Nutritional plasticity in female fecundity: The impact of protein on egg production in *Drosophila melanogaster*

André Nogueira Alves

MSc Biology of Evolution and Development

A thesis submitted for the degree of Doctor of Philosophy at
Monash University in 2022
School of Biological Sciences

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Thesis abstract

Nature is a system in constant change. Nowadays more than ever, with humankind's actions accelerating climate change. Facing this increasing variation in the wild, it is up to organisms to adjust their phenotype and evolve. One strategy used by many animals and plants to cope with environmental change is plasticity. This phenomenon is characterized by the generation of distinct phenotypes without any alteration of an organism's genome. The past half century has seen a resurgence in interest and importance of this phenomenon to evolution. Plasticity is a source of phenotypic variation among individuals and contributes to diversification of populations. It is also thought to play a possible role in the colonization of new ecological niches. With climate change, this ability will become increasingly important for populations to persist. To better understand how plasticity shapes an organism, and consequently a population, we need to explore how environmental variation alters mechanisms both at a molecular and at a behavioural level to create phenotypic variation.

My thesis work explored proximate mechanisms underlying variation in nutritional plasticity for female fecundity, a major trait determinant of a species persistence, in *Drosophila melanogaster*. The goal of this thesis was three-fold: first, to characterize nutrient sensing mechanisms that generate variation in nutritional plasticity in fecundity; second, to observe the genetic variation in nutritional plasticity in an isogenic panel generated from a wild population and explore the capacity of plasticity to evolve; and third, to explore the proximate behavioural and physiological mechanisms underlying genetic differences in the plastic responses of egg laying to nutrition.

I found that individual amino acids have distinct effects on egg production in *D. melanogaster*. Deficiencies for some amino acids cause egg production to stop rapidly. Deficiencies for other amino acids are not accurately sensed by these pathways, and therefore, do not inhibit egg production, allowing the flies to produce eggs for a prolonged period of time. This difference in response is regulated by the Target of Rapamycin signalling pathway. Next, using a newly generated isogenic panel of fly lines, I found significant genetic variation for nutritional plasticity for fecundity, due to differences in genetic correlations between diets. However, lines adapted to nutritional stress did not evolve changes in nutritional plasticity for fecundity. Finally, I uncovered the proximate mechanisms through which plasticity in female fecundity is achieved. *D. melanogaster* flies characterized by high nutritional plasticity for fecundity do not differ in ovary morphology. Instead, they achieve higher plasticity by increasing food intake and having a higher food-to-egg conversion efficiency.

The results from this thesis illustrate how, in *D. melanogaster*, variation in nutritional plasticity for female fecundity can be regulated at several levels, from molecular to behavioural. Furthermore, the way plasticity varies within a population determines if adaptation to new environments will result in a population that persists or goes extinct. This thesis also highlights the little that is known about how environmental factors impact plasticity, and persistence of species, and emphasizes that further research needs to be done to understand nature as an ever-changing system that generates biodiversity.

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.

Print Name: André Nogueira Alves

Date: 31/1/2022

Publications during enrolment

Mirth, C.K., Alves, A.N. and Piper, M.D. (2019). Turning food into eggs: insights from nutritional biology and developmental physiology of *Drosophila*. Current opinion in insect science, 31, 49-57.

Alves, A.N., Sgrò, C.M., Piper, M.D. and Mirth, C.K. (2022). Target of Rapamycin drives unequal responses to essential amino acid depletion in egg laying. Frontiers Cell and Developmental Biology, 10,

Havula E., Ghazanfar S., Lamichane N., Francis D., Hasygar K., Liu Y., Alton L.A., Johnstone J., Needham E.J., Pulpitel T., Clark, T., Niranjan H.N., Shang V., Tong, V., Jiwnani N., Audia G., Alves A.N., Sylow L., Mirth C., Neely G.G., Yang J., Hietakangas V., Simpson S.J. and Senior A.M. (2022). Genetic variation of macronutrient tolerance in *Drosophila melanogaster*. Nature Communications, 13.

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 *no* original papers published in peer reviewed journals and (*1)* accepted publications. The core theme of the thesis is evolutionary and developmental 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 Dr. Christen Mirth, Dr. Matthew Piper and Prof. Carla Sgrò.

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 2, 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	Target of Rapamycin drives unequal responses to essential amino acid depletion in egg laying	Published	60%. Concept, collecting data, data analysis, and writing manuscript	15% C. K. Mirth: concept, data analysis, edits to manuscript. 15% M. D. W. Piper: concept, edits to manuscript. 10% C. M. Sgrò: concept, edits to manuscript	No

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

Student name: André Nogueira Alves

Date: 31/1/2022

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: Christen Mirth

Date: 31/1/2022

Acknowledgements

I am still flabbergasted by the relativity of time as I write this. It's been four years since this adventure started, and yet, it feels like I was jumping on a plane to come to Melbourne and do a PhD just a few days ago. It feels like time ran through me, and if, to an outside viewer, it seemed that everything was a smooth trip, to those who experienced it, it was a rollercoaster. And it was sometimes as fast as the blink of an eye and at times as slow as watching paint dry.

However, if there is one thing that is not relative, but rather absolute, it is the contribution of so many people to this degree. Although my name will be on the front page of this thesis, this thesis belongs just as much to every single person I will mention in this section, because without them I would not have had the resilience, strength, support, wisdom and love to do it. And what better to express my gratitude for these past four years if not with four pages of acknowledgements?

I would like to start by acknowledging Monash University. Without their financial support, through the Monash Research Scholarship and the Monash International Postgraduate Research Scholarship, nothing of this PhD would have been achieved since I would not be here. A big thank you to the HDR committee for seeing the scientific potential I had and believing I would succeed in this journey. I can say you were right in believing in me, thank you for providing me with this opportunity.

A big thank you must go to the supervisor trifecta: Christen Mirth, Matthew Piper and Carla Sgrò. Throughout these four years they have been unwavering in their support, even when their lives where chaotic, and the world was going through a pandemic, their role as supervisors never faltered. They all helped me with this project as much as they could and as much as I asked, and I asked for a lot. I think it is fair to say that I made them rise to challenges as much as they did me, and I can only thank them for that, since I would not know what I would be capable of and what my skills are had they not done so. Outside of their role as supervisors, knowing them has been enlightening and a pleasure, and I am glad I had the opportunity of having supervisors who were also friends. Each one of them taught me valuable lessons both in the academic world and outside of it, so thank you for everything.

I also experienced the pleasure of having a good PhD thesis panel: Dustin Marshall, Matthew Hall and Damian Dowling. Although afraid of them at the start, they proved to be not only wise and smart, but also friendly and genuinely wanting the best out of this project.

I thank them for their advice on how to run experiments, analyse data and deal with supervisors. A special thank you to Damian, for the amount of extra advice I got and for the support and enthusiasm I got from him about my project.

Throughout this thesis I have done some very lengthy and large experiments. They would absolutely not have been possible if it weren't for the help of a lot of people in my lab. For the isogenics, Avishikta and Fiona, you two were the ones who initiated the isogenic panel and went through the hardest part of it, thank you for doing that. Avi, a big thank you for the number of times you tipped flies over weekends and agreed to swap days so it would suit us both better. Thank you as well for all the conversations we had, they not only killed time but also provided good advice for both of us.

For all my ovary experiments, CAFE assays, and just anything in the lab, I thank Amy Dedman and Tahlia Fulton. Amy, you are an amazing person and worker, and I honestly think you never, not once, declined to help me, and I applaud your work ethic. I don't think many people really value the amount of work you did for them, so I want this part to be a recognition of not only for the help I got, but all the help you gave to everyone else, which meant they would be able to help me. Tahlia, you are such a selfless person that was always up to help me with some experiment, analysis, anything really. You are a bright star, and although sometimes space dust might dim your brightness, you will always be an example on how helping others only makes you shine brighter.

Many other people at the workplace have played some role in this thesis: Josh, Ansa, Jade, Hannah, Mia, among others, a big thank you. Thank you for helping me with experiments, analyses, or just allowing me to have so much cold room space to store all the fly food I needed for the crazy projects I had.

A big thank you to people that we were not always aware of, but are the master pieces that keep the machine running in the background: Fiona Hibbert and Nicole de Rycke. I bet they would say they were only doing their jobs, but they are/were masters at it and amazing people to talk to. Hendrika Duivenvoorden, Christa Meek and Mary Angelopoulos, thank you for all the teaching experience you gave me, it was a completely different side of doing a PhD that not only provided an economical safety net, but also an avenue to realise that research isn't the only thing we are supposed to think about.

