MONASH University

Cross-generational effects of nutrition on life history in *Drosophila melanogaster*

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MBiolAnth (Adv) Hons

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Thesis abstract

By varying the environment of organisms, we can alter their physiology, which in turn can alter their life history, these changes occur due to a plasticity of phenotypes. Parents can make many contributions to their offspring, beyond the effects of passing on their genes alone. Consequently, when the parental environment varies, so do the contributions to the offspring, resulting in variations to offspring life history. A range of environmental changes can mediate cross-generational plastic effects, and nutrition is a major source of environmental variation. Although recent studies have begun to elucidate the effects of nutrition on offspring life history, many outstanding questions remain about the nature of how nutritional changes in parental environments shape offspring life history and physiology. I have addressed these questions in my thesis through four chapters of work, using *Drosophila melanogaster* as an experimental model to address the following aims.

First, little is known about the relative contributions of maternal and paternal dietary-mediated effects and the possibility for parental diets to interact, because most studies investigate parental effects separately, and more focus has traditionally been given to maternal effects. I uncovered complex non-additive interactions between parental diets that shaped the life history outcomes of both parents and offspring—whereby the sucrose content that was optimal for the parents was not optimal for the offspring. Second, whether cross-generational effects of nutrition can be sex-specific in their effects on male and female offspring is currently unknown. I elucidated this aim by finding that grandparental diets had differing effects on life history expression among F2 offspring that were dependent on the sex of the F2. Third, as many studies lack the required full factorial designs to test for adaptive cross-generational anticipatory effects, whether parents or grandparents can prime offspring (via anticipatory effects)—advantaging offspring in matching nutritional environments— is still under debate. I tested this cross-generationally (F0-F1-F2), using a full factorial design, and was unable to detect priming effects; in fact both my studies into this question showed that mismatched combinations of F0 diets, and F0-F1 diets, and F0-F2 diets were more advantageous to offspring life history.

Next, as studies tend to focus on using hypercaloric or obesogenic diets, it is not yet known whether dietary-mediated cross-generational effects are unique to the particular macronutrients manipulated, few studies vary protein level, therefore it is unknown if effects

mediated by manipulation of carbohydrate levels in the diet are similarly induced by protein manipulations. I found that cross-generational effects were specific to the individual macronutrients used, by revealing that F0 protein played a role in shaping female F2 reproductive output, but F0 carbohydrate did not. Finally, the majority of studies exploring the effects of parental nutrition on offspring are intergenerational (F0-F1), with far fewer exploring effects beyond the F1 generation. It is therefore unknown whether the cross-generational effects of nutrition will be concordant across generations. By testing dietary-mediated effects in both inter- and transgenerational contexts, I uncovered that effects were concordant across generations in some contexts, but not in others.

Declaration

This thesis is an original work of my research and contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

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Publications during enrolment

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Camilleri, T.L., (2019). Intrasexual Violence and Aggression. In: Shackelford T., Weekes-Shackelford V. (eds) Encyclopedia of Evolutionary Psychological Science. Springer, Cham. https://doi.org/10.1007/978-3-319-16999-6_259-2

Camilleri, T. L., & Kushnick, G. (2018). Male aggressiveness as intrasexual contest competition in a cross-cultural sample. *Behavioral Ecology and Sociobiology*, *72*(6), 1-9.

Thesis including published works declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes *two* original papers published in peer reviewed journals and *zero* submitted publications. The core theme of the thesis is evolutionary and ecological biology. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the School of Biological Sciences under the supervision of Prof Damian Dowling, A/Prof Matthew Piper, and Prof Rebecca Robker.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of chapters two and three, my contribution to the work involved the following:

Thesis Chapter	**Publication Title**	**Status** *(published, in press, accepted or returned for revision, submitted)*	**Nature and % of student contribution**	**Co-author name(s) Nature and % of Co-author's contribution***	**Co-author(s), Monash student Y/N***
2	Transgenerational obesity and healthy aging in drosophila	*Published*	*55%. Concept and writing first draft, and editing*	*1) Matt Piper input into manuscript concept, drafting and editing 25%* *2) Damian Dowling input into manuscript editing 15%* *3) Rebecca Robker input into manuscript editing 5%*	*No* *No* *No*
3	Maternal and paternal sugar consumption interact to modify offspring life history and physiology	*Published*	*65%. Concept and collecting data, analysing, and writing first draft*	*1) Damian Dowling input into manuscript concept, analysis drafting and editing 25%* *2) Matt Piper input into manuscript editing 10%*	*No* *No*

				3) Rebecca Robker input into concept, and manuscript editing 10%	*No*

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student name: Tara-Lyn Camilleri **Student signature:** **Date:** 30/06/2022

I hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor name: Damian K Dowling

Main Supervisor signature: **Date:** 30/06/2022

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Reflecting upon the past few years is a somewhat complicated endeavour for me. I should stick to the convention of thanking all the people who absolutely deserve it, and be done with it, but I hope the reader will indulge me some reflection. The time since I began my PhD has been full of challenging life events, including struggles with my own health, the health of loved ones, and the loss of loved ones. The transition to living my life with more limitations has coincided with a time when the world has been living with more limitations due to the covid pandemic. It has all taken a toll, and yet, I have endured through to see it to the end.

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Chapter 1 | General Introduction

“Life on earth is such a good story you cannot afford to miss the beginning...”

-Lynn Margulis (founder of endosymbiosis theory)

1.1 | Nutrition, phenotypic plasticity, and life history

In response to modifications in the environment, organisms can change a multitude of phenotypes, such as their behaviour and morphology, in order to cope with the environmental variation. Such plasticity of phenotype can induce changes in an organism's overall fitness, and can affect development in response to variation across a wide variety of environmental conditions, such as climate or temperature, nutrition, parasitism/pathogen presence, and predator presence (Baldwin, 1896; Berg & Ellers, 2010; Bradshaw, 1965; West-Eberhard, 2003, 2005). A major type of environmental variation affecting the expression of organismal phenotypes is nutrition. A diet comprises of a combination of nutrients, and the primary macronutrients are protein, carbohydrate, and fat. Variation in nutrition can fundamentally affect the health and life history of most organisms. Indeed, geographic distributions of species, and mating systems within species, are often determined by nutritional resource distribution and availability (Simpson & Raubenheimer, 2012). Furthermore, patterns of reproduction are also closely tied to the availability of certain nutritional resources. A prominent example is the kakapo parrot (*Strigops habroptilus*), which only mates once every two to five years. This timing coincides with years when fruits are abundant enough for the parents to access adequate nutrition to raise chicks, with dietary variation thus being a large contributor to the highly endangered status of the species (Elliott, Merton, & Jansen, 2001).

While for some species, it is food availability that is the main determinant of variation in the expression of life history traits, for others food may be continually available, but the nutritional composition of their food may vary temporally. For instance, a reduction in breeding pairs of kittiwake gulls (*Rissa brevirostris*) was linked to a lower abundance of lipid-rich fish present, forcing birds to consume fish with a lower lipid content. Even if the caloric

content of the diet was the same, the underconsumption of lipids for a red-footed kittiwake gull resulted in cognitively impaired chicks, which were less able to learn and recall important information about associations between food colours and their nutritional value and location. This ultimately led to birds that were less fit, and less able to survive and reproduce (Kitaysky, Kitaiskaia, Piatt, & Wingfield, 2006). Furthermore, variation in macronutrient balance can result in trade-offs between life-history traits. In fruit flies (*Drosophila melanogaster*), lower protein (and higher carbohydrate) diets can extend lifespan, and reduce reproduction of females, whereas higher protein (and lower carbohydrate) diets can reduce lifespan, but increase reproduction (Silva-Soares, Nogueira-Alves, Beldade, & Mirth, 2017).

Phenotypic plasticity provides animals with a key mechanism to deal with a changing nutritional landscape. Balancing the correct intake of nutrients is complex however, even when seemingly only one food type needs to be consumed. For example, it was thought that adult butterflies need to only consume carbohydrate-rich nectar (sucrose, glucose, fructose and water), because the key nutrients (such as protein) required to sustain adult survival and reproduction are ingested during juvenile development, when butterflies are larvae (Simpson & Raubenheimer, 2012). Recent studies have suggested, however, that there are complex interactions between larval nutrient and adult nutrient consumption that have important effects on fecundity and thus fitness (Geister, Lorenz, Hoffmann, & Fischer, 2008). Indeed, most animals need to consume a range of foods, each that usually contain multiple nutrients requiring consumption at adequate levels, but not resulting in under or over nourishment. Achieving this nutritional balance is multifaceted, and involves both changing nutrient requirements and availability of foods (Simpson & Raubenheimer, 2012).

In recent years, the study of such nutritional complexity has developed with the rise of nutritional geometry, an approach that represents nutrient variation in graphical space.

The framework enables variation in one macronutrient to be interpreted in relation to variation across other macronutrients, rather than the alternative of investigating one nutrient at a time, in isolation of the others. The nutritional geometry framework therefore allows researchers to study complex nutrient interactions, and their effect on organismal trait expression. This enables researchers to precisely define nutritional variation, and allows for scaffolding interpretation of new results to previous studies—informing experimental designs, and facilitating broader comparisons across taxa (Simpson & Raubenheimer, 2012).

1.2 | Inter- and transgenerational plasticity

Parents make many contributions to their offspring beyond the direct effects of passing on their genes. When the parental environment varies, so do the contributions to offspring, and resultant offspring fitness (Russell Bonduriansky, Crean, & Day, 2012; Gluckman, Hanson, & Low, 2019; Marshall & Uller, 2007; T A Mousseau & Dingle, 1991; Timothy A. Mousseau & Fox, 1998; Timothy A Mousseau, Uller, Wapstra, & Badyaev, 2009; Nystrand, Cassidy, & Dowling, 2016; Uller, Nakagawa, & English, 2013). Generational plastic effects, which is plasticity passed from one generation to the next, can be mediated by a vast range of parental environmental changes or stresses. Changes such as variation in parental exposure to light, temperature, toxins, circadian rhythm, immunity, nutrition, and parental age can all elicit a plastic response that can be passed on to subsequent generations (Baker, Sultan, Lopez-Ichikawa, & Waterman, 2019; Bell & Hellmann, 2019; Donelan et al., 2020; Nystrand & Dowling, 2014; Sultan, Barton, & Wilczek, 2009; Wylde, Spagopoulou, Hooper, Maklakov, & Bonduriansky, 2019). Parental effects that are considered ‘non-genetic’ are triggered by either condition-dependant mechanisms such as the direct effects of a variation in parental care or changes in seminal fluid, or through environmentally-induced epigenetic changes in the regulation of genes and gene expression (Curley, Mashoodh, & Champagne, 2017). Plastic responses

that occur across generations are termed intergenerational effects when effects span one generation, and transgenerational effects when effects span multiple generations (and when offspring have no direct experience of the grandparental environment) (Nystrand & Dowling, 2014). Such effects have been found across taxa—in both invertebrate and vertebrate animals, plants, fungi, and bacteria (Dyer et al., 2010; Jablonka & Raz, 2009; Roach & Wulff, 1987). We use the term cross-generational as a catch-all term for any inter- or transgenerational work henceforth.

1.3 | Inter- and transgenerational effects of nutrition

Since gamete investment is larger in females (Kokko, Brooks, Jennions, & Morley, 2003; Trivers, 1974), cross-generational studies of nutritional effects have historically focused on maternal contributions. These studies fit into some main themes of research—in addition to being primarily maternally focused, many studies across taxa (especially model species such as *Mus musculus* and *Drosophila*) are focused on the implications of high sugar and high fat ingestion, thereby seeking to link maternal obesity and metabolic disease predisposition in offspring. These studies are also primarily intergenerational; that is, they study effects from parent (usually mother) to offspring (Bonduriansky, Runagall-McNaull, & Crean, 2016; Guida et al., 2019; Hibshman, Hung, & Baugh, 2016; Matzkin, Johnson, Paight, & Markow, 2013a; Öst et al., 2014; Perez & Lehner, 2019; Polak et al., 2017; Sanchez-Garrido et al., 2018). A few studies look at effects that span more than one generation (Buescher et al., 2013; Deas, Blondel, & Extavour, 2019; Dew-Budd, Jarnigan, & Reed, 2016; Emborski & Mikheyev, 2019; Ivimey-Cook et al., 2021; Krittika & Yadav, 2022). Both inter- and transgenerational studies have uncovered several interesting effects of high sugar and high fat on both parental and offspring phenotypes. They reveal that intergenerational effects

of these diets can change metabolic regulators, body size, body protein, and fat levels, but that effects are often not concordant between generations.

Similarly, few studies investigate whether nutritional variation in the paternal lineage is important, or whether dietary variation in maternal and paternal lineages may interact to shape cross-generational phenotypes. Despite the focus on maternal effects, studies on paternal effects have recently begun emerging (Anwer, Morris, Noble, Nakagawa, & Lagisz, 2022; Crean & Bonduriansky, 2014; Hellmann, Carlson, & Bell, 2020; Öst et al., 2014; Polak et al., 2017; Watkins et al., 2018). For example, an intergenerational study using *D. melanogaster* tested the effects of paternal high sugar on male offspring traits, and observed epigenetic effects via chromatin state alteration that may predispose male offspring to obese-like phenotypes (Öst et al., 2014). Indeed, a recent meta-analysis of transgenerational effects of obesogenic diets in rodents found that only ~21% of studies investigated grandpaternal effects (7 of 33 studies overall). They did however (despite the imbalance in studies) find that the effect of grandpaternal exposure to obesogenic diets (if exposed before mating) had a significant effect on grandoffspring traits (primarily adiposity), albeit weaker than grandmaternal effects, with the grandmaternal mean effect sizes around 23% higher (Anwer et al., 2022).

1.4 | Sex specific nutrient requirements

Studies across taxa show that females and males require different diets to maximise their fitness (Arnqvist & Rowe, 2005; Brommer, Fricke, Edward, & Chapman, 2012; Kokko et al., 2003; Reznick, 1985; Zajitschek, Dowling, Head, Rodriguez-Exposito, & Garcia-Gonzalez, 2018). Generally, female fitness is maximised on a higher relative protein concentration because high protein facilitates egg production, while higher relative carbohydrate content for males provides fuel for attracting and locating a mate (Blanckenhorn

et al., 2002; Camus, Huang, Reuter, & Fowler, 2018; Crudgington & Siva-Jothy, 2000; Gavrilets, Arnqvist, & Friberg, 2001; Reddiex, Gosden, Bonduriansky, & Chenoweth, 2013). Studies in *Drosophila* have been valuable in determining what effect protein in females has on reproduction. Similar to humans, fruit flies require ten essential amino acids from their diet (humans need to ingest nine), and the absence of any of these essential amino acids in the diet halts production of eggs. Although sensitivity to the depletion of each amino acid differs (and is still under investigation), relatively high levels of protein in female fruit flies coincide with higher offspring production (Mirth, Nogueira Alves, & Piper, 2019). One factor that may cause sex differences in nutritional requirements are the differences in gamete size between females and males. Males will often produce a larger number of tiny sperm cells that are energetically cheap to produce, per gamete, whereas females tend to produce a relatively small number of energetically costly and large eggs. Anisogamy describes how the female egg is much larger than the male sperm, and the yolk contains nutritional reserves of lipids, proteins, and polysaccharides that can support zygotic development. Therefore, this difference in gamete size between males and females forms the basis for the assumption that the maternal contribution to offspring early-life success and development will be larger than the male contribution (Clutton-Brock, 2019; Kokko et al., 2003; Trivers & Campbell, 1972).

Although evidence has emerged pointing to sex-specific nutrient requirements within a generation, little is known about sex-specific effects of nutrition cross-generationally. Few studies investigate whether dietary changes can invoke sex-specific inter- or transgenerational effects on offspring phenotypes. Intriguingly, two studies using *D. melanogaster* found that transgenerational diet effects tend to manifest in the opposite grandoffspring sex to that subjected to the grandparental treatment. For example, modification of the grandpaternal environment may enhance or inhibit trait expression among

granddaughters; alternatively modification to the grandmaternal environment may enhance or inhibit trait expression among grandsons (Buescher et al., 2013; Dew-Budd et al., 2016). Some of the inferences of these studies, however, might be limited by their experimental designs. Buescher et al., (2013) found that a high sucrose level administered to adult F0 dams increased the metabolic regulators (regulation of metabolic pathways i.e. glycogen levels) of her F1 an F2 male larvae, but reduced F2 female triglyceride levels. However, F1 female and F0 males were not tested or reported on, making determining the true nature of these sex-specific transgenerational effects difficult.

A further study in *D. melanogaster* by Dew-Budd et al., (2016), employed a full factorial design that was able to trace the transgenerational diet effects through the maternal or paternal lineage. They found sex-specific transmission of effects, whereby dietary-mediated effects mediated through the maternal lineage altered the expression of male offspring traits such as body weight (but not the body weight of female offspring). The converse was true for the paternal line, with the researchers finding that dietary-mediated effects passed through the paternal line effected female offspring weight (but not male offspring weight). Drawing inferences is complicated by the choice of diet in this study, as the focus was obesity; the researchers administered a high fat diet (and a control) to *D. melanogaster.* Flies, however, do not naturally consume fat (in any significant proportions); either in the wild nor in diets provided in the lab, therefore the ecological relevance of these findings may be questioned. Consequently, it is difficult to determine whether effects seen are artefacts of an unusual diet for the fly.

Evidence from other species employing other types of environmental variation however, do concur with the sex and parental lineage specific effects seen in both Buescher et al., (2013) and Dew Budd et al., (2016). An investigation into the cross-generational effects of opioid drug use in mice found sex-specific results, particularly that F0 paternal effects of

morphine ingestion exerted differing effects on the behaviour of F1 male and F1 female progeny. F1 males (from fathers that ingested morphine) showed signs of more anxious behaviours compared to F1 females who showed significantly less indications of anxious behaviours (Brynildsen, Sanchez, Yohn, Carpenter, & Blendy, 2020). Similarly, a study of sticklebacks (*Gasterosteus aculeatus*) found sex-specific transgenerational effects of predator presence. Grandparents were exposed to a predation risk whereby experimenters exposed sticklebacks to a clay model sculpin fish six times over 11 days. When paternal grandfathers were exposed to this predation risk, their female F2 offspring showed reduced behavioural change when under direct predation risk themselves, and had an increased body weight,(compared to F2 male offspring from paternal grandfathers. Conversely, F2 males from maternal grandfathers exposed to the predation risk showed reduced behavioural change in the presence of predators compared to F2 female offspring from maternal grandfathers (Hellmann et al., 2020). Understanding to what degree these effects extend across taxa and nutritional contexts is currently hindered by a lack of studies, especially transgenerational studies.

1.5 | Evidence for anticipatory parental effects of nutritional variation

It has long been predicted that parents may have the ability to anticipate or adaptively prime offspring, through non-genetic means (i.e. epigenetic), to prepare them for the environment they are likely to face (Mousseau et al., 2009). The prediction assumes that the environments that offspring are exposed to are likely to covary with the environments that their parents experienced. Again, like much cross-generational work, this prediction has mostly been tested in the context of maternal effects (Marshall & Uller, 2007). In order to test this prediction, an experimental design must be employed that is full factorial, whereby the parents and offspring are exposed to both matched and mismatched environments. If parents

indeed prime their offspring to have an adaptive response when offspring experience the same environment as their parents (or grandparents), then when the environments match between offspring and parents, offspring should express traits that maximise fitness (Mousseau & Fox, 1998; Uller et al., 2013; Yin, Zhou, Lin, Li, & Zhang, 2019). While some studies have found support for this prediction, the results of meta-analyses (looking at plants, invertebrates and vertebrates) conducted to date have found that evidence for anticipatory parental effects is generally weak, and may be context dependant (Sánchez-Tójar et al., 2020; Uller et al., 2013).

Why some studies have found support for anticipatory effects, while others have not, remains unclear but may likely be partly explained by methodological differences between studies. For example, some studies attempting to infer evidence of anticipatory effects, have failed to test offspring trait expression under matched relative to mismatched combinations of parent-offspring environments. Studies may also suffer from inferential limitations if they are unable to partition condition-dependent effects (e.g. silver spoon effects) from effects that are truly anticipatory in nature, and mediated by epigenetic mechanisms (Bonduriansky & Head, 2007; Bonduriansky & Crean, 2017). Further, one meta-analysis investigating cross-generational effects of environmental change concluded that such effects enhanced offspring phenotypes, in both stressful and benign conditions (so long as conditions between offspring and parents matched), and were likely therefore to be adaptive, but noted these effects occurred primarily in annual plants and invertebrates (Yin et al., 2019). In response, Sanchez-Tojar et al., (2020) reported biases in the analysis and the literature search of the study. The authors found that the literature search conducted by Yin et al., (2019) had limited coverage of the available studies, and was not reproducible, and the analysis did not fully account for non-independence of effect sizes, and non-full factorial studies were included if published after 2013 (but not before). These biases raised doubt about the conclusions made by Yin et

al., (2019) again drawing into question how generalizable parental anticipatory effects might be.

Few studies have tested for anticipatory parental effects in the context of nutrition and life history. Moreover, most studies investigating the cross-generational effects of nutrition to date have not employed the full factorial designs that are needed to test for anticipatory effects (see: (Buescher et al., 2013; Huypens et al., 2016; Matzkin et al., 2013a; Oldham, 2011; Öst et al., 2014; Polak et al., 2017)). One exception is a study that examined the transgenerational consequences of intermittent fasting in *C. elegans,* and found evidence that matching of fasting regimes (between F0 and F1) augmented F1 offspring fitness. The same study however, found several fitness costs of F0 intermittent fasting in subsequent generations, primarily F3 (Ivimey-Cook et al., 2021). As intergenerational adaptive effects may be condition-dependant (i.e. silver spoon effects), this study highlights the potential for nuance and context (or generation) specificity in determining whether a parental effect is adaptive. Furthermore, it is currently unclear what the magnitude of transgenerational effect sizes mediated by changes to nutrition are relative to changes mediated by other sources of environmental heterogeneity, such as climatic variation, or pathogen load (Sánchez-Tójar et al., 2020; Uller et al., 2013; Yin et al., 2019). Still, given high levels of spatial and temporal heterogeneity of food sources naturally available to populations (particularly in species that rely on ephemeral resources to fulfil their dietary requirements), nutritional variation seems an excellent candidate to drive anticipatory effects. Disentangling whether these effects exist may be key to understanding the evolution of cross-generational plasticity (Burgess & Marshall, 2014). Anticipatory effects linked to nutrition are also under investigation in humans; the next section covers this literature in more detail.

1.6 | Cross-generational nutritional effects in humans

Many studies investigating the cross-generational effects of nutrition in humans focus on obesity transmission. These studies use the term metabolic programming, to indicate instances where the diet of the parent results in non-genetic imprinting on the offspring both before and during foetal development (González-Muniesa et al., 2017). Studies report that hypercaloric diets containing high sugar or fat that lead to obesogenic outcomes in parents may predispose offspring (or even grandoffspring) to a greater risk of obesity. These studies also report that parents in nutritionally scarce environments may prime offspring to be more predisposed to obesity, if those offspring find themselves in nutrient abundance, due to a mismatch in environments (Kaati, Bygren, & Edvinsson, 2002). By necessity however, this research is based on cross-generational correlations and historical investigations, therefore drawing causation from these studies is very difficult. Yet, the assumption that parents can predispose or program their children for obesity (beyond the direct effects of their genetic contribution alone) endures.

These fascinating associations in humans have led researchers to turn to model species to try to establish causative evidence for mechanisms that might mediate the patterns. The current cross-generational literature investigating obesity in model species overwhelmingly use mice (*M. musculus*), as non-primate mammalian models. As discussed earlier, full factorial designs are required in laboratory experiments that attempt to test whether anticipatory (priming) parental effects exist. There are however, ethical implications involved in using rodents for experiments that are demanding of high sample size, and due to multiple treatments and full factorial designs, cross-generational experimental designs require large sample sizes. Therefore, most rodent experiments have not employed full factorial designs or requisite sample sizes required to achieve adequate statistical power; limiting their capacity to infer evidence for priming effects (Anwer et al., 2022).

Studies of *Drosophila* provide a powerful tool to validate associations observed in

human and rodent datasets. *Drosophila* have evolutionarily conserved nutritional pathways analogous to mammals, therefore results from studies of *Drosophila* may provide meaningful insights into biological processes across taxa. Studies of *Drosophila* also benefit from the species exhibiting a relatively short life cycle (especially compared to other model species like *M. musculus*), as well as availability of a range of genetic tools, lack of parental care; a likelihood that generations will experience similar nutritional environments, and specifically designed synthetic diets (Piper et al., 2014)—as such, they are a tractable model species for disentangling cross-generational effects of dietary interventions.

1.7 | Mechanisms underpinning cross-generational nutritional effects

Inheritance of epigenetic markers that alter offspring metabolic physiology via transcriptional changes are consistently identified across taxa as being critical to altering transgenerational plasticity. These markers include DNA methylation and histone modifications. These alter the expression of nutrient-sensing pathways such as insulin like-growth factor (IGF), and TOR (Target of Rapamycin) (Anderson et al., 2009). The dysregulation of these nutrient-sensing pathways under obesogenic conditions has been linked to age-related metabolic changes, and altered mitochondrial function (Oldham, 2010; Stegemann et al., 2015; Fleming et al., 2018). Both the TOR and the IGF pathways are evolutionarily conserved, TOR is present in yeast and animals and IGF is present in animals. There is also evidence that small RNAs and other molecular modifiers could be responsible for transmission of transgenerational effects (Ost et al., 2014), but many in vivo studies investigating the role of transgenerational effects are unable to parse these various possible causes.

One study demonstrating the importance of DNA methylation for transmitting phenotypic plasticity in mice used agouti viable yellow (Avy) mutant mice, which are genetically predisposed toward hyperphagic obesity. In this work, maternal obesity

influenced fat accumulation in the offspring to the F3 generation, suggesting a cumulative effect of obesity across generations (Waterland et al., 2008). Interestingly, these effects were ameliorated by providing pregnant mothers with a pro-methylation diet enriched for the methyl donors folic acid, betaine, vitamin B12, and choline. This diet exacerbated the mottled yellow coat of the Avy mice, which is indicative of its efficacy to increase DNA methylation, but the observed effect to suppress transgenerational body weight gain was independent of coat colour variations indicating DNA methylation elsewhere in the genome is important (Waterland et al., 2008). An epigenetic basis of transmission is also supported by mouse studies that have used in vitro fertilization (IVF) with gametes from obese parents and implanted the embryos into normal weight surrogates, thus removing any potentially confounding effects of the environments at conception, in utero, during lactation, or in transmission of the maternal microbiome at birth. These data show that both oocytes and sperm from obese parents can contribute to intergenerational obesity and development of type II diabetes of offspring challenged with a high fat diet (Huypens et al., 2016).

Obese male flies fed a high sugar diet have also been shown to predispose their offspring to obesity, transmitted via epigenetic marks. Unlike mammals, flies possess negligible levels of DNA methylation (Zhang, Huang and Liu, 2015), but they do have a heritable system for modifying DNA accessibility, and thus gene expression, via post-translational modifications of histones (Zhao and Garcia, 2015). Sperm from male flies fed a high sugar diet showed evidence of repressive histone methylation marks and importantly, modifiers of these marks were correlated with sustained repression of lipid biosynthetic genes in the embryo. Moreover, these modifiers are required for intergenerational transmission of a predisposition to obesity when offspring were maintained on a high sugar diet (Ost et al., 2014).

This study on flies provides direct evidence that histone modifications that alter the expression of metabolic genes are required to transmit a phenotype from parent to offspring in a manner similar to what has been shown for mice.

1.8 | Statement of rationale

The literature into cross-generational effects of nutrition that I reviewed above reveals several areas that require empirical attention. First, while it is clear that cross-generational effects exist, and that some studies have examined such effects in a dietary-mediated context, the vast majority of studies conducted to date are intergenerational, focusing on transmission of effects from F0 to F1 only (Anwer et al., 2022; Matzkin, Johnson, Paight, & Markow, 2013b; Öst et al., 2014). More studies that move beyond a single generation are needed to ascertain whether effects seen in in the F1 generation will be concordant with subsequent generations. Indeed, intergenerational effects can often be a result of condition dependence (i.e. silver spoon effects), and therefore transgenerational work is required to explore effects that may be caused by epigenetic mechanisms (Ivimey-Cook et al., 2021; Uller et al., 2013). Furthermore, most of the intergenerational nutrition studies conducted to date are maternally focussed and measure physiological traits, with little attention paid to the capacity for paternal effects to shape offspring life history. Of those that have tested for paternal effects, generally this has been done using an experimental design that does not enable direct comparison of the paternal and maternal contributions to cross-generational phenotypes, or the potential that maternal and parental contributions might interact to shape these phenotypes (Öst et al., 2014). Determining the nature of paternal diet effects, and their magnitude relative to maternal effects, will elucidate whether parental diet effects interact to shape offspring life history phenotypes across multiple generations; currently this is poorly understood (Shenoi et al., 2022). Second, as most studies focus on only one offspring sex, the capacity for dietary variation to exert sex-specific effects on offspring life history phenotypes across generations remains largely unexplored.

Furthermore, a long-standing assumption of many studies posits that offspring are primed by parents (i.e. via anticipatory effects) and will be at a disadvantage if they face a nutritional environment that differs from their parents or grandparents. The evidence for this,

however, within the context of broad environmental changes is mixed, and little attention has been paid to testing nutritional environments specifically. Indeed, most studies lack adequate full factorial experimental designs that would allow parents and offspring to be challenged with both novel and control diets in all possible combinations. This leaves open the question of whether these anticipatory effects, mediated by nutritional variation, actually occur in natural populations of animals. Finally, most studies of cross-generational nutrition have been conducted with a focus on obesity, and have therefore modified regimes of sugar or fat intake to examine the cross-generational consequences. Given this focus on the consequences of obesogenic diets, less attention has been devoted to examining the cross-generational implications in variation in other macronutrients – namely protein variation, and whether variation in both carbohydrates and protein simultaneously may result in dietary imbalance (via negative interactions between macronutrients) that incurs cross-generational consequences.

These open questions inspired my PhD research, and are key to understanding the evolutionary ecology of dietary-mediated cross-generational effects.

1.9 | Aims and chapter summaries

In this thesis, I studied the evolutionary ecology of dietary-mediated cross-generational effects in *D. melanogaster*, investigating how diets varying in carbohydrate and/or protein concentration affect the lifespan, fecundity, and body composition of flies across three generations (F0 to F2). My specific aims were:

(i) To determine the relative contributions of maternal and paternal dietary-mediated effects and the possibility for complex interactions between parents and offspring diets;

(ii) Investigate whether cross-generational effects of nutrition can be sex-specific in their effects on male and female offspring;

(iii) To ascertain whether parents or grandparents can prime offspring (via anticipatory effects)—advantaging offspring in matching nutritional environments;

(iv) To determine whether dietary-mediated cross-generational effects are unique to the particular macronutrients manipulated, testing whether effects mediated by carbohydrate variation are similarly induced by protein variation, or whether effects are specific to the macronutrients or combination of macronutrients used;

(v) To uncover whether the cross-generational effects of variation in nutrition are consistent across generations (comparing direct responses, to intergenerational responses, to transgenerational responses).

Below I provide a summary of each chapter; and outline how each chapter addresses the aims described above.

Chapter 2: Transgenerational obesity and healthy aging in Drosophila, published 2019 Journals of Gerontology- Series A.

My PhD seeks to elucidate dietary-mediated cross-generational contributions to physiology and life history trait expression. It became clear during my initial literature searches on this topic, that much of the relevant research had been conducted in the framework of the cross-generational implications of obesogenic diets. Accordingly, in this chapter, we set out to synthesize evidence for the intergenerational and transgenerational phenotypic effects of parental obesity. We concentrated on the capacity for research utilising the fruit fly *Drosophila melanogaster* to provide formative insights that help advance understanding into the effects of obesogenic diets; proposing *Drosophila* as an excellent model species to study these effects. This chapter comprises a review article that explores how *Drosophila* can be useful for studying the cross-generational effects of obesity, due to their short generation times, genetic tractability, and analogous metabolic pathways. By completing this literature review, I uncovered the major unanswered questions in this field, and realised that using more varied species of model organisms could help to solve these unanswered questions. This chapter helped to formulate the overall aims of my thesis.

Chapter 3: *Maternal and paternal sugar consumption interact to modify offspring life history and physiology, published 2022 in Functional Ecology.*

In this third chapter, I set about testing the thesis aims, experimentally. In this chapter, I implemented an experimental design that would enable me to test Aims i to iii listed above; what are the relative contributions of dietary-mediated maternal and paternal contributions to

offspring phenotypes, and do they interact; are these contributions anticipatory, by augmenting offspring fitness when offspring face environments similar to their parents. Inspired by the research on obesogenic diets, I implemented a full factorial design, in which I altered the carbohydrate concentration in the diets of females, males, and their offspring, in *D. melanogaster*, and then measured the direct and intergenerational (F0 to F1) responses in physiology and life-history (lifespan, fecundity, body weight, triglyceride levels, and feeding behaviour). Specifically, the dietary treatment involved a hypercaloric medication, achieved only by manipulating the sucrose content of the diet (i.e. diets were either high or low in relative sucrose levels). Using sucrose manipulation in the diet, my intent was that the results of this could be scaffolded to the current intergenerational literature on parental priming effects of obesity transmission from parent to offspring. Providing *D. melanogaster* with higher relative sucrose is an ecologically relevant way of ensuring the flies accumulated a higher whole-body triglyceride content (compared to flies who receive lower sucrose). I found complex interactions between parental and offspring diets (support for aim i) that were generally not consistent with parental anticipatory effects (aim iii), but rather suggest a conflict over optimal diets between generations. Notably, when sucrose levels between parents (F0 females and F0 males) matched both parents lived longer, but offspring from these combinations experienced shorter lifespans. My study highlights the need for full factorial designs that consider both sexes of the F0 generation in order to understand the full evolutionary implications of parental effects on offspring life history.

