\sigma = 8.9$). In comparison, for a distribution with minimal stochasticity, a much smaller range is exhibited (blue distribution; {9. . 11}; $\sigma = 0.29$). The full blue distribution is shown inset. The modes of distributions differ only for illustration purposes; both modes can be parameterised to overlap.

This problem is compounded when considering that the parameters of the underlying distribution - Gompertz, for example - have a heritable component (Tatar and Carey, 1994; Promislow *et al.*, 1996) and will differ by genotype. Differences in the probability distribution of death will lead to differential skew and kurtosis of the lifespan distribution - both of these being functions of the underlying putative biological parameters (Lenart, 2014). As such, when considering multiple genetic lines, phenotypic variance of individual lifespans will be genotype-specific; should ageing be regulated at this population level,

analysis of variance in lifespan across genotypes will further convolute biological inference, since both within- and across-genotype lifespan variance will be wholly unrelated to the relevant biological parameters determining lifespan. Any attempt to infer biological meaning from differential variances in individual lifespan will thus adopt artificially inflated phenotypic variance, thereby compressing heritability estimates.

The inherent stochasticity of lifespan thus prompts an interesting question regarding its heritability: should stochastic error ever be considered as a component of phenotypic variance? When considering heritability for the purposes of estimating the response to selection for lifespan, modelling individuals for phenotypic variance (and incorporating stochasticity) is suitable, since individual lifespan is the trait selection acts upon. However, lifespan should not be used as a metric for the ageing process as a matter of course. Lifespan is the stochastic result of ageing physiology that could potentially be wholly genetically determined. The use of different terms with distinct meanings, interchangeably, has previously been noted (van den Berg *et al.*, 2017). As such, the heritability of lifespan could erroneously be interpreted as the heritability of ageing. But when ageing is the object of interpretation, we suggest it requires a view on lifespan whereby the shared and inherited property exists as population-level parameters. In essence, an optimal metric of the heritability of ageing may be one devoid of stochastic noise inherent in lifespan distributions. Inbred lines uniquely allow the measurement of a distribution of ages of death of the same genotype, thereby allowing the separation of the median lifespan of a genotype from the stochastic noise around that median. In this manner, the heritability of ageing can be determined by taking multiple independent measures of a population-level parameter, and treating between-replicate variation as the measure of phenotypic variance, thus removing inherent stochastic variation. This approach does, however, contain a caveat: environmental variance is solely estimated as between-replicate. Likewise, environmental effects of individuals within-replicate (or shared across all replicates) is, by necessity, ignored.

As an example, suppose a heads-biased coin ($P_{(heads)} = 0.7$) is flipped 10 times, with the number of successes (heads) recorded, and this process repeated 10,000 times. Here, our 'biological' determinant ($P_{(heads)}$) is akin to the population-level trait, and we are interested in quantifying the heritability of this underlying trait. As such, we should not assess the within trial variance. Since a coin toss is an inherently stochastic process, the resulting sampling distribution estimating $P_{(heads)}$ ($\mu = 0.7$) will have a mean, variance and skew, partially determined by the extent of bias in the coin. Were we to sample from this distribution ($n_{successes} = \{0. . 10\}$), we would be incorrect to infer anything about the underlying 'biology' ($P_{(heads)}$) by analysing the variance of our sample; in doing so, we would be neglecting the role of stochasticity, and inflating our estimate of phenotypic variance. Furthermore, if we were to compare our variance with sample variances from 100 other differentially-biased coins - which, in this case, is analogous to genotypic-differentiation - we would be making

the additional error of underestimating the degree to which our differentially-biased coins determine outcomes (in this case, H^2 of $P_{(heads)} = 1$). To accurately reflect heritability of ageing, stochastic error should be omitted from estimates of phenotypic variance, and this is uniquely possible when age at death can be measured multiple times from the same genotype.

A number of studies adopting a similar approach to quantifying what has been labelled micro-environmental variance, have previously been carried out for different traits in both inbred (Ordas, Malvar and Hill, 2008; Morgante *et al.*, 2015) and outbred organisms (Sztepanacz, McGuigan and Blows, 2017). Here, we utilise inbred fly lines to assay lifespan at very large sample sizes in both sexes (n = 146 genotypes; 222,542 total flies), modelling population-based metrics of longevity (median lifespan and Gompertz parameters), and partitioning environmental variance at a population level (n = 1,932 cages). We find H^2 of median lifespan to be significantly higher (H^2 = 0.914 - 0.924) than previously recorded lifespan heritabilities (Table 1). We argue that a population-based metric of lifespan may be a useful way of estimating the heritability of an emergent trait, and that the strict genetic control implied by our results gives credence to the possibility of interventions which limit the damage of the ageing process in humans.

Materials and methods

Batches, fly husbandry and experimental protocol

Lifespan experiments were carried out in both sexes of 146 genotypes (n = 222,542 flies total) from the *D. melanogaster* Genetic Reference Panel (DGRP; Mackay *et al.*, 2012) over the course of 15 semi-overlapping batches. A small number of females and males of genotypes were present in multiple batches (n = 28 and 5, respectively). Males were always assayed within the same batch as females of the same genotype; in the small number of cases where males were assayed across batches, their batch distribution was identical to females. Females were assayed at very high sample sizes, with, on average, 10 cages of ~125 flies per genotype (n = 179,787 total). Males were initially assayed at a lower sample size of, on average, 3 cages of ~25 flies per genotype (n = 3,114); samples sizes in males then increased to, on average, 3 cages of ~125 flies per genotype (n = 39,641). Subsequent analysis revealed probable density-dependent effects on mortality and as such, our dataset was partitioned into two, for the purposes of reporting results. Our 'full' dataset contained all genotypes (n = 146 genotypes; 1,932 cages, of which 454 were male, and 1,478 female; 222,542 total flies); our 'restricted' dataset excluded genotypes where fewer than 80 males had been assayed (n = 104 genotypes; 1,391 cages, of which 328 were male, and 1,063 female; 169,398 total flies). Some genotypes (e.g. DGRP-195)

were assayed under identical conditions for other experiments; where appropriate, these data were combined with this experimental dataset.

Flies were maintained on our standard 8% yeast concentration diet (8% autolysed yeast, 13% table sugar, 6% cornmeal, 1% agar and 0.225% [w/v] nipagin). All flies were reared and mated on 8% media for 48 hours, then kept in cages until death. Scoring took place every 48 hours (where dead flies were removed and counted, and food vials were replaced), giving a resolution of 2 days for median lifespan estimates per cage. For further information and discussion on dietary treatments, fly husbandry and experimental protocols, see (McCracken *et al.*, 2020).

Measurements of lifespan central tendency and batch effects

To determine the most appropriate measurement of central tendency for lifespan per cage, we compared median lifespan, mean survival time (MST), and restricted mean survival time (Royston and Parmar, 2013) with a 10% trim on the upper-bound of lifespan. Both MSTs correlated very highly with median lifespan (r = 0.988) and to each other (r = 0.999) despite some bias exhibited towards the tails of the distribution. Given the congruence between these measurements, we used median lifespan for cage-based estimates, since the median is a more reliable estimator of skewed distributions. Linear regression and simulations determined no statistically significant effect of an even/odd age scoring pattern on median lifespan.