This thesis would not have been completed if it weren't for the help of all the people outside of the labs I worked at. A body might be able to do tasks, but if the mind is not working, nothing can be done. And for that I have to thank many friends, past and present, that supported and encouraged me to keep striving. Eduarda Pinto, you were my Portuguese anchor for the first year of my PhD, you were there for what seemed every hour

of it, always available to listen and provide advice and cheer me up. Nuno Soares, the number of hours we spent talking about books and science cannot be counted, and your input has always been excellent and refreshing, you are one of the wisest minds to dissect a problem. Scott Scott for the honest worry, interest and conversations we have had, and for the laughs you gave me. Mike Chong, for the friendship and all the good times we have spent to make me stress less about my PhD. Lachlan Cauchi for being such a good friend, allowing me to have a space to vent my frustrations, and for always believing in me and being the first to cheerlead me into every challenge. I reckon we created a feedback loop of hope between us, where seeing each other succeed fed the other some hope and strength to keep going, and I hope this loop continues outside both our degrees.

And as for the ones closest to my heart, I am the most grateful for. A key person in making all this possible is Brendan Houston. Not only was he a role model for how to be professional, efficient and successful in science, he also helped me so much with experiments, weekdays and weekends, drafting emails, reviewing drafts and all without expecting anything in return. Outside of “Postdoc Brendan”, “Partner Brendan” was an even greater help. Taking care of our home life while I was busy doing my own PhD isn’t easy. Doing a PhD through a pandemic was more tolerable thanks to you. For believing in me, encouraging me to go for it, for being that safe space I could go to when the river of time was flooding through me and hitting me with all the debris of a PhD. Through anxiety, stress, nervousness, sadness, you were always there to anchor me and secure me for better times. And for making me smile through the worst times, even when you were going through the same chaotic circumstances. An eternal thank you.

Before I move to a final paragraph in Portuguese, I would like to formally acknowledge my family, scattered all around the world, that collectively are proud of me for being the first person in two families to reach this far; that no matter where they are, they claim to have a cousin/nephew/grandson in Australia who is studying to be a doctor. They don’t even know what I really do, but they believe in me, and I thank them for that faith.

Neste último parágrafo quero começar por agradecer não a uma pessoa mas a um pequeno ser, Bolinha. Embora já não esteja connosco, esta cadelita foi uma felicidade e o meu pequeno animal de estimação por muitos anos, e todas as semanas eu quis vê-la por Skype e saber que ela ainda ficava curiosa ao ouvir a minha voz. Muitas tardes passadas no meu colo enquanto eu me candidatava a este doutoramento e foi uma grande tristeza quando partiu, mas o tempo que passou cá foi bem passado, um obrigado a toda a felicidade que ela me trouxe. E, finalmente, tenho de agradecer às pessoas que suportaram toda a tristeza de não me puderem ver e não me terem por perto para que eu fosse feliz.

Angela Nogueira e Eduardo Alves, os meus pais, um muito obrigado por tudo o que fizeram por mim. Vocês sempre apostaram em mim, acreditaram que eu fosse conseguir tudo o que pusesse o meu esforço e que seria bem sucedido. Vocês podem não entender nada do que faço, mas fazem todo o esforço para entender e para mostrarem quão orgulhosos estão. Não tenho mais palavras para descrever o quão agradecido estou por tudo. Amo-vos e Obrigado.

To all these people, remember: just like time is relative, even though the word that expresses my gratitude is said within a heartbeat, the feeling will be everlasting. Obrigado.

Chapter 1 | General Introduction

“In the beginning the Universe was created.
This has made a lot of people very angry and been widely regarded as a bad move.”

By Douglas Adams in The Restaurant at the End of the Universe

Nature is an ever-changing system, and plasticity allows organisms to adjust their phenotypes to new conditions (Schmalhausen, 1949). Phenotypic plasticity, the capacity of one genotype to produce multiple phenotypes when exposed to environmental variation, exists in a wide range of traits, from animals that dramatically alter their colouration with changing seasons to much subtler, but not less impressive, changes such as modifications in organ size to adapt to the new environmental conditions. Importantly, plasticity in fecundity, the ability of an organism to change its reproductive output with environmental conditions, is of vital importance. Plasticity in fecundity determines if a species is able to mould the number of offspring it can produce to adjust and persist for future generations. With insufficient plasticity, populations run the risk of dwindling in numbers as they cannot adjust to keep up with the unrelenting pace of environmental change, which eventually results in another extinct branch of the tree of life.

Changes in nutritional availability and/or quality are particularly important, as they have the potential to initiate a cascade that breaks numerous trophic links in an ecosystem (Scheffers et al., 2016; Houghton et al., 1990). Nutritional stress one of the most common stressors animals face in nature, and will become increasingly so under climate change (Long et al., 2014). In addition, changes in other abiotic components, such as temperature and humidity, tend to impact nutrition (Rosenblatt & Schmitz, 2016). If changes in nutrition happen at the level of primary consumers within an ecosystem, this can potentially mean that all trophic levels above primary consumer will have to adjust to a new nutritional environment. Plasticity in fecundity to nutrition is, therefore, a critical precursor for organisms to be able to adapt and persist in new environments.

The aim of this thesis was to enrich our knowledge on the role of nutritional plasticity in female fecundity, and the capacity of plasticity to generate mechanistic diversity in response to protein availability. In this chapter, I introduce how the concept of plasticity arose from a historical perspective. Next, I present the proximate mechanisms that generate nutritional plasticity and outline how these contribute to variation in nutritional plasticity between individuals. I then focus on the importance of genetic variation in plasticity and the potential of genetic variation to shape the evolution of plasticity in fecundity. Finally, I conclude the chapter with a discussion of the gaps in our understanding of plasticity which this thesis will address.

1.1 | The history of phenotypic plasticity

The idea that plastic responses to the environment could facilitate population fitness was first suggested in the nineteenth century by Baldwin, who thought that individuals would need to have a capacity to change traits such as behaviour, morphology, and physiology for adaptation to occur (Baldwin, 1896; Morgan, 1986). But it wasn't until more than half a century later that researchers started realizing the importance of plastic responses for evolution. With the coining of terms such as reaction norms, which visualise the relationship between the expression of a phenotype across an environmental gradient (Woltereck, 1909), or genotype-by-environment interactions, where genotypes react differently to environmental conditions (Johannsen, 1911), empirical evidence for plasticity, and its role in how populations respond to changing environments, grew.

In 1949, Schmalhausen proposed that environmental changes could induce plastic shifts in traits, and that these could be adaptive (Schmalhausen, 1949), thus the concept of phenotypic plasticity was born. This concept was further improved in 1965, when Bradshaw proposed that phenotypic plasticity is genetically controlled, and thus, there is genetic variation in the capacity to be plastic (Schlichting and Pigliucci 1998; Lande 2009). Because of this variation, Bradshaw recognised that plasticity itself could evolve. Although initially phenotypic plasticity was thought to be only a process that hinders evolution, or to be less important than selection (Williams, 1966; Charlesworth et al., 1982), in the past half century the importance of plasticity in generating phenotypic variation and driving adaptation has been increasingly acknowledged.

1.2 | The role of plasticity in adapting to environmental change

By its modern definition, phenotypic plasticity is the capacity for a genotype to produce multiple phenotypes when exposed to different environments (Bradshaw, 1965; Gause, 1947; Ørsted et al., 2017; Schlichting & Pigliucci, 1998; Pigliucci, 2001; Pigliucci et al., 2006; Garland & Kelly, 2006; West-Eberhard, 2003; West-Eberhard, 2005). Phenotypic plasticity has the capacity to underpin species' persistence when facing ongoing climatic changes, since it may buffer organisms from the immediate effects of environmental changes and allows them to persist in a short- to medium-term (Sgrò et al 2016). Global warming has made this capacity increasingly vital, since climate change is exposing natural populations to environmental stresses which impact all levels of biodiversity, including the

distribution and abundance of species (Berg et al., 2010; Scheffers et al., 2016; Houghton et al., 1990; Dawson et al., 2011; Thomas et al., 2004; Foden et al., 2013).

While increasing temperature is often assumed to be the main stressor caused by climate change, a number of other components of the environment will also change. In particular, changes in CO_2 levels and precipitation will have major impacts on primary producers (Morison and Morecroft, 2007). Climatic changes will alter species ranges, interactions between species, and overall productivity, which will impact the timing and abundance of food sources for herbivorous and frugivorous animals (Pecl et al., 2017; Rosenblatt & Schmitz, 2016).