Chapter 4: Sex-specific transgenerational effects of diet on offspring life history and physiology

The fourth chapter of my thesis builds directly on to the third, with the intent to address the same aims; but testing aims (i), (ii) and (iii) in a transgenerational content (across two

generations, F0 to F2). Therefore, in this chapter, I examine whether the effects of sucrose variation in F0 males and F0 females on F2 offspring will be concordant with results seen in F1, thereby also enabling me to address whether cross-generational effects are consistent across generations (aim v). Again, both sexes in the F0 and F2 were provided with diets that were either high or low in (relative) sucrose levels (providing the F1 with a common garden diet). This enabled me to see how these combinations of diets affected F2 offspring lifespan, fecundity, body weight, and triglyceride levels. Again, I used a full factorial design across both generations so that F2 offspring were challenged with high or low relative sucrose concentrations that either matched or mismatched one or both of their grandparents. I reveal grandmaternally mediated transgenerational effects of sucrose variation that exerted opposing effects in the F2 grandoffspring sexes, in which ingestion of a lower sucrose diet by grandmothers increased female F2 lifespan, but decreased male F2 lifespan. Additionally, although I found complex interactions between grandparents and grandoffspring diets affected grandoffspring life history traits, again as seen in chapter three, the direction of these patterns was not consistent with these transgenerational effects being anticipatory.

Chapter 5: Dietary protein enhances transgenerational reproductive success

In both Chapters four and five, I uncovered complex interactions between grandparental and grandoffspring diets that shaped grandoffspring life history in sex- and diet-specific ways. In both chapters, I had manipulated variation solely in carbohydrate concentrations in the diet, keeping protein concentrations constant. The goal of this chapter was to determine whether the cross-generational patterns I had uncovered in previous chapters were specific to variation in dietary carbohydrate, or whether similar patterns could be invoked through modification of protein concentration (aim iv). Furthermore, the design enabled me to test whether interactions between carbohydrate and protein levels were key

to cross-generational fitness effects. In previous chapters, I had concentrated on measuring consequences of dietary variation on lifespan and physiology, with less focus on reproductive consequences. Here, I sought to focus closely on female reproductive output given reproduction is the most closely aligned trait to evolutionary fitness.

I achieved this by altering protein to carbohydrate levels and ratios in such way that maximises the dietary variation across nutritional space, and assaying female reproductive output over a six-day period of early life, in both F0 and F2 females. The diet challenge resulted in 25 F0 combinations of diets, but the F1 and F2 diets were held constant on a common garden diet. I demonstrated that both F0 grandfathers and F0 grandmothers contribute independently to their F2 granddaughter's reproductive output, with higher protein levels consumed by each grandparent increasing granddaughter (F2) reproductive output. I found that the transgenerational effects of protein were larger than the effects of carbohydrate. Moreover, the direct effect of protein on the F0, and transgenerational effects of F0 protein on the F2 were remarkably similar—high protein consumed by the F0 generation increased female reproductive output. This work suggests transgenerational nutrition is important for the fitness of subsequent generations.

Chapter 6: General discussion

Finally, in Chapter six, I synthesize and discuss the main findings from my investigations, and describe how these studies have helped to elucidate the nature of inter- and transgenerational effects of nutrition on organismal fitness. I also discuss further avenues for research. Although this is my thesis, and I carried out the much of the planning, experimental design, data collection, analysis, and manuscript preparation, it was in collaboration with, and under the guidance and advice of my supervisory team, and therefore

the language henceforth will read “we” rather than “I”, but will revert back to “I” in Chapter six for the general discussion.

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Chapter 2 | Transgenerational obesity and healthy aging in *Drosophila*

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Julia Gillard (First female Prime Minister of Australia)

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Review

Transgenerational Obesity and Healthy Aging in *Drosophila*

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Abstract

Substantial evidence suggests that individuals born to overweight and obese parents suffer detrimental health consequences that dramatically decrease healthy aging. The number of obese individuals worldwide now exceeds the number of under- and malnourished individuals. This obesity epidemic is responsible for approximately 4 million deaths worldwide each year, and predisposes sufferers to a range of age-related diseases such as cardiovascular diseases, and metabolic syndrome. Additionally, obesity is associated with an accelerated onset of age-related ailments, such as cancers and inflammation. The importance of dietary interventions to reduce the incidence of obesity is magnified by emerging evidence that parental physiology can predispose future generations to poor health outcomes. Characterizing and understanding these effects, and how they are mediated, is important if we are to continue to drive improvements to population health. In this article, we synthesize evidence for the intergenerational and transgenerational phenotypic effects of parental obesity. We concentrate on how the fruit fly *Drosophila melanogaster* can be used as a model to study these effects. Fruit flies are highly tractable, and their conserved nutrient signaling and metabolic pathways make them an ideal model for studying nutritional effects on metabolic, reproductive, and aging phenotypes.

Keywords: Fecundity, Life span; Obesity

Over the last century, average life expectancy has been steadily increasing, and shows no signs of slowing (1,2). Dietary interventions to further extend and improve healthy life span into the future are a major focus of biogerontology (3). In general, moderate restriction of food intake, through either restricting calories or restricting overall diet nutrients (CR, DR) or modification of dietary nutrient balance, can augment life span—an effect evident in model organisms from yeast to primates (4). Furthermore, a growing body of evidence points to beneficial metabolic and physiological effects of CR, including reductions in body fat, in both lean and overweight humans when administered during early- to mid-life (5,6). These findings are exciting because they highlight the promise of deploying dietary interventions as a tool to improve healthy aging within the general population. Moreover, the practical utility of such dietary interventions is amplified by recent reports of negative health effects in children born to overweight and obese parents. Negative intergenerational effects of obesity are not well understood, but researching the prevalence and magnitude of these effects will be key to addressing the health and economic costs associated with obesity in contemporary human populations (7,8).

Here, we synthesize evidence from experimental studies that have investigated the effects of parental obesity on reproduction and life span of future generations. In particular, we highlight the utility of the fruit fly *Drosophila melanogaster* as a model for the study of inter- and transgenerational obesity, given its short generation time, small containment footprint, conserved metabolic pathways, well characterized diet, and a readily available suite of advanced genetic tools. We address whether experiments conducted in *Drosophila* can be used to inform likely responses in humans, by critically evaluating whether experimental findings from studies of *Drosophila* are consistent with those that have come from studies of mice. We conclude that the study of *Drosophila* offers a powerful means to better understand the mechanisms involved in the regulation between nutrition and health, across generations.

Why Do We Eat Unhealthy, Imbalanced Diets?

The role of nutrition in health is complex. Diets consist of dozens of components that are required in proportions that vary as a function

of the supply of other nutrients, as well as the consumer's genome, microbiota, life stage, and condition (9). In general, too little or too much of a nutrient is detrimental to an organism's health (10). Thus to maximize fitness, organisms must perform a multinutritional balancing act to ingest and absorb nutrients in suitable proportions. First, this is achieved by nutrient-specific appetites that can alter the relative consumption of different ingredients to achieve and maintain homeostasis (11). When organisms eat, however, the proportion of nutrients in their food may not match requirements. Consumers must therefore make compromises on the balance of nutrients they ingest, and where possible, ameliorate those compromises by mixing foods with complementary nutritional profiles (12,13). The precise nature of these compromises is determined by the relative priority with which each nutrient effects appetite and satiety—a function of the specific evolutionary history of each organism (14).

Of the many nutrients in a diet, carbohydrate, fat, and protein are the three major energy-contributing nutrients. These play a key role in determining evolutionary fitness and are important determinants of diet choice (15). The exact strategy for nutrient mixing varies across species, and not all nutrients have an equal influence on feeding behavior. For some primates (including humans), mice and *Drosophila* larvae, data indicates that protein is the primary determinant of appetite and satiety (14,16–20). Thus, within limits, food is consumed to keep protein intake within a tight range of values, and a much broader range of lipid and carbohydrate intake is tolerated. This can be understood in light of the fact that protein underconsumption leads to a decrease or cessation in reproductive output, while overconsumption can carry metabolic and physiological costs associated with excretion (3,21). By contrast a broader range of sugar and fat consumption can be tolerated as excess energy can be stored as body fat and be advantageous as a buffer against seasonal variations in energy supply (22,23). The capacity for protein levels to shape feeding behavior can be exploited to facilitate loss of body fat by curtailing appetite through consumption of high protein diets. By contrast, meeting a protein intake target when consuming low protein foods can result in overconsumption of sugar and fat, which in the long term can lead to obesity (18). Together, this has led to the hypothesis that the current obesity epidemic in developed countries is fuelled (at least in part) by the unprecedented abundance of low protein, energy dense foods that are highly palatable, yet have little satiety value (24).

Health and Body Fat

The number of obese individuals worldwide now exceeds the number of under- and malnourished individuals. Obesity predisposes sufferers to a plethora of age-related diseases such as type 2 diabetes, cardiovascular diseases, and metabolic syndrome, and is responsible for approximately 4 million deaths worldwide each year (25,26). Additionally, obesity sufferers are more likely to be afflicted by age-related conditions, such as cancers and chronic inflammatory diseases (26).

Body mass index (BMI) estimates body fat, and is calculated by body mass in kilograms divided by the square of the person's height in meters, and is a useful correlate of health outcomes. BMI from 18.5 to 24.9 ("normal" range) is considered "healthy" and without weight-related adverse health consequences (26). The categories of underweight (BMI <18.5), overweight (BMI 25–29.9) to obese (BMI ≥ 30), however, are associated with increased risks of certain diseases, costs to reproductive performance, and reduced life expectancy (27–31). Importantly, for studying the mechanistic bases of these poor outcomes, the obese state can be modeled in mice and flies to varying degrees of accuracy. By providing highly palatable, energy dense diets with low satiety value to model species, the percentage of body fat increases and is accompanied by negative outcomes for reproduction and life span, analogous to the pattern observed in humans (32–34).

Modeling Obesity in Murids and Flies

Murids

Feeding high-energy diets, supplied as excess fat or sugar, to mice or rats leads to a higher percentage of body fat compared to those fed a control diet. These obesogenic diets also lead to a suite of altered metabolic markers similar to changes found in humans, such as: increases in rates of diabetes; poor insulin sensitivity; high circulating blood glucose; and increased incidences of some cancers (35–37). Furthermore, fertility and life span also decrease in overweight mice, similar to what is observed in humans (38–40). These detrimental effects of obesity on female fertility can occur whether the mice are hyperphagic (over-eating), or feeding on diets high in fat and or sugar (41–43). Although obesity has generally negative effects, some recent studies have shown that altering dietary macronutrient balance can modify the propensity of an obese individual to suffer from later-life pathologies such as type II diabetes and heart disease (40,44,45). These effects are fascinating and warrant further investigation.

Flies

Similar to murids, it is possible to manipulate the proportion of adult body fat in flies. In the wild, flies consume a diet of rotting fruit, sourcing carbohydrates from the fruit and the remainder of their nutrients (including additional carbohydrates) from yeast (46). These diets contain very little fat (approximately 1% of mass), and in the lab, growth, reproduction, and life span can be readily supported with diets containing only sugar and yeast. A typical example of one such diet used in our laboratory supplies energy from protein: carbohydrate: fat in proportions ~55:40:5 (corresponding to ~45 g/L protein, 35 g/L carbohydrate, and 2 g/L fat) (47,48). Data from studies that have supplemented this natural nutritional profile with fat show universally detrimental physiological outcomes and shortened life span (49–52). However, it is not yet possible to discern if these unfavorable changes mimic the costs that mammals suffer on high fat obesogenic diets, or if they simply reflect novel pathologies caused by dietary fat levels that are well above what flies have evolved to experience in the wild. We postulate a more ecologically relevant approach to increasing body fat in flies is by increasing the carbohydrate (sugar) component of the diet (53,54), and for this reason we will restrict our discussion to those studies that manipulate parental sugar. Both female and male flies get fatter with increasing dietary sugar concentrations and this does not appear to be acutely toxic since there is no cost to life span for values of dietary carbohydrates of up to ~80% of total energy (3,34,47,55,56). In contrast, female fecundity is maximized at intermediate dietary carbohydrate levels (up to ~50% of total energy), when body fat remains low. As dietary sugar and body fat rise beyond this point, egg laying sharply declines (Figure 1) (3,34,47,55,56).

Inter- and Transgenerational Consequences of Parental Obesity

It is becoming increasingly apparent that obesity susceptibility is transmitted from generation to generation. These effects can be intergenerational (transmitted from parent (F0) to offspring (F1))

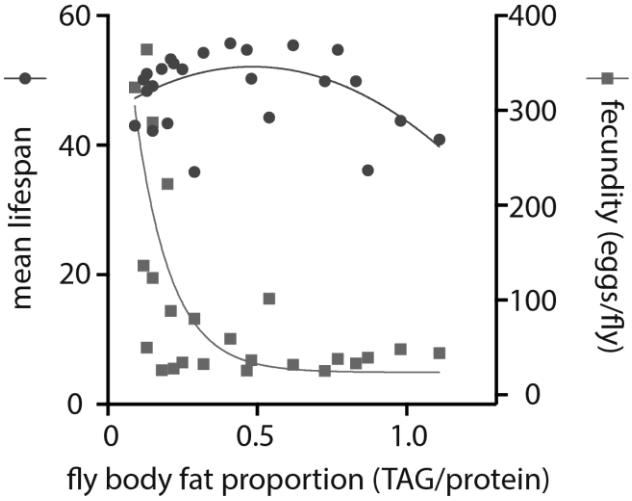

Figure 1. Relationship between body fat, egg laying and life span in an inbred lab strain of female Drosophila melanogaster. Altering the relative proportion of sugar and yeast in a fly diet alters body fat composition. Maximal female egg laying corresponds to nutrient compositions at which body fat content is very low, and as body fat rises, egg laying declines. In contrast, life span remains long, showing only mildly compromised at nutrient compositions that promote higher body fat content (data from Skorupa et al. (34)).

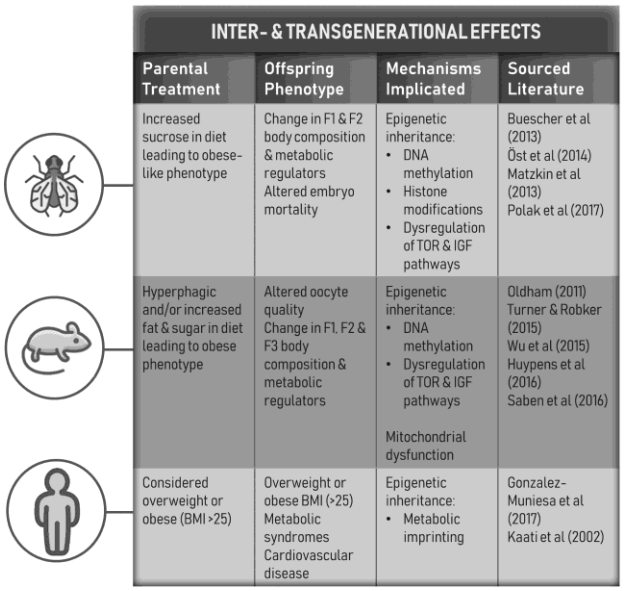

Figure 2. Obesogenic diets in parents can confer detrimental metabolic phenotypes on offspring to the second and third generations. These effects appear to be evolutionarily conserved, meaning we can start to examine their mechanisms in short-lived, easily housed model organisms. The common mechanism to be implicated across taxa involves epigenetic marks that may alter the expression of key nutrient signaling pathways. It will be important in future work to explore additional possible mechanisms (eg, mito-nuclear interactions), using nutrient explicit diets. We also recommend the use of fully-factorial diet designs on mothers, fathers and their offspring in order to parse the effects of maternal and paternal contribution to health of males and females in future generations.

and even transgenerational (transmitted from parent (F0) to the F2 generation and beyond; Figure 2). Distinguishing between these inheritance modes is important for understanding the mechanisms of susceptibility transfer, since in the case of intergenerational effects, the offspring may experience the predisposing parental environment (eg, while in utero), while for transgenerational effects, the affected offspring have no direct experience of the predisposing grand-parental environment. Thus, in oviparous species, like fruit flies, transgenerational effects are those transmitted to the grand offspring (F2) generation, but in mice and humans, they are the effects transmitted to the great-grand offspring generation (F3) because the primordial germ cells of the F2 generation are present in the F1 female fotus while in utero, and are thus subject to the grand-maternal environment.

Transgenerational Plasticity

Inter- or transgenerational phenotypic plasticity describes the situation when the parental environment or phenotype impacts the phenotype of the offspring, beyond the effects of gene transfer alone (57,58). The most commonly studied effects are those of maternal diet on offspring physiology, which are thought to result from transmission of epigenetic markers, antibodies, hormones, and/or nutrients (59). Since selection typically favors the total lifetime reproductive success of parents (rather than reproductive success during any one bout), both negative and positive maternal effects can be favored if they enhance maternal fitness (57). This will depend on the life expectancy of the mother, resource availability, environmental conditions, and the interaction between the costs of producing an offspring phenotype and the benefits of that phenotype (57). Maternal effects that confer a positive impact on offspring quality are often referred to as "adaptive maternal effects." These are more likely to occur in predictable environments when mothers prime offspring to be suited to the same environmental stresses she experienced (59). Maternal transgenerational plasticity may also be disadvantageous, however, if the postnatal environment differs from the intrauterine one, such as if a fetus is subjected to a malnourished environment and is thus "programmed" for a more energy efficient metabolism, but is raised in a nutrient abundant environment, predisposing the offspring to obesity (60). Although evidence is mounting for adaptive transgenerational anticipatory effects, progress has been hindered by a paucity of experimental studies testing that employ fully-factorial designs in which both parents and offspring are challenged with both a control diet and a novel diet (58).

Inter- and Transgenerational Effects of Parental Obesity in Humans

Several recent studies have shown that many individuals born to overweight and obese parents suffer detrimental later-life health consequences, particularly when born into a nutrient rich environment (61,62) (Figure 2). Developing obesity becomes more likely due to critical developmental periods whereby metabolic imprinting (programming of metabolism) can occur (26). Critical periods identified in the development of obesity are the pre- and neonatal periods (up to 2 years old). In the prenatal period, maternal disproportionate gestational weight gain, especially in the first 20 weeks of pregnancy has been identified as a risk factor in the development of obesity later in the child's life (63–65). Even parental weight gained (or BMI) prior to fertilization is associated with a child's later-life BMI. A mother's prepregnancy and early-pregnancy BMI explains most of the variance in a child's BMI—even when controlling for pregnancy complications such as gestational diabetes, and other lifestyle factors (26). Similarly, paternal BMI could be an important factor for later-life progeny health. Historical records have shown that males

exposed to excess food during the slow growth period of childhood (8–12 years old) exhibit a fourfold greater risk of their grandsons developing later-life type II diabetes and cardiovascular disease (61). We note that parallel poor late-life outcomes have also been reported for individuals that were in utero at the time of extreme energy deprivation (66). In addition, suboptimal fetal nutrition, both from under- or overnourishment leads to an increased risk of cardiovascular diseases and type 2 diabetes (67).

While the evidence for intergenerational effects of obesogenic diets in humans is interesting, it is challenging to separate the effects of this transmission from effects of shared environment. This is because the children are likely to be exposed to the same obesogenic lifestyle as their parents. This is where nonhuman models become critically valuable in helping to us to disentangle trait transmission from shared environmental effects.

Compelling evidence for inter- and transgenerational inheritance of obesity comes from mouse studies where obesity can be restricted to particular windows of offspring development. Specifically, even when oocytes from obese females are used for IVF or embryos transferred to lean mothers for gestation, they exhibit altered fetal development (42,68,69), and become obese and insulin resistant in adulthood (70).

Inter- and Transgenerational Effects of High Sugar Diet-Induced Obesity in *Drosophila*

Most studies that have investigated the effects of high sugar diets in *Drosophila* have focused on the direct effects on trait expression that manifest within an individual's lifetime (3,34,47,55,56,71). A few studies, however, have investigated the effects of altering parental diets on offspring and grand-offspring traits (summarized in Table 1 and Figure 2). Feeding *Drosophila* larvae with isocaloric diets that differed in protein: sugar ratio is sufficient to elicit phenotypic differences in their offspring, even when the offspring were maintained on a common diet (32). The offspring from parents that had received a low protein/high sugar (obesogenic) diet during development, exhibited a lengthened period for metamorphosis, produced less eggs, and had altered body composition (protein, glycogen, TAG), when compared to the offspring from parents that had been raised on a high protein/low sugar diet. Interestingly, many of these effects differed between isofemale genotypes, and in some cases were reversed, indicating intergenerational effects are genotype dependent (32).

In another report, Buescher et al. (53) showed that high sugar diets provided to females produced an obese-like phenotype that persisted in male offspring to the F2 generation. Upon challenge with a high sugar diet, F1 sons of mothers exposed to high sugar exhibited increased levels of trehalose, glycogen, glucose and TAG, as well as exaggerated changes in expression of key genes involved in carbohydrate and fat metabolism. Furthermore, the grandsons (F2) of those same high sugar diet fed females also exhibited a higher proportion of body fat, glycogen and trehalose than the grandsons of females fed a low sugar diet, even though the mothers from the intermediate generation (F1) did not experience high sugar diets. Although no data for the daughters were reported, grand-daughters displayed a higher level of trehalose but not of whole-body TAG. Together, these data suggest that flies exhibit a sex-specific metabolic and gene regulatory response to energy challenge, and that this is sensitive to their grand-mothers' dietary experience.

Although most studies using *Drosophila* focus on how maternal diet affects trajectories of offspring health, the paternal diet can have effects also. Remarkably, even transient changes in the sugar content of the paternal diet, for periods as short as 48 hours prior to mating, can lead to intergenerational obesity and metabolic reprogramming of the offspring (33). When males fed either very high or low sugar diets were crossed to control diet fed females, their sons showed an increase in body fat percentage on high sugar diets when compared to sons from crosses where both mothers and fathers were maintained on diets with intermediate sugar content. This effect was attributed to altered chromatin markers in the sperm of the fathers

Table 1. Inter- and Transgenerational Effects of High Sugar Diets in *Drosophila*.

	F0	F1	F2
Maternal high sucrose Buescher et al. (53).	*Adult mothers*: ↑Trehalose, Glycogen, TAG ↓Body weight	*Larval sons*: ↑Glucose, trehalose, circulating sugars, gene expression: gluconeogenesis, fat body lipolysis ↓Glycogen, cholesterol, gene expression: dFOXO, glycolysis, sugar transport *Adult sons*: ↓Body weight[a], Glucose ↑Trehalose, Glycogen, TAG[a] *Larval and adult daughters:* Not reported	*Larval grandsons*: ↑Glucose and trehalose *Larval granddaughters*: ↑Trehalose ↓TAG
Paternal high sucrose Öst et al. (33).	*Adult fathers*: ↑TAG	*Adult sons*: ↑Body weight TAG, lipid droplet size, glucose ↓Trehalose Altered sperm chromatin state *Adult daughters:* Not studied	*Adult grandsons*: No transgenerational effects found *Adult granddaughters:* Not studied
Both Parents high sucrose (isocaloric) Matzkin et al. (32).	*Adult parents*: ↑Glycogen ↓Protein, fecundity	*Adult offspring*: ↑Glycogen, development time ↓Fecundity, protein, TAG	*Adult grand offspring*: Not studied

Note: TAG = Whole body Triacylglyceride content. [a]Worsened with a high sucrose diet challenge; ↑increased, ↓decreased or reduced.

(33). More recently, it was found that offspring viability during embryogenesis is sensitive to alterations in the ratio of protein: carbohydrate, the caloric density, and the quality of carbohydrates in the paternal diet (72). Exactly how these factors affected the offspring differed when comparing the offspring sired by the father's first mating to the offspring sired by his second mating to a different virgin female. This study is particularly interesting because unlike others that only vary parental dietary sugar, this systematically varied both the dietary protein: carbohydrate ratio as well as total energy density. In doing so, it shows that the better the paternal condition (the sum of energy contained in the father's fat, glycogen, and protein reserves), the higher the proportion of his sired offspring that survived embryogenesis. It would be interesting to examine the mechanistic basis of this transmission to understand if each of the nutrient combinations operate through the same mechanisms to affect the next generation (72).

Taken together, these studies provide evidence that varying the composition of diets consumed by *Drosophila* parents not only affect their own phenotype but also that of their offspring and grand offspring, and that these effects can be altered by genetic background. While interesting, these data are the result of relatively few studies and each differs in experimental design. Furthermore, because the traits being investigated are sensitive to environmental conditions, transgenerational effects can be difficult to reproduce, even within the same laboratory (73). Thus, a limitation is that it is currently difficult to measure the magnitude of these inter- and transgenerational effects, and to identify specific nutritional triggers. This highlights the need for further research, and for future studies to make explicit the nutritional composition of the diets they employ, and ideally adopt a range of diets over which to generate the effects. This will enable phenotypic responses to be mapped to diet within a structured framework, such as the geometric framework of nutrition (9) as reported in Polak et al. (72). In this way, transgenerational phenotypes whose manifestation might appear to be weak or variable between two studies can be unified into a continuum of responses across nutrient space. This will give us the power to understand what nutritional treatments are required to elicit transgenerational plasticity and therefore target work to understand the mechanisms.

Mechanisms Underpinning Intergenerational Effects of Diet-Induced Obesity

Determining the molecular mechanisms underlying inter- and transgenerational metabolic imprinting using human clinical samples is challenging, due (at least in part) to the variable nature of conditions and environments from which samples originate. We therefore look to model organisms for answers. For murids and flies, both genetic and dietary models of obesity are available. Here, we concentrate on diet-induced obesity and evolutionarily conserved mechanisms. Although our understanding is far from complete, inheritance of epigenetic markers that alter offspring metabolic physiology via transcriptional changes are consistently identified across taxa as being critical to altering transgenerational plasticity. These markers include DNA methylation and histone modifications (Figure 2). These alter the expression of nutrient-sensing pathways such as insulin-like-growth factor (IGF), and TOR (Target of Rapamycin) (74). The dysregulation of these nutrient-sensing pathways under obesogenic conditions has been linked to age-related metabolic changes, and altered mitochondrial function (75–77). Both the TOR and the IGF pathways are evolutionarily conserved, TOR is present in yeast and animals and IGF is present in animals. There is also evidence that small RNAs and other molecular modifiers could be responsible for transmission (33) but many in vivo studies investigating the role of transgenerational metabolic imprinting are unable to parse these various possible causes.

One study demonstrating the importance of DNA methylation for transmitting phenotypic plasticity in mice used agouti viable yellow (A^{vy}) mutant mice, which are genetically predisposed toward hyperphagic obesity. In this work, maternal obesity influenced fat accumulation in the offspring to the F3 generation, suggesting a cumulative effect of obesity across generations (78). Interestingly, these effects were ameliorated by providing pregnant mothers with a pro-methylation diet enriched for the methyl donors folic acid, betaine, vitamin B12, and choline. This diet exacerbated the mottled yellow coat of the A^{vy} mice, which is indicative of its efficacy to increase DNA methylation, but the observed effect to suppress transgenerational body weight gain was independent of coat color variations indicating DNA methylation elsewhere in the genome is important (78). An epigenetic basis of transmission is also supported by mouse studies that have used in vitro fertilization (IVF) with gametes from obese parents and implanted the embryos into normal weight surrogates, thus removing any potentially confounding effects of the environments at conception, in utero, during lactation, or in transmission of the maternal microbiome at birth. These data show that both oocytes and sperm from obese parents can contribute to intergenerational obesity and development of type II diabetes of offspring challenged with a high fat diet (70).

Obese male flies fed a high sugar diet have also been shown to predispose their offspring to obesity, transmitted via epigenetic marks. Unlike mammals, flies possess negligible levels of DNA methylation (79), but they do have a heritable system for modifying DNA accessibility, and thus gene expression, via post-translational modifications of histones (80). Sperm from male flies fed a high sugar diet showed evidence of repressive histone methylation marks and importantly, modifiers of these marks were correlated with sustained repression of lipid biosynthetic genes in the embryo. Moreover, these modifiers are required for intergenerational transmission of a predisposition to obesity when offspring were maintained on a high sugar diet (33). This study on flies provides direct evidence that histone modifications that alter the expression of metabolic genes are required to transmit a phenotype from parent to offspring in a manner similar to what has been shown for mice.

In evidence of other mechanisms, mitochondrial and nuclear genotypic interactions have also been shown to alter whole-body metabolism and gene expression under diet-induced obesogenic conditions (81,82). In murids, obesogenic prenatal diets that lead to metabolic syndrome are associated with reduced mitochondrial DNA (mtDNA) content (reduced copy number of mtDNA molecules per cell) and altered expression of the mtDNA genome in offspring (43,83). It is not clear from these studies if the reduced mtDNA frequency is due to an overall loss of—or dysfunction of—the mitochondria. Studies attempting to elucidate what causes the loss have implicated mitochondrial dysfunction in oocytes of obese mothers as causal in mitochondrial loss in offspring, due to endoplasmic reticulum stress. Endoplasmic reticulum stress reduces protein secretion and disrupts mitochondrial activity in oocytes, thus impairing its function. This indicates that a maternal obesogenic diet can influence offspring metabolism by altering the mitochondrial content and quality in the oocyte at periconception (43,84). It is interesting to note that another study has also shown altered mitochondrial morphology and cellular metabolism in the muscle of F2 and F3 offspring of female mice fed high fat or high sugar diets (69). How

mitochondria may interface with the epigenetic alterations observed in offspring of obese mothers is yet to be determined (68). Emerging evidence using fruit flies has demonstrated that the mtDNA interact with the nuclear genome of the organism to affect phenotypic outcomes that can have long-term consequences on components of health and fitness, such as life span (85,86). Building upon this work will be revealing to understand how mito-nuclear interactions contribute to inter- and transgenerational effects of obesogenic diets.

Perspectives and Future Directions

Transgenerational effects of obesity may cap healthy aging improvements and life expectancy outcomes into the future, but to what extent and by what mechanisms is currently unknown. By studying model organisms, we have learned that both the quantity and quality of nutrients consumed by either parent can contribute to varying offspring phenotypes via epigenetic mechanisms that modify offspring nutrient-sensing pathways (Figure 2). We note also a connection between the metabolic pathways affected and mitochondrial metabolism as well as a possible connection between affected pathways and interactions between the mitochondrial and nuclear genomes, the importance of which is well worth further exploration.

We also note that future studies can benefit from employing a fully-factorial manipulation of both the parental prereproduction diets and those used to challenge the offspring, so that the specific and potentially synergistic effects of obesogenic diets can be identified (59). To date, only one study has attempted this in *Drosophila* (53) but it manipulated maternal diet only. By mating the parental generation in a fully-factorial design and providing offspring with diet challenges that are either matched or mismatched to the diet their parents received, we can parse several effects. First, we can determine the relative contributions of maternal and paternal diet on offspring health. Second, we can evaluate whether offspring health and fitness improves when the diet is matched to their mother's or their father's diet (when compared to mismatched), which elucidates any potential parental anticipatory effects. Conversely, if offspring from parents fed obesogenic diets display poorer health and fitness when challenged with an obesogenic diet, this may indicate obesogenic diets have a compounding effect across generations. It is also imperative for future studies to define both the energy content as well as the macronutrient balance administered in inter- and transgenerational obesity studies, since parental diet quality has the power to impact transgenerational phenotypes, even when diets are isocaloric (32). Using a structured framework, such as the geometric framework of nutrition (9) may reveal obesogenic phenotypes that afford differing inter- and transgenerational risks to health and longevity.

Finally, examining the interactive effects of genotype and diet on transgenerational obesity by using genetic models, such as those used in (53), may be helpful to elucidate mechanisms. Palu et al. (2017) (73) studied metabolic phenotypes in the wild-type grandoffspring of a fly with obesity that is caused by loss of the glucagon receptor orthologue adipokinetic hormone receptor (AKHR). These flies have a defect in fat catabolism. The data show that wild-type grand-offspring had an altered fat phenotype only when they descended from AKHR null grandfathers and heterozygote mothers. Thus, different metabolic causes of obesity may have different modes of transmission. By combining carefully designed diet studies with genetic models of obesity, it will be possible to generate greater resolution for mechanistic studies so we may better understand these complex phenotypes in humans.

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Conflicts of Interest

None reported.

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Chapter 3 | Maternal and paternal sugar consumption interact to modify offspring life history and physiology

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"However complicated you think it is, everything is always more complicated than that."

-Naomi Alderman (The Power)

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RESEARCH ARTICLE

Maternal and paternal sugar consumption interact to modify offspring life history and physiology

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Abstract

1. Intergenerational effects on offspring phenotypes occur in response to variation in both maternal and paternal nutrition. Because the combined maternal and paternal effects are rarely considered together, however, their relative contributions, and the capacity for interactions between parental diets to shape offspring life history and physiology are not understood.
2. To address this, we altered the sucrose levels of adult fruit flies (*Drosophila melanogaster*) prior to mating, across two generations, producing parent–parent and parent–offspring combinations that were either matched or mismatched in dietary sucrose. We then measured life span, fecundity, body mass and triglyceride levels in parents and offspring.
3. We reveal complex, non-cumulative interactions, which involve diets of each parent and offspring, shape offspring phenotypes, but the effects were generally not consistent with an adaptive response to parental diet.
4. Notably, we find that interacting parental flies (sires and dams) lived longer when their sucrose treatments were matched, but they produced shorter lived offspring.
5. These results are suggestive of intergenerational conflict over optimal diets, and call for further research into the capacity, and mechanisms, for mismatches in parental environments to enhance offspring phenotype generally.
6. Our study also indicates that studies of maternal and paternal effects will need embrace experimental designs with power to test for interactions between maternal and paternal environments if they are to fully understand the ecological and evolutionary significance of parental effects on offspring fitness.