To determine whether batch was a significant predictor of median lifespan, we ran linear models on within-sex cage data, with effect size of batch (as an additive variable) reported as omega-squared (ω^2), and partial omega-squared (ω_p^2)' and model selection performed by BIC. For males, batch was non-significant (F = 2, df = 3; p = 0.11). For females, batch was highly significant (F = 17.36, df = 11; p < 0.001), but a very poor determinant of median lifespan variability in comparison to genotype (batch $\omega^2 = 0.01$, genotype $\omega^2 = 0.915$; batch $\omega_p^2 = 0.12$, genotype $\omega_p^2 = 0.924$). Additionally, predicted values from the model output correlated very highly with observed values (r = 0.97; r = 0.96 for genotype-only model). Experimental batches were not balanced by genotype, and thus, a certain degree of variability was expected. Given this, we report raw uncorrected values of median lifespan. Heritability estimates are also based on these data.

Density-dependent effects of male lifespan

Since males had been assayed at both low and high sample sizes per cage (n = ~25 and ~125, respectively) we partitioned our dataset into two - one with the inclusion of all genotypes ('full') and another which omitted genotypes with low male sample size ('restricted'). To test for possible density-dependent effects of lifespan, we used linear models where we tested the effect of low sample size male cages on mean median lifespan of genotypes, in males and females separately. Mean median lifespan refers to the mean of

by-sex median lifespan of cages. Genotypes were assigned a categorical variable of 'low' sample size when males of that genotype were assayed at sample sizes lower than 80; otherwise they were considered 'high'. When partitioning the dataset by sex, there was a significant effect of low sample size male cages only for male mean median lifespan (females: F = 1.2, df = 1, p = 0.28; males: F = 13.8, df = 1, p = < 0.001). This moderate effect in male lifespan (ω^2 = 0.08) was confirmed by BIC. More moderate thresholds for male sample size were also tested, with no significance determined. Additionally, the R_{MF} (or male / female correlation; Bonduriansky and Chenoweth, 2009) of mean median lifespan increased (0.58 to 0.72) when excluding genotypes with low sample size male cages. We thus interpret there to be significant confounding effects of low male sample size in male lifespan estimates, possibly as a result of density-dependence. Hereafter, we report results for both our full and restricted datasets, respectively, to show that qualitative conclusions do not change.

Measure of heritability effect size

We compared the frequently used measure of effect size eta-squared ($\eta^2 = SS_{genotype} / SS_{total}$), to the bias-corrected ($\omega^2 = (SS_{genotype} - (df_{genotype})*(MS_{residual})) / (MS_{residual} + SS_{total})$) in null models, to quantify the extent of bias inherent to these estimations. Despite known issues with upwards bias when applying η^2, it remains a commonly used calculation. Simulations (n = 1,000 runs) used the same number of grouping variables as genotypes within our full dataset (n = 146), and a range of sample sizes (n = 3; 10; 30; 50; 100; 150) to sample from the same Gaussian or Gompertz distribution. Estimates and 95% credible intervals (CIs) were manually derived from 1000 simulations, with the estimate representing the mean of the sampling distribution. CIs represent the 2.5 and 97.5 percentile bounds of the sampling distribution.

In alignment with previously published work (Keselman, 1975; Okada, 2013), effect size bias rose with a decrease in sample size when using η^2, in both Gaussian and Gompertz distribution simulations, but no similar upwards bias was observed with ω^2 (Fig. S1). H^2 estimates for our dataset were thus more conservative when using ω^2, compared with other estimates which have used η^2. While dispersion around the mean sampled effect size was larger when estimated with ω^2, this degree of uncertainty only carries the potential for significant upwards bias at low sample size, or when genotype variances are heterogeneous (Troncoso Skidmore and Thompson, 2013). As such, we rely on ω^2 to generate estimates of H^2.

ANOVA-based heritability estimates

Measures of heritability for our dataset were partitioned into male and female estimates, given the degree of heteroskedasticity present, and unequal sample sizes of the whole dataset. For population-based estimates, these were generated from one-way ANOVA cage-level lifespan data, where each cage parameter represented a data point. Both

Gompertz parameters (separately, for a two parameter Gompertz model), and median lifespan were modelled for each cage. The two parameter Gompertz has a hazard function $u(x) = a.e^{\beta x}$, where $u(x)$ represents mortality rate (or risk) at age x, a denotes the level of initial mortality, and β is mortality increase by age. Gompertz distribution parameters of rate and shape, are analogous to a and β parameters, respectively. Gompertz parameters were natural log transformed prior to ANOVA. Extreme outliers were defined as either values below (Q1 - 3 * IQR) or above (Q3 + 3 * IQR), and were removed from the analysis to reduce heteroskedasticity. Extreme outlying cages represented < 1.5% of data (Table S1, S3); their removal did not affect qualitative conclusions.

Individual age of death was also modelled for our dataset, to allow comparison to population-level estimates and previous work. For a fair comparison with this previous work (Table 1), no outliers were removed despite severe heteroskedasticity and non-normally distributed residuals. While severe violations of homogeneity of variance assumptions are liable to inflate both Type I and Type II errors (Hayes and Cai, 2007), no bias in regression coefficient estimation by ordinary least squares necessarily occurs (White, 1980). Right-censored individuals (< 10%) were excluded from this analysis since no age of death is available. For both individual- and population-level models, 95% confidence intervals were estimated using the noncentrality parameter (NCP) method, which is appropriate for estimating non-central CIs of effect sizes based on the F distribution (Table S3; Steiger, 2004; Kelley, 2007).

To evaluate if any additional bias is introduced when assaying at low sample sizes, individual- and population-based estimates were downsampled and reanalysed. Data were derived from one-way ANOVA of either individual-level lifespan data, where each individual represents a data point (Fig. 3A; Table S4), or from population-level median lifespan estimates, where each cage represents a data point (Fig. 3B). Note, when downsampling population-level data, median lifespan of cages was recalculated prior to ANOVA. In essence, downsampling population data altered both cage, and genotype, average lifespans. Estimates and 95% credible intervals were manually derived from 1000 simulations, with the estimate representing the mean of the sampling distribution. CIs represent the 2.5 and 97.5 percentile bounds of the sampling distribution.

SNP-based heritability estimates

SNP-based measures of narrow-sense heritability were carried out on genome-wide SNPs, and performed in GCTA (Yang *et al.*, 2011) where phenotypic variance was the mean by-cage median lifespan. Estimates were derived via genomic-relatedness-based restricted maximum likelihood (GREML) where the first 10 eigenvectors computed from a GRM of SNP and non-SNP variants were included in the model as quantitative covariates. Principal component analysis revealed the top eigenvectors were capturing large-scale inversions. Genotype files were taken from publicly-accessible Freeze 2.0 (Huang *et al.*, 2014). No

genotypes or SNPs were excluded from this analysis via quality control, in order to generate the uppermost bound of h^2 estimates for the most conservative comparison with H^2.