One of the most common stressors in nature is nutrition (Simpson & Raubenheimer, 2012). Nutritional stress will become increasingly common and acute under climate change. Studies have concluded that under elevated CO_2 levels and warmer temperatures, plants will decrease in nitrogen content, which is a proxy for protein concentration, and mineral content, especially in organs such as leaves and roots (DaMatta et al., 2010; Sardans et al., 2017; Lenhart, 2017). Protein will, therefore, become a limiting macronutrient for animals that feed on these plants. Evidence from a wide variety of research suggests that climate change alters the nutritional value of plants both in terms of macronutrient and micronutrient compositions (DaMatta et al., 2010; Sardans et al., 2017; Lenhart, 2017). Shifts in the nutrient composition of plants may have severe consequences on a wide range of traits of herbivorous and frugivorous animals such as feeding rate, the efficiency of nutrient assimilation, growth rate, and many others (Koricheva et al, 1998; Zvereva and Kozlov, 2006; Dijkstra et al., 2012).

To understand how populations can persist under climate change, the role of plasticity in fitness and how it varies across individuals and populations must be investigated (Atkins & Travis, 2010). Fitness is a property of a genotype and can be defined as the capacity of a genotype to survive and leave the most copies of itself in future generations (Haldane, 1926; Fisher, 1930). Selection acts on variation in traits that affect fitness, or on fitness directly. All phenotypic traits contribute to a genotype's fitness, albeit not all contribute to the same degree. The impact of changing environments on a number of traits related to organism development, morphology, behaviour, or stress-resistance have been well studied (Chakraborty et al., 2020; Klepsatel et al., 2020, Sgrò et al., 2010; Chevin & Hoffmann, 2016). However, fitness is difficult to measure, and so research tends to focus on life-history traits that represent good proxies for fitness such as lifespan and fecundity (Haldane, 1926).

1.3 | Fecundity as a proxy for fitness

Fecundity is defined as the potential to reproduce, i.e., the ability to produce functional gametes, fertilize them, and carry the gestation to full term (Grebenik, 1958). This trait is tightly correlated with fitness since changes in the number of offspring produced impact fitness directly (Fisher, 1930). Furthermore, in oviparous animals, female fecundity is easily quantifiable, since the number of eggs laid corresponds almost linearly with the number of offspring (Fisher, 1930). Fecundity is also affected by nutrition (Bongaarts, 1980). Both the quantity and quality of nutrients available affect fecundity (Kilham et al., 1997). This means that organisms need sufficient calories, but also those calories need to contain the right proportion of macro and micronutrients. This makes fecundity a key trait to understanding not only species' persistence, but how species will react to changes in nutrition caused by climatic change.

While the concentration of most nutrients influences fecundity, protein plays a major role in regulating this trait. For instance, in several species of fish, such as *Oreochromis niloticus* and *Poecilia reticulata*, the concentration of protein in their diet correlates positively with the number of offspring produced (Dahlgren, 1980; Hafedh et al., 1999). This is also true for insects; reducing the concentration of protein in the food decreases egg production in several species including carabid beetles (Wallin et al., 1992), Queensland fruit flies (Fanson & Taylor, 2012), and *Drosophila melanogaster* fruit flies (Lee et al., 2008). This shows that protein limits fecundity across the animal kingdom. Since fecundity directly affects fitness (Fisher, 1930), changes in fecundity might dictate if a species is able to persist or if it will go extinct. Further, because environmental change is predicted to decrease the protein content of many plants on which animals feed (DaMatta et al., 2010; Sardans et al., 2017), understanding the effect of protein restriction in fecundity will be paramount to comprehend how species can persist when facing climate change.

Drosophila melanogaster has proven to be an excellent model organism for exploring how diet affects life-history traits (Lüersen et al., 2019). It is also a great model to understand the mechanisms regulating plasticity at a cellular, organ, or organism-level (Mirth et al., 2019). This is in part because of its rapid development and short lifespan, which provides an inexpensive system to study the effects of quantitative variation in diet composition. Also, many powerful tools have been developed in this animal. From genetic tools that allow researchers to manipulate gene expression at precise times and in specific organs (Greenspan, 2004), to isogenic panels that permit the use of individual genotypes to quantify variation in plasticity such as the *Drosophila melanogaster* Genetic Reference Panel

(DGRP) and the *Drosophila* Synthetic Population Resource (DSRP) (Mackay et al., 2012; King et al., 2012), the tools available in *D. melanogaster* allow researchers to delve deeper into the effects of nutrition on fecundity.

In the wild, *D. melanogaster* flies obtain protein by eating yeast proliferating in rotting fruit. Climate change will likely alter macro and micronutrient content of primary producers (DaMatta et al., 2010), which will also alter the quality of fruits where yeast proliferate. It is through this yeast that fruit flies obtain their ten essential amino acids, without which they would not be able to produce eggs (Sang and King, 1961). An amino acid is considered to be essential if it has to be obtained from the animal's diet, as it cannot be synthesized by the organism (Wu et al., 2013). In the absence of any of these essential amino acids, fecundity is heavily impaired (Grandison et al., 2009).

1.4 | Proximate mechanisms that generate plasticity in fecundity

Plasticity in fecundity can be achieved by regulating the proximate mechanisms that control egg production and laying. Proximate mechanisms can include behavioural, physiological, developmental, and genetic mechanisms (Figure 1). Behavioural mechanisms alter food-related behaviours like food intake. Physiological mechanisms control the efficiency of nutrient absorption and assimilation. Developmental mechanisms encompass traits like egg development rate. Finally, genetic mechanisms include the molecular mechanisms that underpin trait responses, including nutrient sensing pathways and the pathways that regulate cell physiology and fate. Each of these mechanisms can be regulated to allow an animal to adjust egg production with protein availability. Further, these mechanisms are not mutually exclusive. Changes in the genetic mechanisms have the potential to impact multiple other mechanisms simultaneously, such as feeding behaviour and egg development rate (Avery, 1993).

Behavioural mechanisms are important to achieving plasticity in fecundity. Differences in food intake, for example, can determine the extent to which individuals experience nutritional stress across environments (Lee et al., 2008). In many *Drosophila* species, larvae regulate their food intake, and reduce the amount they eat if they experience diets with increased protein or caloric content (Carvalho and Mirth, 2017; Silva-Soares et al., 2017). Even among populations, variation in food intake underpins some of the differences in female body size (Chakraborty et al., 2021). Food intake determines the number of calories and macronutrients ingested, which are known to be critical for egg production and development (Sang and King, 1961; Grandison et al., 2009). Regulating food

intake is vital for an individual to reach its nutritional goal and maximize life-history traits such as fecundity (Simpson & Raubenheimer, 2009). More strikingly, sexes differ in their dietary requirements, and this is confirmed by differences in food consumption across sexes and by the fact that reproductive success is maximised on different diets between the two sexes (Camus et al., 2017).

Physiological mechanisms also play a role in plasticity in fecundity. The efficiency with which an organism absorbs and assimilates nutrients, as well as the efficiency with which it allocates these nutrients to traits related to fecundity, will be important for regulating fecundity in different environments. While we have a limited understanding of how these processes regulate egg production, differences in the absorption or assimilation of nutrients is known to affect traits like body size (Sibly, 1981). Juvenile animals that ingest large quantities of nutrients, but are not able to assimilate them efficiently, or are unable to allocate them to growing tissues, end up with smaller adult body sizes (Urabe and Watanabe, 1991; Neat et al., 1995). The ability to convert a fixed nutrient quantity into body size has been shown to diverge across populations of *D. melanogaster* (James and Partridge, 1995). Even within a population, individuals are likely to vary in their capacity to assimilate nutrients (Lee, 2017).

Both behavioural and physiological mechanisms have the potential to impact developmental processes. Egg development and production in *D. melanogaster* depends on protein (Drummond-Barbosa and Spradling, 2001; Armstrong et al., 2014). Thus, the amount of food eaten will correlate with the amount of nutrients an organism has to devote to fecundity (Partridge et al., 2005). At the same time, nutrient assimilation and allocation can impact the ovary's access to nutrients necessary for egg development to occur (Armstrong, 2020). Observing developmental mechanisms such as egg development and oviposition rate informs the extent to which these mechanisms regulate plastic responses to new environments.