KEYWORDS
adaptive priming, diet, intergenerational, maternal effects, parental effects, paternal effects, transgenerational

1 | INTRODUCTION

Parents contribute to the development of their offspring beyond the direct genotypic effects of gene transfer (Bonduriansky et al., 2012; Gluckman et al., 2019; Nystrand et al., 2016). Non-genetic parental effects can arise through either condition-dependant mechanisms (e.g. direct effects of variation in parental care) or through changes in the regulation of gene expression via environmentally mediated epigenetic mechanisms (Curley et al., 2017). Consequently, when the environment of a parent varies, this can affect parental contributions to their offspring and shape offspring fitness (Marshall & Uller, 2007; Mousseau & Dingle, 1991; Mousseau & Fox, 1998; Mousseau et al., 2009; Uller et al., 2013). These effects are plastic responses that occur across generations, termed intergenerational plasticity (when effects span one generation) and transgenerational plasticity (when effects span multiple generations and offspring have no direct experience of the grandparental environment). Intergenerational plasticity has been documented broadly—from bacteria, to fungi, to plants, and in both invertebrate and vertebrate animals (Dyer et al., 2010; Jablonka & Raz, 2009; Roach & Wulff, 1987). Such plasticity can be triggered in response to a wide range of parental environmental stresses or changes, such as parental age and challenges to immunity, nutrition, temperature, toxins, circadian rhythm and light quality (Baker et al., 2019; Bell & Hellmann, 2019; Donelan et al., 2020; Nystrand & Dowling, 2014; Sultan et al., 2009; Wylde et al., 2019).

Currently, two questions remain unresolved when it comes to understanding the broader implications and mechanisms underpinning environmentally mediated (non-genetic) intergenerational plasticity. The first question is whether this plasticity is adaptive to offspring. Numerous empirical studies have suggested that individuals exposed to particular environmental stresses can prime their offspring through mechanisms of non-genetic inheritance to cope with these same stresses (anticipatory parental effects), thereby augmenting offspring resilience and fitness (Marshall & Uller, 2007; Rowiński et al., 2020). Recent, meta-analyses, however, have generally found that evidence for anticipatory parental effects is limited (Radersma et al., 2018; Sánchez-Tójar et al., 2020; Uller et al., 2013). For example, while a meta-analysis by Sánchez-Tójar et al. (2020) established that offspring do on average 11% better in environments that match their parents compared to those in mismatched environments, heterogeneity in effects was high, however, and therefore question the generality of anticipatory parental effects (Sánchez-Tójar et al., 2020). These results align closely to an earlier meta-analysis by Uller et al. (2013), who established a weakly positive point estimate in effect size associated with matching, which cannot be statistically distinguished from zero (error overlaps with zero due to high heterogeneity in the data). Furthermore, the presence and strength of anticipatory effects appears to be moderated by many factors; Uller et al. (2013) reported the evidence was stronger in animals than plants, although Sánchez-Tójar et al. (2020) suggested that weaker effects in plants might be specific to those that are perennial, rather than annual. Sánchez-Tójar et al. (2020) also reported evidence that such effects are more likely to manifest when passaged through either the paternal or maternal lineage, but not through both, and when the ancestral developmental period of exposure to the treatment was at the adult or embryonic stage, but not at the juvenile stage. Thus, although currently the evidence is weak with high heterogeneity across studies, signatures of anticipatory effect might exist under some ecologically relevant scenarios in certain taxa. Indeed, progress in resolving the question of whether parental effects are adaptive has been somewhat hindered by a lack of experimental studies with the power to satisfactorily disentangle adaptive from non-adaptive intergenerational responses (Sánchez-Tójar et al., 2020; Uller et al., 2013). These designs require both parents and their offspring are provided with both a control treatment and a novel environmental treatment, in all possible matched and mismatched combinations, thus enabling determination of whether offspring fitness is higher when offspring environment matches that of their parents (Burgess & Marshall, 2014; Sánchez-Tójar et al., 2020; Uller et al., 2013). Notwithstanding, it is important to note that even the use of such designs may be ineffective at partitioning out transfer from parent to offspring of condition-dependent effects, from transfer of adaptive anticipatory effects (Bonduriansky et al., 2012; Bonduriansky & Head, 2007; Engqvist & Reinhold, 2016), and thus results require careful interpretation.

Second, the relative magnitude of paternal effects to maternal effects on offspring phenotypic expression remains ambiguous. Traditionally, it has been predicted that the relative contribution of maternal effects would be larger than paternal effect, due to the larger gamete size of females (Camus et al., 2019). While maternal effects are known to be pervasive, and have been studied for decades (Mousseau & Fox, 1998), the possibility for non-genetic paternal effects to shape phenotypic expression in offspring received much less attention until recently (Crean & Bonduriansky, 2014; Immler, 2018). In the past decade, however, it has become clear that males contribute to offspring phenotypes beyond that of their direct genotypic contributions, and indeed some studies even point to paternal effects as being greater in magnitude than maternal effects (Crean & Bonduriansky, 2014; Evans et al., 2019; Immler, 2018). Despite recent progress, however, the relative contributions of paternal and maternal effects on offspring performance remain elusive, as does the question of whether paternal contributions interact non-additively with maternal contributions to shape intergenerational fitness in ways that may not be captured simply by measuring maternal or paternal contributions in isolation.

Dietary variation refers to heterogeneity across individuals in the quality or quantity of macronutrients ingested, and represents a major source of environmental influence in natural populations. Dietary variation affects a wide range of fitness-related traits—from physiological measures of obesity, to reproductive success, and life span (Duxbury & Chapman, 2020). Many of these effects appear to be conserved across invertebrates and vertebrates, and modifications to diet in one generation have been shown to trigger indirect effects on the metabolic performance and body composition of offspring and grand offspring (Camilleri-Carter et al., 2019;

Dunn & Bale, 2009; Ivimey-Cook et al., 2021). Research into dietary-mediated intergenerational inheritance has focussed on mice and flies, where studies have explored the intergenerational consequences of obesogenic diets. Recent insights in each system reveal persistent parentally mediated effects of high-fat (in mice) or high-sugar (in flies) diets on offspring phenotypes, with effects that can transcend multiple generations (Buescher et al., 2013; Huypens et al., 2016; Öst et al., 2014). In particular, a recent study by Huypens et al. (2016) in mice showed that maternal and paternal diets can interact to confer complex effects on offspring phenotype. These effects are not simply caused by additive contributions of each parent's diet. Buescher et al. (2013) similarly demonstrated that maternal and offspring diets interact in ways that are not always additive in *D. melanogaster*, and shape F1 and F2 physiological measures of sugar and fat contents, as well as gene regulation linked to lipid metabolism. While intriguing, the broader evolutionary consequences and generality of these maternal-by-paternal diet interactions and maternal-by-offspring interactions revealed in each study remain unanswered, since each measured only early life physiological parameters of offspring, and it is possible that offspring are able to compensate for these early life effects throughout the life course.

Motivated by the questions of whether parental diet effects on offspring fitness phenotypes interact, and whether they may be adaptive for offspring, we tested the relative contributions of variation in adult maternal and paternal diets to offspring life-history traits (adult life span and fecundity) as well as body composition traits (triglycerides and body mass) in the fruit fly *D. melanogaster*. We provided experimental flies with one of two diets that varied in the concentration of sucrose (2.5% and 20%) relative to the other ingredients of yeast, agar and water. The diets were administered using a fully factorial design, in which the two diets were assigned to mothers, fathers and offspring in all possible combinations, such that female–male and parent–offspring diet combinations were either matched or mismatched. This design enabled us to test the prediction of whether dietary-mediated intergenerational effects are adaptive (with offspring produced by parents of a matching diet having higher fecundity and life span), and whether these effects primarily manifest as maternal or paternal effects, or via interactions between male and female parents.

2 | MATERIALS AND METHODS

2.1 | Study species and generating experimental flies

We sourced flies from a large laboratory population of *D. melanogaster* (Dahomey), originally sourced from Benin, West Africa (Puijk & de Jong, 1972). No ethical approval was required for the use of *D. melanogaster*. The flies have been maintained in large population cages, with overlapping generations in the Piper laboratory at Monash University since 2017, and prior to that in the Partridge laboratory at University College London (Mair et al., 2005). Prior to the beginning of the experiment, we collected ~3,000 eggs from the cages, and distributed them into 250-ml bottles containing 70 ml of food, at densities of 300–320 adults per bottle. Food comprised 5% sucrose (50 g of sucrose, 100 g of yeast and 10 g of agar per 1 L solution with an estimated protein to carbohydrate [P:C] ratio of 1:1.9 and 480.9 kcal/L [see Table S12; Figure S2 for further diet details]). Each generation, we admixed adult flies, emerging from across different bottles, together before redistributing back into bottles at a density of 300–320 adults per bottle, repeating this for seven generations. To control for potential sources of variation in their environment during these seven generations, we controlled the age of flies at the time of ovipositioning (all flies were within 24 hr of eclosion into adulthood when producing the eggs that propagated the subsequent generation) and the egg density within each bottle (~300 eggs per bottle).

2.2 | Dietary treatments

The diet media we used consist of sucrose, autolysed brewer's yeast powder (sourced from MP Biomedicals SKU 02903312-CF) and agar (grade J3 from Gelita Australia), as well as preservatives—propionic acid and nipagin. We prepared two dietary treatments, differing in relative sucrose concentration; 2.5% sucrose (that we refer to as a lower sucrose treatment, relative to the 5% concentration usually provided to the population of flies used in this experiment) and 20% sucrose (that we refer to as a higher sucrose treatment) of overall food solution. The 2.5% sucrose diet contains 25 g of sucrose, 100 g of yeast and 10 g of agar per litre of food prepared, with an estimated P:C ratio of 1:1.4 and 380.9 kcal/L of food. The 20% sucrose treatment contains 200 g of sucrose, 100 g of yeast and 10 g of agar per litre of food prepared, with an estimated P:C ratio of 1:5.3 and 1080.9 kcal/L of food. The diets thus differed not only in sucrose concentration, but overall macronutrient balance and their total caloric content. The use of varied levels of sucrose in our experiment is justified on an ecological basis, as fruit flies in a natural setting can experience temporal and spatial heterogeneity in diet, depending on what is available, and usually feed upon rotting fruit, which at varied levels of decomposition produce differing levels of sucrose (Kristensen et al., 2016). Moreover, the high-sucrose concentration was selected based on preliminary experiments that we conducted, which caused the flies to accumulate a higher content of triglycerides but still allowed for offspring production and viability (relative to other sucrose levels), consistent with results from previous work in *D. melanogaster* (Buescher et al., 2013; Skorupa et al., 2008). All diets contained 3 ml/L of propionic acid and 30 ml/L of a nipagin solution (100 g/L methyl 4-hydroxybenzoate in 95% ethanol) and were prepared according to the protocol described in Bass et al. (2007). Each vial is 40 ml and contained 7 ml of food.

2.3 | Experimental design

Male and female virgin flies were assigned to one of the two dietary treatments prior to mating (we refer to this generation of flies as

F0), and then the offspring produced (we refer to this as the F1 generation) were also assigned to one of the two treatments. All possible combinations of dam × sire × offspring diet treatment were represented (= 2 × 2 × 2 = 8 combinations). Specifically, we collected flies of the F0 generation as virgins and placed them in vials of 10 flies across 30 vial replicates per treatment (×2 sexes) (1,200 flies, 600 of each sex), in their respective sexes, onto either the high-sucrose (20%) or the low-sucrose (2.5%) diets for the first 6 days of their adult life (Figure S1). We transferred flies to vials containing fresh food of the designated diet every 48 hr during this 6-day period.

At day 6, we randomly sampled six vials from each treatment and snap-frozen (using liquid nitrogen) the flies of these vials, storing them at −80°C for later triglyceride and body weight assays. Cohorts of flies in the remaining vials then entered a cohabitation phase to enable female and male flies to mate. Cohorts of males and female flies were combined, in vials of 10 pairs, in each of all four possible diet combinations: Lower sucrose females × lower sucrose males; higher sucrose females × higher sucrose males; lower sucrose females × higher sucrose males; higher sucrose females × lower sucrose males. During this phase, flies cohabited for 96 hr, allowing them to mate. They were transferred to a new vial with fresh food of standard 5% sucrose diet every 24 hr during this time.

Following the cohabitation phase of 96 hr, the F0 flies were separated back into their respective sex-specific cohorts, and placed back onto the high or low-sucrose diets that they were originally assigned prior to the cohabitation phase, in vials of 20 flies. Flies of these vials were then monitored for longevity (the longevity assay is described below). The vials from the 6-day old F0 flies (i.e. the vials from day 1 of the 96-hr cohabitation phase) were retained, and the eggs that had been laid by females of the respective vials were trimmed to 80 per vial (by removing excess eggs with a spatula). The remaining eggs were left to develop into adult offspring over 10 days at 25°C (on a 12:12 light/dark cycle in a temperature-controlled cabinet; Panasonic MLR-352H-PE incubator). These adult flies constituted the F1 offspring in the experiment, all F1 were reared on standard media (5% sucrose). We collected virgin F1 adults from each of the four combinations of parental diet treatments, and placed them in their respective sexes in vials of 10 flies, across 30 vial replicates per treatment per sex (2,400 flies) (Figure S1). We then assigned these F1 flies, produced by each dietary treatment combination of F0 flies, to either the lower sucrose or higher sucrose diet. At day 6 of adulthood, we snap-froze F1 flies of six randomly chosen vials per dam × sire × offspring diet combination. On the same day, 10 virgin focal F1 flies were placed together with 10 *tester* flies of the opposite sex (age standardised), collected from the Dahomey stock population, entering into a cohabitation phase of 96 hr (during which time the number of eggs laid by females of each vial was assessed). After 96 hr, flies were separated again into their respective sexes (in vials of 20 flies), and assigned back onto either the lower sucrose or higher sucrose diets that they had been on prior to cohabitation, and a longevity assay was carried out.

2.4 | Longevity

We scored the longevity of experimental flies of both parental (F0) and offspring (F1) generations. Cohorts of each sex were assayed separately. Each vial in the assay commenced with 20 flies each, and we included 10 vial replicates per treatment combination (dam × sire) for the F0 (800 flies), and seven vial replicates per treatment combination (dam × sire × offspring) for the F1 (2,240 flies). The number of dead flies per vial was scored three times per week (Monday, Wednesday and Friday), and surviving flies at each check transferred to vials with fresh food of the assigned diet treatment—until all flies were deceased. During the life span assay, vials were stored in boxes of up to 100 vials each, which were moved to randomised locations in the (25°C) control temperature cabinet every few days to decrease the potential for confounding effects of extraneous and random environmental variation from affecting the results.

2.5 | Fecundity

We measured the egg output of female flies from generations F0 and F1 at day 8 following the females' eclosion to adulthood, and used these egg counts as a proxy of female fecundity. On day 8, female flies oviposited for a 23-hr period, and were then transferred to fresh vials. Day 8 was selected because fecundity over 24 hr at this age has been shown to correlate with total lifetime fecundity of females in this Dahomey population (Nguyen & Moehring, 2015). Additionally, day 8 aligns with the peak period in reproductive output in the species (Bass et al., 2007) and early, short-term measures of reproduction of between 1 and 7 days can be used to accurately predict total lifelong fecundity in *D. melanogaster* (Nguyen & Moehring, 2015). Moreover, prior data show that modification of sucrose concentrations does not alter the timing of the reproductive peaks between treatments (Bass et al., 2007; see Figure S8 for more information). For the F0 generation, we counted eggs from 12 vial replicates per sire × dam combination, each containing 10 female flies, that had been mated with 10 male flies, across two different sucrose levels (2.5% and 20% sucrose), as above. We also counted eggs from F1 female flies, sampling 14 vial replicates per sire × dam × offspring combination, each containing 10 focal females (females from the experiment) combined with 10 tester male flies.

2.6 | Feeding behaviour

A separate experiment was set up to assess the feeding behaviour of the adult flies. Flies from the same wild-type Dahomey population were used as in the previous fecundity and longevity assays, and kept under the same conditions as they were for previous assays (in the same parental and offspring diet combinations as described above), with the exception of the number of flies per vial. For

this assay, flies were kept in vials of five individuals, separately by sex, except during the 96-hr cohabitation window when they were kept in vials of 10 flies, five males and five females. These flies were transferred to new food every 24 hr, always transferring them to new food the night before an observation.

We measured feeding behaviour of the flies of the F0 and F1 generations of each combination of diet treatment, using previously reported protocols of Wong et al. (2009). In short, feeding behaviour of the flies of each vial (number of proboscis extensions into the food) was observed over a 2-hr observation period, commencing at 10 a.m., including 30 min of time acclimating flies to the observer's presence. Observations were run four times for each vial over the first 3 weeks of adult life at 8, 11, 15 and 17 days of age for the parental generation, and 8, 11, 15 and 20 days of age for the offspring generation. This was performed for each of the two different dietary treatment levels (2.5% and 20% sucrose), and for each of the parental dietary treatment combinations (dam × sire × offspring), to address whether the flies moderated their feeding behaviour according to the dietary treatment they were subjected to, or the treatment of their parents.

2.7 | Body weight

Adult flies that had been snap-frozen at day 6 post-eclosion were individually weighed with a Mettler Toledo ultra-microbalance (Model: XP2U/Z). In the F0 generation, 220 (110 females and 110 males) flies were weighed from the two different dietary treatments. In the F1 generation, 879 flies (439 males and 440 females) were weighed from the two dietary treatments (219–220 per parental diet treatment combination).

2.8 | Lipids and protein

Whole-body triglyceride levels were measured in adult flies, prior to mating, from the (F0) parental generation. Adults, six days of age were used, and triglyceride levels were divided by body weight and protein levels respectively (full protocols reported in Supporting Information). A separate experiment was set up to generate additional samples of the (F0) parental generation, this time, after they had cohabited (mated) for 96 hr, and snap-frozen at day 9 following eclosion. Flies were kept under the same conditions as they were for previous assays (as described above). We also added an additional assay to measure whole-body protein. This was done to determine whether the protein and triglyceride content of the (F0) parental flies would be altered after mating, using protein as a proxy for the amount of metabolically active tissue.

Six biological replicates (from different vials) per treatment level were used to conduct the triglyceride and protein assays in the F0, as well as three technical replicates (repeated aliquots from the same sample of adult flies). For the F1 generation, three biological replicates per treatment level, with three technical replicates per biological replicate, were used. Five female flies and eight male flies were used for each biological replicate in the assay.

2.9 | Statistical analyses

We used R (Version 3.6.1) and RStudio (Version 1.2.1335) (R Core Team, 2019) for statistical analyses. We modelled dietary effects on both the F0 and F1 flies, running separate models for each generation and each trait (life span, fecundity, feeding behaviour, body weight and triglyceride level). We fitted linear mixed-effects models, using the R package lme4 (Bates et al., 2015), to test the effects of fixed factors parental diet, offspring diet, mate diet and sex, and possible interactions between these fixed effects, on life span of the F0 flies (offspring diet and offspring sex was not included in F0 analyses) and F1 flies respectively. We included the vial identification number as a random intercept in the longevity models. Since we monitored flies for life span thrice weekly (Monday, Wednesday and Friday), the age of recorded death for each individual fly was estimated within a margin of 72 hr (e.g. a life span of 30 days indicates that a fly died between 27 and 30 days post-eclosion).

To test the effects of parental diet, offspring diet, mate diet and sex on female fecundity, we fit a linear model to the egg output data for both generations. We included parental diet, offspring diet, mate diet and sex as fixed effects, and we explored interactions between these factors. The fecundity models only included one observation per vial because we counted the overall number of eggs laid per group of females of a given vial, and divided by the number of females in the vial, to derive an average per female, and therefore no random intercept was required in these models. We used sum-to-zero constraints in all models. Our data are available from Dryad Digital Repository: https://doi.org/10.5061/dryad.mcvdnck21.

We first fit full models to the data, including all fixed effects and their interactions (up to the level of second-order interactions). We reduced each model down to a final (minimum adequate) model using an approach based on parsimony reduction, in which the least-significant terms were removed sequentially, starting with the highest order interactions. We tested whether the reduction of each term led to a significant change in the deviance between models with log-likelihood ratio tests, and an alpha criterion of 0.05. The final models for life span, body weight, feeding behaviour and triglyceride level were fit by restricted maximum likelihood, applying type III *F* tests with Kenward-Roger's approximation of degrees of freedom. Fecundity measures were fit using *F* tests and Type III sum-of-squares ANOVA. We visually inspected diagnostic plots for the linear mixed effect models, to ensure that the assumptions of normality and equal variances were met. To investigate the relationships between fitness indicating traits (egg production and life span) with body composition (body mass and triglyceride content), we calculated Pearson's pairwise correlations and then bias-corrected bootstrapped confidence intervals. We also tested correlations between expression of triglyceride levels, fecundity and life span of female and male F1 offspring; these results are presented in Supporting Information.

3 | RESULTS

3.1 | Effects of sucrose treatments on the parental (F0) flies

3.1.1 | Life span and fecundity

The effect of dietary sucrose on longevity of the F0 flies was moderated by sex ($F_{1,38}$ = 57.00, p < 0.001, Figure 1a; Table S1), with female longevity exhibiting high sensitivity to sucrose (~35% increase in longevity on a lower sucrose diet relative to the higher sucrose diet). Whereas male longevity *decreased* ~3% on a lower sucrose diet relative to the higher sucrose diet. Notably, the longevity of the F0 males and females was in part dependent on the diet of the flies that they mated with during the brief 96-hr cohabitation phase early in life ($F_{2,37}$ = 3.16, p < 0.05, Figure 1a; Table S1). Specifically, when the diets of the cohabiting flies were matched for sucrose content, the flies lived longer.

Females on the lower sucrose diet produced less eggs than those on a higher sucrose diet ($F_{1,46}$ = 20.73, p < 0.001, Figure 1b; Table S2), but there were no effects of the diet of the males that they mated with on fecundity ($F_{1,46}$ = 0.15, p = 0.699).

3.1.2 | Lipid and protein measurements

Before mating

Females accumulated more triglycerides than males, both when triglycerides were normalised (divided by protein levels) to protein levels ($F_{1,12}$ = 7.90, p < 0.05, Table S3a) and normalised to body mass ($F_{1,9}$ = 12.14, p < 0.01, Table S3b). When normalised to protein content,

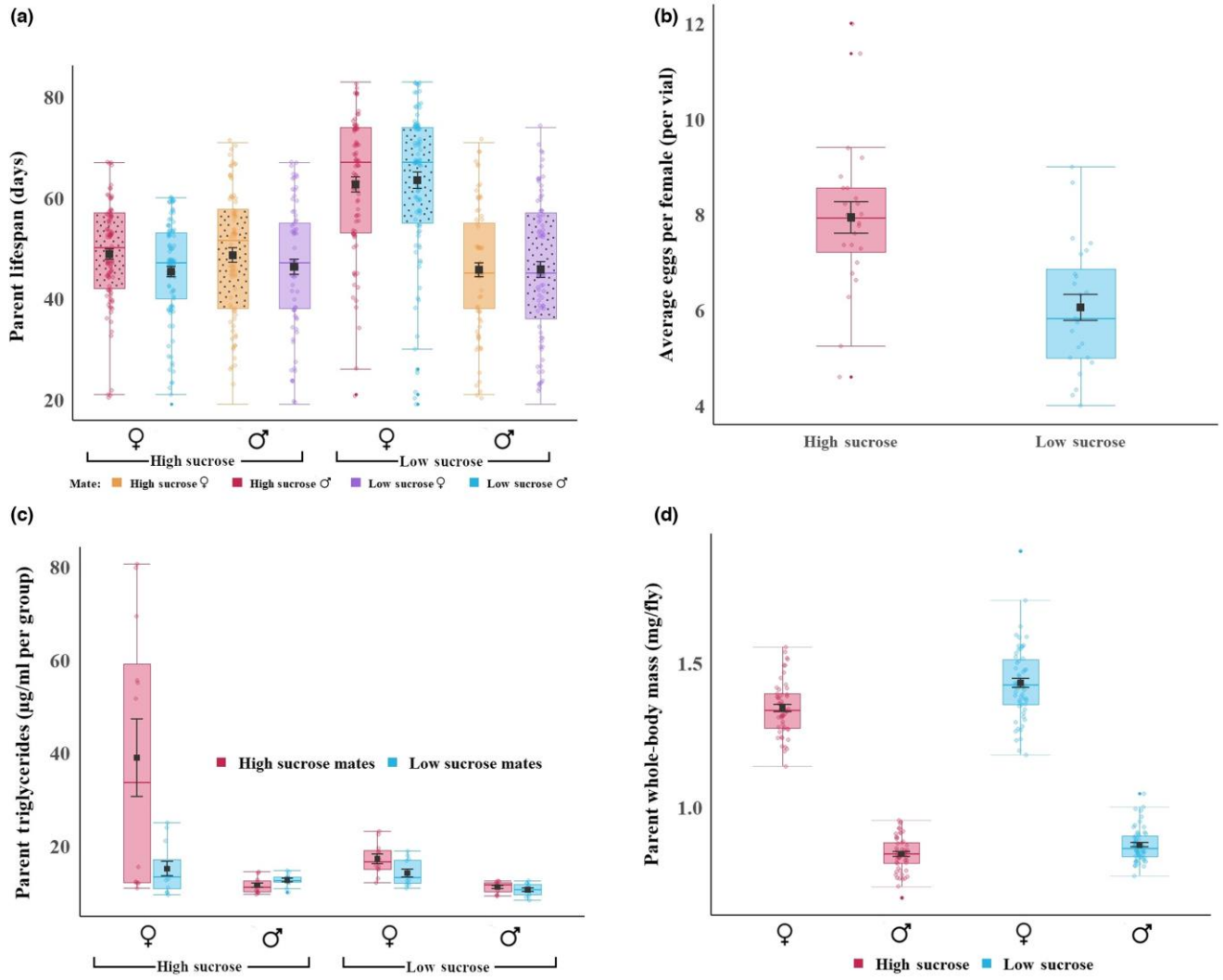

FIGURE 1 Effects of high sucrose (20% of overall solution) and low sucrose (2.5% of overall solution) on F0 (parent) life span, egg production, triglyceride levels and body mass. Plots show means, and standard error bars inside boxplots show medians and quartiles. (a) Life span of F0 flies (*y*-axis), their diet (*x*-axis) and diet of the mate (indicated by colour of the boxplots) on life span, matching parent diets are indicated by dotted pattern (interaction: Diet of F0 × Diet of their mate). (b) Average eggs per F0 female (*y*-axis), across both diets (*x*-axis) (main effect: Diet of F0 female). (c) F0 whole-body triglycerides (*y*-axis), sex of F0 and their diet (*x*-axis), with the diet of their mate indicated with colour (interaction: Diet of F0 × Sex of F0). (d) F0 body mass (*y*-axis), sex of F0 and their diet (*x*-axis), with their diet indicated with colour (interaction: Diet of F0 × Sex of F0)

triglyceride levels were also affected by the interaction between diet and sex of the flies ($F_{1,12}$ = 11.66, p < 0.001). Females fed lower sucrose diets had higher triglyceride levels than those fed higher sucrose (mean ± *SE*: $female_{low\ sucrose}$ = 3.7 μg/ml ± 0.24 and $female_{high\ sucrose}$ = 2.7 μg/ml ± 0.19), with the reverse pattern in males ($male_{low\ sucrose}$ = 1.6 μg/ml ± 0.24 and $male_{high\ sucrose}$ = 1.22 μg/ml ± 0.10).

After mating

Triglyceride levels (divided by protein levels) of parents after they mated were affected by their own diet (in a pattern consistent with their pre-mating triglyceride levels) (*lmer* analysis with Kenward-Roger's *F* test $F_{1,27}$ = 8.07, p < 0.01, Table S4a), and by their sex ($F_{1,27}$ = 8.31, p < 0.001, Table S4a). Intriguingly, the triglyceride levels after mating were also affected by the diet of their mate ($F_{1,27}$ = 6.09, p < 0.05). These outcomes were unchanged when the data were divided by body weight, and again triglyceride levels in parents are affected by their sex ($F_{1,27}$ = 6.60, p < 0.05, Table S4b), and by the diet of their mate ($F_{1,27}$ = 4.70, p < 0.05, Table S4b). With the exception of focal males on higher sucrose diets, focal flies of both sexes had higher whole-body triglyceride levels if they mated with a tester fly provided with higher sucrose diet (Figure 1c).

3.1.3 | Body mass

Both dietary sucrose content and sex, and their interaction, affected the body mass (measured prior to mating) of the parental F0 flies ($F_{1,206}$ = 5.27, p < 0.05 Table S5). Flies assigned to the lower sucrose diet were heavier compared to flies assigned to the higher sucrose diet, with the difference in body mass across the two diets greater in females than males (Figure 1d).

3.1.4 | Feeding behaviour

Sucrose content did not affect feeding behaviour (number of proboscis extensions onto the food) for the F0 flies, but an interaction between age and sex affected feeding behaviour ($F_{1,118}$ = 12.14, p < 0.001, Table S6; Figure S4), with females feeding more than males at days 8 and 11, but with sex differences dissipating at later life stages.

3.2 | Effects of parental diets on offspring (F1)

3.2.1 | Life span

The life span of F1 flies was shorter for flies produced by parents whose diets were matched for sucrose content than those born to parents whose diets were mismatched (*lmer* analysis, maternal diet × paternal diet, $F_{1,103}$ = 4.82, p < 0.05, Table S7, Figure 2a). Dietary sucrose intake of the F1 females also directly affected their longevity in a manner that mimicked the effects in the F0 flies; high-sucrose diets greatly decreased the life span of females relative to the low-sucrose diet. Intriguingly, the pattern was reversed in F1 males, with males on the higher sucrose diet outliving those on the lower sucrose diet ($F_{1,103}$ = 249.37, p < 0.001, Table S7). There was no interaction between offspring diet and either the maternal or paternal diet, indicating no signatures of an adaptive intergenerational effect for longevity.

3.3 | Fecundity

On average, female offspring flies assigned to a low-sucrose diet generally oviposited more eggs than those on a high-sucrose diet (Figure 2b). This general pattern differed to that observed for fecundity of the F0 females, where flies on the higher sucrose had a greater egg output. Notably, F1 egg output was shaped by a complex interaction between the F1 diet, maternal diet and paternal diet ($F_{2,103}$ = 8.02, p < 0.001, Table S8), albeit the pattern was not in the direction predicted under a scenario of an adaptive intergeneration effect, in which matched parent–offspring combinations would be expected to outperform mismatched combinations. Rather, we observed a large effect of one particular parental diet combination on intergenerational fecundity, in which F1 fecundity was higher when the dams were exposed to high sucrose, and the sires low sucrose (Figure 2b).

3.4 | Whole-body triglycerides

A complex interaction between F1 diet, sex and the diets of both parents shaped whole-body triglyceride levels in the F1 offspring (*lmer* analysis with Kenward-Roger's method $F_{3,32}$ = 3.93, p < 0.01, Figure 2c; Table S9). Triglyceride levels were lower when the F1 female diet was matched to the maternal diet, but these patterns were not observed for F1 males (Figure 2c). Both F1 females and males assigned to the low-sucrose treatment were generally characterised by low triglyceride content when produced by parents that had both consumed low sucrose, and high triglyceride content when produced by parents that had both consumed high sucrose. Conversely, F1 females and males that had been provided with a high-sucrose diet were characterised by the highest triglyceride content when produced by parents that had both consumed low sugar.

3.5 | Body mass

An interaction between the three diets, that is, the sucrose content of the dam, sire and offspring affected the body mass of the F1 offspring ($F_{2,86}$ = 4.50, p < 0.05, Table S10). Flies that consumed the lower sucrose diet, but that were produced by parents whose diets were matched to each other (i.e. either both parents consumed high sugar, or both consumed low sugar) weighed less than flies whose parents consumed diets that were mismatched for sucrose (Figure 2d).