Comparisons of heritability estimates

Two-tailed Z-tests for the difference between means were performed to compare heritability estimates, given suitably large sample size for cages. Given CIs for ANOVA-based estimations were estimated by NCP method (see above), SEs were inferred for Z-tests by dividing CIs on either side of the mean by 1.96. SEs and CIs were often unequal either side of the mean, given the NCP method of estimation (Fig. 2; Table S1, S3-S4). Given this, the SEs nearest the mean being compared, were used. Unless otherwise specified, the following comparisons are reported: Reported comparisons of females were performed on our full dataset; to exclude the experimental confound of density-dependent effects on lifespan, reported comparisons of males were based on our restricted dataset. When comparing sexes, our restricted dataset was used to ensure parity of genotypes for both sexes.

Repeatability estimates

Repeatability (or intra-class correlation) for median lifespan was performed using rptR (Stoffel, Nakagawa and Schielzeth, 2017), which uses a mixed-effects model framework to partition variance into group-level (genotype), and residual (Table S2). No outliers were removed from this calculation. Repeatability was also measured by regressing 28 genotypes' female mean median lifespan estimates from one batch, against another (Fig. S2). These data represented all female genotypes assayed over more than one batch; where genotypes were assayed over more than two batches, the highest two sample-size batches were used.

Results

Broad-sense heritability of ageing is high

We used median lifespan data from individual cages as a population-based metric of lifespan to generate estimates of H^2 for males and females across lines. Estimates were high, and ranged from 0.914 to 0.924, with no significant differences between males or females, or between our full and restricted datasets (Table S1). Additionally, we note that the margins of error in males, although estimated with a lower sample size, were not substantially larger. By using a cage-based measurement of lifespan to estimate heritability, we force environmental variance to be considered between-cage variance exclusively. Therefore our estimates of heritability are similar to analysing repeatability of median lifespan across cages (Nakagawa, Johnson and Schielzeth, 2017). Consequently,

we find similar results to repeatability values (Table S2, 0.909 - 0.915; Fig. S2, R = 0.86), despite experimental batches being assayed up to 22 months apart.

Gompertz models closely fit demographic data within and between-species, and have been used to interpret ageing by partitioning risk into an age-related component, and an overall vulnerability to the ageing process. As Gompertz parameters explicitly model the stochastic process that determines the distribution of ages of death, we also fitted each cage with these models to demonstrate our results are not dependent on the use of median lifespan. In addition, this analysis provides information on the heritability of the separate Gompertz parameters, which, although correlated (Strehler and Mildvan, 1960; Simons, Koch and Verhulst, 2013), could reveal a differentially heritable component of each. Estimates of heritability for both parameters in both sexes were significantly lower compared to median lifespan (Fig. 2; Table S1, S3; H^2 shape = 0.797 - 0.806; rate = 0.817 - 0.865), but still substantially higher than previous lifespan heritability estimates in the fly (Table 1). In addition, there were differences between male rate and shape heritability estimates ($Z = 3.06$; $p = 0.0022$), with no significant deviations recorded between female parameters. Male rate heritability also exceeded female rate heritability ($Z = 2.02$; $p = 0.043$).

Modelling individual lifespan yields low estimates of heritability

To allow a comparison of our results with previously published work (Table 1), we also estimated heritability of individual ages of death in our analysis of variance, treating each individual as a data point (Table S4). Notably, there was a significant drop in H^2 estimates (H^2 = 0.4 - 0.46) relative to cage-based estimates, as a result of substantially increased phenotypic variance when incorporating lifespan stochasticity (and potentially, local environmental effects not estimated when using median lifespan). Our highest estimate here showed a significant deviation from the closest population-based estimate of both sexes (Fig. 2; $Z = 10.5$; $p < 0.001$).

We note that these results are in line with previously published work that used the DGRP to estimate heritability of lifespan (Table 1). Given this, we suggest that modelling population-level data may yield more useful heritability of ageing estimates, since it relies upon a more optimal measure of phenotypic variance - one without stochasticity - to draw conclusions about the ageing process.

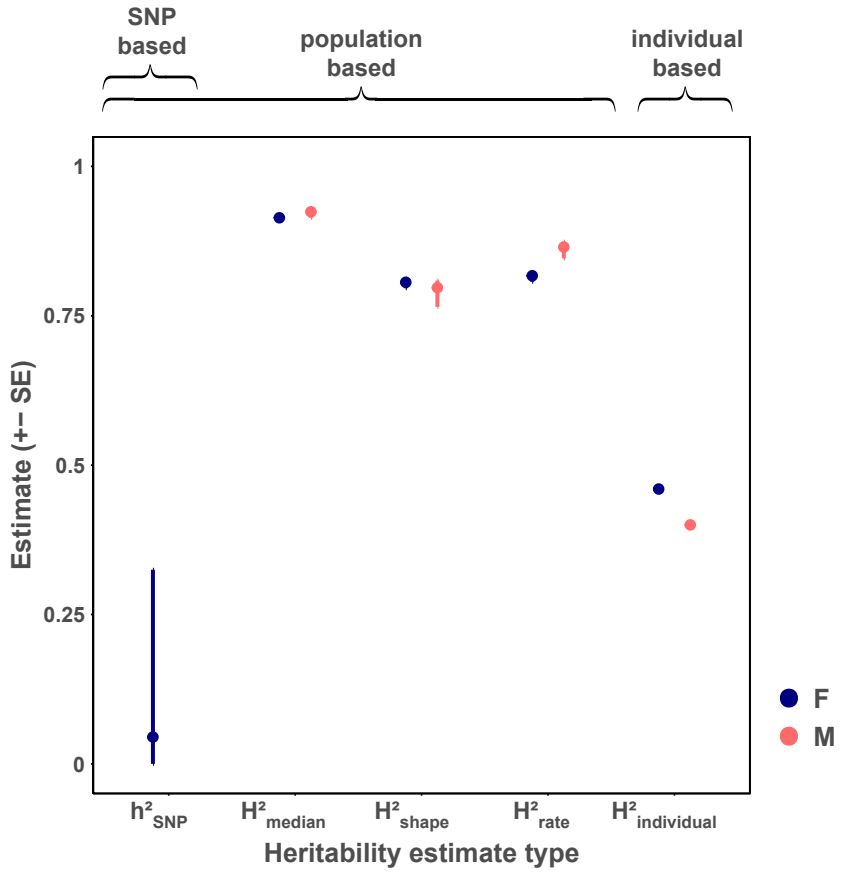

Figure 2. Comparison of different heritability estimates of the dataset. Population-based methods of interpreting the phenotypic variance of ageing result in significantly higher broad-sense heritability estimates. For females, estimates are based on the full dataset. To exclude the experimental confound of density-dependent effects on lifespan, male estimates are based on the restricted dataset. Shown are narrow-sense heritability estimates via GREML of genotype mean median female lifespan (n = 146 genotypes), broad sense heritability of population-based metrics estimated via ANOVA (median lifespan; Gompertz shape; Gompertz rate. See supplementary tables for sample sizes), and broad-sense heritability estimated via ANOVA when modelling individual age of death (females: n = 168,941 flies; males: n = 37,828 flies).