Like in all other animals, egg development in *D. melanogaster* happens in the ovaries, which are made up of 16-22 ovarioles (David, 1970; Hodin and Riddiford, 2000). An ovariole is a string of developing eggs surrounded by somatic cells. At the anterior most tip of every ovariole there is a germ line stem cell niche called the germarium (Spradling, 1993). As the germ line stem cells divide, one daughter-cell stays behind keeping its stemness, while the other daughter cell develops into a cystoblast (Koch and King, 1966). Once they exit the germarium, these cystoblasts are called egg chambers. Egg chamber development proceeds through 14 stages before it generates a fully-developed oocyte (King et al., 1956; Cummings and King, 1969). The first six stages of egg chamber development result in the

differentiation of one of the sixteen germline cells into an oocyte, while the remaining cells will become nurse cells (Brown and King, 1964). Stages 8 to 11 are termed vitellogenic, or yolk-forming, stages due to the uptake of yolk proteins by the oocyte. In the last 3 stages of egg development, egg membranes are deposited and the dorsal filaments, which are tubes that project from the anterior part of the egg and allow for gas exchange, form (Cummings and King, 1969).

The rate at which egg development occurs is of vital importance for fecundity. Lower number of ovarioles correspond to lower number of eggs being produced within the same time frame (David, 1970). Additionally, if egg development is slowed down, fewer eggs are produced (McMillan et al., 1970). Slight changes in egg development can have huge impacts on an animal's fecundity, and consequently on a population's capacity to persist (Robertson

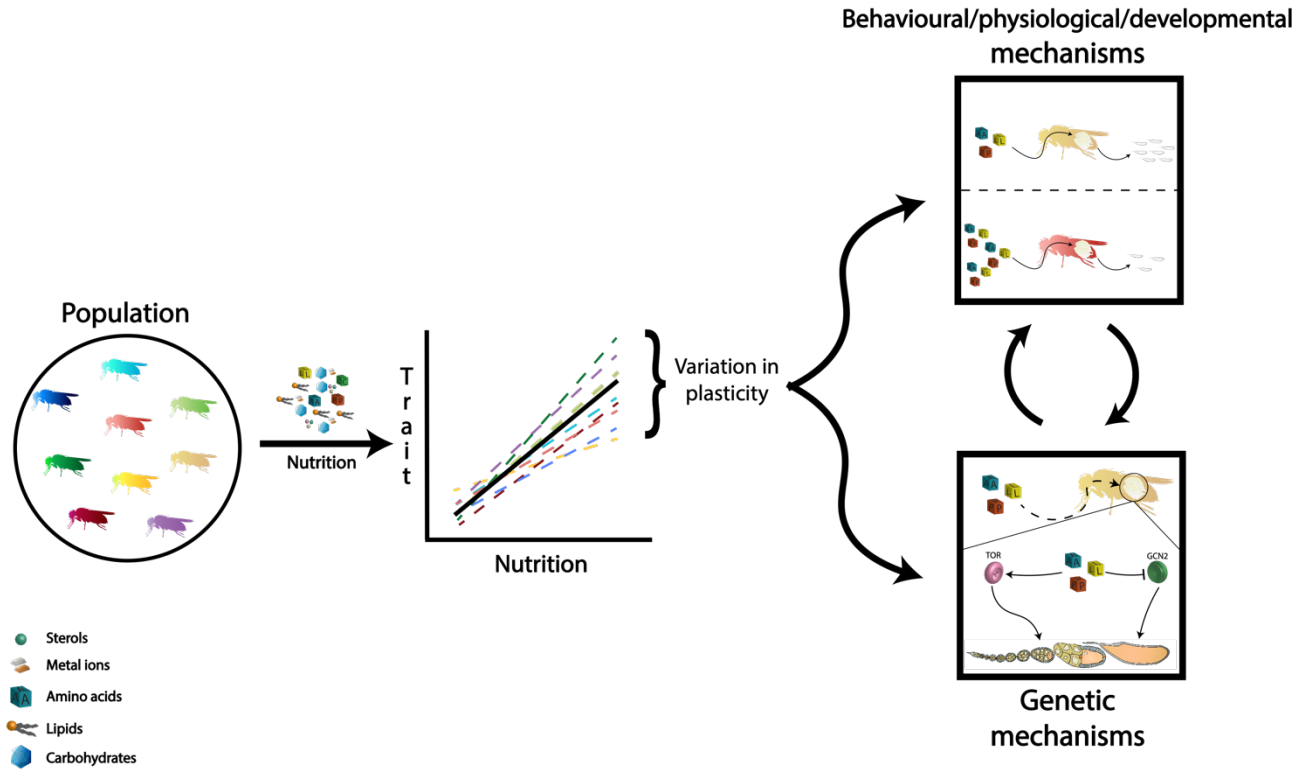

Figure 1 - Proximate mechanisms regulating plasticity in fecundity. A population is composed of several genotypically-different individuals. When these individuals are subjected to variation in nutrition, individual respond diffferently. The variation in response to diet is a representation of variation in plasticity. This variation in plasticity can be regulated by behavioral, physiological, and developmental mechanisms. Difference in genetic mechanisms, including nutrient sensing pathways, underpin differences in behavioral, physiological, and developmental mechanisms.

& Sang, 1944). Plasticity in fecundity is achieved when egg development is adjusted in response to changes in environmental conditions.

1.5 | Genetic mechanisms as generators of variation in plasticity

Genetic mechanisms can also drive differences in fecundity by affecting behaviour, physiology, and/or development simultaneously. Organisms have a number of genetic pathways dedicated to sensing different nutrients and activating or inhibiting the production of signals that will regulate fecundity in response to changes in the organism's nutritional status (Partridge et al., 2005). Pathways like the Target of Rapamycin (TOR) or General Control Non-derepressible 2 (GCN2) are responsible for sensing amino acids (Layalle et al., 2008; Gallinetti & Mitchell, 2013) and are known to regulate feeding behaviour and egg development in females (Ribeiro & Dickson, 2010; Hoedjes et al., 2017; Mirth et al., 2019). Other pathways like the highly conserved Insulin pathway is responsible for signalling when carbohydrate or amino acid sensing pathways like TOR become active (Grewal, 2009).

The activity of the TOR pathway increases with increasing intracellular concentration of amino acids, stimulating cell survival, growth, and proliferation (Wang & Proud, 2009). The absence of essential amino acids regulates protein synthesis by reducing TOR signalling (De Virgilio & Loewith, 2006; Solon-Biet et al., 2014). Although TOR signalling in the gut and fat body of *D. melanogaster* regulates traits such as lifespan (Fan et al., 2015; Bjedov et al., 2010), TOR is also known to act in the ovaries to regulate fecundity (Armstrong et al., 2014). When the intracellular concentration of amino acids is high in the ovaries, the TOR pathway is highly active and it promotes germ stem cell maintenance, growth, and proliferation, leading to an increase in egg production (LaFever et al., 2010). Furthermore, there is evidence that the TOR pathway triggers responses across organs, as knocking down this pathway in adipocytes causes a partial block in ovulation, with no effect seen in the maintenance of germ stem cells (Armstrong et al., 2014). Additionally, researchers have suggested that TOR signalling, coupled with insulin signalling, might drive a diet-dependent regulation of fecundity, further proving the role of TOR in mediating the response of life history traits to diet (Camus et al., 2019).

The GCN2 pathway is mainly known for regulating protein synthesis, however, it also regulates other processes such as autophagy and metabolic homeostasis (Yuan et al., 2017; Falcón et al., 2019). When the intracellular concentration of amino acids is low, and the presence of uncharged tRNAs becomes higher, GCN2 activates (Van Hook, 2015). It is still unclear if GCN2 activates upon accumulation of uncharged tRNAs for any amino acid, or if only depletion of certain amino acids is capable of activating this pathway. Nonetheless,

in *Drosophila* GCN2 plays a major role in gut and fat body function, regulating lifespan when faced with amino acid restriction (Kim et al., 2020). The effect of GCN2 in the ovaries, and its role in regulating fecundity and plasticity in fecundity is still unclear. However, lack of amino acids leads to an activation of GCN2 in the fat body, which leads to the loss of germ line stem cells (Armstrong et al., 2014; Sonenberg and Hinnebusch, 2009). The nature of the signal that communicates the low concentration of amino acids in the fat body to the ovary is still unknown and deserves further investigation.

While the TOR and GCN2 pathways have opposing effects when active, recent research has started to understand the interactions between them. Under amino acid deprivation, the GCN2 pathway suppresses TOR signalling by upregulating Sestrin2 (Ye et al., 2015; Gallinetti et al., 2013). On the other hand, studies in yeast have found that TOR suppression can also promote GCN2 activation via TAP42/SIT4 signalling (Cherkasova & Hinnebusch, 2003). This shows the potential for these two pathways interact to regulate fecundity when amino acids are absent.