3.6 | Feeding behaviour

Sucrose content of either the parental diets or the offspring diets did not have a significant effect on offspring feeding behaviour

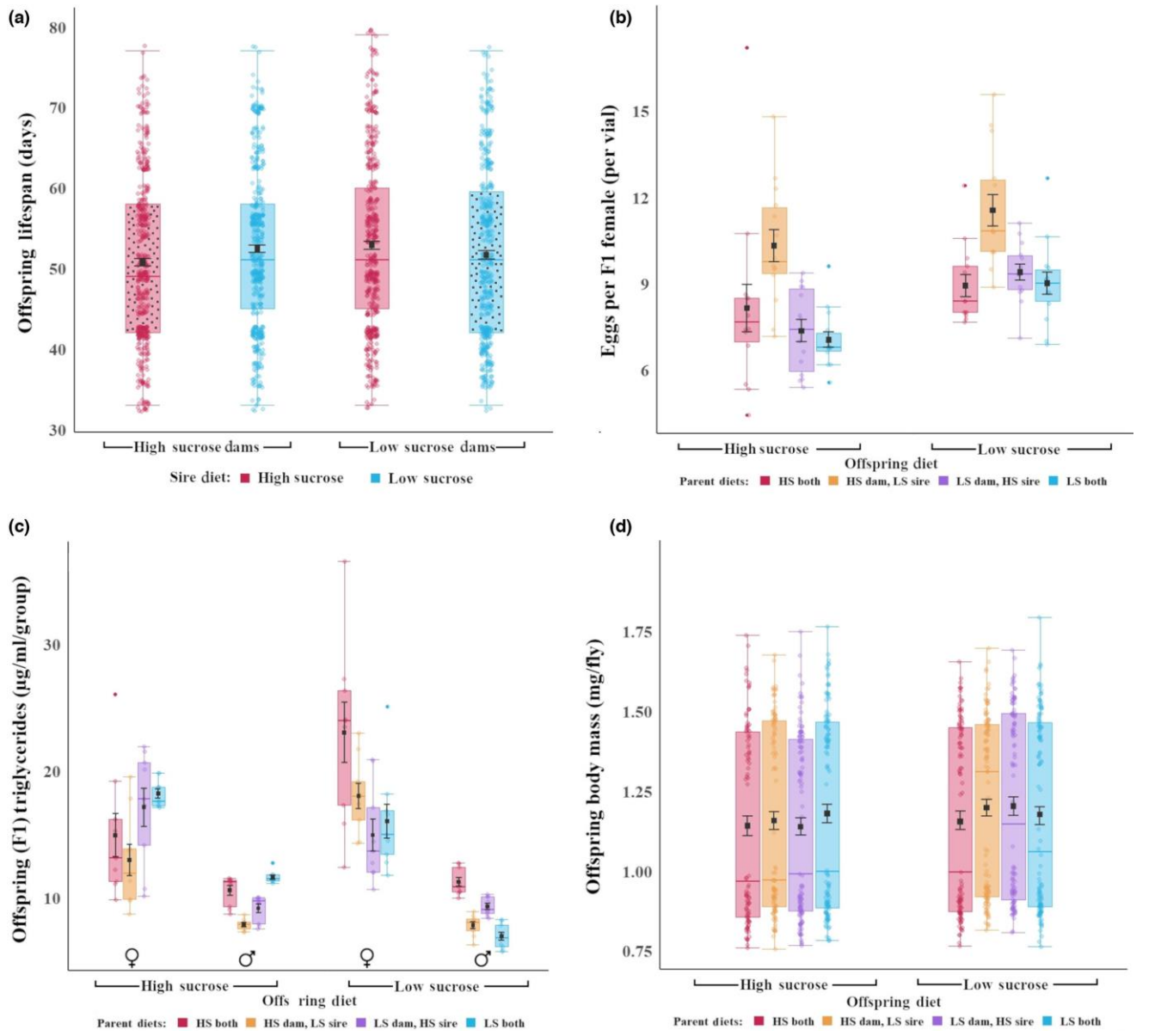

FIGURE 2 Effects of high sucrose (20% of overall solution) and low sucrose (2.5% of overall solution) on F1 (sexes combined) life span, egg production, triglyceride levels and body mass. Plots show means, and standard error bars inside boxplots show medians and quartiles. (a) Life span of F1 flies (*y*-axis), their dam's diet (*x*-axis) and diet of their sire indicated by colour of the boxplots, matching parent diets are indicated by dotted pattern (interaction: Maternal diet × Paternal diet). (b) Average eggs per F1 female (*y*-axis), their diets (*x*-axis) and the parental diet combination indicated by colour (interaction: Offspring diet × Maternal diet × Paternal diet). (c) F1 whole-body triglycerides (*y*-axis), sex of F1 and their diet (*x*-axis), with the diet combination of their parental indicated with colour (interaction: Offspring diet × Offspring sex × Maternal diet × Paternal diet). (d) F1 (sexes combined) body mass (*y*-axis), diet of F1 (*x*-axis), with the parental diet combination indicated with colour (interaction: Offspring diet × Maternal diet × Paternal diet)

(number of proboscis extensions onto the food). However, feeding behaviour differed across the sexes, with females feeding more than males, especially during peak reproductive periods of 8 and 11 days post-eclosion ($F_{1,43}$ = 24.85, p < 0.001, Table S11; Figure S4).

3.7 | Correlations

In our dataset, we tested associations between triglyceride levels, fecundity and life span, and found them non-significant in all cases, indicating that if associations exist they are weak. Further information can be found in Supporting Information.

4 | DISCUSSION

We varied the concentration of sucrose relative to all other nutrients in the diet of female and male *D. melanogaster*, across two generations, and examined the response in the expression of both life-history and physiological traits. We used a fully factorial design in

which sires, dams and their offspring were provided with diets that were either higher (20% of overall solution) or lower (2.5% of overall solution) in relative sucrose, such that combinations of parental diets and parent–offspring diets were either matched or mismatched, in all possible combinations. This design provided an opportunity to screen for adaptive dietary-mediated anticipatory parental effects on offspring phenotypes, and an opportunity to explore relative maternal and paternal contributions to offspring performance following dietary manipulation.

Our study revealed several new findings. First, although we detected parent-by-offspring diet interactions on offspring fecundity, triglyceride content and body mass, rarely were these in a direction consistent with predictions of the effects being anticipatory and adaptive. Moreover, generally these interactions were complex, with offspring trait values contingent on the diets of all interacting parties—sire, dam and offspring. As such, dietary-mediated parental contributions to offspring phenotypes were typically non-additive rather than cumulative, with particular combinations of mismatched dam–sire diets conferring heightened trait expression in offspring. Second, we identified unexpected dietary-mediated effects on the life span of both F0 and F1 generations. Notably, the life span of F0 flies was affected by the diets of their mates, with flies paired to mates that had been fed a diet matched in sucrose content to their own diets exhibiting longer life span than flies paired to mates fed a mismatched diet. Remarkably, however, while interacting dams and sires whose diets were sucrose matched enjoyed longer lives, their offspring suffered a longevity disadvantage relative to offspring produced by dams and sires whose diets were mismatched for sucrose. This suggests potential for a parent–offspring conflict over optimal dietary sucrose ingestion.

4.1 | Evidence for anticipatory parental effects

The key prediction underpinning the hypothesis of anticipatory parental effects is that components of offspring fitness will be higher when the offspring environment matches the parental environment—a prediction that has been most often tested in the context of maternal effects on offspring fitness (Mousseau & Fox, 1998; Uller et al., 2013; Yin et al., 2019). Testing this requires a particular experimental design whereby parents and offspring are exposed to matched and mismatched environments, and predicts that matched combinations (between parent and offspring) will result in the expression of offspring trait values that maximise fitness, and are hence adaptive, relative to mismatched combinations (Burgess & Marshall, 2014; Uller et al., 2013). While this prediction has received support from both classic and recent studies that used match–mismatch designs (Agrawal et al., 1999), meta-analyses aimed at synthesising patterns across species have however produced only weak evidence that such effects exist generally (Radersma et al., 2018; Sánchez-Tójar et al., 2020; Uller et al., 2013; Yin et al., 2019). Some evidence suggests the failure to detect general effects might be due to methodological deficiencies across studies (Burgess & Marshall, 2014; Uller et al., 2013), for example, a failure to test intergenerational outcomes in both matched and mismatched combinations, or the inability to partition anticipatory effects from condition-dependent parental effects. More generally, partitioning condition-dependent parental effects from cases that are genuinely anticipatory is likely to represent an ongoing challenge. Even in cases where researchers employ match–mismatch designs, given that condition-dependent effects may be context dependent in some cases and mimic patterns expected under the prediction of an anticipatory scenario. For example, this might occur in the case of 'silver-spoon' scenario, in which parents in better condition may produce offspring in better condition, compared to their lower conditioned counterparts, but only under certain environmental conditions (Bonduriansky & Crean, 2018; Engqvist & Reinhold, 2016).

Currently, it is unclear how often anticipatory parental effects might be triggered by environmental heterogeneity in the quality of food available to individuals. Many studies investigating dietary-mediated intergenerational or transgenerational effects in model organisms (in the context of nutritional and metabolic programming) have not implemented the requisite full factorial designs required to test the hypothesis (Buescher et al., 2013; Huypens et al., 2016; Matzkin et al., 2013; Oldham, 2011; Öst et al., 2014; Polak et al., 2017). Moreover, meta-analyses conducted to date have not sought to disentangle the relative strength of different classes of environment (e.g. dietary, climatic and pathogenic) on the magnitude of effect sizes associated with inter- or transgenerational anticipatory effects (Sánchez-Tójar et al., 2020; Uller et al., 2013; Yin et al., 2019). Notwithstanding, heterogeneity in the food environment would seem to be an excellent candidate to drive intergenerational anticipatory effects, given that macronutrient availability is likely to be relatively stable across generations for many species, and such predictability is a key theoretical requirement underpinning the evolution of anticipatory parental effects (Burgess & Marshall, 2014). We thus tested for anticipatory parental effects in the context of dietary sucrose environments, testing whether offspring life span, fecundity and physiology were sensitive to parent–offspring interactions.

While we found that fecundity, triglyceride content and body mass are sensitive to such interactions, only the patterns for female triglyceride content were consistent, and only weakly so, with the prediction that parent–offspring matches might result in a superior phenotype. In particular, female offspring that had been assigned to higher sucrose diets had lower triglyceride contents if their mothers had also been assigned to the higher sucrose treatment. Similarly, female offspring on a lower sucrose diet had a lower triglyceride content if their mothers were also provided lower sucrose. These signatures of intergenerational anticipatory effects were not evident in male offspring, and were only transmitted through dams. Whether or not these signatures of anticipatory effects are adaptive would depend generally on the association between triglyceride levels and fitness. The association would need to assume that low triglyceride levels confer higher lifetime reproductive success, and there is some evidence in *D. melanogaster* to suggest this may be the case. Studies that

use *Drosophila* as a model for studying effects of diet, obesity and exercise have shown that heightened activity, simulating exercise, in flies leads to reductions in triglyceride content (Sujkowski & Wessells, 2018), and a previous study has shown a sharp decline in female fecundity with increasing triglyceride levels (Skorupa et al., 2008). Notwithstanding, in our dataset, associations between triglyceride levels, fecundity and life span were non-significant in all cases, indicating that if associations exist they are weak. Thus, we conclude that if mechanisms of anticipatory parental effects, regulated by dietary sucrose variation, are at play in this species, the effects are weak and dwarfed by complex and non-additive maternal-by-paternal diet interactions, we discuss in more detail below.

4.2 | Non-additive maternal and paternal effects on both parent and offspring life spans

We observed a contradictory pattern across generations, whereby dietary matching between males and females extended life span of the interacting flies, but reduced life span among their offspring. Such a result is intriguing and indicative of potential antagonism between generations in terms of the optimal macronutrient balance underpinning the expression of key fitness-related traits. Our result is concordant with results of two recent transgenerational dietary restriction studies in *C. elegans*. In these studies, the authors revealed what they termed 'missing costs' of dietary restriction, demonstrating that a parental optimum for temporary fasting (restricted food for 6 days) that increased their own survival, reproduction and heat tolerance incurred negative effects on offspring fitness, and notably increased the mortality risk in the great-grandparental (F3) generation. Female offspring produced by long-lived mothers that had fasted had lower lifetime reproductive output, smaller body size and slower development than daughters from mothers that had not fasted (Ivimey-Cook et al., 2021; Mautz et al., 2020). It is possible that our results, together with other recent work, point to a mechanistic process whereby a trade-off may be driving the results we observe between generations: such as a trade-off between parental investment in offspring quality versus offspring quantity under certain dietary conditions or combinations. Our results cannot be extrapolated in this way, however, and we reaffirm the contention that intergenerational effects, mediated by dietary restriction or modification to macronutrient balance, may differ not only in their relative magnitude from one generation to the next, but also in their direction. Avenues for further research may investigate the role of whether matching between parental diets produces offspring of a lower quality (and therefore shorter lived), compared to offspring from parental diets that mismatch. Indeed, these effects could also be extended to an exploration of modifying other macronutrients in addition to sucrose.

The observation that an individual's life span is shaped in part by the diet of their mate is remarkable, especially given that the flies used in our experiments only cohabited for a period of 4 days early in life. This begs the question of what underlying physiological processes may mediate these effects. One possibility is that the diets of flies directly affected their condition, and subsequently shaped the levels and intensity of sexual interaction between males and females. Increases in sexual interaction have been shown to decrease the life span of female *D. melanogaster* (Bretman & Fricke, 2019; Liddle et al., 1995; Wigby & Chapman, 2005) and these effects also carry over to the next generation, resulting in a decreased life span among offspring (Dowling et al., 2014). A previous study has also confirmed that dietary quality of males (levels of yeast in the diet) affects their reproductive competitiveness under sexual selection, in a nonlinear pattern (Fricke et al., 2008). Another possibility is the effects we observed may be partly mediated by triglyceride levels of the interacting flies. Our analyses of triglyceride levels of flies provide some insight, since females that cohabited and mated with males subjected to higher sucrose diets had higher whole-body triglyceride levels post-mating when compared to those that cohabited with males provided with lower sucrose mates. This suggests some capacity for males to directly alter the physiological status of their mates through transfer of seminal proteins during mating, but this cannot explain the effects that are universal across both sexes and diet conditions (Chapman et al., 1995). The capacity for dietary variation to mediate patterns that shape the outcomes of interacting phenotypes—phenotypes that are partly mediated by non-genetic effects among conspecifics—and the role of triglycerides in moderating effects on female life history following mating warrants further investigation.

Finally, we note that the nature of the parent–offspring diet interactions we observed were generally complex and contingent on the diets of interacting flies. For example, the main determinant of female offspring fecundity was an interaction between maternal and paternal diet, which affected female offspring fecundity independently of the diet of the female offspring. In particular, female F1 offspring produced by mothers fed higher sucrose and fathers fed lower sucrose had substantially higher fecundity than female offspring produced by any other combination of parental diet. These interactions suggest that effects of maternal and paternal diet on offspring phenotypes will not be simply cumulative, but rather the result of non-additive interactions.

5 | CONCLUSIONS

We suggest that future work expand the range of diet treatments that we used here to investigate whether the antagonistic effects mediated by dietary matching that we observed across generations are specific to the dietary treatments we used, or whether they can be generalised across a broader range of protein to carbohydrate ratios and caloric contents. There have been recent calls for such experiments that utilise the nutritional geometric framework within a transgenerational context (Bonduriansky & Crean, 2018). Additionally, our study measured reproductive consequences of the different dietary treatments in females only, and over a short period early in adult life. We suggest

that future studies focus on the intergenerational effects of diet on reproductive success. This is important because negative intergenerational effects that we reported on F1 life span may indeed be adaptive if accompanied by overall increases in reproductive output across the F1 life span. Exploration of effects beyond the F1 would facilitate interpretation of whether the patterns reported here are more likely mediated by direct condition transfer from parents to offspring, or via epigenetic mechanisms that are more likely to be anticipatory in nature (Sánchez-Tójar et al., 2020). Finally, further study is needed to determine how general the effects we see here in fruit flies are to other taxa and other diets. We suggest that the insights gained here may have relevance to mechanisms underpinning nutritional programming in mammalian systems including humans, given that many of the genes and metabolic pathways involved in nutrition, obesity and ageing are generally conserved (Camilleri-Carter et al., 2019).

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

AUTHORS' CONTRIBUTIONS

T.-L.C., D.K.D., M.D.W.P. and R.L.R. conceptualised the study and designed the experiment; T.-L.C. planned and carried out the experiment and wrote the first draft of the manuscript; T.-L.C., D.K.D., M.D.W.P. and R.L.R. contributed to the writing and editing of the subsequent drafts. All authors gave final approval for publication.

DATA AVAILABILITY STATEMENT

Data are available from Dryad Digital Repository: https://doi.org/10.5061/dryad.mcvdnck21 (Camilleri et al., 2022).

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Functional Ecology

Supplementary Material

Maternal and paternal sugar consumption interact to modify offspring life history and physiology

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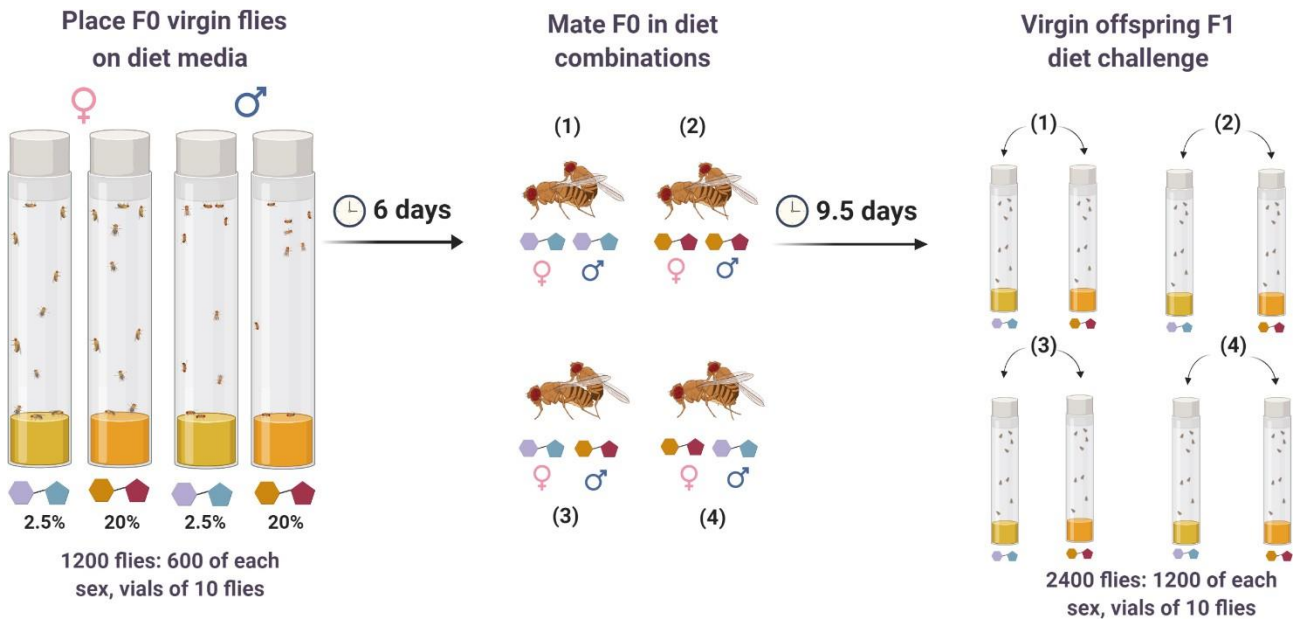

Figure S1. Experimental design, sample sizes, and diet challenges for each generation.

We collected flies of the F0 generation as virgins and placed them in vials of 10 flies across 30 vial replicates per treatment (x 2 sexes) (1200 flies, 600 of each sex), in their respective

sexes, onto either the high sucrose (20%) or the low sucrose (2.5%) diets for the first 6 days of their adult life (Figure S4, supplementary information). We transferred flies to vials containing fresh food of the designated diet every 48 hours during this 6 day period.

Results

F0 Lifespan

Table S1. Table S1. Effects of diet of the focal fly (Diet), sex, and diet of the mate (Mate) on longevity of the focal flies. General Linear Mixed Model, with fixed effects parameters calculated via F test with Kenward-Rogers approximation of degrees of freedom, and variance associated with random effect (Vial identity) estimated via REML.

*** indicates significance at $p < 0.001$, ** indicates significance at $p < 0.01$, * indicates significance at $p < 0.05$.

Fixed Effects	***F***	***p-value***
(Intercept)	1031.32	< 0.001 ***
Diet	92.88	< 0.001 ***
Sex	1.73	0.185
Mate	2.07 [a]	0.122
Diet: sex	57.47	< 0.001 ***
Diet : mate	3.16[a]	< 0.05 *
Random Effects	***Variance***	
Vial identification	0.0	
Residual	200.8	

[a]df=1, all other df=1, df res=38

F0 Fecundity

Table S2. Statistical results (analysis of deviance) of the linear model (Gaussian error distribution) after model reduction for predictors of female (F0) egg output.
*** indicates significance at $p < 0.001$, ** indicates significance at $p < 0.01$, * indicates significance at $p < 0.05$.

Fixed Effects	***Sum Sq***	***F***	***p-value***
(Intercept)	1507.02	758.84	< 0.001 ***
Female diet	41.94	20.73	< 0.001 ***

Male diet	0.31	0.151	0.699

all *df* =1, df res=46

F0 TAG & Protein

Table S3a. Analysis of deviance table with Kenward-Rogers F test, linear mixed model (Gaussian error distribution) after model reduction for predictors F0 whole-body triglycerides, normalised by protein—before mating.
*** indicates significance at $p < 0.001$, ** indicates significance at $p < 0.01$, * indicates significance at $p < 0.05$.

Fixed Effects	***F***	***p-value***
Intercept	115.28	< 0.001 ***
Diet	0.48	0.50
Sex	7.87	< 0.05 *
Diet : Sex	11.65	< 0.01 **
Random Effects	***Variance***	
Vial identification	0.1475	
Residual	0.01126	

All df =1, df res=12

Table S3b. Analysis of deviance table with Kenward-Rogers F test, linear mixed model (Gaussian error distribution) after model reduction for predictors F0 whole-body triglycerides, normalised by body weight—before mating.
*** indicates significance at $p < 0.001$, ** indicates significance at $p < 0.01$, * indicates significance at $p < 0.05$.

Fixed Effects	***F***	***p-value***
Intercept	28.09	< 0.001 ***
Diet	0.88	0.37
Sex	12.14	< 0.01 **
Random Effects	***Variance***	
Vial identification	0.000	
Residual	0.000	

All df =1, df res=9

Table S4a. Analysis of deviance table with Kenward-Rogers F test, linear mixed model (Gaussian error distribution) after model reduction for predictors F0 whole-body triglycerides, normalised by whole-body protein—after mating.

*** indicates significance at $p < 0.001$, ** indicates significance at $p < 0.01$, * indicates significance at $p < 0.05$.

Fixed Effects	*F*	*p-value*
Diet	8.074	< 0.01 **
Sex	8.312	< 0.001 ***
Mate diet	6.095	< 0.05 *
Random Effects	***Variance***	
Vial identification	0.00026	
Residual	0.00027	

All df =1, df res=27

Table S4b. Analysis of deviance table with Kenward-Rogers F test, linear mixed model (Gaussian error distribution) after model reduction for predictors F0 whole-body triglycerides, normalised by body weight—after mating.

*** indicates significance at $p < 0.001$, ** indicates significance at $p < 0.01$, * indicates significance at $p < 0.05$.

Fixed Effects	*F*	*p-value*
Diet	8.07	< 0.10
Sex	8.31	< 0.05 *
Mate diet	6.09	< 0.05 *
Random Effects	***Variance***	
Vial identification	0.0000017	
Residual	0.00000033	

All df =1, df res=27

F0 Body mass

Table S5. Statistical results (analysis of deviance) of the linear mixed model (Gaussian error distribution) after model reduction for predictors of whole-body weight for the parental generation. *** indicates significance at $p < 0.001$, ** indicates significance at $p < 0.01$, * indicates significance at $p < 0.05$.

Fixed Effects	*F*	*p-value*
(Intercept)	7128.55	< 0.001 ***
Diet	15.51	< 0.001 ***
Sex	862.63	< 0.001 ***
Diet: sex	5.28	< 0.05 *
Random Effects	***Variance***	
Vial identification	0.000	
Residual	0.007	

All df =1, df res=206

F0 Feeding behaviour

Table S6. Statistical results (analysis of deviance with Kenward-Rogers's method) of the linear mixed model (Gaussian error distribution) after model reduction for predictors of feeding behaviour for the parental generation. *** indicates significance at $p < 0.001$, ** indicates significance at $p < 0.01$, * indicates significance at $p < 0.05$.

Fixed Effects	***F***	***p-value***
Diet	1.36	0.2506
Sex	19.88	< 0.001 ***
Age	18.32	< 0.001 ***
Sex : Age	12.14	< 0.001 ***
Random Effects	***Variance***	
Vial identification	0.8546	
Residual	5.5823	

All df =1, df res=118

F1 Lifespan

Table S7. Analysis of deviance table with Kenwood-Rogers F test, linear mixed model (Gaussian error distribution) after model reduction for predictors of offspring age.
*** indicates significance at $p < 0.001$, ** indicates significance at $p < 0.01$, * indicates significance at $p < 0.05$.

Fixed Effects	***F***	***p-value***
(Intercept)	2926.96	< 0.001 ***
Offspring diet	381.54	< 0.001 ***
Offspring sex	14.99	< 0.001 ***
Maternal diet	1.92	0.154
Paternal diet	1.47	0.209
Offspring diet : offspring sex	249.37	< 0.001 ***
Maternal diet : paternal diet	4.82	< 0.05 *
Random Effects	***Variance***	
Vial identification	3.2	
Residual	122.1	

A*ll df =1,* df res=103

F1 Fecundity

Table S8. Statistical results (analysis of deviance) of the linear model (Gaussian error distribution) after model reduction for predictors of female offspring egg output. *** indicates significance at $p < 0.001$, ** indicates significance at $p < 0.01$, * indicates significance at $p < 0.05$.

Fixed Effects	***Sum Sq***	***F***	***p-value***
(Intercept)	927.63	282.48	< 0.001 ***
Offspring diet	4.13	1.26	0.265
Maternal diet	4.27	1.30	0.257
Paternal diet	33.05	10.06	< 0.001 ***
Offspring diet : maternal diet	5.41	1.65	0.202
Offspring diet : paternal diet	-19.50	266.53	0.649
Offspring diet : maternal diet :paternal diet	52.70	8.02	< 0.001 ***

All df =1, df res=103

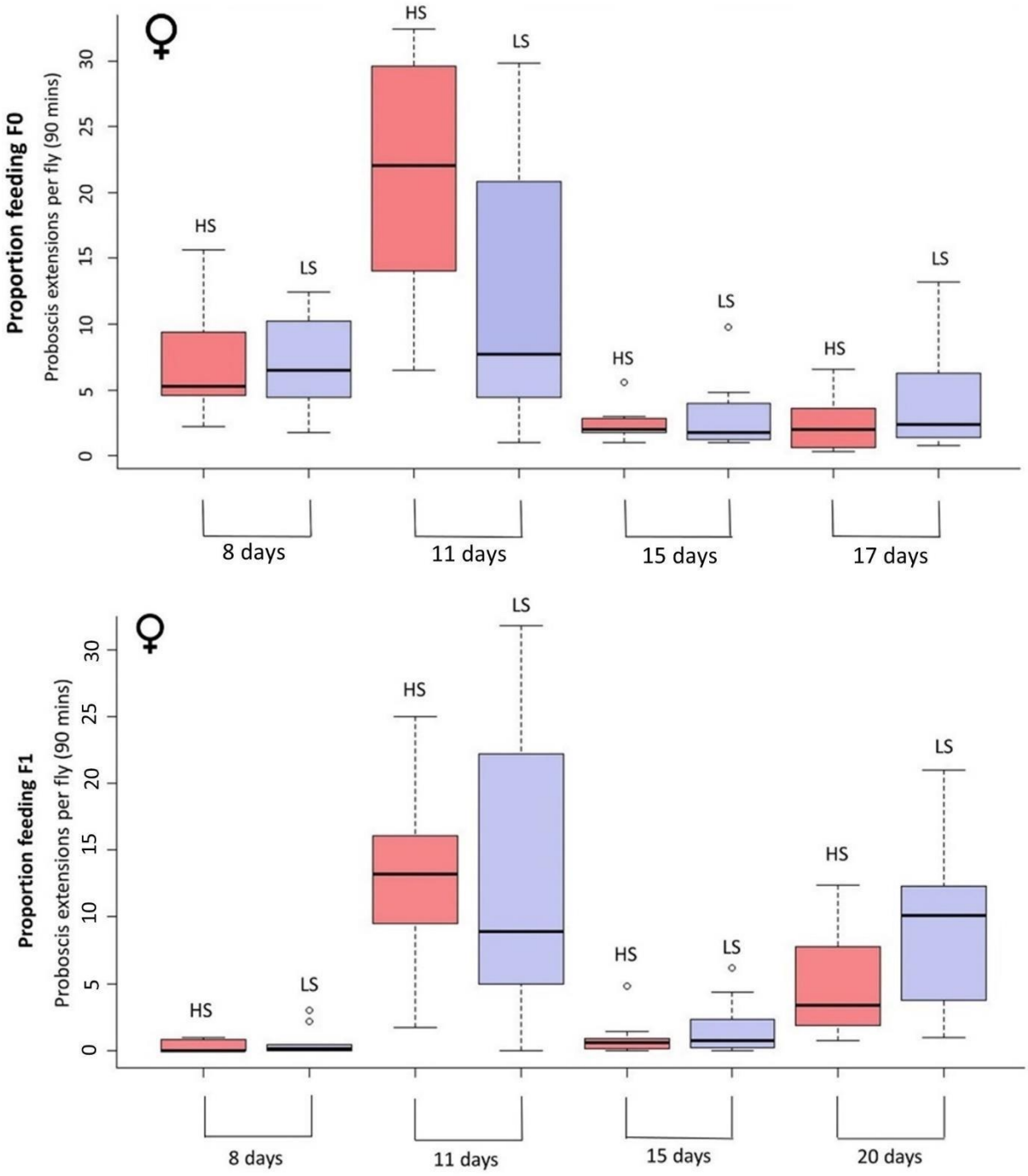

Figure S2. Median and quartiles of observed feeding for both generations, across 4 times periods. This is the total number of proboscis extensions into food media within 90 minutes, for the males and females of the parental generation at each age assayed. HS indicates a high sucrose diet of 20% (P:C ratio 1:5.3), LS indicates a low sucrose diet of 2.5% (P:C ratio 1:1.4)

F1 TAG

Table S9. Analysis of deviance table with Kenwood-Rogers F test, linear mixed model (Gaussian error distribution) after model reduction for predictors of offspring whole-body triglycerides.

*** indicates significance at $p < 0.001$, ** indicates significance at $p < 0.01$, * indicates significance at $p < 0.05$.

Fixed Effects	***F***	***p-value***
Diet	26.73	< 0.001 ***
Sex	4.09	< 0.05 *
Maternal diet	2.76	0.106
Paternal diet	1.22	0.278
Diet : sex	1.845	0.183
Sex : maternal diet	3.49	0.070
Sex : paternal diet	0.243	0.625
Diet : maternal diet	23.78	< 0.001 ***
Diet : paternal diet	2.23	0.144
Diet : maternal diet : paternal diet	4.28	< 0.01 **
Sex : maternal diet : paternal diet	1.19	0.283
Diet : sex: maternal diet : paternal diet	3.96	< 0.01 **
Random Effects	***Variance***	
Vial identification	0.000	
Residual	0.011	

All *df* =1, df res=32

F1 Body mass

Table S10. Statistical results (analysis of deviance) of the linear mixed model (Gaussian error distribution) after model reduction for predictors of offspring weight (milligrams).
*** indicates significance at $p < 0.001$, ** indicates significance at $p < 0.01$, * indicates significance at $p < 0.05$.

Fixed Effects	***F***	***p-value***
(Intercept)	10560.11	< 0.001 ***
Offspring diet	0.044	0.947
Offspring sex	3936.54	< 0.001 ***
Maternal diet	1.50	0.224
Paternal diet	1.30	0.256
Maternal diet : paternal diet	0.8921	0.347
Offspring diet : maternal diet	7.08	< 0.01 **
Offspring diet : mat diet : pat diet	4.50	< 0.05 *
Random Effects	***Variance***	
Vial identification	0.001	
Residual	0.006	

All *df* =1, df res=86

F1 Feeding behaviour

Table S11. Statistical results (analysis of deviance with Kenwood-Roger's method) of the linear mixed model (Gaussian error distribution) after model reduction for predictors of offspring feeding behaviour.
*** indicates significance at $p < 0.001$, ** indicates significance at $p < 0.01$, * indicates significance at $p < 0.05$.

Fixed Effects		***F***	***p-value***
Diet		4.89	0.54
Sex		24.85	< 0.001 ***
Maternal diet		5.57	0.89
Paternal diet		24.45	0.38
Age		2.95	0.12
Random Effects	***Variance***		
Vial identification	0.000		
Residual	5.5		

All *df* =1, df res=43

Correlations between body composition and fitness

Female F1 offspring

There is a weak negative relationship between female offspring triglyceride content and the amount of eggs they output, (r = -0.152, p = 0.718, BCa CI: -0.9519, 0.4283), and a moderate positive relationship between their triglyceride content and their lifespan (r = -0.399, p = 0.328, BCa CI: -0.5317, 0.9062). The relationship between female offspring body mass and egg output is a weak positive correlation (r = 0.214, p = 0.610, BCa CI: -0.6457, 0.8833), so too for female offspring body mass and lifespan, a weak positive correlation (r = 0.180, p = 0.668, BCa CI: -0.6298, 0.7948).

Male F1 offspring

For male offspring, a weak positive correlation was found between their triglyceride content and their lifespan (r = 0.196, p = 0.641, BCa CI: -0.7640, 0.8620), whereas a weak negative relationship was found between their body mass and their lifespan (r = -0.301, p = 0.469, BCa CI: -0.8132, 0.3609).