<u>High sample sizes are required for accurate estimates of the heritability of ageing</u>

To evaluate if any additional bias is introduced when assaying genotypes at low sample sizes, we downsampled our full female dataset and reperformed our analyses. When modelling individual age of death, we found downsampled H^2 estimates led to a marginal upward bias in mean heritability estimates (Fig. 3A; Table S4), possibly as a result of non-complete balance within our dataset. We observed an increase in the margin of error

surrounding mean heritability estimates; this increase is expected when sample size is lower, due to a reduction in statistical power. However, no bias was observable in mean heritability estimates even at a very low sample size. The degree of stochasticity of genotype-specific lifespan will be determined by the shape of the distribution of lifespan. Given this, we suggest these lower (relative to population-based) estimates may simply be a function of the skew and kurtosis of underlying distributions, and as such, thresholded by the inherent variability of genotypes assayed.

In contrast, when modelling cage median lifespan using downsampled data, we found that mean estimates of heritability decreased, while their respective margins of error increased, as sample size decreased. (Fig. 3B). At the most extreme sample size simulated (n = 5, per cage; which still represents n = ~50, per genotype), we observed a downward bias of ~22% in H2 estimate (mean ω^2 = 0.717). When the cage mean is estimated with lower precision, heritability of the population-level trait - median lifespan - reduces, since the estimated mean of cages is subject to more variability. Downsampling then demonstrates that precision of the measurement of the population-level trait, determines the upper bound of heritability that can be estimated. Our approach is therefore contingent upon substantial within-environment sample size.

Genome-based narrow-sense heritability is negligible across the DGRP

To compare our broad-sense estimates with narrow-sense, we estimated SNP-based h^2 from complete female genomic data, using genotype-level measures of lifespan. We found the explanatory power of additive genetic variance to be low (full dataset: h^2 = 0.0449, SE = 0.28; restricted dataset: 0.0151, SE = 0.397) with high margins of error, given low genotype sample size. Here, we observed no difference between SNP h^2 and individual-based H^2 estimates (Fig. 2; Z = 1.04). In contrast, SNP-based h^2 was significantly lower than other population-based measurements of H^2 (Fig. 2; female shape; Z = 2.04; p = 0.041). From this, we tentatively conclude a large portion of a large portion of genetic variance accountable for the heritability of ageing is non-additive.

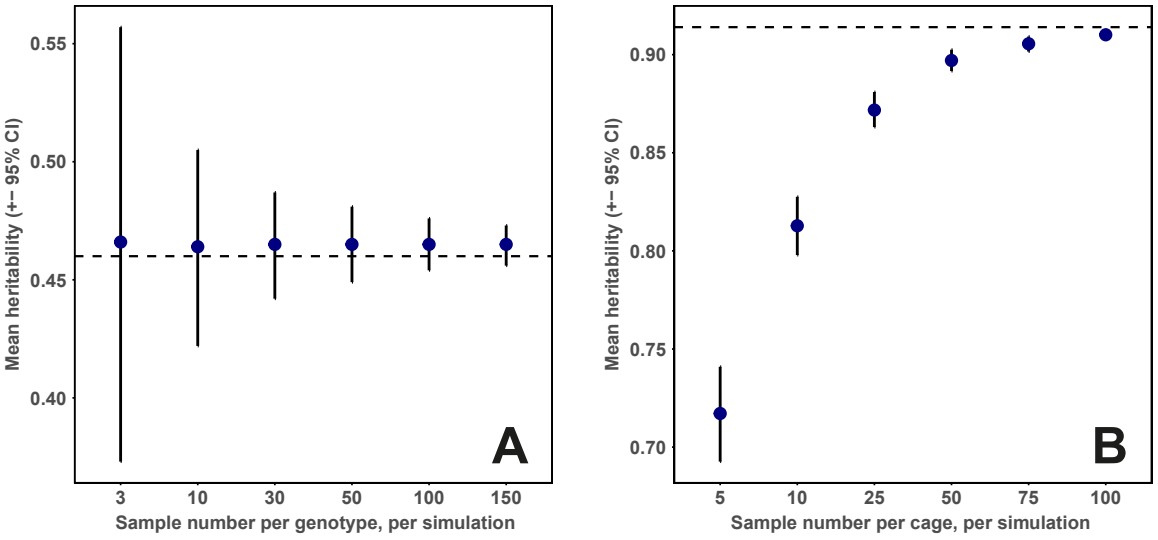

Figure 3. Effects of sample size on H^2 in individual- and population-level data. A - When using individual age of death to measure phenotypic variance, mean H^2 estimates, as expected, remain static when downsampling, even to an extreme degree (n = 3, per genotype). **B** - When using cage-based median lifespan to measure phenotypic variance, mean H^2 estimates fall in line with sample size reductions per cage. Both panels report downsampled estimates from our full female dataset, using ω^2 as a measure of effect size. Horizontal lines represent non-downsampled estimates of full female datasets (see Supplement).

Discussion

Lifespan has widely been determined to have low broad- and narrow-sense heritability (Table 1; Johnson and Wood, 1982; Herskind *et al.*, 1996; Kerber *et al.*, 2001; Mitchell *et al.*, 2001; Martin *et al.*, 2002; Kaplanis *et al.*, 2018) - a finding which has puzzled researchers, given both the wealth of genetic manipulations which extend lifespan (Friedman and Johnson, 1988; Kenyon *et al.*, 1993; Kapahi *et al.*, 2004) and differential ageing rates of species (Jones *et al.*, 2014). Quantifying the heritability of lifespan is commonplace, but almost exclusively derived from modelling individual age of death (Table 1; Herskind *et al.*, 1996; Kerber *et al.*, 2001; Mitchell *et al.*, 2001; Martin *et al.*, 2002; Kaplanis *et al.*, 2018). However, stochasticity is a highly prevalent mediator of this outcome, and population-based metrics are necessary to tease apart biological relevance from statistical noise to determine lifespan's relevance to ageing. Indeed, the demographic interpretation of ageing - mortality rate, or shape of the Gompertz distribution - is contingent upon a population-level analysis of an individual trait. Lifespan's relevance to ageing is therefore restricted to how individuals of a particular genotype, en masse, perform.

Identifying a population-based metric of lifespan for a single genotype, necessitates the use of inbred lines. Here, we quantified heritability of ageing, by partitioning inbred genotypes into discrete environments, allowing us to generate discrete measures of variance in population-based metrics of lifespan. We found genetic variance to be responsible for ~90% of this phenotypic variance in median lifespan - a more than two-fold increase over previous lifespan estimates from the DGRP (Table 1). Similarly, heritability of cage-level Gompertz parameters (H^2 = 0.797 - 0.865) were two-fold higher than these previous individual-level estimates. One median lifespan estimate can incorporate a range of Gompertz parameters (Garratt, Nakagawa and Simons, 2016); we suggest this may be the reason for lower Gompertz estimates, relative to median lifespan. As a direct comparison to population-level estimates, we included stochastic error into our estimates of phenotypic variance and re-analysed our data using individual lifespan. Here, we found a reduction in H^2 of ~50%, due to stochastic error being apportioned to environmental variance. We conclude that modelling population-level data may yield more useful heritability of ageing estimates, since it relies upon a more optimal measure of phenotypic variance - one without stochasticity - to draw conclusions about the ageing process..