1.6 | Genetic variation in plasticity and its capacity to evolve

Genetic variation in plasticity results in differences in reaction norms across individual genotypes (Mirth et al., 2016). If different genotypes have horizontal and parallel reaction norms (with a slope of 0), then this indicates the existence of genetic variation for the trait observed, but no plasticity for that trait (Figure 2A). Slopes significantly greater or less than zero indicate plastic responses to the environment. If genotypes have parallel, but non-horizontal reaction norms then this indicates that all genotypes are equally plastic (Figure 2B). This means there is no genetic variation in plasticity for the trait under the given environmental conditions. (Figure 2B). When genotypes have non-parallel reaction norms, this indicates that there is genetic variation in plasticity for the trait (Figure 2C). This means genotypes react differently in response to changing environments. Variation in plasticity associated with genotypes is common, and often described as Genotype-by-Environment interactions (Schlichting and Pigliucci, 1998; Lande, 2009). This variation in plasticity is thought to be key for a population to respond to selective pressures imposed by environmental change (Sgrò et al 2016; Chown et al., 2007).

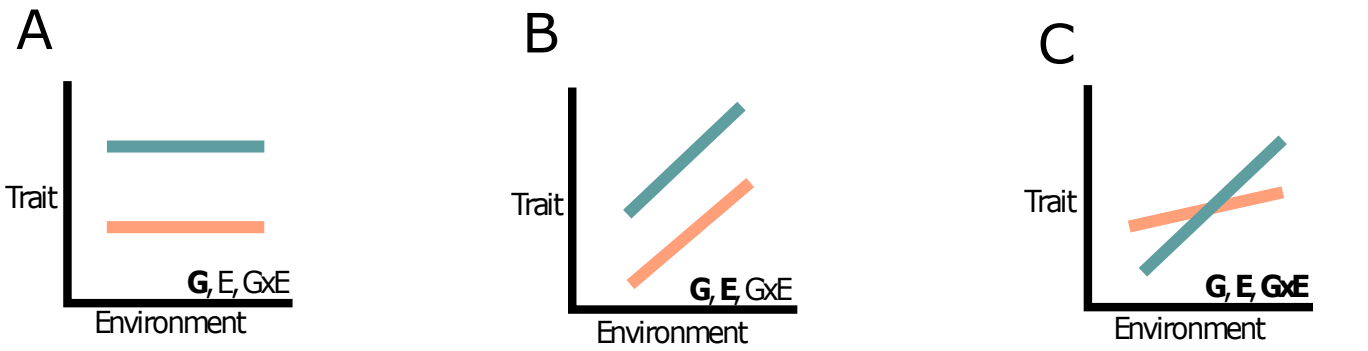

Figure 2 – Reaction norms illustrating significant Genotype, Environment, and Genotype by Environment interactions on trait expression. A – Significant effects of Genotype alone on the focal trait result when genotypes have different trait means but do not show changes in trait mean across environments. B – Significant effects of both Genotype and Environment result in distinct trait means across genotypes and significant positive or negative correlations with the environmental gradient. However, if there is no genetic variation in plasticity the reaction norms will be parallel with similar slopes. C – Significant Genotype, Environment, and Genotype-by-Environment interactions will result in reaction norms whose slopes differ across genotypes, in addition to genetic difference in trait mean and significant correlations between the trait and the environmental condition. Orange and green lines represent different genotypes.

Genotype-by-Environment interactions have been studied extensively in populations sampled from along an environmental gradient. These studies have revealed genetic variation in plasticity in response to nutrition for viability, development time, and body size (Chakraborty et al., 2020; Klepsatel et al., 2020, Sgrò et al., 2010; Chevin & Hoffmann, 2016). Differences in plasticity between populations are important, because they allow for populations to colonize new environments, or to better adapt if their ecosystem suffers environmental changes, such as fluctuations in food resources (Chakraborty et al., 2021).

Plasticity can also vary within a population. Recent research has found that genetic variation in plasticity within a population could play an important role in adaptation to environmental change as it can shape the population-level response to selection (Dreyer et al., 2016; Frankino et al., 2019). Individuals within a population will vary in their response to environmental conditions, resulting in genetic variation in reaction norms. Because a population's reaction norm is the mean of the reaction norms across all individuals within that population, the population reaction norm masks this genetic variation (Dreyer et al.,

2016). Research into reaction norms back in the twentieth century acknowledged the existence of variation in plasticity within a population, however, it understated the importance of genetic variation in plasticity in determining population-level responses to selection (Dreyer et al., 2016). Theoretical studies have demonstrated that the outcome of selection depends on the distribution of reaction norm slopes within a population (Dreyer et al., 2016). This has been applied to body size traits to understand how the relationship between the growth of two organs within the same individual evolve (Frankino et al., 2019). In principle, the distribution of genotype-specific reaction norms has the potential to shape the evolution of any trait, including fecundity.

1.7 | Aims and thesis scope

In this thesis, I implemented a multidisciplinary strategy involving molecular genetics, developmental biology, quantitative genetics, and behavioural approaches to investigate how nutrition can impact fecundity, a trait highly correlated with fitness and, therefore, important for understanding species' persistence to global change. Specifically, the aim of this thesis was to investigate i) how the developmental processes underlying fecundity are modified by the environment, especially nutrition; ii) how variation in plasticity for fecundity can impact selection for new nutritional environments, and iii) the proximate mechanisms underlying genetic differences in nutritional plasticity for fecundity.

Despite the knowledge that female fecundity is sensitive to changes in nutrition, few studies have delved into the impact of the individual components of diet on this trait. This gap in knowledge was addressed in Chapter 2. Here I examined the role of individual amino acids in triggering egg development in the ovaries of *D. melanogaster*. Next, I examined how different protein sensing pathways regulate egg development to produce the varied responses to nutrition that were observed. This represents an important step in elucidating the genetic mechanisms generating plastic responses to nutrition for fecundity.

How genetic variation in plasticity for fecundity determines the response to selection to a different nutritional environment was explored in Chapter 3. In this chapter, I asked whether a population of *D. melanogaster* harboured genetic variation for plasticity in fecundity when faced with protein restriction. First, I characterized genetic variation in nutritional plasticity for female fecundity using a newly generated panel of isogenic *D. melanogaster* lines. I then explored how nutritional plasticity for fecundity evolves using experimental evolution lines derived from the same source population and adapted to low calorie diets during larval development. This approach sheds an intriguing light on the role

of variation in plasticity in contributing to population responses to selection and adaptation to new environments.

Chapter 4 examined the proximate mechanisms shaping genetic variation in plasticity for female fecundity. To do this, I characterized plasticity in female fecundity for the isogenic lines previously used (Chapter 3), and selected lines that portray high plastic responses or low plastic responses to protein restriction. I then examined if these differences in plasticity for fecundity are due to differences in ovariole number or protein ingestion. Differences in these mechanisms will help us better understand how plasticity can be regulated to buffer animals from harsh climatic changes.

Finally, in Chapter 5, I will discuss the main findings of my thesis work and future research directions that would further increase our understanding of plasticity, and how the environment can shape a species' abundance and persistence. Although I was responsible for the planning, experimental design, data collection, analysis, and manuscript preparation for the three following chapters, the first-person plural, "we", is used throughout to reflect the collaborative nature of this research.

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Chapter 2 | Target of Rapamycin drives unequal responses to essential amino acid depletion in egg laying

“’The truth.’ Dumbledore sighed. ‘It is a beautiful and terrible thing, and should therefore be treated with great caution.”

By J. K. Rowling in *Harry Potter and the Philosopher’s Stone*

André N. Alves, Carla M. Sgrò, Matthew D. W. Piper*, Christen K. Mirth[1]*
School of Biological Sciences, Monash University, Melbourne, VIC 3800, Australia

[1] Corresponding author: Christen K. Mirth, email: christen.mirth@monash.edu
* MDWP and CKM are co-senior authors, meaning they equally supervised the research. Senior author order was determined by coin toss.