Further diet information

Table S12

Sucrose	S:Y	P:C:F
0.25%	1:40	1 : 0.9 : 0.02
2.5%	1:4	1 : 1.4 : 0.02
5%	1:2	1 : 1.9 : 0.02
10%	1:1	1 : 3.1 : 0.02
20%	2:1	1 : 5.3 : 0.02
40%	4:1	1 : 9.8 : 0.02

Calculated by overall mass of ingredients and not calories. (If calculating by calories the kcals of protein and carb are the same 4kcal/g and fat yields about 9ckal/g)

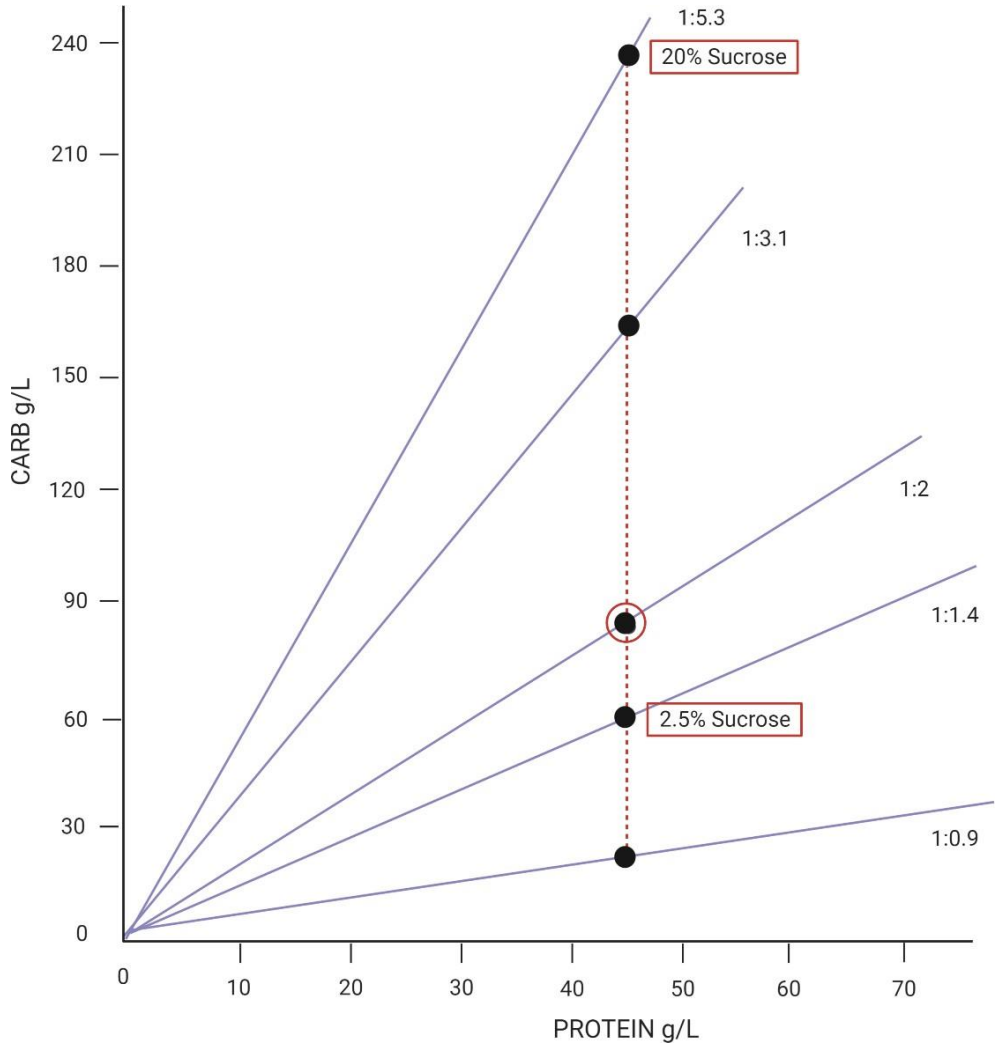

Figure S3. Sucrose levels in nutritional space

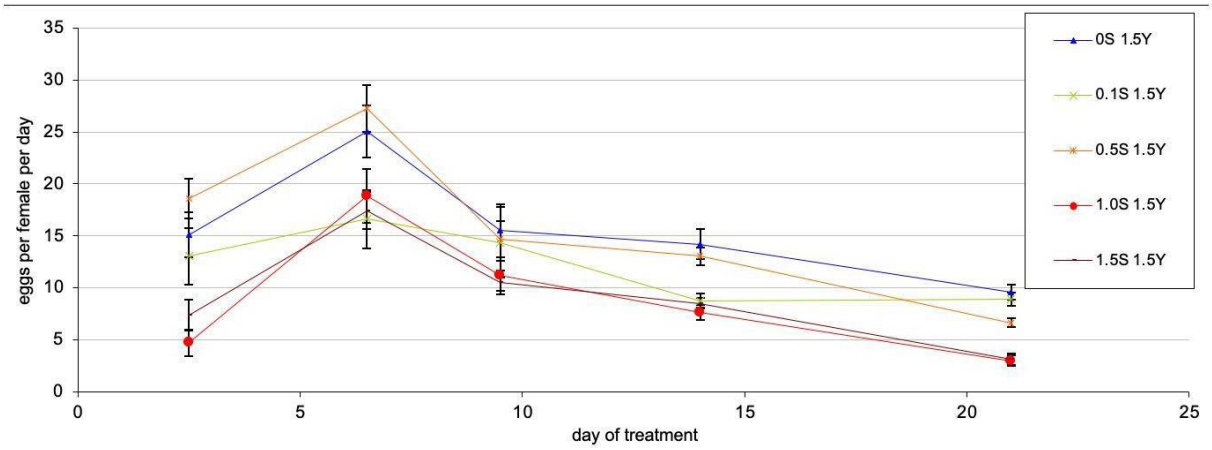

Figure S4. Reproductive peaks, and time points, under differing sucrose concentrations, with data from Bass et al., 2007.

Lipid and protein Assay protocol

Frozen flies (stored at -80C) were homogenized, using 5 flies per Eppendorf tube. Using a pestle, flies frozen in liquid nitrogen were crushed and 1 ml 0.05% Tween 20 lysis solution was added. Microtubes were then vortexed for 20 seconds each. We heated samples at 70°C in a waterbath for 5 min, and centrifuged at 5,000 rpm for 1 minute. We transferred supernatant (500 µl) from each sample to new Eppendorf tubes and centrifuged at 14,000 rpm for 3 minutes. For the protein assay, all above steps were followed, and PIERCE kit #23225 was used. Make working regent from kit with 50 parts of reagent A, and 1 part of reagent B. Add 10ul of protein samples or bovine serum albumin (BSA) standard (2g/ml) or samples per well in replicates.

For TAG: Supernatant (50 µl) from each sample was then transferred to a 96-well plate, and 200 µl of Thermo Infinity Triglyceride solution (pre-warmed at 37°C) was added to each well. For TAG: Samples were incubated for 5 minutes (37 degrees Celsius), and absorbance was measured at 540 nm.

For protein: Samples were incubated for 10 minutes, and absorbance measured at 562nm.

Chapter 4 | Sex-specific transgenerational effects of diet on offspring life history and physiology

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"I hate a Barnacle as no man ever did before, not even a Sailor in a slow-sailing ship."

-Charles Darwin (letter to William Darwin Fox)

Sex-specific transgenerational effects of diet on offspring life history and physiology

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Abstract

Dietary variation in males and females can shape the expression of offspring life histories and physiology. However, the relative contributions of maternal and paternal dietary variation to phenotypic expression of latter generations is currently unknown. We provided male and female *Drosophila melanogaster* diets differing in sucrose concentration prior to reproduction, and similarly subjected grandoffspring to the same treatments. We then investigated the phenotypic consequences of this dietary variation among grandsons and granddaughters. We demonstrate transgenerational effects of dietary sucrose, mediated through the grandmaternal lineage, which mimic the direct effects of sucrose on lifespan, with opposing patterns across sexes; low sucrose increased female, but decreased male, lifespan. Dietary mismatching of grandoffspring-grandparent diets increased lifespan and reproductive success, and moderated triglyceride levels, of grandoffspring, providing

insights into the physiological underpinnings of the complex transgenerational effects on life histories.

Keywords

Transgenerational effects, sucrose, life history, drosophila

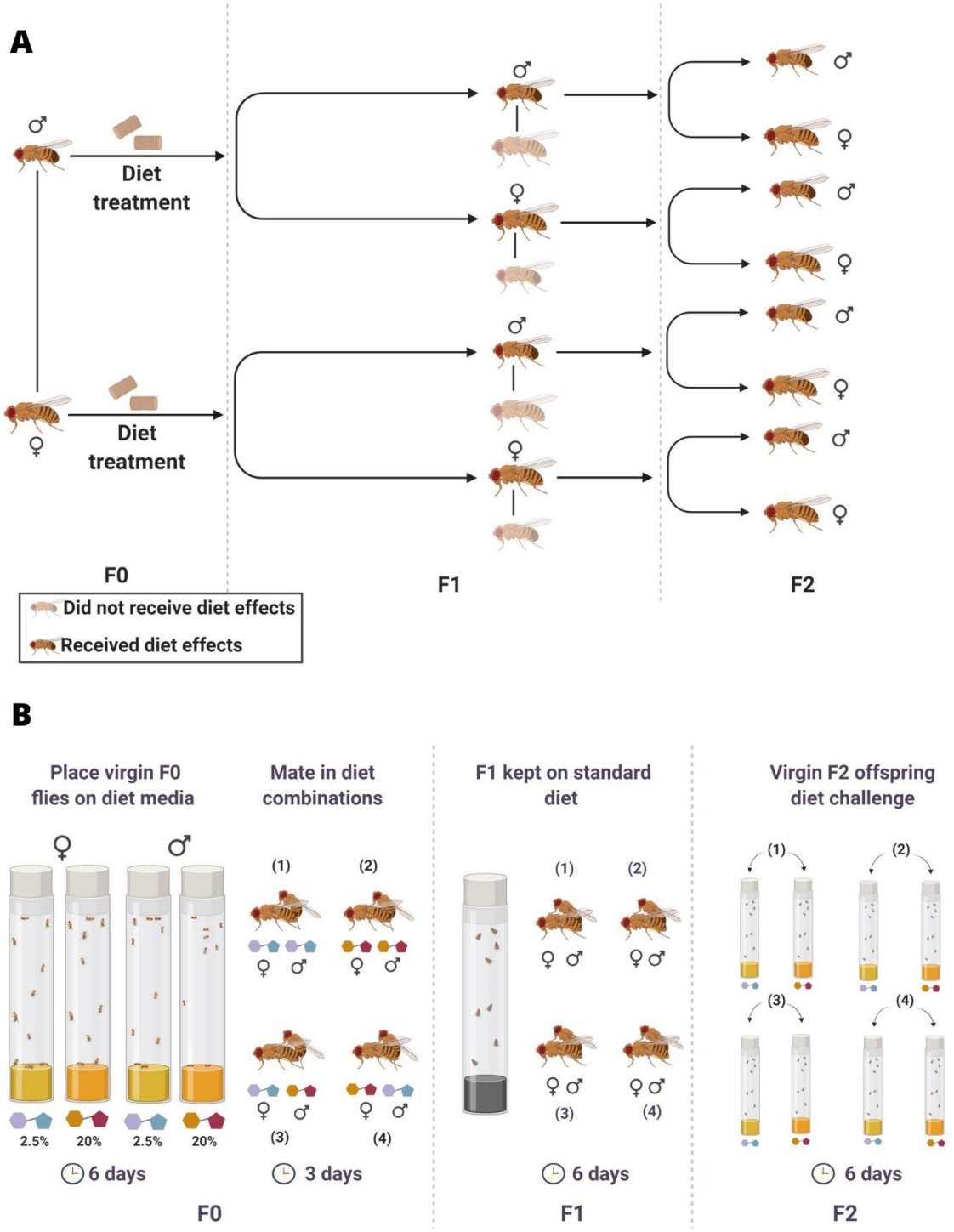
Main

From nematodes to primates, parental environments may shape the phenotypes of their offspring through non-genetic mechanisms that are either condition dependent or epigenetic in origin[1–3 4]. Consequently, when individuals are subjected to environmental heterogeneity prior to reproduction, their exposure to these environments can shape components of fitness in offspring and subsequent generations (transgenerational effects)[5–9]. Recent experiments have shown that variation in environmental factors, such as predation risk and levels of sexual conflict, among parents may catalyse transgenerational effects that differ in magnitude or direction across sexes, and which may also be lineage (genotype) specific[10]. Notwithstanding, currently it remains unclear whether such transgenerational effects are consistently instigated across diverse environmental stresses, whether they generally act to enhance or depress offspring performance, and whether they are transferred primarily through maternal or paternal lineages or hinge on interactions between both.

Nutrition is a pervasive and critical source of environmental variation that shapes phenotype. Variation in macronutrient balance or caloric content has been shown to confer direct effects on lifespan, fecundity, and underlying physiology[11–14]. Studies from diverse species have demonstrated that females and males require different diets to maximise their fitness[15–19]. Female fitness is maximised on a higher relative protein concentration because

high protein facilitates egg production, while higher relative carbohydrate content for males provides fuel for attracting and locating a mate[20–24]. Recent studies have also shown dietary-induced intergenerational effects across a variety of species; for example, changes to sugar content of the parental diets in fruit flies[25,26] or dietary fat content in mice[27] induces phenotypic changes in parents that are transmitted to their offspring. Intriguingly, when the sucrose content of both male and female parents are altered, then parental contributions to offspring phenotypes may involve complex dam-by-sire interactions that are non-cumulative and dependent upon the sucrose content of the offspring diet [28]. It is less clear, however, whether these dietary-mediated parental effects are epigenetic in origin, and thus inherited across multiple generations [10,25,29–32], and if so, whether phenotypic consequences for males and females are divergent.

Here, we experimentally tested the capacity for dietary sucrose variation among male and female fruit flies (*Drosophila melanogaster*) to precipitate transgenerational effects on components of life-history and physiology in their grandoffspring. Flies were administered one of two diets that varied in the concentration of sucrose (2.5% or 20% sucrose). The diets were administered using a full factorial design: males and females were each assigned to one of the two diets prior to reproduction, and then their grandsons and granddaughters were administered the same dietary treatments. All male-female-grandoffspring dietary combinations were represented, resulting in female-male and grandparent-grandoffspring diet combinations that were either matched or mismatched (Figure 1, panels A and B). This design enabled us to test whether dietary-mediated transgenerational effects exist, to decipher the relative grandmaternal and grandpaternal contributions, and the capacity for interactions between grandparental diets and those of the grandoffspring to shape grandoffspring phenotype, and to determine whether such effects are sex specific.



74

75 **Figure 1. A) Diet effects lineage.** Diet treatments were administered to both parents in the

76 F0; and they were mated to create the F1 offspring, and received a standard diet (both

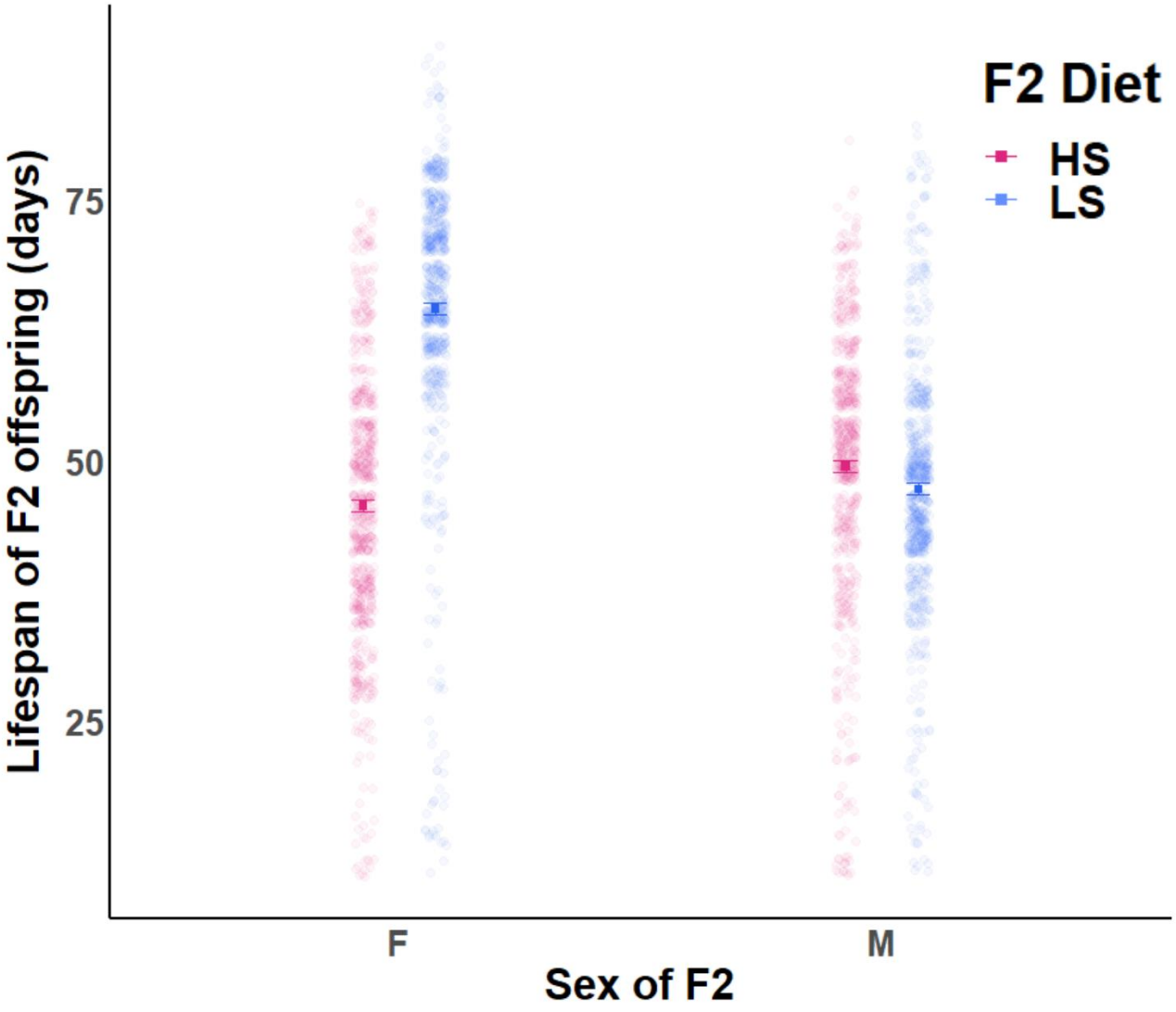
77 males and females for each parent), and F1 offspring were mated with flies outside of the

experiment that received a standard diet. This allowed us to track which F1 sex was passing on the diet effects to the F2 generation. **B) Experimental design.** The F0 generation was administered either higher (20% of overall solution) or lower (2.5%) relative sucrose in adulthood, and kept on this diet in sex-specific cohorts for 6 days as virgins before a subsequent three day cohabitation (on common garden media) that allowed mating to occur. Male and female F0 flies were combined in all possible diet combinations. The F1 generation was reared, maintained (6 days again), and cohabited (3 days) on common garden media (an intermediate sucrose content of 5%). The F2 generation was reared from egg-to-adulthood on common garden media, and then challenged as virgins with either the higher or lower sucrose such that their diet either matched or mismatched one or both of their grandparents (F0).

Results

Direct and indirect effects of dietary sucrose on grandoffspring lifespan are sex-specific

The diets of the grandoffspring (F2) flies conferred direct and sex-specific effects on their lifespan ($F_{1,148} = 369.80$, $p < 0.001$, Table S2, Figure 2). Female F2 flies assigned to the low sucrose diet lived longer than females or males assigned to any other treatment, and 30% longer than females on the high sucrose diet. Females assigned to a high sucrose diet exhibited the shortest lifespan of any group of flies. In contrast to the large negative effect of high sucrose on female lifespan, high dietary sucrose conferred a moderate increase in male lifespan relative to males assigned to a low sucrose diet (Table S2; Figure 2).



102 **Figure 2.** Direct effects of dietary sucrose on the lifespan (plots show emmeans ± standard error, and data points, each data point is an individual fly) of F2 granddaughters (F) and grandsons (M). HS indicates a high sucrose diet of 20% (P:C ratio 1:5.3), LS indicates a low sucrose diet of 2.5% (P:C ratio 1:1.4).

105

The lifespan of F2 flies was also in part mediated by the diets of their grandmothers, with

106 the pattern of effects differing across F2 males and females ($F_{1,148} = 9.35$, $p < 0.01$, Table

107 S2, Figure 3, panel A). The transgenerational effects of sucrose concentration mimicked

108 the direction of direct effects described above. That is, F2 females descended from

109 grandmaternal lineages assigned to a low sucrose diet lived longer than those descended

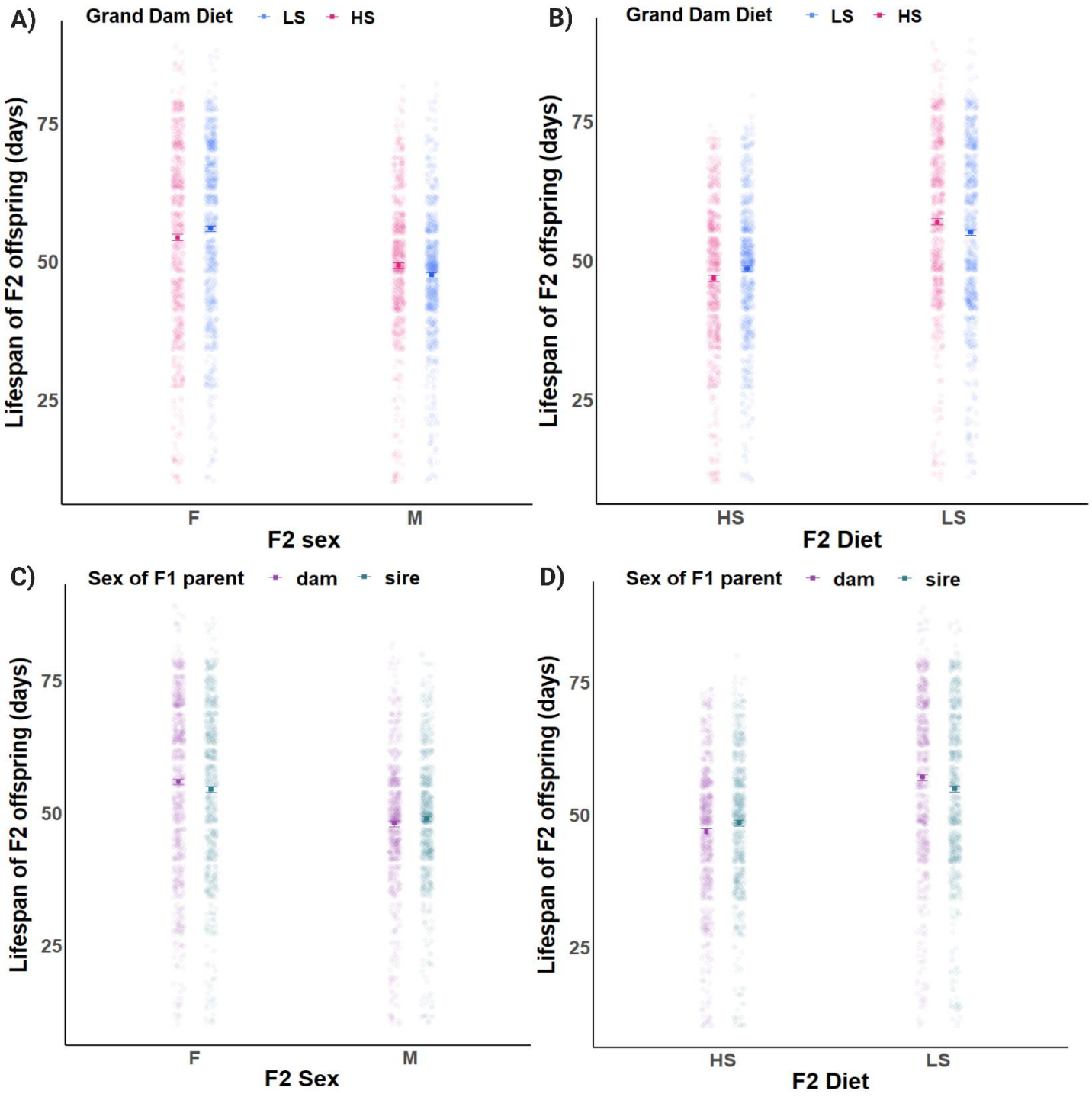
110 from high sucrose lineages, while the opposite pattern was observed in F2 males, whereby

111 those descended from high sucrose grandmaternal lineages outlived those from low

112 sucrose lineages (Figure 3, panel A). Additionally, matching combinations of

grandmaternal-grandoffspring dietary sucrose led to shorter F2 lifespan than mismatched combinations (Figure 3, panel B).

In our experimental design, grandparental flies were manipulated, and F2 phenotypes measured. This involved transfer of effects across an intermediate generation – the F1 parents. Although the diets of F1 parents were never manipulated (they received a standard diet of 5% sucrose, an intermediate sucrose content), our experimental design ensured the grandparental effects were transferred through either male F1 or female F1 flies (but not both, Figure 1). Thus, we could track whether the sex of the *transferring F1 parents* affected the pattern and direction of the transgenerational effects. Indeed, the interaction between the sex of the F2 flies and the sex of the transferring F1 parents affected F2 lifespan ($F_{1,148}$ = 4.44, p <0.05, Table S2); female F2 lived longer if the grandparental dietary treatments were transferred through F1 females, while male F2 lived longer when the effects were transferred through F1 males (Figure 3, panel C). The sex of the transferring F1 parent flies also moderated the direct effects of the F2 diet on F2 lifespan, Table S2, Figure 3, panel D, $F_{1,148}$ = 12.42, p <0.001). F2 flies assigned directly to a high sugar diet lived longer if grandparental dietary treatments were transferred through F1males rather than through females, while F2 flies assigned to a low sugar diet lived longer if grandparental dietary treatments were transferred through F1 females than males.



133 **Figure 3.**

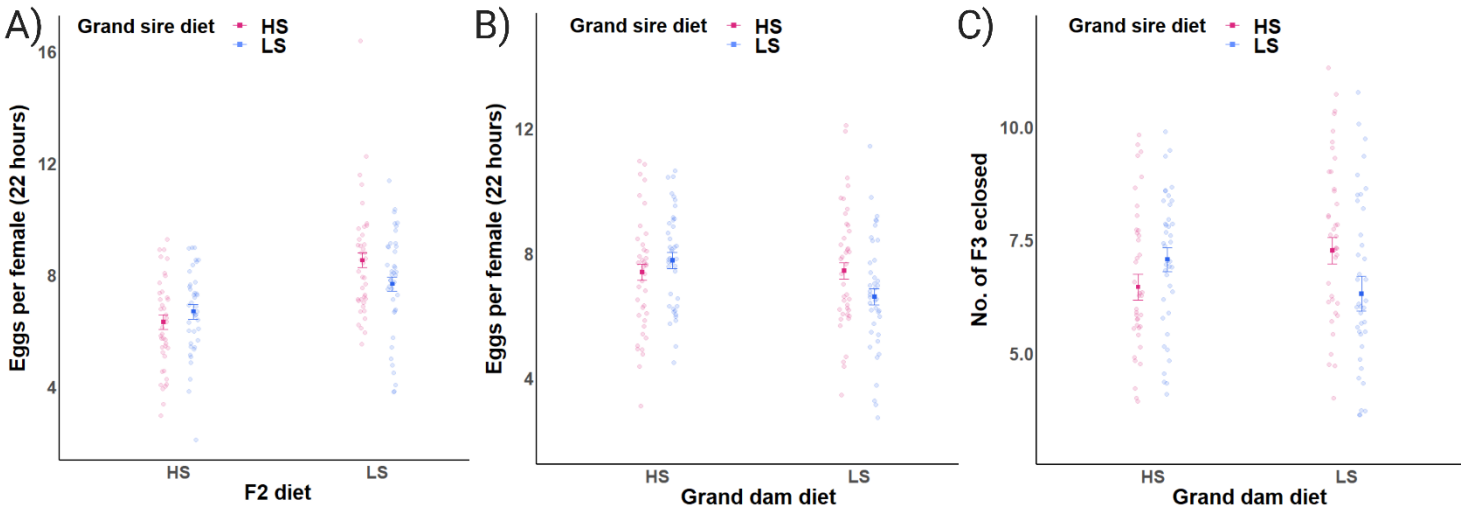
134 Effects of high sucrose (HS, 20% of overall solution) and low sucrose (LS, 2.5% of overall solution) on F2 lifespan (F=female, M=male). Plots show emmeans, standard error bars, and data points, each data point is an individual fly. (A) Lifespan of F2
135 flies (y-axis), their grand dam's diet (colour), their sex (x-axis), (interaction: grand dam diet× F2 sex). (B) Lifespan of F2 flies (y-axis), their grand dam's diet (colour), their diet (x-axis), (interaction: grand dam diet × F2 diet). (C) Lifespan of F2 flies (y-axis), the sex of the parental linage that received a diet treatment, (colour), their sex (x-axis), (interaction: F1 sex × F2 sex). (D) Lifespan of F2 flies (y-axis), the sex of the parental linage that received a diet treatment (colour), their diet (x-axis), (interaction: F1 sex × F2 diet).

142

Grandoffspring fecundity, viability, and triglycerides are mediated by grand maternal and grand paternal diets

Fecundity & viability

Direct dietary effects were observed in the F2 generation; F2 Females had higher fecundity when ingesting the low sucrose than high sucrose diet. These direct effects of diet were, however, shaped by the grand paternal, but not grand maternal diet (Table S3, $F_1 = 5.49$, $p < 0.05$). Mismatched combinations of grandpaternal-F2 female diet resulted in F2 granddaughters producing more eggs than matched combinations (Figure 4, panel A). Female F2 fecundity was also shaped by an interaction between the grand maternal and grand paternal diets (Table S3, Figure 4, panel B, $F_1 = 14.77$, $p < 0.05$); F2 females that descended from matched grandmaternal-grandpaternal combinations tended to have lower fecundity than those arising from mismatched combinations, and in particular F2 females descended from grandparents that were each assigned to low sucrose diets exhibited lowest fecundity (Figure 4, panel B). The reproductive success (as gauged by the number of adult offspring produced) of the F2 females was also shaped by a similar interaction between grandmaternal and grandpaternal diet, in which the clutch size was lower for F2 females descended from matched, relative to mismatched, combinations of grandmaternal-grandpaternal diet (Table S4, Figure 4, panel C, $F_1 = 5.25$, $p < 0.05$).



164 **Figure 4**

165 Effects of high sucrose (HS, 20% of overall solution) and low sucrose (LS, 2.5% of overall solution) on female F2 reproductive output. Plots show emmeans, standard error bars and data points, each data point is the number of eggs or offspring adults produced per female F2. (A) Number of eggs laid by F2 flies (y-axis), their grand sire's diet (colour), their diet (x-axis), (interaction: grand sire diet × F2 diet). (B) Number of eggs laid by F2 flies (y-axis), their grand sire's diet (colour), their grand dam's diet (x-axis), (interaction: grand sire diet ×grand dam diet). (C) Number of F3 flies eclosed per vial (y-axis), their grand sire's diet (colour), their grand dam's diet (x-axis), (interaction: grand sire diet × grand dam diet).

166 *Triglyceride levels*

167 An interaction between the diet of F2 offspring and the grandmaternal diet affected the

168 triglyceride level of the F2 flies (Table S5, Figure 5, panel A, $F_{1,98}$= 8.56, p <0.01). F2 flies

169 fed high sucrose diets that descended from grandmothers assigned to high sucrose,

170 exhibited much higher triglyceride levels than F2 flies from any other combination of

171 grandmaternal-F2 offspring diet (Figure 5, panel A). Similarly, the interaction between F2

172 diet and grandpaternal diet shaped triglyceride level; however in this case, F2 offspring

173 assigned to a high sucrose diet and descended from grandfathers assigned to low sucrose,

exhibited much higher triglyceride levels than any other combination of grandpaternal-F2 diet (Table S5, Figure 5, panel B, $F_{1,98}$= 12.75, p <0.001).

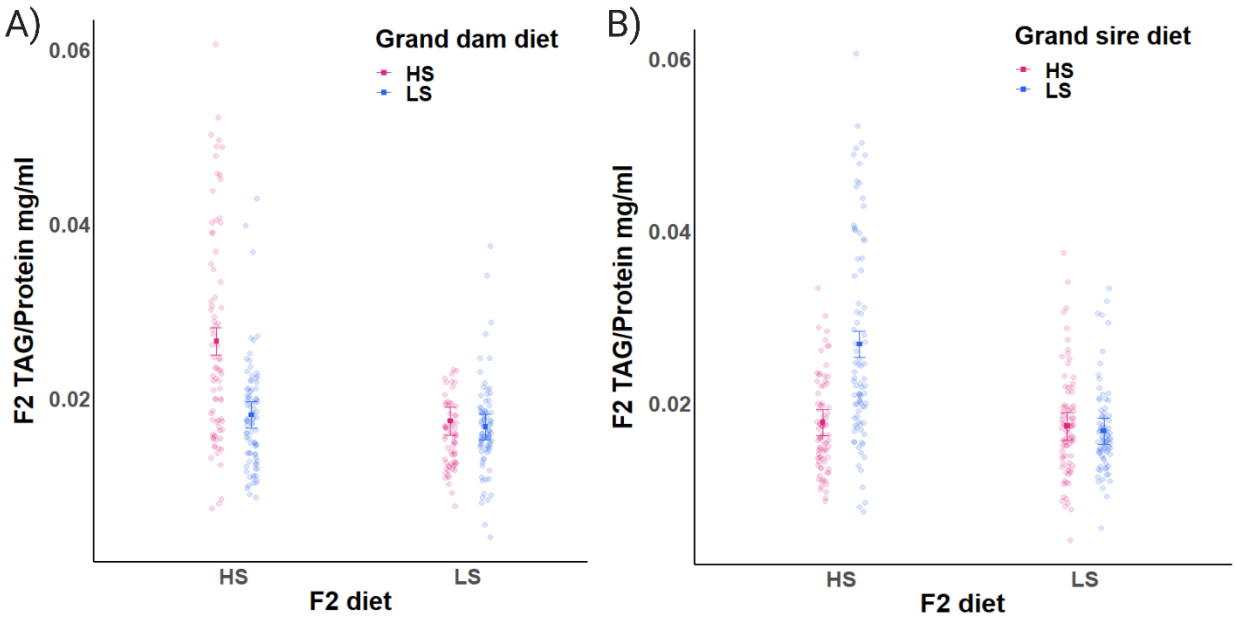


Figure 5.