Individual age of death often bears no resemblance to the emergent trait of ageing: an individual fly dying at age 10 days could be part of a population with widely-varying group-level parameters. The resulting inflation of phenotypic variance when modelling individuals from populations with group-regulated parameters (Fig. 1) will always compress heritability to a marked degree - even if heritability of the population-level trait were to be fixed at 1 and measured at very high sample sizes. The extent to which heritability estimates will be compressed will be determined by the inherent variance of the trait within the population. Given this, when modelling individual age of death to determine the heritability of ageing, rather than lifespan, we suggest estimates will simply be a function of the underlying skew and kurtosis of underlying distributions, and as such, thresholded by the inherent variability of genotypes assayed.

In accordance with this idea, in humans there are indications that heritability increases when lifespan is thresholded at increasing ages, in studies which stratified their data by age (Ljungquist *et al.*, 1998; Hjelmborg *et al.*, 2006; Gögele *et al.*, 2011; van den Berg *et al.*, 2019). Similarly, studies which have restricted their analysis to the heritability of extreme longevity have found some relatives of the long-lived to outlive their peers (Perls *et al.*, 2002; Montesanto *et al.*, 2011; van den Berg *et al.*, 2018), suggesting heritability of long life may be higher than previously reported twin or pedigree studies. These results have generally been interpreted as an increasingly larger positive genetic component being necessary to survive to very old age (Sebastiani and Perls, 2012). However, by dichotomising lifespan in a case-control manner, stochasticity inherent within the distribution of lifespan will be reduced - much like our use of the population-based metric of median lifespan. In this sense, when measuring the heritability of longevity in particular

(by thresholding for a long life), estimates may be substantially higher than those derived from whole-life data, precisely because this is a truer representation of the heritability of ageing.

Despite our very high estimates of broad-sense heritability of ageing, we find that additive variance accounts for a very small proportion of total genetic variance. This could be because the genetic architecture regulating the trait is highly epistatic and/or dominant; indeed epistatic interactions play a significant role in quantitative trait expression in *Drosophila* (Huang *et al.*, 2012) and humans (Mackay and Moore, 2014). For human lifespan, there is also evidence that non-additive variance does have a modest contribution to its regulation (McGue *et al.*, 1993; Herskind *et al.*, 1996). However, the relevance of epistatic variance in natural populations is disputed, with evidence pointing to additive variance comprising the vast majority of total genetic variance (Hill, Goddard and Visscher, 2008), and a strong additive basis for lifespan in *Drosophila* has previously been observed (Lehtovaara *et al.*, 2013). It is indeed possible that our genotype sample size resulted in inadequate power to capture additive variance, as evidenced by our large standard errors, and this issue may have been compounded by GREML SNP-based estimates' sensitivity to missing, rare causal variants (Yang *et al.*, 2017; Wainschtein *et al.*, 2019).

Alternatively, in estimating broad-sense heritability, we may be capturing additional, non-genetic variance in our estimates. Our approach, may therefore represent an upper limit of the heritability of ageing, and presents some caveats. First, we make an implicit assumption that any variation within-population is due to stochastic error, and omit it on that basis; the corollary being that any genuine environmental variance captured in our data will be partitioned into genetic variance. In this instance, the strength of within-cage environmental effects - perhaps due to heterozygosity, parental effects, or incomplete genetic control of ageing at a population-level - will be muted by recording a population-based estimate. Second, despite lack of complete homozygosity of genotypes within the DGRP (Huang *et al.*, 2014), our estimates invariably capture a degree of inbreeding depression (Charlesworth and Willis, 2009; Schou *et al.*, 2018) - this has the potential to significantly augment our estimates of genetic variance (but see Lee *et al.*, 2017), and is potentially a poor representation of natural variation. Third, our estimates are generated via a singular environment; by contrast, estimates of non-model organisms, like humans, encompass substantial environmental heterogeneity. Heterogeneity could result in a compression of estimates, due either to an increase in environmental variance, or gene-by-environment interactions - both of which would cause a relative reduction in genetic variance. The consistency with which environmental variance is controlled in our study is irregular in the natural world and thus, the translational relevance of our study could be questioned. Lastly, we acknowledge that our two day resolution of lifespan has the potential to artificially inflate estimates when compared to a resolution of one day.

Under the likely assumption that lifespan is stochastic and that the distribution of lifespan of a genotype represents the biology more closely associated to the ageing process, heritability of individual ages of death should not be interpreted as heritability of ageing. If ageing is indeed modulated by biological parameters only observable at a population-level, our estimates will represent a much more accurate assessment of heritability of *ageing* than those previously published. These previous low estimates of lifespan heritability may have cast doubt on the genetic determination of ageing. However, the high heritability we find when we analyse ageing as a population level trait across genotypes, implies an almost complete genetic control of ageing, despite our finding that a much of this genetic variance may be non-additive. Such considerations are key to translating knowledge from the biology of ageing to the clinic, especially when considering gene therapy.

In keeping with our observations, one previous study in *S. cerevisiae* estimated heritability, and performed downstream analysis on a population-based metric of longevity, finding broad-sense heritability between 0.72 and 0.9, depending on growth conditions (Jung *et al.*, 2018). Another study has partially detailed results which partitioned environmental variance in a manner akin to this study (Huang *et al.*, 2020). Line replicates were randomly assigned into two micro-environmental groups, but with lower estimates than we detail here (H^2 = 0.66 - 0.74). To our knowledge however, no studies, including the above, have emphasized the need to consider the heritability of ageing as a population-level emergent trait. The idea that ageing has a strong genetic component deserves renewed attention, and provides promise for personalised gene therapy to overcome specific genetic predispositions for a short life.

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Author Contributions

A.W.M. and M.J.P.S. designed and interpreted the experiments. A.W.M. drafted the first version of the manuscript. A.W.M. and M.J.P.S. revised the manuscript. A.W.M. led the data acquisition with help from M.J.P.S., G.A., and L.H. M.J.P.S. supervised the project.