Keywords: Cellular signalling, egg laying dynamics, Target of Rapamycin (TOR), GCN2, egg chamber development

2.1 | Abstract

Nutrition shapes a broad range of life-history traits, ultimately impacting animal fitness. A key fitness-related trait, female fecundity is well known to change as a function of diet. In particular, the availability of dietary protein is one of the main drivers of egg production, and in the absence of essential amino acids egg laying declines. However, it is unclear whether all essential amino acids have the same impact on phenotypes like fecundity. Using a holidic diet, we fed adult female *Drosophila melanogaster* diets that contained all necessary nutrients except one of the 10 essential amino acids and assessed the effects on egg production. For most essential amino acids, depleting a single amino acid induced as rapid a decline in egg production as when there were no amino acids in the diet. However, when either methionine or histidine were excluded from the diet, egg production declined more slowly. Next, we tested whether GCN2 and TOR mediated this difference in response across amino acids. While mutations in GCN2 did not eliminate the differences in the rates of decline in egg laying among amino acid drop-out diets, we found that inhibiting TOR signalling caused egg laying to decline rapidly for all drop-out diets. TOR signalling does this by regulating the yolk-forming stages of egg chamber development. Our results suggest that amino acids differ in their ability to induce signalling via the TOR pathway. This is important because if phenotypes differ in sensitivity to individual amino acids, this generates the potential for mismatches between the output of a pathway and the animal's true nutritional status.

2.2 | Introduction

Diet impacts most life-history traits in organisms, determining their ability to survive, resist stress, grow, and even dictatating how many offspring they will have (Simpson and Raubenheimer, 2012). The term diet typically refers to a combination of nutrients, each of which have distinct impacts. For example, a high protein diet can reduce lifespan by negatively affecting gut function, whereas a high carbohydrate, low protein diet prolongs lifespan by reducing egg production (Zanco et al., 2021; Regan et al., 2016). It is important for organisms to sense the components of the diet accurately, so that they can adjust their phenotypes in a manner appropriate to the nutrients available. Failure to do so would lead to a mismatch between the phenotype and the diet. However, how diet alters these life-history traits has been difficult to pin down, because different components of the diet are sensed by distinct signalling pathways and because the effects of diet differ across organs (Mirth et al., 2016).

The cells within organs respond to nutrients via nutrient-sensing signalling pathways. Several signalling pathways that respond to macronutrients have been identified, including those that respond to protein concentrations. Cells directly sense amino acid concentration through the general control non-derepressible 2 (GCN2) and Target of Rapamycin (TOR) pathways (Layalle et al., 2008; Gallinetti & Micthell, 2013). GCN2 is a ubiquitous protein kinase activated in the presence of uncharged tRNAs, which occurs when there is an insufficiency of amino acids (Armstrong et al., 2014; Sonenberg and Hinnebusch, 2009). In these conditions, GCN2 becomes active and signals to inhibit general protein translation (Sonenberg and Hinnebusch, 2009). The TOR pathway also responds to the intracellular concentration of amino acids, increasing cell survival, growth, and proliferation in the presence of amino acids (Wang and Proud, 2009). While there is some suggestion in the literature that GCN2 and TOR might not respond with the same sensitivity to all amino acids, most of these differences have only been characterised in cell culture (Yuan et al., 2017; Ye et al., 2015).

One way to uncover differences in sensitivity in the GCN2 and TOR pathways to amino acids is to examine how life-history traits that are easy to quantify respond to deficits in individual essential amino acids. Female fecundity is an excellent choice for such studies. The effect of protein on female fecundity has been extensively studied (Mirth et al., 2019; Lee et al., 2008; Piper et al., 2014; Armstrong et al., 2014). This trait is convenient because we can trace all the effects of dietary protein to a single output, the number of eggs laid, or offspring produced. The effects of protein concentration on fecundity are well documented,

consistent, and transferable across different classes of animals. For example, in several species of fish, such as *Oreochromis niloticus* and *Poecilia reticulata*, the concentration of dietary protein in females is positively correlated with the number of offspring generated (Dahlgren, 1980; Hafedh et al., 1999). This is also true for insects, where reducing the concentration of protein in the food decreases egg production in carabid beetles, locusts, crickets, Queensland fruit-flies, and *Drosophila* fruit flies (Wallin et al., 1992, Behmer et al., 2001, Maklakov et al., 2008, Lee et al., 2008, Fanson & Taylor, 2011). Indeed, protein is often a limiting nutrient for fecundity across the animal kingdom (White, 1993).

Studies in the fruit fly *Drosophila melanogaster,* have contributed significantly to what we know about how protein exerts its effects on egg laying. The rate of egg laying is highest when flies ingest protein-rich food (Lee et al., 2008; Grandison et al., 2009; Piper et al., 2014). Thus, protein availability limits fecundity in *D. melanogaster* like it does in other animals. *D. melanogaster* bear the additional advantages of a fast life cycle, a range of genetic tools, a fully synthetic diet (Piper et al., 2014), and in depth understanding of the development of eggs. This makes it tractable to test whether fecundity differs in sensitivity across amino acids and to identify the signalling pathway responsible for these differences. *D. melanogaster* require twenty common amino acids to make up all proteins encoded in the genome. Of these, there are ten essential and ten non-essential amino acids. An essential amino acid has to be obtained from the diet, since it cannot be synthesised by the organism (Wu et al., 2013). In the wild, *D. melanogaster* must obtain essential amino acids by eating the yeast that grows in rotting fruit. The absence of any one of the ten essential amino acids stops egg development (Sang and King, 1961), and females stop laying eggs (Grandison et al., 2009).

Egg development in *D. melanogaster* occurs within the ovaries but depends on yolk protein and other signals from the fat body and hormones from the brain and *corpora allata* (Armstrong et al., 2014). *D. melanogaster* ovaries are made up of 16-22 ovarioles (David, 1970; Hodin and Riddiford, 2000; Mendes and Mirth, 2016; Sarikaya et al., 2011), strings of developing eggs and their surrounding somatic cells. At the anterior tip of each ovariole, the terminal filament cells, cap cells, and escort cells compose the stem cell niche for 2-3 germline stem cells (Spradling, 1993). These germline stem cells adhere to the supporting cap and escort cells, which play an important role in ensuring the stem cells remain undifferentiated (Koch and King, 1966). As the germline stem cells divide, the daughter cell that remains attached to the cap cells remains a stem cell, while the other daughter cell develops into a cystoblast (Koch and King, 1966). The cystoblast will divide 4 times without completing cytokinesis, resulting in 16 cells that share cytoplasmic connections called