Effects of high sucrose (HS, 20% of overall solution) and low sucrose (LS, 2.5% of overall solution) on F2 whole body triglyceride (TAG) levels divided by their whole body protein levels, per fly. Plots show emmeans, standard error bars, and data points, each data point is the amount of TAG divided by the amount of protein for each group of flies—five females and 8 males. (A) F2 TAG per fly (y-axis), their grand dam's diet (colour), their diet (x-axis), (interaction: grand dam diet × F2 diet). (B) F2 TAG per fly (y-axis), their grand sire's diet (colour), their diet (x-axis), (interaction: grand sire diet × F2 diet).

No direct effect of dietary sucrose on female F0 fecundity

Neither the male, nor female diet, affected egg output of the F0 females (Table S1).

Discussion

Here, we show opposing effects of dietary sucrose on the lifespan of each sex, in *D. melanogaster*—low sucrose enhances female lifespan, but decreases male lifespan relative to high sucrose. Notably, these effects were observed in both direct and indirect (i.e. transgenerational) contexts. Moreover, the dietary-mediated transgenerational effects on lifespan mimicked the observed direct effects for each sex: a low sucrose grandmaternal diet conferred elevated F2 female lifespan, but decreased male F2 lifespan, relative to a high sucrose grandmaternal diet. We also revealed strong effects of specific combinations of grandparental and grandoffspring diet, and between grandmaternal and grandpaternal diets, in shaping the measured traits; all of which exhibited a similar pattern—a mismatch in diet enhanced trait expression in subsequent generations. We highlight inherent complexity in the nature of the transgenerational effects. The effects are generally sex-specific, and unexpectedly, are affected by the sex of the transferring F1 parent. Finally, we note that interactions between grandparental diet and F2 diet affected triglyceride levels of F2 flies, suggesting that dietary-mediated modifications of triglyceride levels, across generations, may contribute to the observed transgenerational effects on life history phenotypes.

Studies investigating sex-specificity of transgenerational effects across a range of taxa have observed instances in which environmental modification such as dietary challenges, presence of predators, or behaviour-modifying drugs of the grandparental environment triggered sex-specific effects on grandoffspring phenotype. Intriguingly, in these cases, transgenerational effects tend to manifest in the opposite sex to that subjected to the grandparental treatment; that is, modification of the grandmaternal environment may enhance or inhibit trait expression among grandsons, or conversely, modification to the grandpaternal environment may enhance or inhibit trait expression among granddaughters [10,25,30,32,33]. Our findings are consistent with previous research, revealing opposing

directions of sucrose-mediated grandmaternal effects in each of the sexes. Notably, we have uncovered further levels of complexity in the nature of the transgenerational effects.

First, we revealed sex differences in the magnitude of transgenerational effect (the effect transmitted from dietary-treated F0 flies to F2 flies) are dependent on the sex of the transferring F1 parent. Second, we observed that the outcomes of transgenerational effects depend on interactions between the diets of the grandparents and those of the grandoffspring; dietary mismatching across generations tends to enhance lifespan (mediated by a grandmaternal-by-grandoffspring interaction) and fecundity (mediated by a grandpaternal by granddaughter diet interaction). Whether or not these effects are mediated by underlying triglyceride levels of the experimental flies remains unclear; yet one pattern was notable, suggestive of a possible transgenerational link between physiology and lifespan. F2 offspring assigned to a high sucrose treatment, and descended from high sucrose grandmaternal lineages exhibited the highest triglyceride levels and the shortest lifespans.

Investigations into life history traits are imperative in assessing the adaptive significance of transgenerational effects on offspring, given the close link between these traits and lifetime fitness[5]. Our experiments, across three generations, with the diet challenge also given to the F2 generation had the requisite power to address these previous knowledge gaps. Our finding that dietary mismatching (between both grandparents and between grandparents and grandoffspring) tends to enhance trait expression adds new insight to studies investigating transgenerational effects of diet, and of transgenerational effects of environmental change more generally. Previous studies of dietary-mediated transgenerational effects have tended to focus on changes in metabolite profiles and physiology across generations, rather than on changes to expression of life history traits[25,26]. Moreover, these designs typically do not have the requisite power to partition relative influences of (grand)maternal and (grand)paternal effects on transgenerational

phenotypes, nor the factorial design required to determine whether transgenerational mismatches enhance or depress performance [9].

The prevailing prediction is that a matching of environment between grandparents and grandoffspring may augment offspring fitness-related traits, because the matching environments may allow parents to prime offspring to cope with environments that their parents faced (anticipatory effects). The evidence for anticipatory effects across contexts and taxa is, however, mixed and weak[9,29], and many studies that have leveraged experimental designs with the power to test for these effects have primarily focused on intergenerational effects (from F0-F1[29]), with very few studies classified as transgenerational where grandoffspring should have no direct experience of the grandparental environment[34]. Our study generally revealed patterns that were contrary to the predicted pattern – dietary mismatching, rather than matching, between grandparents and F2 offspring tended to augment offspring performance. This begs the question of whether cross-generational dietary mismatching may be a general phenomenon that extends across the diets used in our study.

Two recent studies shed some light on this question. Deas et al. (2019) manipulated dietary quality across three generations (F0 to F2) in *D. melanogaster*, providing flies of each generation with a 'rich' diet (rich in calories and supplemented with yeast) or a poor diet (calorie diluted, with no yeast supplementation), in all combinations, and then measuring phenotypic expression in the grandoffspring (F2). They reported that a mismatch between the diet quality ("poor vs "good" diet) of granddams and granddaughters led to a faster development time in the pupal stage of the granddaughters, but this effect did not hold for the entire development time[35]. This study also focused on females, and therefore was not able to capture sex specificity in any generation. On the other hand, Camilleri et al. (2022) tested effects of dietary mismatching of F0 flies and their F1 offspring, manipulating the

diets of parents of each sex and their offspring, and utilising the same sucrose diets used in the current study. We found that dietary mismatching between parents and F1 offspring led to an increase in lifespan, and fecundity of the offspring[28]. Here, we advance these findings by demonstrating that these effects of dietary mismatch are carried over for multiple generations, are also dependent on the sex of F1 lineage. Because the effects are unambiguously transgenerational (extending from F0 to F2), they are less likely to result from differences in condition of the grandparents, suggesting instead possible epigenetic mechanisms regulating the effects.

In sum, our work uncovers dietary-mediated transgenerational effects that are on the one hand remarkably consistent across generations – transgenerational effects of sucrose tend to mimic the direct effects. We have also extended previous work to demonstrate that dietary mismatching across generations tends to augment phenotype in a manner that is unlikely to be directly linked to condition-dependence. Future work should focus on uncovering the ecological and evolutionary significance of these results, and the underpinning mechanistic drivers. We suggest that a process in which transgenerational dietary mismatching promotes fitness of future generations could buffer populations from future changes in environment, and be particularly adaptive for species that live and depend on ephemeral resources for their source of nutrients. If this is the case, then populations evolving in fluctuating environments may be more likely to evolve mechanisms that promote the fitness of offspring encountering novel environments.

Methods

Study species and generating experimental flies

We sourced flies from Dahomey, a large laboratory population of *D. melanogaster*, originally sourced from Benin West Africa[36]. The flies have been maintained in large population cages, with overlapping generations in the Piper laboratory, Monash University, Australia, since 2017, and prior to that in the Partridge laboratory, University College London [37]. Prior to the beginning of the experiment, we collected ~3000 eggs from the cages, and distributed them into 250mL bottles containing 70mL of food. Food comprised 5% sucrose (50 grams sucrose, 100 grams yeast, 10 grams agar per 1 litre solution with an estimated protein to carbohydrate [P:C] ratio of 1:1.9, and 480.9 kcal per litre (see Supplementary Material Figure S4 for further diet details). Every generation (for 7 generations), adult flies eclosing from multiple bottles were admixed prior to redistributing the flies across new bottles. To control for potential sources of variation in their environment, during these 7 generations we strictly controlled both the age of flies at the time of ovipositioning—all flies were within 24 h of eclosion into adulthood when producing the eggs that propagated the subsequent generation, and their population density was 300-320 adult flies within each bottle in each generation.

Dietary treatments

The diet media we used consists of sucrose, autolysed brewer's yeast powder (sourced from MP Biomedicals SKU 02903312-CF), and agar (grade J3 from Gelita Australia), as well as preservatives—propionic acid, and nipagin. We prepared two dietary treatments, differing in relative sucrose concentration; 2.5% sucrose (that we refer to as a lower

sucrose treatment relative to the 5% concentration usually provided to the population of flies used in this experiment), and 20% sucrose (that we refer to as a higher sucrose treatment) of overall food solution. The 2.5% sucrose diet contains 25 grams of sucrose, 100 grams of yeast and 10 grams of agar per litre of food prepared, with an estimated P:C ratio of 1:1.4 and 380.9kcal per litre of food. The 20% sucrose treatment contains 200 grams of sucrose, 100 grams of yeast, and 10 grams of agar per litre of food prepared, with an estimated P:C ratio of 1:5.3 and 1080.9kcal per litre of food. The diets thus differed not only in sucrose concentration, but overall macronutrient balance and their total caloric content, resembling differences typically observed between obesogenic and healthy diets in humans. The higher sucrose concentration was selected based on preliminary experiments that we conducted, and which elicited an obese-like phenotype in the flies, consistent with results from previous work in *D. melanogaster* [11,25,38]. All diets contained 3ml/l of propionic acid and 30ml/l of a Nipagin solution (100g/l methyl 4-hydroxybenzoate in 95% ethanol) and were cooked according to the protocol described in Bass *et al.* (2007) [39]. Each vial is 40mL in volume, and contained 7mL of food.

Experimental design

Male and female virgin flies were assigned to one of two of the dietary treatments prior to mating (we refer to this generation of flies as F0), and then the grandoffspring produced (F2 generation) were also assigned to one of the two treatments. All possible combinations of grand dam × grand sire × grandoffspring diet treatment were represented (= 2 × 2 × 2 = 8 combinations). Specifically, we collected 1280 flies of the F0 generation as virgins and placed them onto either the high sucrose (20%) or the low sucrose (2.5%) diets for the first 6 days of their adult life. They were in vials of 10 flies across 64 vial replicates per treatment, and per sex (High sucrose: 32 vials of males and 32 vials of females; low sucrose, 32 vials of males and 32 vials of females, 128 vials in total;1280 flies, 640 of each sex). They were kept

342 in their respective sexes. We transferred flies to vials containing fresh food of the designated
343 diet every 48 hours during this 6 day period.
344
345 At day 6, we randomly sampled six vials from each treatment, and snap froze (using liquid
346 nitrogen) the flies of these vials, storing them at -80°C for subsequent measures of
347 triglyceride levels. Cohorts of flies in the remaining vials then entered a cohabitation phase to
348 enable female and male F0 flies to mate. Cohorts of males and female flies were combined, in
349 vials of 10 pairs, in each of all four possible diet combinations: lower sucrose females ×
350 lower sucrose males; higher sucrose females × higher sucrose males; lower sucrose females ×
351 higher sucrose males; higher sucrose females × lower sucrose males. During this phase, flies
352 cohabited for 96 hours. They were transferred to a new vial with fresh food of standard 5%
353 sucrose diet every 24 hours during this time.
354
355 The vials from the 6 day old F0 flies (i.e., the vials from Day 1 of the 96 h cohabitation
356 phase) were retained, and the eggs that had been laid by females of the respective vials were
357 trimmed to 80 per vial by removing excess eggs with a spatula. The remaining eggs were left
358 to develop into adult offspring over 10 days at 25°C (on a 12:12 light/dark cycle in a
359 temperature-controlled cabinet; Panasonic MLR-352H-PE incubator). These adult flies
360 constituted the F1 offspring in the experiment, and F1 flies developed on standard 5%
361 sucrose media. We collected 2080 virgin F1 flies from each of the four combinations of
362 parental diet treatments, and placed them in sex-specific cohorts of 10 individuals per vial, on
363 standard 5% sucrose media for 6 days. We then allowed these F1 males and F1 females to
364 cohabit and mate with male or female *tester* flies (creating 10 pairs per vial) that had been
365 collected from the same Dahomey stock population (but not subjected to a dietary sucrose
366 treatment) to create the F2 generation. The diet treatments applied to the F0 flies were thus

transferred to the F2 generation via either F1 males or F2 females, but never through both sexes. The F1 flies were 6 days of adult age when laying the eggs that produced the F2 generation.

We then collected virgin F2 flies – the grandoffspring of the F0 flies – from each of the four combinations of F0 diet treatments (per sex), and placed them in their respective sexes in vials of 10 flies, across vial replicates per treatment per sex (4080 flies, 2040 male, 2040 female). We then assigned these F2 flies, produced by each dietary treatment combination of F0 flies, to either the lower sucrose or higher sucrose diet. At day 6 of adulthood, we snap froze F2 flies of six randomly chosen vials per grand dam × grand sire × grandoffspring combination. On the same day, 10 virgin focal F2 flies of each grand dam × grand sire × grandoffspring combination and each sex were placed together with 10 age-matched tester flies of the opposite sex from the Dahomey population, entering into a cohabitation phase of 96 h (during which time the number of eggs laid by females of each vial was assessed). After 96 hours flies were separated again into their respective sexes (in vials of 20 flies), and assigned back onto either the lower sucrose or higher sucrose diets that they had been on prior to cohabitation, and a lifespan assay carried out.

Lifespan

We scored the lifespan of experimental flies of the F2 generation. Each vial in the assay commenced with 20 flies of single sex in each, and we included 10 vial replicates per treatment (grand dam × grand sire × grandoffspring) (3400 flies total, the original amount collected, minus the snap frozen samples). The number of dead flies per vial was scored three times per week (Monday, Wednesday, Friday), and surviving flies at each check transferred to vials with fresh food of the assigned diet treatment—until all flies were deceased. During

the lifespan assay, vials were stored in boxes (of 85 vials per box) that were moved to randomised locations in a (25°C) control temperature cabinet every few days to decrease the potential for confounding effects of extraneous sources of environmental variation within the cabinet from affecting the results.

Fecundity

We measured the egg output of female flies from generations F0 and F2 at eight days following eclosion, as a proxy of female fecundity. On day eight, female flies oviposited for a 23 hour period, and were then transferred to fresh vials. Day eight was selected because fecundity over 24 hours at this age has been shown to correlate with total lifetime fecundity of females in this Dahomey population[39] and early, short term measures of reproduction of between one and seven days can be used to accurately predict total lifelong fecundity in *D. melanogaster* [40]. Moreover, data shows that varying the range of sucrose concentrations did not alter the timing reproductive peaks between treatments [39].

For the F0 generation, we counted eggs from vials, each containing 10 female flies, that had been mated with 10 male flies, across 2 different sucrose levels (2.5% and 20% sucrose), and different mate combinations, as above. For the F2 generation, we counted eggs from each grand dam × grand sire × grandoffspring dietary treatment combination; each combination was represented by 10 vial replicates, each containing 10 focal females (females from the experiment) combined with 10 tester male flies. Additionally, we counted the number of adult flies that eclosed within 10.5 days from the eggs laid by F2 females (a composite of clutch viability and juvenile developmental speed). F2 females cohabited and mated with age-matched tester males of the Dahomey population (in the experimental process described above, rather the standard medium of 5% sucrose), for 24 hours at 6 days of life, and the vials

containing these eggs were left to develop into adult offspring, for 10 days at 25°C; 12:12 light/dark cycle in a temperature-controlled cabinet (Panasonic MLR-352H-PE incubator).

Lipids and protein

Whole-body triglyceride levels were measured in adult flies from the F2 generation (six days of adult age, corresponding with six days of exposure to the relevant F2 dietary treatment, prior to mating) and normalized to protein content (full protocols reported in the Supplementary Material). Three biological replicates per treatment level, with three technical replicates per biological replicate were used. Five female flies and eight male flies respectively, were used for each biological replicate in the assay.

Statistical Analyses

We used R (Version 3.6.1) and RStudio (Version 1.2.1335) (R Core Team, 2019) for statistical analyses. To test the effects of F0 female diet, F0 male diet, F2 diet, and sex on lifespan, TAG, and F2 offspring production, we fitted linear mixed effects models, using the R package lme4[41], to the lifespan data for the F2 generation. We use the term lifespan to denote the age of recorded death for each individual fly within a margin of 72 hours (for example, a lifespan of 30 days indicates that a fly died between 27-30 days post eclosion). To test the effects of grand maternal diet, grand paternal diet, grand-offspring diet, and sex on female fecundity, we fit a linear model to the egg output data for both generations.
We included F0 male, F0 female, F2 diets, and F2 sex as fixed effects in each model, exploring interactions between these factors. We included the vial identification number as a

random effect in the lifespan models. The fecundity models only included one observation per vial because we counted eggs per vial, and divided by the number of females in the vial (approx. 10 females); therefore no random effects were included in this model. We used log-likelihood ratio tests that reduce the full model, via the sequential removal of highest order terms that did not (significantly) change the deviance of the model, using a *p* value significance level of <0.05. The final reduced models (except fecundity measures) were fit by restricted maximum likelihood, applying type III ANOVA with Kenwood-Roger's F test and approximation of denominator degrees of freedom. We used sum to zero constraints in all models, and we visually inspected diagnostic plots for the linear mixed effect models, to ensure that the assumptions of normality and equal variances were met.

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Conflicts of Interest

The authors have no conflicts of interest to declare.

Author contributions

TLC, DKD, MDWP & RLR designed the experiment, TLC planned and carried out the experiment, and wrote the initial draft of the manuscript, and TLC, DKD, MDWP & RLR

all contributed to the writing and editing of the manuscript. TLC performed statistical analysis under the guidance of DKD.

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Sex-specific transgenerational effects of diet on offspring life history and physiology

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Supplementary information: Results

Tables

Table S1. Statistical results (analysis of deviance) of the linear model (Gaussian error distribution) after model reduction for predictors of female F0 egg output.
*** indicates significance at $p < 0.001$, ** indicates significance at $p < 0.01$, * indicates significance at $p < 0.05$.

Fixed Effects	***Sum Sq***	***F***	***p-value***
(Intercept)	1143.66	364.07	< 0.001 ***
Male diet	8.97	2.979	0.09117
Female diet	0.64	0.2155	0.6539
Residual	135.53	45	

All *df* =1

Table S2. Statistical results (analysis of deviance with Kenward-Roger method) of the linear mixed model (Gaussian error distribution) after model reduction for predictors of F2 offspring lifespan.

*** indicates significance at $p < 0.001$, ** indicates significance at $p < 0.01$, * indicates significance at $p < 0.05$.

Fixed Effects	***F***	***Df.res***	***p-value***
Intercept	2639.15	136.92	< 0.001 ***
F2 diet	430.37	145.66	< 0.001 ***
F2 sex	15.73	147.58	< 0.001 ***
Grand maternal diet	13.16	145.25	< 0.001 ***
Parental (F1) sex	0.2757	146.11	0.6003
F2 diet : F2 sex	369.80	148.33	< 0.001 ***
Grand maternal diet : F2 sex	9.35	148.35	< 0.01 **
F2 diet : grand maternal diet	11.01	148.44	< 0.01 **
F2 sex : F1 sex	4.44	148.47	< 0.05 *
F2 diet : F1 sex	12.42	148.56	< 0.001 ***
Random Effects	***Variance***		
Vial identification	1.423		
Residual	12.73		

df =1

Table S3. Statistical results (analysis of deviance) of the linear model (Gaussian error distribution) after model reduction for predictors of female F2 offspring egg output.

*** indicates significance at $p < 0.001$, ** indicates significance at $p < 0.01$, * indicates significance at $p < 0.05$.

Fixed Effects	***Sum Sq***	***F***	***p-value***
(Intercept)	1053.44	383.39	< 0.001 ***
F2 fem diet	97.71	35.56	< 0.001 ***
Grand maternal diet	0.05	0.02	0.8973
Grand paternal diet	13.04	4.75	< 0.05 *

F2 fem diet : grand paternal diet	15.08	5.49	< 0.05 *
Grand maternal diet : grand paternal diet	14.77	5.38	< 0.05 *
Residual	423.15		

df =1, *Res df*=154

Table S4. Statistical results (analysis of deviance with Kenward-Roger method) of the linear mixed model (Gaussian error distribution) after model reduction for predictors of female grand offspring (F3) viability (offspring produced by F2 females).
*** indicates significance at $p < 0.001$, ** indicates significance at $p < 0.01$, * indicates significance at $p < 0.05$.

Fixed Effects	***F***	***Df.res***	***p-value***
Intercept	321.888	5.754	0.20190
Grand maternal diet	4.145	33.062	< 0.05 *
Grand paternal diet	2.826	151.714	0.09479
F2 fem diet	31.59	107.955	< 0.001 ***
Grand maternal : Grand paternal diet	5.255	9.464	< 0.05 *
Random Effects	***Variance***		
Counter	0.00		
Residual	252.8		

df =1

Table S5. Statistical results (analysis of deviance with Kenward-Roger method) of the linear mixed model (Gaussian error distribution) after model reduction for predictors of F2 whole-body TAG divided by protein.
*** indicates significance at $p < 0.001$, ** indicates significance at $p < 0.01$, * indicates significance at $p < 0.05$.

Fixed Effects	*F*	*Df.res*	*p-value*
Intercept	180.64	99.614	< 0.001 ***
F2 diet	3.32	98.185	0.064
F2 sex	2.25	96.717	0.136
Grand maternal diet	21.84	99.352	< 0.001 ***
Grand paternal diet	24.74	99.352	< 0.001 ***
F2 diet : Grand mat diet	8.56	98.832	< 0.01 **
F2 diet : Grand pat diet	12.75	98.848	< 0.001 ***
Random Effects	***Variance***		
Plate reading replicate	0.0031018		
Technical replicate	0.0015873		
Vial identification	0.0063383		
Residual	0.0007295		

All *df* =1

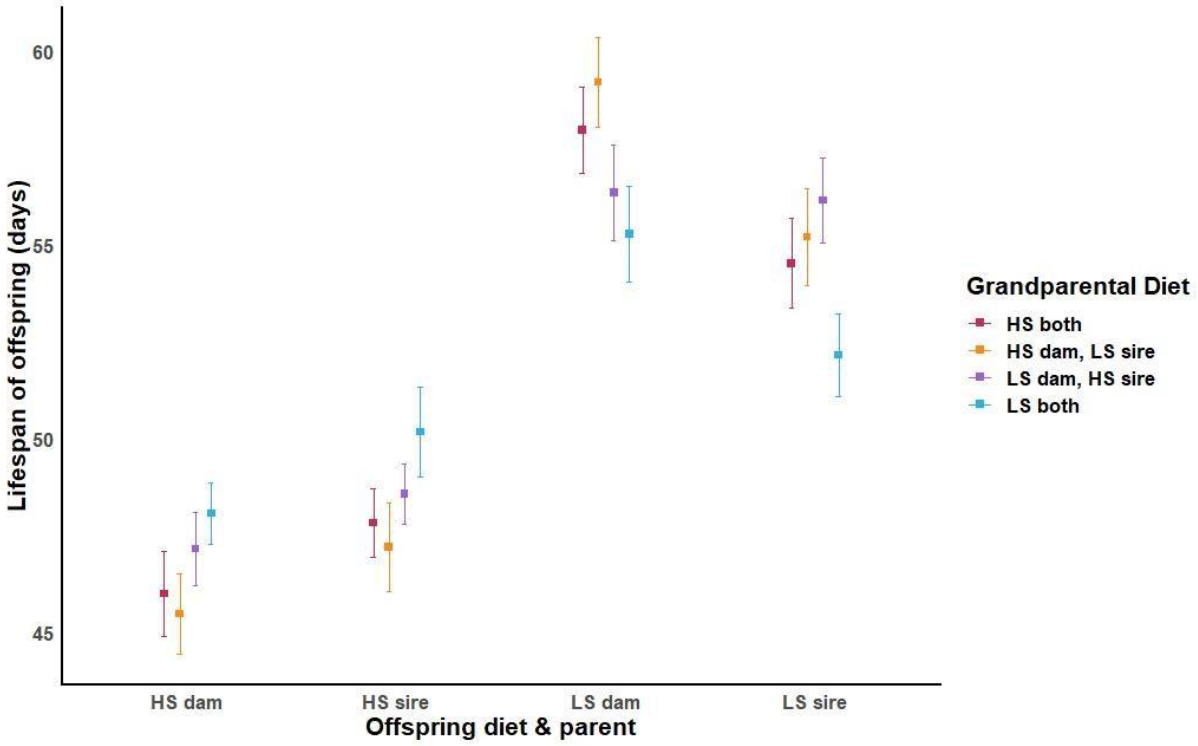

Figure S1. Mean longevity ± Standard Error (95% Confidence Interval) for the lifespan of the F2 generation. The dietary effects in the F0 generation (grandparental diets) were transferred to the F2 offspring either via F1 males or F1 females (but never via both sexes).

This figure the combination of the F2 and F1 combinations as well as the F2 combinations. HS indicates a high sucrose diet of 20% (P:C ratio 1:5.3), LS indicates a low sucrose diet of 2.5% (P:C ratio 1:1.4).

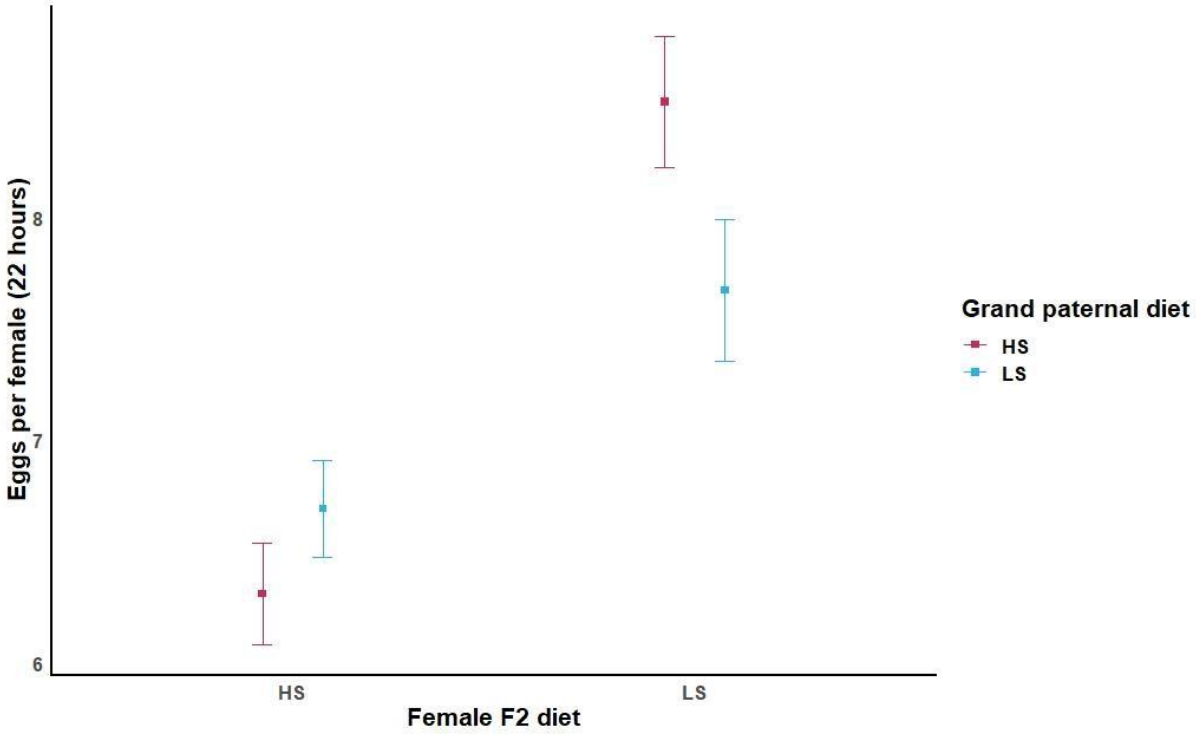

Figure S2. Mean egg output per female ± Standard Error (95% Confidence Interval) for the F2 generation, showing the grand paternal diet and the diet of the female F2 offspring. HS indicates a high sucrose diet of 20% (P:C ratio 1:5.3), LS indicates a low sucrose diet of 2.5% (P:C ratio 1.1.4).

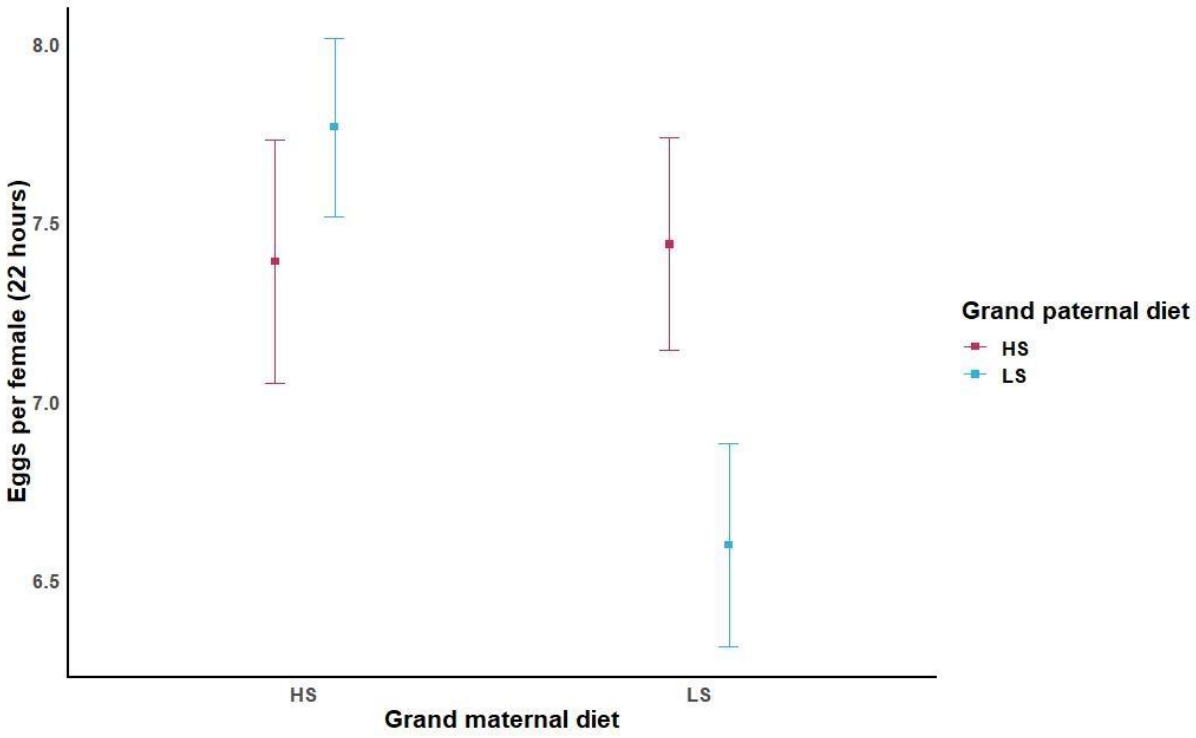

Figure S3. Mean egg output per female ± Standard Error (95% Confidence Interval) for the F2 generation, showing the grand paternal diet grand maternal diet. HS indicates a high sucrose diet of 20% (P:C ratio 1:5.3), LS indicates a low sucrose diet of 2.5% (P:C ratio 1:1.4).