Supplement

Figures

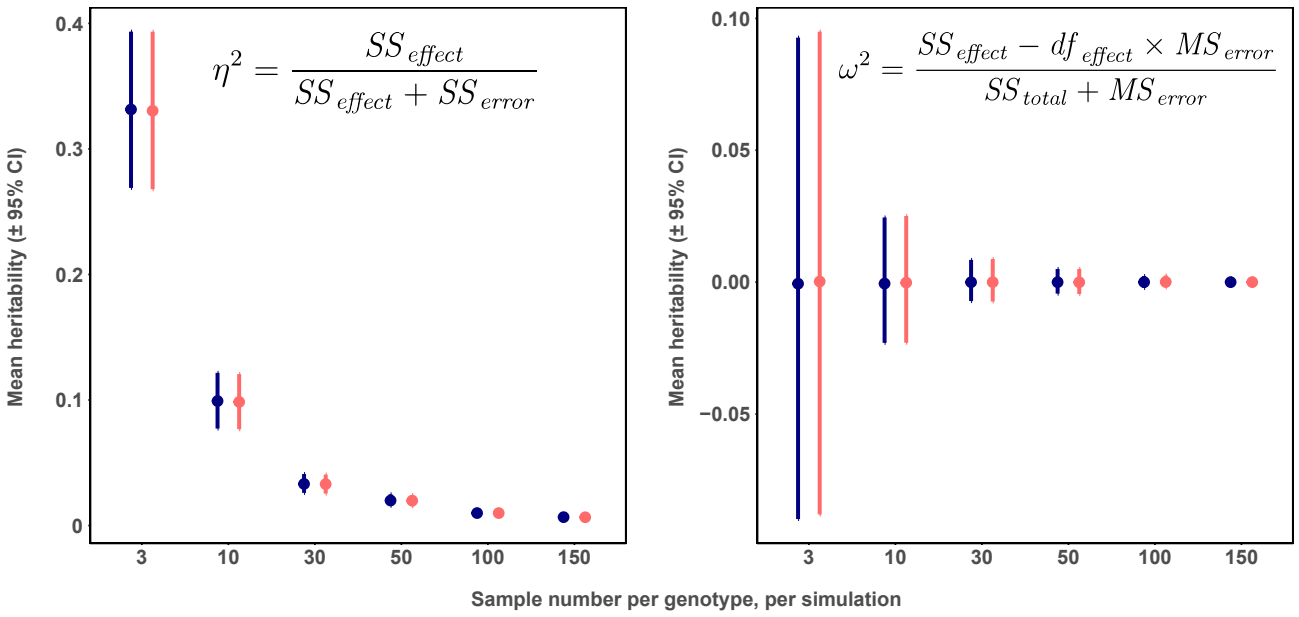

Figure S1. Eta-squared is a highly biased measure of effect size. Comparison of the typically used effect size estimator eta-squared (η^2; **left panel**) with the bias-corrected omega-squared (ω^2; **right panel**). All groups were sampled from the same Gaussian (blue) or Gompertz (orange) distribution to provide a null model. Mean effect size and variance increase in response to lowering sample size for η^2; only variance of the sampling distribution increases for ω^2. Note that ω^2 estimates can be negative when F < 1. N = 146 grouping variables for all simulations.

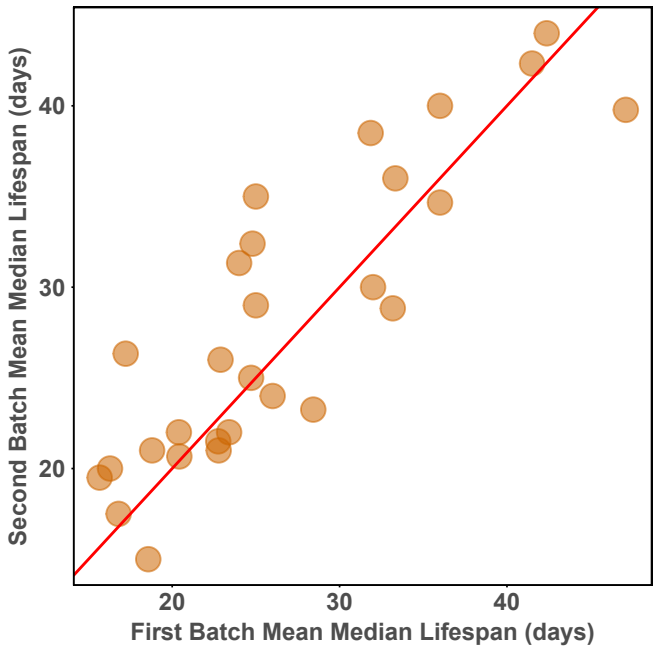

Figure S2. Mean median lifespan across batches is highly repeatable within genotypes. Regression of 28 genotypes' female mean median lifespan estimates from one batch, against another. These data represent all female genotypes assayed over more than one batch; where genotypes were assayed over more than two batches, the highest two sample-size batches were used. $R = 0.86$; $R^2 = 0.74$.

Table S1. Estimates of median lifespan heritability ($omega^2$) from across observed datasets, where each cage represent a data point.

	Full dataset					Restricted dataset				
	estimate	lower CI	upper CI	n	outliers	estimate	lower CI	upper CI	n	outliers
male	0.916	0.877	0.927	453	1	0.924	0.904	0.935	327	1
female	0.914	0.906	0.920	1459	19	0.914	0.905	0.921	1048	15

Table S2. Estimates of median lifespan repeatability (R) across datasets, where $R = V_G/(V_G + V_R)$.

	Full dataset				Restricted dataset			
	estimate	lower CI	upper CI	n	estimate	lower CI	upper CI	n
male	0.908	0.882	0.929	454	0.909	0.878	0.932	328
female	0.915	0.894	0.931	1478	0.917	0.892	0.936	1063

Table S3. Heritability estimates ($omega^2$) of Gompertz parameters from across observed datasets, where each cage represent a data point.

	Full dataset					Restricted dataset				
	estimate	lower CI	upper CI	n	outliers	estimate	lower CI	upper CI	n	outliers
male shape	0.711	0.448	0.730	454	0	0.797	0.734	0.820	328	0
male rate	0.783	0.723	0.802	453	1	0.865	0.828	0.883	327	1
female shape	0.806	0.786	0.816	1467	11	0.824	0.804	0.837	1053	10
female rate	0.817	0.799	0.827	1471	7	0.825	0.805	0.837	1060	3

Table S4. Estimates ofindividual lifespan heritability ($omega^2$) from across observed datasets, and downsampled estimates (n = 30), where each individual represents a data point.

	Observed estimates from dataset				Downsampled estimates		
	estimate	lower CI	upper CI	n	estimate	lower CI	upper CI
male (full)	0.407	0.400	0.413	40628	0.425	0.403	0.445
female (full)	0.460	0.457	0.463	168941	0.465	0.442	0.487
male (restricted)	0.400	0.393	0.407	37828	0.405	0.380	0.434
female (restricted)	0.452	0.449	0.456	122435	0.455	0.430	0.480

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Discussion

Summary

In this thesis, I studied the biology of ageing using inbred *Drosophila melanogaster* as a model, with a particular focus on the interplay between longevity, diet and genetic variance. I utilised a demographic-based approach, measuring age-specific mortality across different dietary regimes and genetic lines, using large sample sizes.