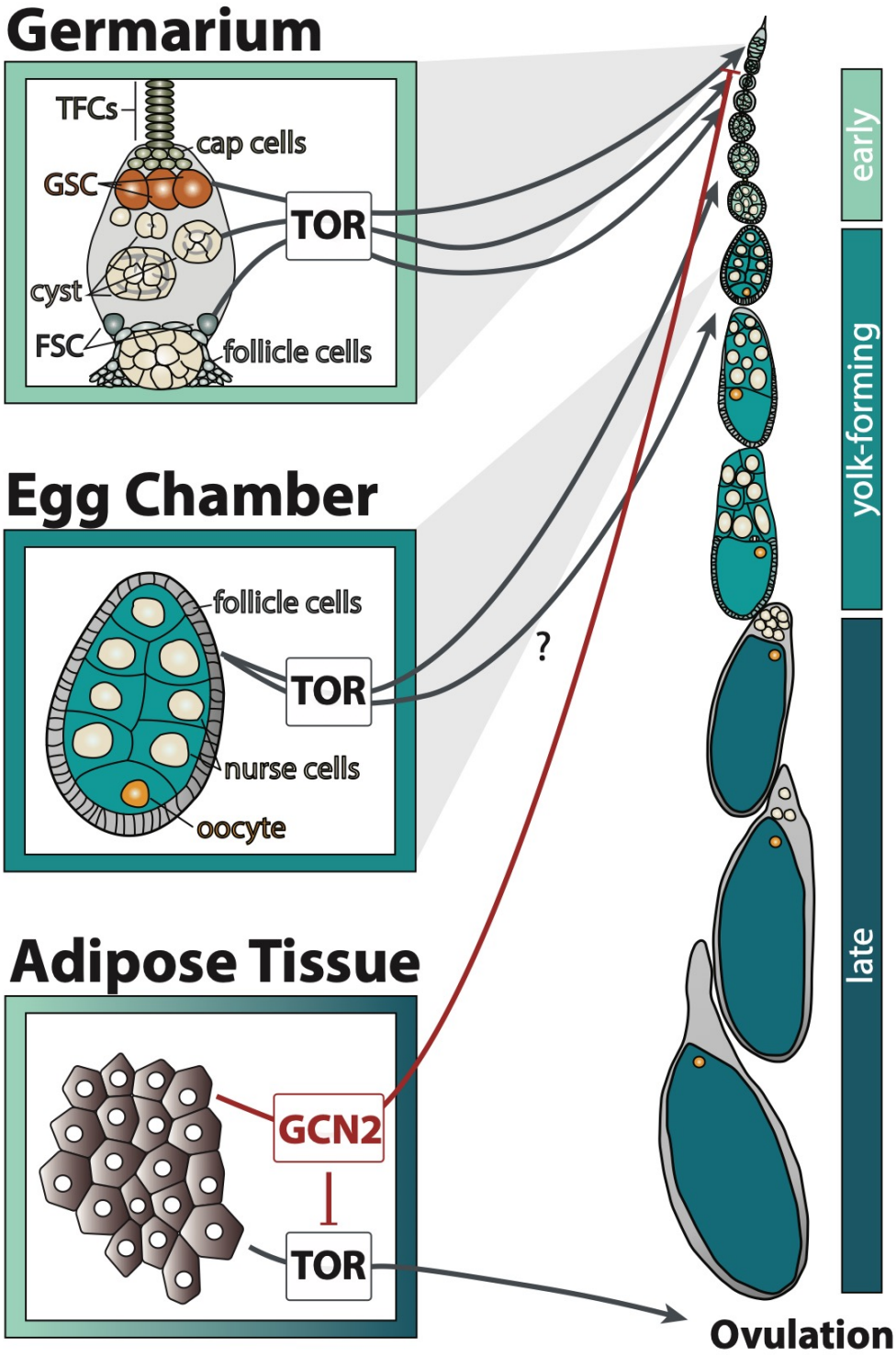

Figure 3: GCN2 and TOR signalling in the adipose tissue and ovary transmit amino acid availability by affecting distinct processes of egg chamber development. Early stages include stages 1-7, yolk-forming includes stages 8-11, and late includes stages 12-14 of egg chamber development. The question mark indicates that it is unclear through which cell type TOR regulates the yolk-forming stages. TFCs: terminal filament cells, GSC: germline stem cells, FSC: follicle stem cells.

germline cysts (Koch and King, 1966). As the cyst moves out of the germarium, it becomes encapsulated by a single layer of follicle cells, which are produced from divisions of the follicle stem cells (Koch and King, 1966). Once encapsulated, the cysts are known as egg chambers. Egg chambers go through 14 stages of development to become fully formed oocytes (King et al., 1956; Cummings and King, 1969, Figure 1). The first seven stages can be broadly grouped as the early stages of egg chamber development. These early stages are characterised by growth of the egg chamber, and differentiation of one of the sixteen germline cells into an oocyte while the remaining cells take on the role of nurse cells (Brown and King, 1964). Stages 8-11 are the vitellogenic, or yolk-forming, stages. During the yolk-forming stages, the oocyte fills up with yolk produced by the fat body and follicle cells. The nurse cells begin to deposit protein and mRNA into the oocyte that will be necessary for embryogenesis (Cummings and King, 1969). The late stages of egg chamber development involve depositing the egg membranes, the death of the follicle and nurse cells, and building the dorsal filaments, which are tubes that protrude from the anterior of the egg (Cummings and King, 1969). Early, yolk-forming, and late stages of egg development all depend on dietary protein to progress (Drummond-Barbosa and Spradling, 2001).

Like other cells in the body, GCN2 and TOR signalling regulates egg chamber development in response to amino acid availability. TOR is required in many cell types of the ovary, and also in the fat body, to promote egg development. When intracellular amino acids are present at high concentrations, TOR is active in the germline stem cells to promote the maintenance of stem identity and to induce their division (LaFever et al., 2010, Figure 1). Before the cyst leaves the germarium, TOR is important for cyst growth (LaFever et al., 2010). It also promotes follicle stem cell division and follicle growth, necessary for egg chamber growth (LaFever et al., 2010). The egg chambers of TOR mutant animals never initiate yolk formation. It is unclear if this is due to TOR activity in the follicle cells, fat body, or in other tissues (LaFever et al., 2010).

Amino acid sensing in the fat body further regulates germline stem cell maintenance and ovulation (Armstrong et al., 2014). The fat body regulates germline stem cell number in response to low amino acids via GCN2, but not TOR (Armstrong et al., 2014, Figure 1). Low amino acid concentrations activate GCN2 in the fat body, which in turn represses TOR and blocks ovulation (Armstrong et al., 2014, Figure 1). The signal secreted by the fat body to control these processes in the ovary is unknown. Nevertheless, because GCN2 and TOR perform distinct roles in regulating egg chamber development (Figure 1), we can use these differences to discern if these two pathways differ in sensitivity to individual amino acids.

To investigate differences in the sensitivity of egg production across essential amino acids, we used a fully synthetic diet that allows us to eliminate individual amino acids and determine their specific impact on egg laying (Piper et al., 2014). We hypothesized that if amino acids differed in their ability to trigger cellular signalling, then eliminating individual amino acids could induce different rates of decline in egg laying. If egg laying did decline at different rates, we could then probe whether this resulted from the activity of either the GCN2 or TOR signalling and define their precise effects on egg development. Our work highlights that these key nutrient signalling pathways are limited in their ability to faithfully represent amino acid availability, which has the potential to lead to mismatches between diet and phenotype.

2.3 | Methods

2.3.1 | Stocks and Fly Maintenance

We used three outbred populations of flies in this study: a population of wild-type flies (Red Dahomey), an outbred Dahomey population that also carries a mutation in the white gene (w^{1118}, hereafter referred to as white Dahomey - wDah), and a white Dahomey population that harbours a null mutation in GCN2 (GCN2Δ, supplied from Linda Partridge and Sebastian Grönke). Both Red Dahomey and White Dahomey were maintained in population cages with overlapping generations at 25 °C on a 12h light:dark cycle. GCN2Δ were maintained in vials at 18 °C on a 12h light:dark cycle. All populations were maintained on sugar, yeast, agar food (SYA) as described in Bass et al., 2007.

2.3.2 | Staging and collecting adults

To control larval rearing density and to synchronize adult emergence time, we collected eggs from the parental generation by leaving them to lay in embryo collection cages (Genesee Scientific) on 90mm petri dishes half-filled with apple juice/agar medium for 24 h at 25ºC, as described in Linford et al., 2013. 250 eggs were then distributed into each food bottle, which contained SYA food at 25ºC.

The adult flies that emerged from these cultures were collected over a 48-hour period, transferred to new bottles containing SYA medium, and left to mate for 48 h. Once mated,

five female flies were transferred into vials that contained a fully synthetic diet (100N, named for having 100 mM of biologically available nitrogen) (Piper et al. 2017), grouped in ten replicates using drosoflippers (http://drosoflipper.com/), and left to acclimatize for one week, replacing the vials with fresh food every three days. Synthetic diets were made according to Piper et al. 2014.

2.3.3 | Diets, drug treatments, and egg counts

After a week of acclimatization to the synthetic diet, females were transferred to new vials that contained one of twelve diets. In addition to two control diets - all amino acid diet (All AA) and no amino acid diet (No AA) - we used ten experimental diets that contained all but one of ten essential amino acids: arginine$^-$, histidine$^-$, isoleucine$^-$, leucine$^-$, lysine$^-$, methionine$^-$, phenylalanine$^-$, threonine$^-$, tryptophan$^-$, and valine$^-$. We established ten replicate vials per diet. Females were transferred to fresh diet every 24 h, after which the eggs laid over the previous 24 h period were counted manually. This was done for 7 to 9 consecutive days.

Mutations in genes from amino acid sensing pathways are known to reduce ovariole number, thereby limiting the maximum number of eggs a female can produce (Green & Extavour, 2014). To account for differences in ovariole number between the GCN2Δ and control genotypes (wDah), the ovaries of 10 to 15 females of each genotype were dissected out in cold PBS and their ovarioles were counted.

To inhibit the mTOR pathway, Rapamycin was diluted in pure ethanol to make a 900 μM stock solution. For each diet, 100 μL of the solution was dispensed on the surface of 3mL of food to a final concentration of 30 μM. The same quantity of the carrier, pure ethanol, was added to a control set of diets.

2.3.4 | Ovary Dissection, Imaging, and Staging

For ovary dissections, flies were collected as described above, and after a week of acclimatization on an all amino acid diet (All AA) additional groups of females from the three genotypes mentioned above were transferred to new vials that contained one of six diets. In addition to two control diets – complete amino acid diet (All AA) and no amino acid diet (No AA) – we used four experimental diets that contained all but one of four essential amino acids: arginine$^-$, histidine$^-$, methionine$^-$ and phenylalanine$^-$. Females were transferred to fresh diet every 24 h. At days zero, three, and seven, five replicates of five flies were

removed and their abdomens dissected. Red Dahomey flies were divided between Rapamycin- and ethanol-laced diets, as described above.