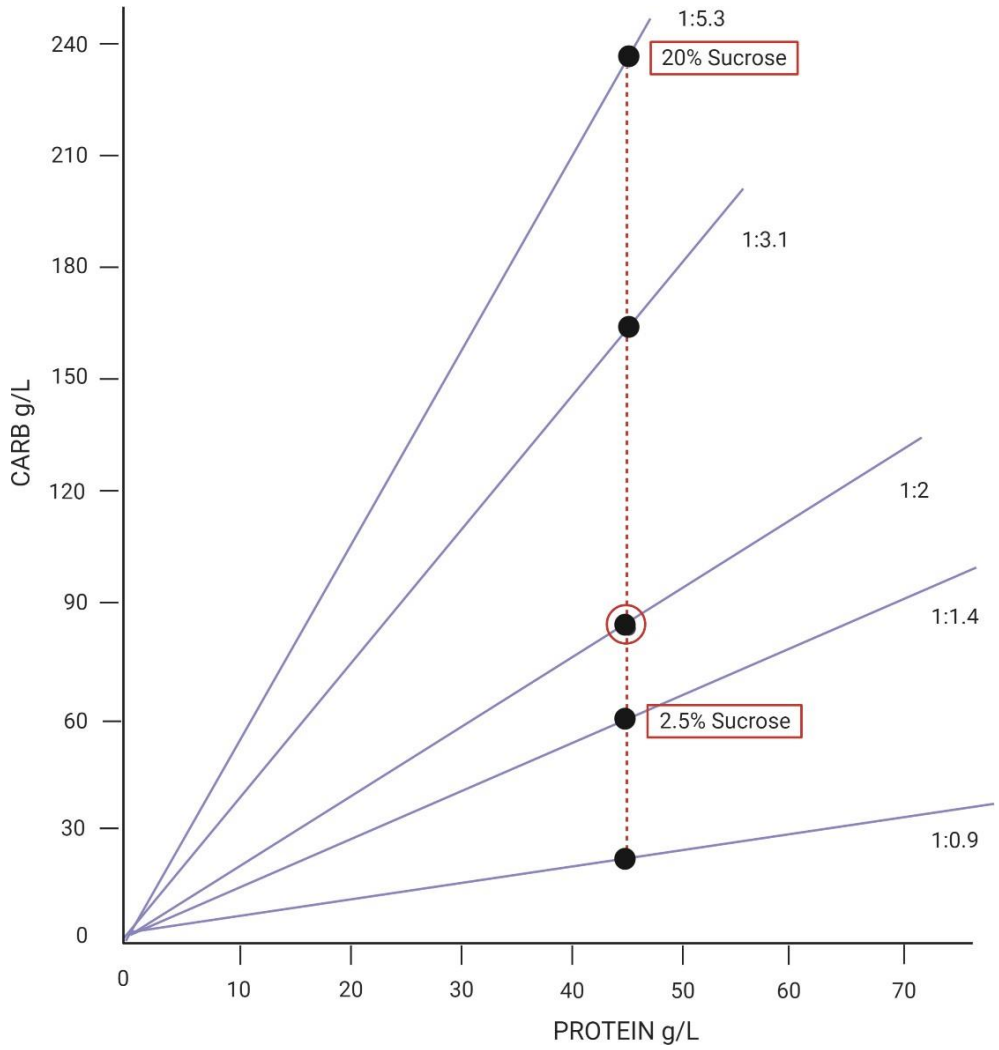

Figure S4. The diets used in the experiment according to their carbohydrate and protein contents, the diet in the middle that is circled represents the standard media in which flies were reared on prior to the experiment, while they were mating and for the full durations in the case of the F1 generation. The 20% sucrose is what we refer to as higher relative sucrose, and 2.5% sucrose we refer to as lower relative sucrose treatments.

Chapter 5 | Dietary protein enhances transgenerational reproductive success

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"For any biologist, or indeed anyone with a passion to follow their own burning questions, I would say follow your heart. The path will not be smooth but there will be magic in it."

B. Rosemary Grant (Known for her work on Darwin's finches)

Dietary protein enhances transgenerational reproductive success

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Abstract

Changes in the macronutrient contents of the diet lead to direct effects on the reproductive output of females and males. Currently unknown however, is whether variation in macronutrient ingestion, prior to reproduction, can shape the fitness of future generations. We provided adult male and female fruit flies, *Drosophila melanogaster*, with diets differing in protein and carbohydrate concentrations, prior to reproduction, and then reared their F1 and F2 descendants on a common garden diet. We investigated the consequences of dietary variation in the F0 flies on the reproductive output of both F0 and F2 females, measured across six days in early life. We demonstrate transgenerational effects of dietary protein variation that pass independently through each F0 sex. High relative protein ingestion by F0 females or F0 males prior to reproduction increased the reproductive output of F2 females. The positive effects of F0 female protein were sustained across the six days of the assay, but the F0 male effects, while initially large, eroded quickly with time. F0 female and F0 male protein ingestion did not interact to shape patterns of reproduction, but rather were independent. Notably, the effects of F0 female protein ingestion were concordant across generations, with higher F0 protein conferring direct and transgenerational increases on reproductive output. Our results suggest that variation in dietary protein ingestion is a key contributor to variance in reproductive success across generations. Establishing the evolutionary implications of these results should be a priority for future research.

Keywords

Transgenerational effects, protein, life history, reproduction

Main

Environmental changes regularly induce phenotypic plasticity, leading to modifications to physiology, morphology, or function that may have cascading effects on organismal fitness. Plasticity may be induced by change to a wide range of environmental conditions, such as temperature, nutrition, and the presence of parasites, pathogens or predators[1]. One

axis of environmental variation that has pervasive effects on organismal phenotype is nutrition. Across animal taxa, dietary modification leads to direct effects on key fitness-related traits including lifespan, reproductive output, stress tolerance, development time, and body size[2]. In particular, the effects of caloric restriction on lifespan extension are well documented. These effects have been experimentally validated, for example, the reduction of the caloric density of mouse chow to 70% of normal levels was shown to extend lifespan of wild-type mice by one year, and extend lifespan of the already longer-lived Ames dwarf mice by 3-4 months[3]. More recently, it has become clear that dietary-mediated plasticity is not determined solely by calorie modification of the diet, but also by modification of the balance of particular macronutrients (i.e. protein, carbohydrate, and fat). Indeed, it is now clear that many previously observed effects of caloric restriction might not be mediated by reduction in calories per se, but by the restriction of specific nutrients[4]. For example, per calorie, a reduction in yeast, the only source of protein in most lab based *Drosophila* diets, extends lifespan by more than the reduction of sugar[5].

In many species, protein is the limiting factor for females in offspring production[6]. In fish such as the guppy *(Poecilia reticulata)*, there is a positive correlation between the protein content females consume and the number of offspring they produce[7]. Similarly, positive correlations exist between protein ingestion and female fecundity across many insects, from fruit flies (*Drosophila melanogaster)*, to locusts (*Locusta migratoria)*, and carabid beetles (*Pterostichus cupreus , Pterostichus melanarius)*[8,9]. In general, a lower protein (and correspondingly higher carbohydrate) diet is associated with extended lifespan, and reduced reproductive output for females across many taxa, whereas a higher protein (and lower carbohydrate) diet reduces lifespan, but increases reproductive output[10,11]. Relationships between protein and reproductive success in males are less clear, and in insects, different components of male reproductive success are optimised under different

66 macronutrient balances, but total male offspring production is generally maximised under
67 higher carbohydrate and lower protein diets[12–14].

68 Environmental changes can also trigger transgenerational plasticity in phenotypic
69 expression, whereby environmentally mediated effects are transmitted through parents to
70 offspring and even to latter generations[15,16]. Indeed, numerous studies have now
71 demonstrated effects of dietary variation that are transmitted as non-genetic parental
72 effects (e.g. mothers to offspring, fathers to offspring), or transgenerational effects (e.g.
73 across multiple generations mapping to maternal or paternal lineages)[17–20]. These include
74 studies of parental effects of diet across a range of taxa, such as changes to the dietary fat
75 content in parental mice[21], or sugar concentration in parental fruit flies[17,20]. Much focus has
76 been devoted to studying the effects of variation in ingestion of dietary sugar or fat, since
77 excess consumption of carbohydrates and fat can lead to obesity, a health challenge facing
78 most contemporary human populations. These studies tend to measure traits such as body
79 size, longevity, triglyceride levels, levels of circulating sugars, and other metabolic
80 regulators to provide insights into direct and putative transgenerational consequences of
81 carbohydrate-rich hypercaloric diets in humans[20,22–27]. Whereas, the transgenerational
82 consequences of protein modification are not well studied.

83 Major questions remain when it comes to the current understanding of the ecological and
84 evolutionary significance of dietary-mediated transgenerational effects. First, most studies
85 to test for such effects have adopted designs whereby diets are defined by a small number
86 of divergent categories (e.g. high vs low quality, sugar, or fat[20,21,25–28]), and therefore it
87 remains unclear whether previously observed instances of dietary-mediated
88 transgenerational effects are likely to manifest more generally across the broader range of
89 dietary variation. Second, most studies of non-genetic transgenerational inheritance focus
90 on understanding maternal contributions to phenotypic variation across generations. Fewer

91 studies have focused on the capacity for dietary-mediated variation to manifest through
92 paternal lineages[17,29], and scant attention has been provided to the capacity for interactions
93 between maternal and paternal lineages to shape patterns of cross-generational
94 plasticity[25,26]. Third, while much attention has focused on the physiological and lifespan
95 consequences of differing diets, less focus is given to the implications of how dietary
96 variation affects reproductive output, both within and across generations. Understanding
97 these patterns seems imperative for understanding the evolutionary implications of such
98 effects, given that traits linked to reproductive output align most closely with evolutionary
99 fitness. Finally, it remains unknown whether the balance of macronutrients required to
100 optimise fitness components in one generation (via direct effects on fitness) lead to
101 optimisation of fitness components of latter generations (via transgenerational effects), or
102 whether optimisation of diets in one generation may invoke transgenerational costs in
103 subsequent generations.

104 Here, we address these questions by experimentally testing the direct and transgenerational
105 implications of modifications to dietary protein and carbohydrate concentrations to patterns
106 of early-life reproductive output in female fruit flies (*D. melanogaster*). We administered F0
107 female and male flies with one of five diets that varied in protein to carbohydrate (P:C) ratio
108 (1:5.3, 1:4.9, 1:2, 1:1. 1:0.88), achieved through multiple combinations of three different
109 carbohydrate levels (239.5, 89.5, 39.5 g/L) and three different protein levels (90, 45, 18 g/L).
110 The diets were administered using a full factorial design: F0 adult males and females were
111 each assigned to one of the five diets for six days, prior to the flies of each diet being paired
112 to flies of the opposite sex to accommodate mating, resulting in 25 maternal-by-paternal diet
113 combinations. We then examined the direct effects of these diets on the reproductive output
114 of the F0 females measured over a six-day period early in adult life, as well as the

transgenerational effects on the reproductive output of F2 female descendants measured over a six-day period in early life.

Results

Direct effects of protein and carbohydrate levels on F0 reproductive success

Protein and carbohydrate concentrations in the diets of the F0 females affected the number of offspring they produced. The reproductive output of F0 females across the six days of the assay (i.e. with female age across six days) was characterised by a quadratic function ($F_{2, 310.09} = 323.43$, $p < 0.001$, Table 1, Figure 1). This relationship differed according to the level of protein ingested ($F_{2, 305.62} = 93.36$, $p < 0.001$, Table 1), and the level of carbohydrate ingested ($F_{2, 313.25} = 9.36$, $p < 0.001$, Table 1) by females prior to reproduction. Dietary protein had a large positive effect early in the reproductive assay of the F0 females, but this effect waned over the six days of the assay, resulting in a steeper decline with age for those females administered higher protein diets (Figure 1, panel A). High levels of dietary carbohydrate were associated with low reproductive output in the first days of the assay, resulting in shallow declines in reproductive output over the course of the assay relative to females that ingested low or intermediate levels of carbohydrate (Figure 1, panel B). Both the main and the age-dependent effects of carbohydrate ingestion (accounting for ~8% and 2.6% of the variance in the model respectively) were much weaker than those of protein ingestion (~35% and 19% of the variance respectively, Table 1). Finally, we note that the levels of F0 male protein and carbohydrate ingested prior to mating conferred no effects on the reproductive output of their F0 female mates (0.03% and 0.32% of the variance respectively, Table 1).

Table 1. Effects of the day of the assay (i.e. age of F0 females), assay day squared, carbohydrate and protein content of the F0 male and F0 female fly diets, on F0 female reproductive output (number of eclosed pupae produced per F0 female fly). General Linear Mixed Model, with sum-to-zero contrasts, and fixed effects parameters calculated via F test with Kenward-Rogers approximation of degrees of freedom, and variance associated with random effects (vial identity, and random slopes allowing variation in the relationship of Assay day, Assay day ^{2} and Assay day^3 across vials) estimated via REML. There were six days of the assay; flies were aged 7-12 days old. The protein concentrations were either 18, 45, or 90 g/L; the carbohydrate concentrations were either 35.5, 89.5, or 239.5 g/L. *** indicates significance at $p < 0.001$, ** indicates significance at $p < 0.01$, * indicates significance at $p < 0.05$.

Fixed Effects	***F***	***Df***	***Df res***	***p-value***	***Effect size***	***Lower bound***	***Upper bound***	***Variance %***
(Intercept)	878.01	1	323.31	< 0.001 ***				
Assay day	355.26	1	310.37	< 0.001 ***	0.03	0	0.07	1.98
Assay day^2	323.43	1	310.09	< 0.001 ***	0.02	0	0.05	1.32
F0 female carb	20.56	2	311.15	< 0.001 ***	0.12	0.06	0.19	7.92
F0 female protein	177.61	2	308.03	< 0.001 ***	0.53	0.46	0.59	34.98
F0 male carb	0.70	2	300.95	0.49	0.004	0	0.03	0.32
F0 male protein	0.30	2	299.60	0.74	0.0003	0	0.01	0.03
Assay day: F0 female carb	14.05	2	313.78	< 0.001 ***	0.07	0.03	0.12	4.62
Assay day: F0 female protein	132.34	2	307.18	< 0.001 ***	0.41	0.34	0.48	27.06
Assay day^2 : female carb	9.36	2	313.25	< 0.001 ***	0.04	0.01	0.08	2.64
Assay day^2 : female protein	93.36	2	305.62	< 0.001 ***	0.29	0.22	0.35	19.14

Random Effects	***Variance***	***Std. Dev.***	***Corr***	
Vial identification (Intercept)	138.58	11.77		
Assay day | Vial identity	24.50	4.95	-1.00	
Assay day^2 | Vial identity	0.29	0.54	1.00	-1.00
Residual	25.57	5.06		

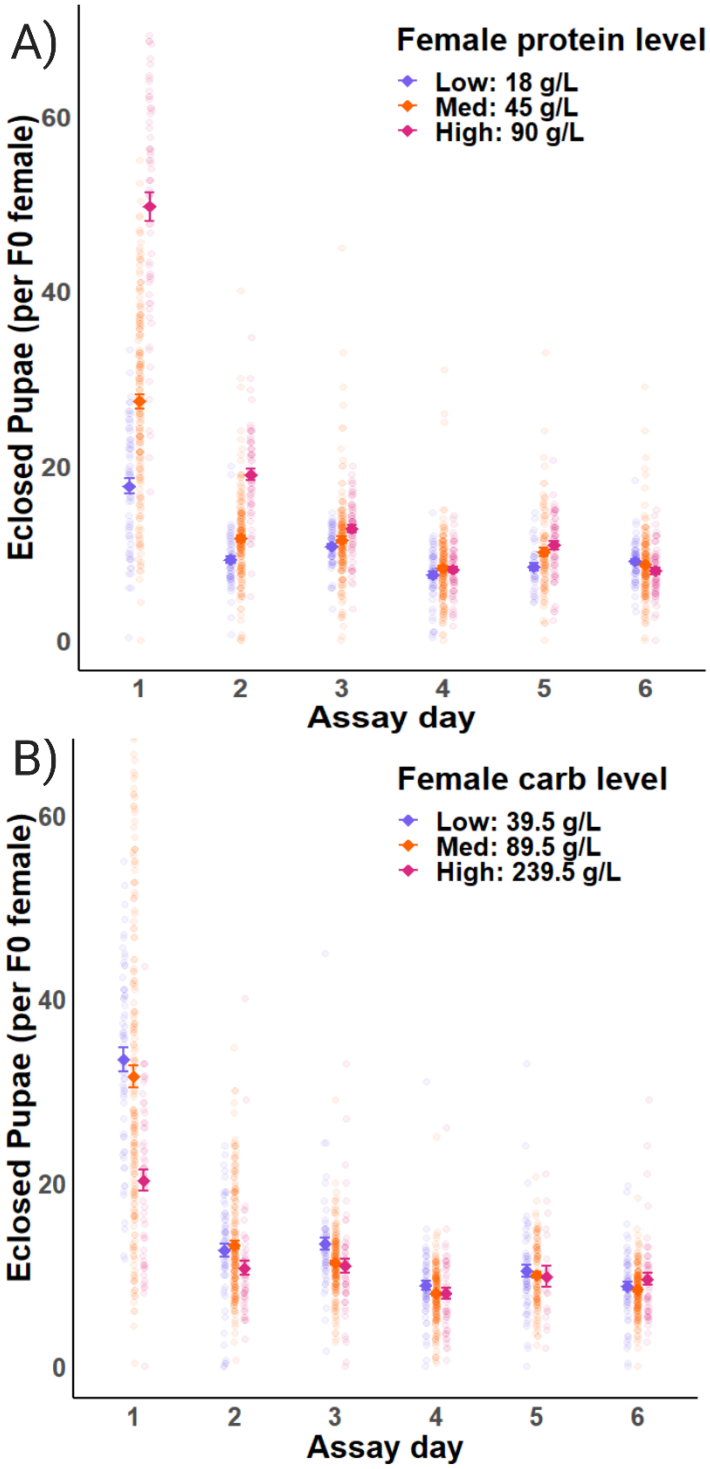

Figure 1. Effects of female F0 protein (low 18 g/L; medium 45 g/L; high 90 g/L) and carbohydrate (low 39.5 g/L; medium 89.5 g/L; high 239.5 g/L) levels on female F0 reproductive output. Flies are 7-12 days old during the assay. Plots show means, and standard error bars. (A) Number of pupae eclosed from F0 female flies (y-axis), their protein level (colour), the day of the assay (x-axis), (interaction: Female F0 protein × Assay day). (B) Number of pupae eclosed from F0 female flies (y-axis), their carbohydrate level (colour), the day of the assay (x-axis), (interaction: Female F0 carb × Assay day).

Transgenerational effects of protein on F2 reproductive output are transferred through males and females

Higher protein levels in F0 females boosted reproductive output of the F2 females, and this effect was general across the six days of age in which F2 females were assayed ($F_{1, 310.78}$ = 17.83, p <0.001, Table 2, Figure 2, panel A). The reproductive output of F2 females across the six days of the assay was characterised by a cubic function ($F_{1, 320.37}$ = 222.99, p <0.01, Table 2, Figure 2, panels A and B), with this relationship differing according to levels of protein ingested by F0 males ($F_{1, 320.43}$ = 9.37, p <0.01, Table 2, Figure 2, panel B). High protein ingestion of F0 males had a positive effect on F2 female reproductive output, with the effect particularly strong at Day 1 of the assay, leading to a steeper slope to the cubic function. However, unlike the effects of differences in F0 female protein ingestion on F2 reproductive output, which persisted across the six days of the reproductive assay, the effects of F0 male protein ingestion were negligible by Day 5 of the assay (Figure 2, panel B). These transgenerational effects of protein were much smaller than the direct effects on F0 reproductive output reported above. The main effects of F0 male protein and F0 female protein accounted for the same amount of variation within the model ~3.5%, and the F2 age-specific reproductive effect of F0 male protein accounted for ~1% (cubed term) of the variation with the model. There were no interactions between F0 female and male diets on F2 reproductive output. Furthermore, transgenerational effects linked to F0 carbohydrate ingestion were not statistically significant.

Table 2. Effects of F0 protein and carbohydrate level, assay day (i.e. age of F2 females), assay day squared, assay day cubed on reproductive output (number of eclosed pupae produced) of female F2 flies. General Linear Mixed Model, with sum-to-zero contrasts, and fixed effects parameters calculated via F test with Kenward-Rogers approximation of degrees of freedom, and variance associated with random effects (vial identity, and random slopes allowing variation in the relationship of Assay day, Assay day^2 and Assay day^3 across vials) estimated via REML. There were six days of the assay; flies were aged 7-12 days old. The protein concentrations were either 18, 45, or 90 g/L; the carbohydrate concentrations were either 35.5, 89.5, or 239.5 g/L. *** indicates significance at $p < 0.001$, ** indicates significance at $p < 0.01$, * indicates significance at $p < 0.05$.

Fixed Effects	***F***	***Df***	***Df res***	***p-value***	***Effect size***	***Lower bound***	***Upper bound***	***Variation %***
(Intercept)	509.99	1	340.90	< 0.001 ***				
Assay day	412.62	1	318.36	< 0.001 ***	0.25	0.21	0.29	29.46
Assay day^2	277.83	1	319.57	< 0.001 ***	0.24	0.2	0.29	28.28
Assay day^3	222.99	1	320.37	< 0.001 ***	0.25	0.2	0.3	29.46
F0 female carb	3.69	1	323.42	0.06	0.006	0	0.03	0.77
F0 female protein	17.83	1	310.78	< 0.001 ***	0.03	0.01	0.07	3.53
F0 male carb	0.80	1	310.56	0.37	0.002	0	0.02	0.26
F0 male protein	32.74	1	360.24	< 0.001 ***	0.03	0.01	0.06	3.53
F0 male protein : Assay day	18.90	1	317.76	< 0.001 ***	0.02	0	0.03	2.36
F0 male protein : Assay day^2	12.26	1	319.36	< 0.001 ***	0.01	0	0.03	1.18
F0 male protein : Assay day^3	9.37	1	320.43	< 0.01 **	0.01	0	0.03	1.18

Random Effects	***Variance***	***Std. Dev.***	***Corr***		
Vial identification (Intercept)	38.17	6.18			
Assay day	6.54	2.54	-0.30		
Assay day^2 | Vial identity	1.61	1.27	-0.36	-0.78	
Assay day^3 | Vial identity	0.02	0.15	0.48	0.69	-0.99
Residual	10.46	3.23			

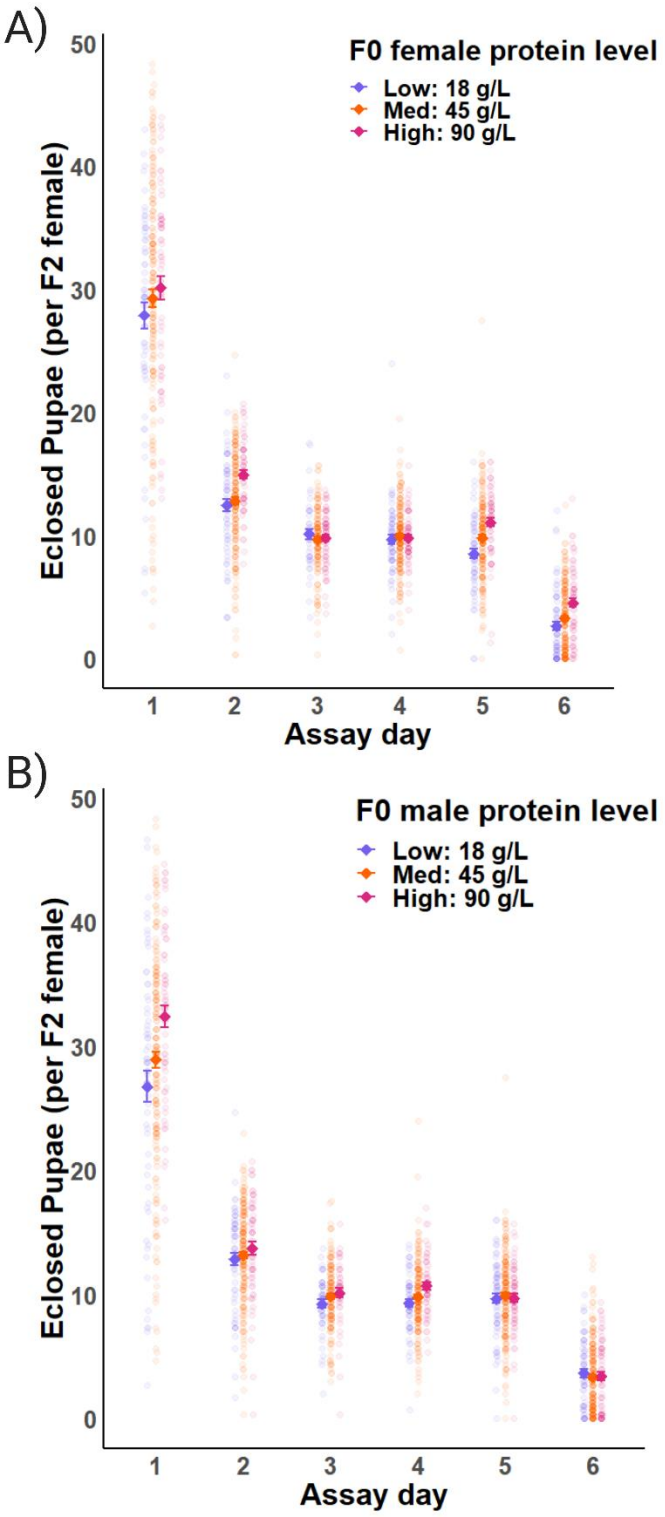

Figure 2. Effects of F0 female protein (low 18 g/L; medium 45 g/L; high 90 g/L) and F0 male protein levels on female F2 reproductive output. Flies are 7-12 days old during the assay (7 days old at Assay Day 1, and 12 days old at Assay Day 6). Plots show means, and standard error bars. (A) Number of pupae eclosed from F2 female flies (y-axis), F0 female protein (colour), Assay day (x-axis), (interaction was not significant, data depicted in this way to show day-specific means as a point of comparison to panel B; F0 female protein levels effected F2 reproductive output regardless of assay day/F2 female age). (B) Number of pupae eclosed from F2 female flies (y-axis), F0 male protein level (colour), Assay day (x-axis), (plot depicts interaction between Male F0 protein × Assay day).

Discussion

While high levels of protein and low levels of carbohydrate each independently conferred direct effects on F0 female reproductive output, dietary protein variation made a much larger contribution to F0 reproductive output than carbohydrate variation. Furthermore, the effect of dietary protein consumed by the F0 generation invoked transgenerational effects on F2 female reproductive output. In contrast, varying carbohydrate variation in the F0 diet did not affect F2 female reproductive output. Notably, these transgenerational effects of protein ingestion by F0 females on F2 female reproductive output mimicked the direct effects on F0 reproductive output—high protein given to the F0 female increased reproductive success of both generations, albeit the transgenerational effects were predictably weaker than the direct dietary effects. This suggests that effects of protein variation on patterns of reproduction are likely to be reinforced across generations. Moreover, although protein intake of F0 males did not affect the reproductive output of their F0 female mates, protein levels in F0 males did affect the reproductive output of their F2 female descendants. The effects on F2 female reproduction associated with protein variation in F0 females were general across the six days of the reproductive assay, whereas the transgenerational effects associated with protein variation in F0 males declined in magnitude with F2 female age (i.e. declined in final days of the assay). Our results draw attention to the differing contributions that distinct macronutrients may make to shaping transgenerational plasticity in fitness. They also highlight a large role for protein variation in mediating patterns of non-genetic inheritance through both maternal and paternal lineages. We discuss the evolutionary and ecological implications of these findings below.

Studies investigating the effects of diet on female reproductive output have shown that consuming protein rich food increases reproductive output in many insect and fish species[6–9].

Indeed, adequate ingestion but not over consumption of dietary protein intake has been linked to the reproductive health of livestock[30], mice[31,32], and humans[33]. Our findings for the F0 females are consistent with previous research, including research in *D. melanogaster*[9,11], revealing a higher ingestion of protein, and relatively lower ingestion of carbohydrate enhanced the reproductive output of F0 females. We note that in our previous research, we found the F0 male diet (either high or low in dietary sucrose) affected the lifespan of the F0 females with whom they mated. This was an intriguing result that suggested that sucrose variation may have conferred effects on components of the male ejaculate, with this variation in ejaculate composition exerting effects on the physiology and ultimately life history of females[26]. In this study however, we found no such dietary-mediated F0 male effect on the reproductive output of F0 females, which suggests that the effects of the male diet on females may be trait-dependent, or otherwise not detectable on early life components of female life history.

Notably however, we have uncovered further levels of complexity associated with dietary variation by revealing transgenerational effects linked to protein variation in the diets of F0 flies. Previous studies in humans, mice, pigs, and sheep have linked sufficient protein consumption in mothers to improved cell signalling, stable uterine growth, and foetal development[31]. Few studies exist that examine the effect of maternal protein consumption on daughter reproductive output; one notable exception by Matzkin et al.,[27] found that adult fruit flies administered with high protein diets during juvenile development produce F1 female offspring that were more fecund[18]. However, this study examined the effect of protein variation in both parents combined, and cannot elucidate what their respective effects may be on offspring reproduction. Moreover, this study was unable to partition out the respective effects of protein and carbohydrate levels, as the higher relative protein content was always

coupled with a lower relative carbohydrate level (and vice versa). Furthermore effects beyond the F1 generation were not investigated, leaving the question open of whether dietary effects of protein variation may invoke effects that are truly transgenerational, and thus more likely to be mediated via epigenetic mechanisms, rather than simply by variation in condition-dependent effects amongst mothers. Here, we have revealed that paternal contributions to protein-mediated transgenerational plasticity are of approximately equal magnitude to the maternal contributions. This is an intriguing insight, given the large disparity in the size of the cytoplasmic components of the male gamete, relative to the female gamete in *Drosophila*. Such anisogamy is a key reason why traditionally research attention focused on the maternal rather than paternal non-genetic effects in shaping transgenerational phenotypes[34–36] . While the mechanisms that regulate these maternal and paternal influences across generations in our study remain unclear, they likely involve invoking epigenetic changes to the inherited DNA sequence.

Most previous studies to examine the cross-generational implications of diet have focused on the consequences of obesity transmission, given the reported prevalence of dietary-induced obesity in humans[24] . Accordingly, these studies have utilised diet interventions given to model species (primarily *Mus musculus* and *Drosophila*) that have focused on binary diet schemes of high or low fat, sucrose, or calories[17,20,27,28,37,38]. Such studies have investigated the effects of these diet changes on the physiology (i.e. triglyceride levels, body weight, circulating sugars, and metabolic regulators) on F1 offspring, but rarely investigated effects beyond one generation or expanded diets to include a range of macronutrient variation[17–20,39]. Notable studies using mice that do investigate the effects of obesogenic diets on reproductive indicators have found that increased fat or sugar in the diet (inducing obese-like phenotypes) alters measures such as oocyte quality, and increased embryo mortality in F1 offspring[40,41].

The effects of protein variation however, were not investigated, nor was the capacity for transgenerational effects beyond the F1. Indeed, how the paternal lineage diet affects the F2 female reproduction has remained largely elusive as studies overwhelmingly focus on maternal dietary contributions [17,42].

One notable insight from our study is that despite the identification of F0 female (grandmaternal) and F0 male (grandpaternal) effects on F2 reproductive output, these effects did not interact. This finding contrasts with our two previous studies that reported complex interactions involving F0 female and F0 male diets, as well as interactions between F0 female, F0 male, and F1 offspring and F2 offspring diets (respectively), which differed in relative sucrose concentration, affecting offspring lifespan, fecundity, and physiology in *D. melanogaster*[25,26]. While the underlying reasons for the differences in these results between studies remain cryptic, we suggest these discrepancies are likely explained in the different methodologies and foci of the studies. First, the measure of reproductive success used here was one that screened adult offspring production by females over a six-day period of early life, thus converging on an accurate estimate of early life female reproductive success. In contrast Camilleri et al.,[25,26] focused on fecundity, as measured by the number of eggs produced over a narrow window (22 hour period) early in female life. Second, the diets used by Camilleri et al.,[25,26] were based on two divergent sucrose diets, that held protein constant[25,26], whereas here we used five different diets that differed both in carbohydrate and protein concentrations. This enabled us to have greater insight into the relative contributions of protein and carbohydrate in shaping shape transgenerational reproductive success. Third, unlike in Camilleri et al.[25,26], in this study the diets of F2 females was held constant, and only the diets of F0 females and males manipulated, thus preventing us from screening for potential interactions between parental and offspring diets.

The findings of our study suggest that increases in dietary protein in both females and males confer transgenerational benefits to female reproductive success. Remarkably, these non-genetic effects were large (~3,5%), noting that effect sizes for heritable loci identified through GWAS deemed important for influencing phenotypic change and disease in humans are often around 1%[43]. Our study highlights the need for further study into the mechanisms that underpin these transgenerational contributions, deciphering whether there is a shared mechanism at play in both parental lineages. Finally, our study was limited to quantifying transgenerational dietary effects on female reproductive output. This raises the question of whether male reproductive success is equally sensitive to transgenerational effects of macronutrient variation, and if so whether these effects are similarly mediated through protein variation. Previous research has indeed identified sex specificity in transgenerational effects of sucrose variation on lifespan in *D. melanogaster*[25]. Given that the direct effects of protein variation differ across sexes, with male reproductive success in insects generally augmented by higher carbohydrate relative to protein levels[44], we predict that parent-offspring conflict may arise over optimal parental macronutrient balance, with a resolution that differs between the sexes.

Methods

Study species and generating experimental flies

We sourced flies from Dahomey, a large laboratory population of *D. melanogaster*, originally sourced from Benin, Africa [45]. The flies have been maintained in large population cages, with overlapping generations at Monash University since 2017, and prior to that in the Partridge laboratory, University College London [5]. Prior to the beginning of the experiment, we collected ~3000 eggs from the cages, and distributed them into 10 250mL bottles containing 70mL of food medium. Food comprised 5% sucrose (50 grams

sucrose, 100 grams yeast, 10 grams agar per 1 litre solution with an estimated protein to carbohydrate [P:C] ratio of 1:2, and 480.9kcal per litre) (Figure 3, and Table 3 for further diet details)—admixing adult flies between bottles every generation, for 3 generations. To control for potential sources of variation in their environment, during these three generations we strictly controlled both the age of flies at the time of ovipositioning—all flies were within 24 h of eclosion into adulthood when producing the eggs that propagated the subsequent generation, and their population density, 300-320 adult flies within each bottle in each generation. All flies were maintained under standard laboratory conditions (25°C, 12:12 h light: dark photoperiod).

Dietary treatments

The diet media we used consisted of sucrose, autolysed brewer's yeast powder (sourced from MP Biomedicals SKU 02903312-CF), and agar (grade J3 from Gelita Australia), as well as preservatives—propionic acid, and nipagin. We prepared five dietary treatments, differing in relative sucrose and yeast concentrations summarised in Table 3.