The value of reaction norms

Phenotypic plasticity - where individuals within a population exhibit temporal variation in their traits, in response to environmental conditions - is considered key in underpinning much of the within- and between-individual variability observed in nature. These plastic responses may have evolved as an adaptive response to particular environments (Nijhout, 2003), or as a means of evolutionary bet-hedging in highly unpredictable environments (Haccou and Iwasa, 1995). At its most extreme, plasticity can result in discrete phenotypes, or polyphenisms (Simpson, Sword and Lo, 2011). Phenotypic plasticity in response to diet (either caloric, or nutrient intake) has been well-studied, and perturbations can elicit robust effects on physiology and morphology (Liu and Wang, 2007; Naya *et al.*, 2008; Brzek *et al.*, 2009). Nonetheless, the value of lifespan reaction norms to diet within a DR paradigm remain underappreciated. In particular, several studies which indicate lifespan can be refractory to diet, or even curtailed in response to DR, base these conclusions on experiments that use two, rather than a range of diets (Liao *et al.*, 2010; Dick, Ross and Yampolsky, 2011; Swindell, 2012; Mitchell *et al.*, 2016; Jin *et al.*, 2020). Such conclusions have been drawn in the absence of genotype-specific reaction norms to diet, despite an existing theoretical framework as to why DR-driven longevity responses can sometimes be obscured (Flatt, 2014).

In chapter one, I detailed how diet-induced lifespan plasticity can vary substantially by genotype, and emphasised how an appreciation of dietary reaction norms is crucial in the interpretation of diet effects across genotypes and environments - especially when results seem to indicate an absence of the DR longevity effect. I showed for the first time in wild-derived inbred lines, how the typical bell-shaped response to diet - where lifespan reaches a maxima under DR conditions, and declines on either side of this optimal yeast concentration - may obscure the longevity effect when a dietary dyad is used. Indeed, in chapter two, the responses of lines to different temporal dietary regimes consisting of two diets, showed substantial genetic variation - including starvation. Acknowledging a dietary reaction norm would be overly laborious in some model systems, I suggested that diets differ minimally from one another in dietary dyad experiments, to reduce the likelihood of starvation or nutritional toxicity responses occurring. This suggestion will be of particular importance for studies focusing on genetic variance in the DR response. My results show

there to be strong genetic elements to dietary reaction norms, and that considerable appreciation of a reaction norm framework is necessary when interpreting diet effects across genotypes.

If reaction norms to diet vary strongly between individuals, measuring longevity or health outcomes in a single environment may only provide a narrow or biased perspective. Such considerations could be key to understanding differences in longevity within a biomedical context. Some individuals may be more prone to accelerated ageing because of a certain susceptibility to environmental factors - like diet - that shape ageing. The fundamental study of the evolutionary significance of phenotypic plasticity could prove valuable in understanding why there is standing genetic variation in humans for such responses.

Water is a nutrient

Nutritional targets are highly regulated, and organisms will pursue an intake pattern of nutrients which favours maximal lifetime reproductive success, which often means investing in reproduction at the expense of longevity (Raubenheimer and Simpson, 1997; Simpson *et al.*, 2004; Lee *et al.*, 2008). In several species it has now been demonstrated that the protein:carbohydrate ratio is the primary response axis of longevity (Lee *et al.*, 2008; Maklakov *et al.*, 2008; Solon-Biet *et al.*, 2015; Simpson *et al.*, 2017). Flies, specifically, strictly regulate intake of protein and carbohydrate to maintain a constant ratio of 1:4 (Lee *et al.*, 2008; Jensen *et al.*, 2015) presumably to maximise their fitness. Deviations from nutritional targets constitute a cost; the most advantageous strategy is therefore the minimisation of deviation between target and intake (Simpson *et al.*, 2004; Cheng, Simpson and Raubenheimer, 2008). However, most experimental systems which utilise *Drosophila* as a model organism when investigating DR, offer no choice in nutritional intake: micro- and macro-nutrient composition of the diet is decided by the investigator. Besides nutritional choice, it is highly unusual for flies to be provided with supplementary water. This lack of choice may compel a compromise, whereby the consumption of nutrients, or calories, continues beyond a saturation point, to meet certain minimal nutritional requirements (Raubenheimer and Simpson, 1997; Simpson *et al.*, 2004; Ja *et al.*, 2009).

Previous studies have suggested flies routinely advance past their caloric saturation point to adequately hydrate (Dick, Ross and Yampolsky, 2011; Fanson, Yap and Taylor, 2012); one previous study even indicated that desiccation was the proximate cause of the DR longevity effect (Ja *et al.*, 2009). This had been disputed by Piper *et al.*, who observed no rescue of mortality by water in their experimental system (Piper *et al.*, 2010). In chapter one, I provided additional, robust evidence against the causal nature of desiccation in the

DR longevity response, by detailing strong lifespan reaction norms to diet remained in the presence of water supplementation.

My results did show, however, that water can indeed play an extensive confounding role in DR experiments, with desiccation significantly reducing lifespan in some genotypes, under high yeast conditions. This is in line with previous research showing flies consider water to be a nutrient (Fanson, Yap and Taylor, 2012), and routinely consume around 1-2µl per day (Lehmann, Dickinson and Staunton, 2000), with even higher consumption at higher dietary yeast (Fanson, Yap and Taylor, 2012) and sugar consumptions (van Dam *et al.*, 2020). As smaller flies, Drosophila melanogaster are particularly susceptible to desiccation: glycogen oxidation only accounts for ~20-25% of water replenishment when active and ~9% when at rest (Lehmann, Dickinson and Staunton, 2000). By means of comparison, other insects are capable of either complete evaporative water loss restoration (Roberts, Harrison and Hadley, 1998), or manufacture of a substantial excess via metabolic water production (Bertsch, 1984). My results therefore demonstrate water has a considerable importance in studies in the fly, and a potentially significant role as an experimental confound in lifespan experiments. These considerations could also impact on the longevity data presented in chapter three, since some genotypes may be especially susceptible to desiccation. Reaction norms are likely to arise at many levels of physiology and thus measuring individuals in one environment will likely capture genetic variance for separate, non-distinguishable reaction norms.

The cost of dietary restriction

In chapter two, I tested the most direct prediction that follows from the evolutionary model of DR (Shanley and Kirkwood, 2000; Kirkwood and Shanley, 2005) - that diet restricted individuals, when returned to a nutrient-rich environment, should be better equipped to survive and/or reproduce, given increased investment in somatic maintenance. In contrast to these expectations of this model, flies maintained on DR for a long period of time, or only intermittently, suffered a loss of fitness when fed a rich diet. I observed genetic variance for both these novel mortality phenotypes. In an attempt to tease apart the nature of these costs, I performed asymmetric dietary switches. Here, I found the magnitude of the mortality exacerbation increased according to the duration of a DR diet - something indicative of direct costs of the DR state. Most strikingly (and in line with the idea of costs of DR) the reduction in lifespan and fecundity occurring when returning to a rich diet was observable even in genotypes which suffered a starvation response under a restricted diet. The hidden costs associated with DR are therefore independent of it facilitating a pro-longevity response. Attempting to identify a mechanism for the mortality phenotypes, I performed a dietary switch under conditions of microbiome depletion, water supplementation, and social isolation - finding mortality exacerbation to be independent of

all. While the microbiome is often considered an important factor mediating the effect of diet, a manipulation of the microbiome and DR together has rarely been conducted (but see Snyder *et al.*, 1990; Tazume *et al.*, 1991) and as such, the microbiome has not yet been established as causal in the DR prolongevity response. Similarly, exacerbation of mortality observed in these dietary regimes was not contingent upon sex. I therefore posit that DR contributes to, as yet unknown, costs to the organism, which are only observable upon resumption of a nutrient-rich diet. It may be that a degree of adaptation to the DR state renders the organism more susceptible to costs of a rich diet. Alternatively, these costs may be more direct in nature, more in line with the lethal protein hypothesis.