Table 3. Breakdown of the five diets; S:Y is sucrose to yeast ratio; P:C:F is protein to carbohydrate to fat ratio; g/L indicates the grams per litre of carbohydrate and protein. Diet information for diets one to five correspond also to Figure 3.

Diet	S:Y	P:C:F	g/L carb	g/L protein
1	2:1	1 : 5.3 : 0.02	239.5	45
2	0.74:0.4	1 : 4.97 :0.02	89.5	18
3	1:2	1 : 2 : 0.02	89.5	45
4	0.1:2	1 : 1 :0.02	89.5	90
5	0:1	1:0.88 : :0.02	39.5	45

The diets differed not only in sucrose and yeast concentration, but overall macronutrient balance and their total caloric content, maximising distance between each in nutrient space. All diets contained 3ml/l of propionic acid and 30ml/l of a Nipagin solution (100g/l methyl 4-hydroxybenzoate in 95% ethanol) and were cooked according to the protocol described in [46]. Each vial is 40mL in volume and contained 7mL of food.

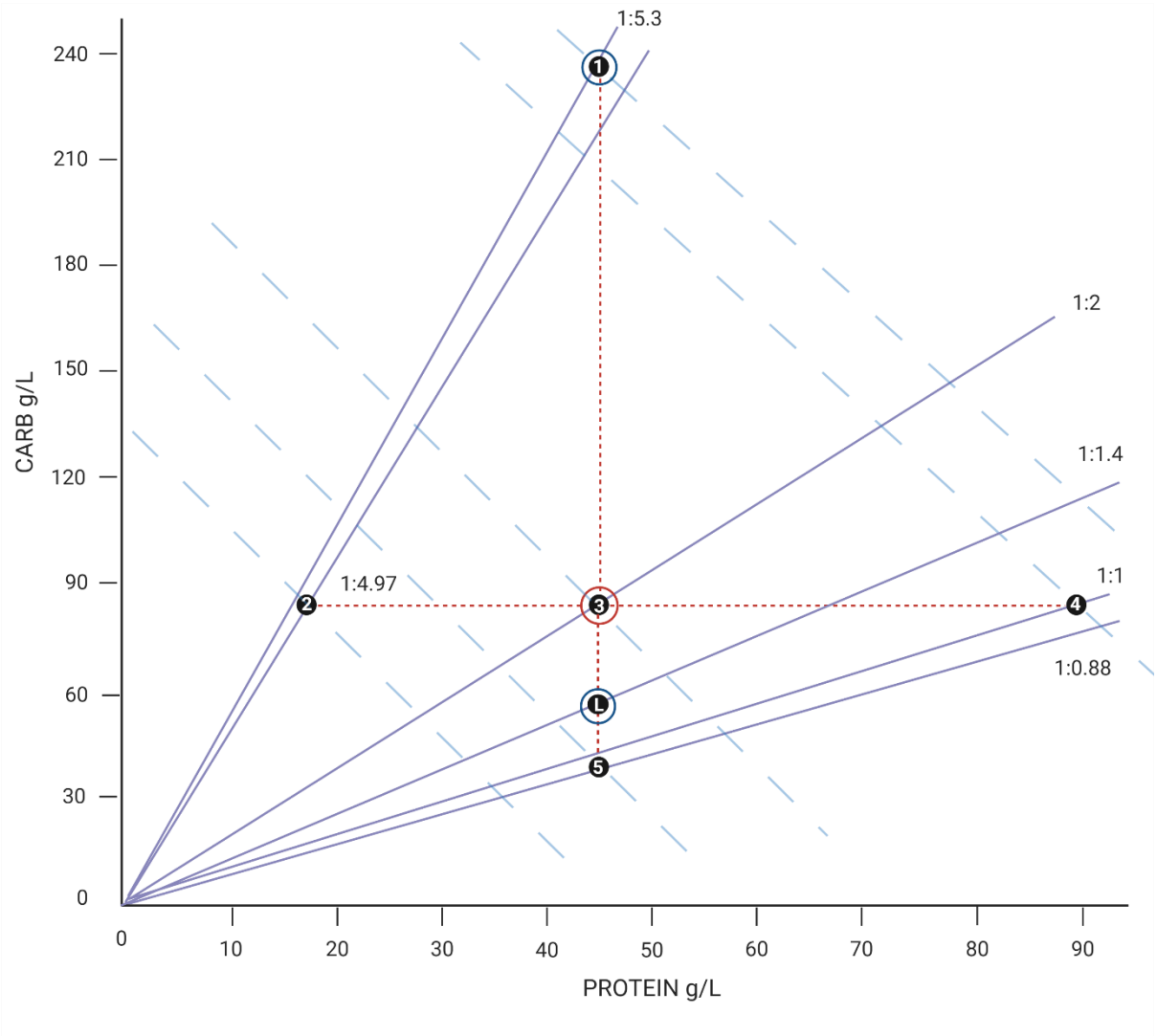

Figure 3. The five diets in nutrient space (carbohydrate and protein), numbers one to five are the diet labels as outlined in Table 1, diet 3 (circled in red) represents the protein and carbohydrate levels of the standard medium (common garden diet) in which flies were

reared on prior to the experiment. Diet 1 and diet L, (circled in blue) are the high and low sucrose diets that were used in Camilleri et al (2022a &b) for reference, but diet L was not used in this paper.

Experimental design

Male and female flies were separately assigned to one of five of the dietary treatments prior to mating (we refer to this generation of flies as F0), and then the grand offspring produced (F2 generation) were reared on diet 3, common garden food. All 25 possible combinations of F0 female diet × F0 male diet treatments were represented (= 5 × 5 = 25 combinations). Specifically, we collected flies of the F0 generation as virgins and placed them in vials of 3 flies each onto their assigned diet treatment, and this was done separately for each sex. These flies then spent first 6 days of their adult life, post eclosion, on their assigned diet. During this time, we transferred flies of each vial to new vials containing fresh food of the designated diet every 24-48 hours.

Flies then entered a cohabitation phase to enable female and male flies to mate for 6 days. Cohorts of males and female flies were combined, in vials of six flies (three males and, three females), in each of the 25 possible diet combinations. They were transferred to a new vial with fresh food (standard media, diet 3) every 24 hours during this time. The eggs that had been laid by F0 females of the respective vials over the six day cohabitation were left to develop into adult offspring over 10 days at 25°C (on a 12:12 light/dark cycle in a temperature-controlled cabinet; Panasonic MLR-352H-PE incubator). All vials were kept and pupae cases counted from the 6 days of the assay. The adult offspring (from day 1 of the F0 assay) constituted the F1 flies in the experiment, and these F1 flies developed on standard media (diet 3 in Table 3). We collected F1 flies from each of the 25 combinations of parental diet treatments, and placed them again on the standard media for 6 days, before allowing males and females to cohabit and mate (with flies collected from the same Dahomey

population, kept on the standard media, diet 3) to create the F2 generation. These F1 flies were all 6-day-old adults at the time of ovipositioning, thus removing the capacity for variation in parental age from shaping subsequent reproductive patterns in F2 flies.

We then collected female virgin F2 flies from each of the 25 combinations of F0 diet treatments, and placed them into groups of 3 flies per vial, across 13 vial replicates per F0 male × F0 female diet combination, again on the standard medium (diet 3). Male tester flies from the Dahomey stock population were also collected as virgins (975 tester males) for the F2 reproductive assay, and their ages were matched to those of the focal F2 females. These tester males were introduced into each vial of females (3 tester males added to each vial of 3 one day-old F2 females), to commence a cohabitation phase for the F2 generation that enabled female focal flies to interact and mate with male tester flies, and lay eggs, across a 6 day time period. During this cohabitation phase, flies were transferred to new vials daily, providing focal females with fresh food media (Diet 3, Table 3) on which to lay eggs. These vials were subsequently retained for 12 days, to enable eggs that had been laid by the females to develop into adult offspring.

Female reproductive output

We scored reproductive output of females from F0 and F2 generations, over six successive days during which the females had cohabited with males. Flies were 7 days old when the reproductive assay began and 12 days old on the final day. To achieve this, we simply counted the number of empty pupal cases per vial, because this parameter corresponds precisely to the number adult offspring produced by the focal females. Short term measures of reproductive output that encompass multi-day periods early in adult life of between one and seven days have been shown to be correlated to total lifelong fecundity in *D.*

melanogaster [47]. In total, we scored 3617 vials (1754 from the F0 assay, and 1863 from the F2 assay).

Statistical Analyses

We used R (Version 3.6.1) and RStudio Version 1.2.1335 (R Core Team, 2019) for statistical analyses. To test the effects of the factors F0 female protein and carbohydrate concentration and F0 male protein and carbohydrate concentration, and the variate assay day (days 1-6 correspond to female age 7-12 days old), on F0 and F2 female reproductive output, we fitted linear mixed effects models (one for the F0 reproductive data, and one for the F2 data), and modelled interactions between the terms, using the R package lme4[48]. We modelled the effect of age as a linear function, but and also fitted quadratic and cubic functions, given that visual inspection of the data suggested a non-linear shape to the relationships of age on reproductive output in both F0 and F2 generations. Our response variables in each model denoted reproductive output, represented as the number of pupae eclosed per vial divided by the number of laying females (for both gens F0 and F2 female), per vial (3 flies per vial), across each 24-hour period, over a 6 day period of sampling. We included the vial identification number as a random intercept, and included random slopes by enabling the linear and non-linear slopes of assay day to vary across vials.

We used log-likelihood ratio tests that reduce the full models, via the sequential removal of highest order interactions that did not change the deviance of the model, using an alpha criterion of 0.05. The final (reduced) models were fit by restricted maximum likelihood, using type III sums-of-squares ANOVA with Kenwood-Roger's F test and approximation of denominator degrees of freedom. We then calculated effects sizes (Eta statistics) and

associated Confidence Intervals on both of the reduced models. We used sum to zero contrasts in each model.

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Conflicts of Interest

The authors have no conflicts of interest to declare.

Author contributions

TLC, DKD, MDWP & RLR conceived of and designed the experiment, TLC planned and carried out the experiment, TLC analysed the data with advice from DKD, and TLC, DKD, MDWP & RLR all contributed to the writing and editing of the manuscript.

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564

Chapter 6 | General Discussion

“But then science is nothing but a series of questions that lead to more questions, which is just as well, or it wouldn’t be much of a career path, would it?”

-Terry Pratchett & Stephen Baxter (The Long Earth)

6.1 | Thesis and aims summary

In this thesis, I explored how modifying diets inter- and transgenerationally can alter the life history and body composition of subsequent generations. My thesis is structured into four chapters, with three research chapters, and each chapter reports the results of a standalone experiment that probes a specific question pertaining to the evolutionary and ecological significance of cross-generational nutritional effects. I began with a review article (Chapter 2) that synthesizes research concerning cross-generational phenotypic effects of parental obesogenic diets. The literature review in Chapter 2 motivated my aims for subsequent chapters by addressing some of the outstanding questions in the field. My first aim was to determine the relative contributions of maternal and paternal dietary-mediated effects and the possibility for complex interactions between parents and offspring diets (addressed in Chapters 3, 4, and 5). My second aim was to investigate whether cross-generational effects of nutrition can be sex-specific in their effects on male and female offspring (addressed in Chapter 4).

My third aim was to ascertain whether parents or grandparents can prime offspring (via anticipatory effects)—advantaging offspring in matching nutritional environments (addressed in Chapters 3 and 4). My fourth aim was to determine whether dietary-mediated cross-generational effects are unique to the particular macronutrients manipulated, and whether effects mediated by carbohydrate variation are similarly induced by protein variation (addressed in Chapter 5). My fifth and final aim was to uncover whether the cross-generational effects of variation in nutrition are consistent across generations, comparing responses across F0, F1 and F2 (address in Chapters 3, 4, and 5). In this, the final sixth chapter, I will review the main findings of this work and indicate possible future avenues of

research to elucidate our understanding of the evolutionary significance of cross-generational effects of nutrition.

6.2 | Both maternal and paternal effects contribute to offspring life history and can be interactive or additive depending on context

My first aim was to determine the relative contributions of maternal and paternal dietary-mediated effects and the possibility for complex interactions between the diets of interacting parents and between parent and offspring diets. Chapters 3, 4, and 5 all address this aim. In Chapter 3, I found complex interactions between parental diets that suggested both parents make significant contributions to their offspring's lifespan, fecundity, body mass and triglyceride levels. In Chapter 4, I build upon the previous chapter by investigating the transgenerational contributions of each grandparent, and the capacity for grandparental and grandoffspring diets to interact. I found complex interactions and sex-specific transgenerational effects whereby grandmaternal diet effects exerted opposing effects on grandoffspring lifespan depending on grandoffspring sex and the diet. Less expected however is that grandpaternal diets, exerted important effects on female grandoffspring reproduction. In Chapter 5, I investigated the capacity for grandmaternal and grandpaternal protein and carbohydrate effects to interact, and I reveal that rather than interacting, both grandparental protein levels were additive, and the higher the protein the grandparents consumed, the higher the F2 female offspring reproductive output. Furthermore, the protein content in grandmaternal and grandpaternal diets made a roughly equal contribution (effect size of
~3.5%) to female F2 offspring reproductive output.

The results from all three of these chapters demonstrate that despite a bias in the literature favouring the study of maternal effects (Galloway, 2005; McCurdy et al., 2009; Mousseau, Uller, Wapstra, & Badyaev, 2009), both parents make significant cross-

generational contributions to their offspring following dietary modification. It may be expected that due to the divergent reproductive strategies of the sexes, that mothers and fathers have differing opportunities to pass (non-genetic) effects onto their offspring (Crean & Bonduriansky, 2014). However, both parents may be able to influence offspring in oviparous species that internally fertilise via epigenetic modifications to the gametes (Curley, Mashoodh, & Champagne, 2017; Gapp, von Ziegler, Tweedie-Cullen, & Mansuy, 2014). Separately, mothers can also contribute to offspring via cytoplasmic content and yolk variation (Newcombe, Moore, & Moore, 2015), and fathers via sperm and seminal fluid (Wong et al., 2007). Therefore, previous literature has studied maternal and paternal dietary effects separately and found that both parents make noteworthy contributions to their offspring (Buescher et al., 2013; Öst et al., 2014).

I have extended significantly on this work by capturing the parental interactions or additive effects, as few studies manipulate both parental diets, and even if they do they are generally unable to partition out the relative contributions of both parents (Ivimey-Cook et al., 2021; Matzkin, Johnson, Paight, & Markow, 2013). Two studies of the neriid fly (*T. angusticollis)* are exceptions, and authors of these studies investigated maternal and paternal effects of larval nutrition on offspring development. The first was able to find only paternal effects (Bonduriansky & Head, 2007) using a binary diet scheme, whereas using a nutritional geometry approach, the second study was able to detect complex non-additive interactions between parental diets that effected offspring growth (Russell Bonduriansky, Runagall-McNaull, & Crean, 2016). Importantly, I extend upon these studies by investigating parental diet effects in the context of lifespan, reproductive output, and crucially, transgenerationally. Moreover, my chapters also extend the results to a different species, showing that these effects might be general, at least across Insecta. Assaying both F0 sexes together in each experiment allowed me to capture complex interactions between

both parental diet effects and parental and offspring diet effects, that held across three generations (F0, F1 and F2), and for all traits assayed including lifespan, reproduction, body mass, and triglyceride levels, and this has implications for further research. Given that here the diets of each parent might interact in complex ways, studies that focus on the diets of only one parent may be drawing incorrect conclusions, since they do not consider the capacity for the diet of the other parent to moderate the patterns in their studies.

Intriguingly, effects of parental and grandparental diets were only involved in interactions in Chapters 3 and 4, but acted additively in Chapter 5. Explanations for this inconsistency likely lie in the differing designs and emphasis of the studies. In Chapters 3 and 4, I manipulated sucrose to be consistent with much of the literature investigating priming (via anticipatory effects) in the context of over-nourishment or obesity, with protein levels held constant. I also focused on lifespan and used a snapshot of fecundity that was measured as the number of eggs laid over just 22 hours. Whereas in Chapter 5, I assayed early life reproductive output over six days, and counted the number of eclosed adult flies produced. In Chapter 5, I also used diets that differed in both carbohydrate and protein concentrations allowing a deeper understanding of how protein and carbohydrate may shape transgenerational reproductive success. It may be that differing macronutrient manipulation may exert opposing cross-generational effects, i.e., the potential differing effects of protein versus carbohydrate. Alternatively, since protein levels were held constant in Chapters 3 and 4, the effects seen in those chapters, may be specific to that protein (and carbohydrate) level; discussion of this is given below in section 6.5. Future cross-generational studies would benefit from more studies that assay both F0 sexes together across a range of nutritional contexts and traits to gain greater insight into which of those contexts or traits results in interactive versus additive parental effects.

6.3 | Cross-generational effects are sex specific

My second aim was to investigate whether cross-generational effects of nutrition could be sex-specific. I was able to capture four types of sex-specificity in Chapter 4. First, I revealed some sex-specific direct diet effects on how higher and lower (relative) sucrose levels affected female and male F2 offspring lifespan. If F2 females ingested a low sucrose diet they experienced a longer lifespan relative to high sucrose, but the opposite was true for F2 males, they experienced a longer lifespan if they consumed a higher sucrose content. Notably, these direct sex-specific effects where mimicked transgenerationally, indeed the second type of sex-specificity I captured was the differing effects that grandparental diets had on F2 male and female offspring. I found that grandmaternal effects of sucrose variation exerted opposing effects in the F2 grandoffspring sexes, in which a lower sucrose concentration (consumed by grandmothers) increased female F2 lifespan, but decreased male F2 lifespan. Only a handful of studies exist that capture transgenerational sex-specific effects, and our results are consistent with the studies that suggest transgenerational effects may be exerted on the opposite offspring sex to the grandparental sex that was exposed to the treatment. That is, modification of the grandpaternal environment may constrain or enhance female offspring phenotypes, and grandmaternal effects may do so on male offspring phenotypes (Brynildsen, Sanchez, Yohn, Carpenter, & Blendy, 2020; Buescher et al., 2013; Dew-Budd, Jarnigan, & Reed, 2016; Hellmann, Carlson, & Bell, 2020; Zizzari, Straalen, & Ellers, 2016).

Next, I was able to uncover a third type of sex-specificity—the differing contributions that grandmaternal and grandpaternal dietary effects made to individual F2 offspring traits. Above I discussed how grandmaternal dietary sucrose effects on F2 offspring lifespan

manifested differently depending on offspring sex. The results for Chapter 4 revealed that grandpaternal diet effects do not contribute to offspring lifespan, they do however contribute to F2 female offspring fecundity. The grandpaternal effects were dependant on the diet the F2 female offspring consumed, and mismatched diets between offspring F2 female and grandsire were the most fecund. I also found that the grandpaternal effects were dependant on grandmaternal effects and that a complex interaction between them contributed to how fecund their F2 female offspring were. This shows in a transgenerational sucrose context that both grandmaternal and grandpaternal diets exerted effects on offspring life history traits of lifespan and fecundity, but whereas grandmaternal diet affected both traits, grandpaternal diet only effected female F2 offspring fecundity. This again fits within the emerging pattern in the literature that grandpaternal effects may augment female offspring fitness (more so than male offspring fitness). I focused here on assaying female reproduction, but future studies could investigate the effects that both granddams and grandsires have on F2 male offspring reproductive success.

Finally, I identified a fourth type of sex-specificity in Chapter 4, which involved tracking the F1 lineage that the transgenerational dietary effects passed through (i.e. whether effects were passed through the F1 maternal or paternal lineages). Transgenerational effects (from F0 grandmothers or F0 grandfathers to F2 grandoffspring) were transferred through either F1 males (fathers) or F1 females (mothers). By including both lineages, I was able to detect sex-specific lineage effects whereby female F2 offspring flies lived longer if effects were passed through the dam lineage and F2 male offspring lived longer if effects were passed through the sire lineage. Strikingly, we saw the sex of the F1 generation (maternal or paternal line) exerting opposing effects on the lifespan of the F2 based on the sex of the F2 or the diet of the F2. For example, the direct effect of consuming the high sucrose diet was more detrimental to offspring who received effects through the F1

dam line as opposed to the F1 sire line. That is, the F2 offspring both ingested high sucrose, but the only difference was whether transgenerational effects were passed through the maternal or paternal lineage (whether the F1 was male or female); remarkably, this interaction was independent of the diet the F0 consumed.

This result follows a similar pattern to the one other study that tracked both maternal and paternal lineages when investigating transgenerational effects of dietary modification. Dew-Budd et al., (2016) used *D. melanogaster* and employed high and low fat diets. They found sex-specific transmission of dietary-mediated effects, whereby such effects transferred through the maternal lineage altered the expression of male offspring traits such as body weight (but not the body weight of female offspring), and the opposite was true for the paternal line (Dew-Budd et al., 2016). A potential limitation of that study was the choice to manipulate fat rather than other macronutrients, however, since consuming fat in any significant proportions is not ecologically relevant for a fruit fly. Notwithstanding, my Chapter 4, and the study by Dew-Budd et al., (2016) both utilised obesogenic diets (high sugar and high fat), therefore future studies could consider tracking the maternal and paternal lineages in a wider range of nutritional contexts.

Why we see such sex-specificity in cross-generational results is currently unknown, but future studies could focus on the interaction between the differing transmission pathways (i.e. sperm vs egg) of the sexes, combined with the differing nutrient requirements for both sexes. It is also likely that due to these divergent requirements and transmission pathways that results may vary according to what nutrients are manipulated.

6.4 | No evidence for cross-generational priming effects

My third aim, was to ascertain whether parents or grandparents can prime offspring (via anticipatory effects)—advantaging offspring in nutritional environments that match their parents, and I addressed this aim in Chapters 3 and 4. The key to testing for priming effects requires a full factorial experimental design whereby offspring and parents are tested with a novel diet and a control diet such that offspring phenotypes from matched and mismatched combinations (between offspring and parents) can be assayed (Mousseau & Fox, 1998; Uller, Nakagawa, & English, 2013). I employed a full factorial design intergenerationally in

Chapter 3, and although matched sucrose contents between parents conveyed a lifespan advantage for the parents, their offspring from those same matched parental combinations were shorter lived, and I found no advantage for offspring who ate diets that matched one or more of their parents. I advanced the intergenerational findings by also employing a full factorial design in a transgenerational context in Chapter 4, and although I found interactions between grandmaternal diets and F2 offspring diets that shaped F2 offspring lifespan, again I found no advantage for offspring who consumed the same diet as one or both of their grandparents. Additionally, I uncovered interactions shaping F2 female fecundity between both grandparental diets, and between grandpaternal diets and F2 female offspring diets, but I could not detect any signatures of anticipatory effects. The results from these two chapters are consistent with recent meta-analyses on cross-generational priming effects that find effects across environments and taxa to be mixed and weak (Sánchez-Tójar et al., 2020; Uller et al., 2013).

Further, not only did I not find signatures of parental anticipatory effects when sucrose content matched between offspring and parents, I found that mismatched combinations between both F0 diets, and mismatches between F0-F1 and F0-F2 diets enhanced the fitness traits of the offspring in both the F1 and the F2 generations. Although the reasons for this remain elusive, there are a few possible explanations. First, there may be a parent-offspring or grandparent-grandoffspring conflict between generations over optimal diet, or more specifically optimal sucrose content. Therefore, mismatching here that enhanced offspring fitness may be specific to sucrose content, and future studies may manipulate other macronutrients such as protein to elucidate this result further. Next, it may be that the specific sucrose concentrations used here (relative high and low), in their combination are simply more beneficial to offspring—the combination creating balance.

However, the fact that the results show complex non-additive interactions between both F0 diets, and between F0-F1 diets, and F0-F2 diets indicate this is probably not the case.

There is a remaining question of why some studies may find evidence for priming effects and others may not. First, if priming effects are detected, they may be context specific, and therefore may differ between taxa and environments. For example, when plants are grown in the same light as their maternal plant, they have higher rates of germination, likely owing to the fact that plants are fixed in place, and are largely unable to move to differing environments (Galloway & Etterson, 2007). Moreover, within a nutritional context it may be that specific diets or nutritional contexts may elicit differing responses. Two recent studies that focus on fasting and/or starvation have found some evidence for intergenerational parental priming effects. The first investigated cross-generational effects of temporary fasting in *C.elegans* and found that if F0 parents and F1 offspring experienced the same temporary fasting environment, the lifespans of both generations were extended (Ivimey-Cook et al., 2021). Importantly, authors did not detect any priming effects in subsequent generations, and in fact found that fasting came at a cost to mortality in the F3 generation. This indicates that whether a parental effect is adaptive may differ between generations.

The intergenerational results of Ivimey-Cook et al., (2021) however, concur with Hibshman et al., (2018) who also found that *C.elegans* offspring from starvation environments that matched their dam, were more like to recover from starvation, grew faster, and had increased fecundity (Hibshman, Hung, & Baugh, 2016). Evidently, in my third chapter, I did not find evidence for intergenerational anticipatory effects, and perhaps this is an artefact of the different diet regimes used in my study versus studies that are investigating starvation or fasting. This may indicate that parental priming effects may differ in dietary environments that provide adequate or over-nutrition compared to contexts where

the organism is forced to fast or starve. It is likely that starvation is a more stressful state for the organism, and therefore may exert a greater effect on phenotypic plasticity both within and between generations.

Future work into adaptive anticipatory effects in a nutritional context might move beyond the sucrose context that I test here in Chapters 3 and 4, and manipulate a wider range of nutrients i.e. protein to see if the effects are consistent across macronutrients. Testing across dietary contexts would be worthwhile, but the accumulating evidence combined with my studies is that, at least when applied to carbohydrate variation, there is little evidence for dietary mediated parental anticipatory effects across generations.

6.5 | Cross-generational effects are specific to individual macronutrients

My fourth aim was to determine whether dietary-mediated cross-generational effects are unique to the particular macronutrients manipulated, and whether effects mediated by carbohydrate variation are similarly induced by protein variation, and I addressed this aim primarily in Chapter 5. I determined that a higher relative protein concentration was more important (than was carbohydrate) to the reproductive output of both F0 females (mediated through direct diet effects), and to F2 females (mediated through transgenerational diet effects). I demonstrated that both F0 grandfathers and F0 grandmothers contribute independently to their F2 granddaughter's reproductive output through variation in protein, but not carbohydrate ingestion, and I did not find evidence of interactions between macronutrients. These results are intriguing as protein is rarely manipulated in cross-generational studies, and when it has been, design limitations have meant that the relative contributions of the parents could not be disentangled (Matzkin et al., 2013). Otherwise, exploration of such interactions were assessed only in an intergenerational (F0 to F1) context (Bonduriansky et al., 2016). Importantly, I extend on these studies in Chapter 5 by assaying the effects transgenerationally (F0 to F2).

My results suggest that dietary protein is of primary importance to female reproductive success, which has long been known when it comes to direct effects (Jensen, McClure, Priest, & Hunt, 2015; Maklakov et al., 2008; Zajitschek, Zajitschek, Friberg, & Maklakov, 2013), but is a novel finding in the transgenerational context.

Studies using crickets (*Teleogryllus commodus)* and flies *(D. melanogaster)* show that high protein consumption tends to reduce lifespan in both sexes, but increases female reproduction, yet tends to have a marginal effect on male reproduction (Jensen et al., 2015; Maklakov et al., 2008; Zajitschek et al., 2013). Similarly, a study using neriid *(*f*T. angusticollis)* found that protein restriction extended adult lifespan in both sexes and produced infertility in females, but had only trivial effects on male reproductive success (Adler, Cassidy, Fricke, & Bonduriansky, 2013). Moreover, another study using neriid flies found that higher relative protein consumption in F0 females enhanced their offspring egg hatching success (how many F1 offspring hatched), but higher protein in F0 males reduced F1 hatching success (Russell Bonduriansky et al., 2016). As there is currently a large disparity in studies investigating female components of reproduction comparative to male components of reproduction, and even fewer that look into the transgenerational effects of F0 diets on male offspring reproduction, future studies could elucidate how the modification of protein in the F0 generation would affect F1 and F2 male reproductive success.

6.6 | Whether effects are concordant across generations is context specific

Finally, my fifth aim was to uncover whether the cross-generational effects of variation in nutrition are consistent across generations (comparing responses across F0, F1, and F2), and I was able to address this aim through comparison of key results across

Chapters 3, 4, and 5. In doing so, I discovered both concordance and discordance between generations. First, in Chapter 3, I uncovered conflict between the F0 and the F1 generations over optimal sucrose concentration for lifespan extension. Indeed, matched parental sucrose concentrations extended parental lifespan but reduced offspring lifespan, and that mismatched parental combinations were more positive for F1 offspring lifespan and fecundity. Whereas, comparing the F1 results from Chapter 3, to the F2 results from Chapter 4, I find a general concordance between these two generations in the direction and pattern of cross-generational effects. In the F2 generation, again a mismatch between grandparental diets conferred the life history advantage to F2 offspring similarly to the effect of parental diet mismatching in the F1 offspring. I again find concordance between generations in Chapter 5, this time between the F0 and the F2 generation. Focusing on female reproductive output, I found that the effects of protein where concordant across generations—higher protein lead to positive direct and transgenerational effects on reproductive output.

The question remains why effects between the F0 and the F2 were concordant in Chapter 5, but at conflict between the F0 and F1 in Chapter 3. The answer could lie in the generations being assayed; indeed other cross-generational nutritional work has also found conflicting patterns in the direction of effects between generations (Buescher et al., 2013; Ivimey-Cook et al., 2021). Alternatively, the answer might lie in the differing foci and diets employed in the studies. In Chapters 3 and 4, I investigated effects of sucrose variation, and challenged both the F0 parents and the F1 (Chapter 3) or F2 (Chapter 4) offspring with either a high or low sucrose content. In Chapters 3 and 4, I also focused primarily on assaying lifespan, and physiological traits associated with body constitution (body mass, and triglyceride levels). My measure of reproductive success was female fecundity measured over a short (22-hour) period in early life. In Chapter 5, however, only the F0

grandparents were challenged with novel diets (not the F2 offspring, who were reared and maintained on a standard diet). The F0 grandparents were challenged with a range of protein and carbohydrate levels rather than the binary diets of Chapters 3 and 4. Variation in female reproductive output across the treatments was then investigated in detail (with offspring production by females measured over a six day period in early life), as the focus of Chapter 5. Although I can only speculate, it is plausible that either the differing diets used in Chapter 5 relative to earlier chapters, or the focus on a different trait (reproductive output relative to lifespan and body constitution), could explain the discrepancies between results here. Moreover, I note that due to the complexity of the interactions I uncovered between generations and phenotypes, pinning down generalities or trends in cross-generational nutritional effects will likely prove to be a difficult endeavour, requiring of more studies before we can attempt to identify general trends between generations (Deas, Blondel, & Extavour, 2019).

6.7 | Conclusions

In my thesis, I set out to characterise the nature of inter- and transgenerational nutritional effects, to see what contributions parents make to their offspring and grandoffspring, and my results were unexpectedly complicated. Particularly Chapters 3 and 4 revealed complex interactions between the diets of maternal and paternal lineages, and offspring diets, and offspring sex, that mediated inter- and transgenerational effects on offspring phenotype. My results have generated a set of new questions that are worthy of testing in the future. Across my chapters, I sampled flies from a mass outbred population of *D. melanogaster* (Dahomey) because my focus was on establishing patterns that were general across varied genotypes. The limitation of this approach however, is that by attempting to establish general patterns by sampling from a population exhibiting high

levels of segregating genetic variance, I am unable to separate epigenetic effects from the effects of an adaptive evolutionary response to selection. My results could have been driven, at least in part, by differences in diets leading to selection on particular genotypes, with only larvae of certain genotypes surviving in subsequent generations. Whether or not the diets I used were sufficiently stressful to have resulted in a genotype-dependent differences in larval mortality or developmental delays, is unclear. Certainly there is some evidence that suggests that genotype likely plays a role in the magnitude and direction of cross-generational effects (Dew-Budd et al., 2016; Matzkin et al., 2013). Thus, future nutritional studies should seek to clarify the mechanisms underlying the cross-generational dietary effects that I have uncovered in my thesis—be they epigenetic or adaptive responses to selection. One means by which these two possibilities could be disentangled is by conducting similar experiments across panels of isogenic lines, removing genetic variation on which selection can act. Such an approach may help to reveal whether patterns are general across, or specific to individual genotypes.

There are documented non-genetic mechanisms by which these cross-generational effects may have been transmitted. First, such effects have been shown to be linked to transmission of epigenetic states involving DNA methylation and modification of histone proteins that affect chromatin structure (Bonduriansky & Day, 2009; Öst et al., 2014). Other studies have identified links involving the transmission of gametic, somatic or cytoplasmic factors, such as hormones, proteins, lipids and RNA (Cuzin, Grandjean, & Rassoulzadegan, 2008; Díaz & Esponda, 2004; Groothuis, Hsu, Kumar, & Tschirren, 2019; Groothuis, Müller, Von Engelhardt, Carere, & Eising, 2005). It is also possible that parents are transferring nutrients to their offspring as nutrition directly effects parental nutrient provisioning which affects the quality and quantity of metabolic resources that are provided to developing offspring, and has been observed in both maternal and paternal studies (Russell

Bonduriansky & Day, 2009). To build upon previous studies, and elucidate the mechanisms at play, future studies that probe the cross-generational implications of dietary variation should move towards embracing a cross-disciplinary tool kit, drawing on transcriptomics to link transgenerational effects to epigenetic mechanisms, and gene expression, thus elucidating the underlying mechanisms for cross-generational dietary effects.

6.8 | References

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