In summary, I was able to conclude that restricted diets can, under certain circumstances, be the origin of particular types of damage to the individual. My results have now pointed towards a more refined explanation of why the DR longevity response occurs - an explanation which does not necessitate DR being seen as intrinsically pro-longevity, or adaptive in origin. This is in line with more mechanistic hypotheses of DR, some of which also challenge the perception of DR being an adaptive response. For example, the DR longevity effect has been suggested to be a passive, hormetic response (Masoro, 1998; Parsons, 2000). Like exercise, DR may induce mild oxidative or metabolic stress; favourable physiological adaptations would then occur as a result of repair processes, which confer an improved capacity to tolerate future, greater insults (Coyle, 2000; Peake *et al.*, 2015). The precise nature of these physiological costs is unknown, but both DR animals, and the ageing phenotype, are associated with the upregulation of stress pathways - like heat shock proteins and antioxidative enzymes (Landis *et al.*, 2004; Pletcher, Libert and Skorupa, 2005; Rattan, 2008). At an organismal level, organisms undergoing DR are known to have impaired immune function and cold tolerance (Puerta and Abelenda, 1987; Kristan, 2008; Carrillo and Flouris, 2011), and it has been pointed out that DR is unlikely to yield any survival advantage in the wild, given DR reduces capacity of the organism to endure these environmental insults (Adler and Bonduriansky, 2014). Since the degree of mortality exacerbation in my flies was in line with the duration of DR preceding it, my data also allowed me to conclude a period of acclimation may be necessary to generate the costs of DR. My results are therefore in line with the idea that the DR longevity response is not adaptive in origin, especially if acclimation to a restricted diet occurs.

The cost of a protein-rich diet

In chapter one, I observed a levelling-off, or decrease, of daily and lifetime reproductive output was observable at the highest yeast concentration, while lifespan continued to decline. Of note, desiccation-induced mortality was able to be rescued by water supplementation, in contrast to the decline in reproductive output. These results allowed me to tentatively conclude that an element of nutritional toxicity, or overfeeding, was

present in most genotypes, since the highest concentration diet was inducing a loss of fitness. Likewise, in chapter two, my overall results also suggested that a DR diet is not intrinsically pro-longevity; indeed perhaps the simplest explanation for the mortality exacerbation observed in flies undergoing dietary switches, was that a DR diet was sensitising the organism to the costs of a rich diet. I therefore suggested a more parsimonious explanation for the DR longevity response: rich diets contribute direct physiological costs to the individual.

The common interpretation of the DR longevity response is that DR confers a protective, pro-longevity effect, operating via an increase in energy apportioned to somatic maintenance. My results therefore imply an inversion of this typical interpretation, instead positioning rich diets as possessing direct physiological costs. This is consistent with the lethal protein hypothesis (Lee *et al.*, 2008; Fanson *et al.*, 2009; Mautz *et al.*, 2019; Moatt *et al.*, 2020), which posits organisms nonetheless consume excess dietary protein to maximise reproductive output. Some limited evidence for this exists empirically (Fanson, Fanson and Taylor, 2012), but further test of key predictions will be necessary.

Heritability and the consistency of environment

The heritability of lifespan in various species has been widely estimated, often as surprisingly low (Johnson and Wood, 1982; Herskind *et al.*, 1996; Mitchell *et al.*, 2001; Martin *et al.*, 2002; Durham *et al.*, 2014). In chapter three, by modelling individual lifespan, I was able to replicate heritability of lifespan estimates previously reported in the literature for the DGRP. I then suggested that heritability of lifespan and that of ageing-related physiology are not necessarily the same. Lifespan is the stochastic result of ageing physiology that could potentially be wholly genetically determined. If ageing is regulated at the population as an emergent trait, then an optimal metric of the ageing process would be one devoid of this stochastic noise inherent in lifespan distributions. To estimate heritability of ageing devoid of stochastic effects I made use of inbred lines that allow the repeated measurement of lifespan from the same genotype. I found broad-sense heritability of ageing to be significantly higher than estimates of heritability of lifespan. This is, at least in part, because lifespan is a highly stochastic trait, and a product of a heterogeneous ageing process. High heritability could therefore mean ageing has a very strong genetic component, in keeping with observations that manipulations of single genes can have profound lifespan extending effects.

However, I found additive genetic variance to have a particularly low explanatory power of this trait. Were additive genetic variance to indeed be negligibly low, this would elicit a very small response to selection (Falconer and Mackay, 1996). Estimates of additive variance may have been compressed by sample size and dependence on the quality of the genomic

relatedness matrix. Alternatively, ageing may indeed be regulated via dominance variance, or a network of epistatic interactions, as has been suggested previously (Huang *et al.*, 2012; Mackay and Moore, 2014). However, I acknowledged this was perhaps suggestive of environmental variance being absorbed into genetic variance in my approach, given the implicit assumption that any within-population variation is due to stochastic error. The stochasticity inherent in lifespan precludes a fair assessment of the heritability of ageing, but my approach - which necessarily minimises the scope of environmental variance - will also represent an upper limit.

Heritability, as an index of relative genetic control of the variance of a trait has been subject to misinterpretation and criticism (Rose, 2006; Moore and Shenk, 2017). Indeed, partitioning the variance is often fraught with complications; where relevant, causal environmental variance is absent, inferring causality can lead to flawed conclusions. This effect could be particularly potent in experimental systems where there is complete environmental homogeneity (Lewontin, 1974). As such, the degree of environmental heterogeneity that exists in nature would be expected to contribute substantially to phenotypic variance. This may mean the heritability of ageing in a naturalistic setting may be substantially lower.

Concluding remarks

This thesis offers novel insights into the mechanism of dietary restriction, the magnitude of diet-induced phenotypic plasticity, and genetic control of the ageing process. My results have cast doubt on the evolutionary mechanisms which underpin the DR longevity response, and may better guide future research in the field. In particular, the dietary-induced mortality phenotypes observed could prove a useful experimental tool to investigate the novel costs of DR. The future elucidation of these costs may afford a substantially greater understanding of the physiological trade-offs which precipitate the ageing process. I have also provided robust evidence both for genetic variance in the plastic response to diet, and for an environmentally-induced confound. These results will assist researchers in the prevention of poor experimental design and drawing of erroneous conclusions. They may also help to reconcile seemingly anomalous results in different experimental designs. Finally, my novel approach for characterising the heritability of ageing has lent credence to the possibility of high genetic control of this trait, and offers renewed optimism for the potential of therapeutic interventions, especially personalised gene therapy.

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