We dissected the abdomens in cold phosphate-buffered saline (PBS). Female abdomens were then fixed in 4% paraformaldehyde (Sigma) overnight at 4ºC. After fixing, abdomens were washed with phosphate-buffered saline with 0.1% Tween (PBST) four times for 15 minutes before RNase (Promega, original concentration of 4 mg/mL) was added in a concentration of 10 µL/mL for 20 minutes. The samples were then washed four times before adding DAPI at a concentration of 0.1 µL/mL for 5 minutes. After staining, two final washes with PBST were performed, followed by two extra washes with PBS. Following staining, ovaries were dissected from abdomens and each treatment placed in one well of a 96-well plate with Fluoromount-G.

Imaging was done with a Leica SP5 5-Channel, using a resonant scanner, with a 20x objective. File conversion from Leica Matrix Screener was done through Python app LM2BS (https://github.com/VolkerH/LeicaMatrixScreener2BigStitcher). H5 files were then read and stitched through the BigStitcher plug-in available in FIJI (version 2.0) and saved as TIFF files.

Ovaries were staged based on characteristics described in (Jia et al., 2016). Only ovaries with clearly visible ovarioles through the z-axis were considered adequate to stage. Staging was grouped into one of three categories: early stages (stages 1-7), yolk-forming stages (stages 8-11), and late stages (stages 12-14).

2.3.5 | Data Analysis

To test for differences in total eggs laid between diets and genotypes, we fit a generalised linear model with the total eggs laid per female as a response variable, with diet and genotype/treatment (where appropriate) as fixed effects, assuming a Poisson distribution with a log link function. Post-hoc comparisons of the means were conducted using the emmeans function (emmeans package).

We identified the models that best fit the effect of amino acid drop-out diets on the number of eggs laid over time by initially fitting the data on the 0N diet using either a linear model, second- or third-degree polynomial models, or a self-starting logistical (SSlogis, $y = asym/(1 + e^{((xmid-day)/scal)})$ regression model using the nls package, where asym is the asymptote, xmid is the mid-point of the curve, and scal is the scaling coefficient (Bates and Watts, 1988, Chambers and Bates, 2017). AIC and BIC were calculated to assess which model best fit the data.

To compare the dynamics of the curve across diets, we used logistic regression (SSlogis), which provides easy to interpret parameters that correspond to rate of change (scaling coefficient) and midpoints (xmid coefficient) of the data. We grouped diets into two groups: the first group where amino acid drop-out diet induced a decline in egg production at a similar rate as the 0N, and a second group where egg production declined more slowly. We then tested whether fitting the logistic regression with coefficients specific to each group improved the model fit over a null model fit with common coefficients. We tested model fit using partial F tests, AIC, and BIC.

Because it is difficult to assess interactions between diets and genotypes/treatments with logistic regression models, we analysed interactions between diet, time (day), and genotype/treatment using third order polynomial regressions. To do this, we fit the number of eggs laid per female as a response variable and a third-order polynomial fixed effect of day interacting with diet. Where appropriate, genotype or treatment was included as a fixed effect. Replicate was used as a random effect within each day. Emtrends was used to obtain statistical differences between slopes of each diet.

To compare egg chamber categories and respective percentages, we fit the data with generalised linear models, assuming a binomial distribution. The proportion of egg chambers at a particular stage was used as a response variable, and day, category, diet and genotype/treatment (where appropriate) were used as fixed effects. We conducted post hoc comparisons of means and slopes using emmeans and emtrends (emmeans package), respectively.

Data was analysed and visualized in R Studio (version 3.4.1). Plots were produced using ggplot2 (tidyverse package, Wickham et al., 2019). All data and scripts are available in Figshare (DOI: 10.26180/16960786).

2.4 | Results

2.4.1 | The number of eggs laid differs between amino acid drop-out diets

Based on previous data (Piper et al., 2014), we hypothesised that the elimination of individual essential amino acids will create different rates of decline in egg laying. To test this, we maintained five female flies on each of twelve different synthetics diets and assessed the effect on the number of eggs laid during nine days of exposure to the treatment diets. These diets included a diet containing all essential and non-essential amino acids (All

AA), a negative control diet with no amino acids (No AA), and ten different drop-out diets containing all except one of the essential amino acids.

We observed that in the absence of all amino acids (No AA), egg laying dropped sharply to zero by the fourth day (Figure 2A). In contrast, female flies fed on diets containing the full complement of amino acids (All AA) showed a gradual decline of ~8% per day in egg laying. The single amino acid drop-out diets induced one of two different responses in egg laying. For seven of these diets, including the arginine, isoleucine, leucine, lysine,

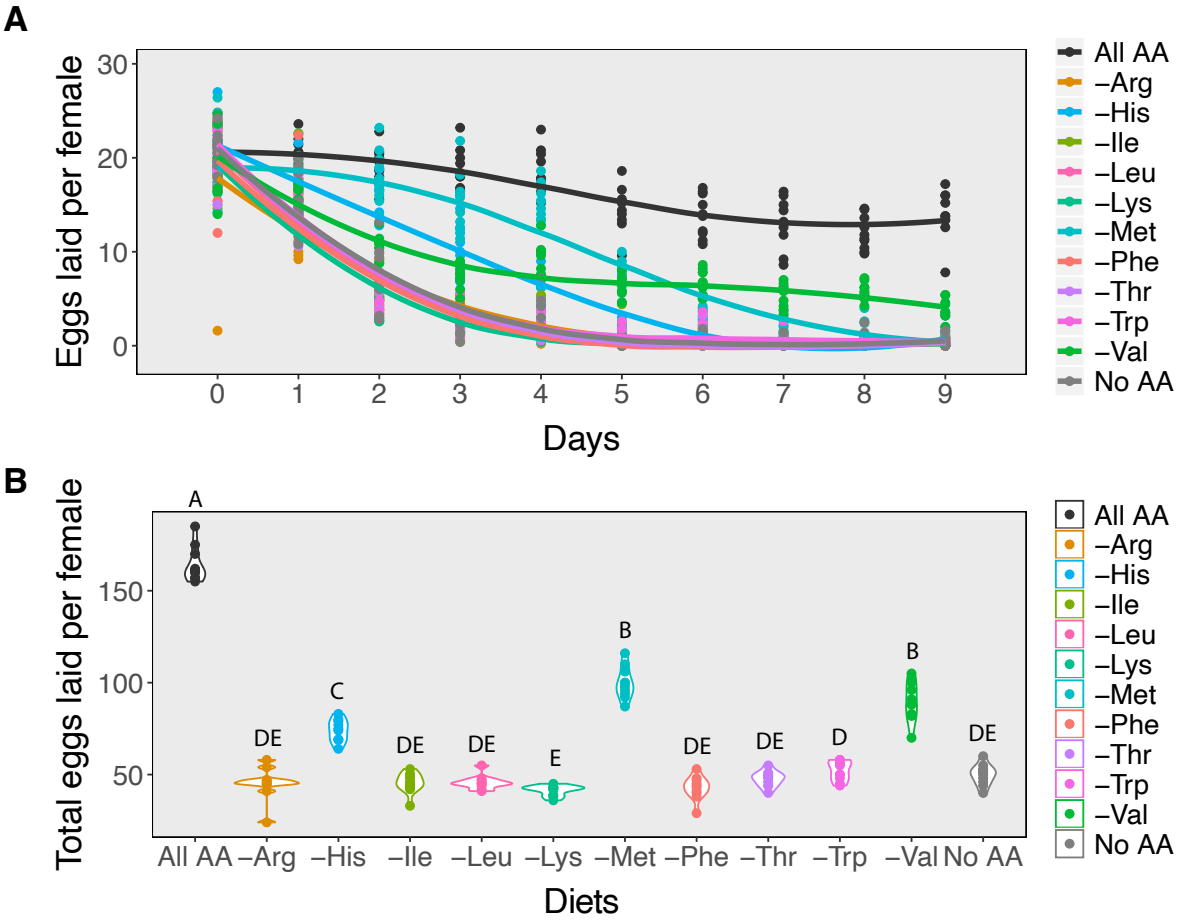

Figure 4: Amino acids differ in their effects on egg laying. A) While in most cases removing individual essential amino acids results in a rapid decline in egg laying at a rate similar to removing all amino acids from the diet, removing valine, histidine, or methionine results in a slower rate of decline. B) For most diets in which a single amino acid is removed, the total number of eggs that females produce over a nine-day period is similar to females on a diet that contains no amino acids. Females on either histidine, valine, or methionine drop-out diets produce more eggs than those on diets missing any of the other essential amino acids. Diets that have differing letters are significantly different, as determined from post hoc pairwise comparisons derived from a generalised linear model assuming a Poisson distribution.

phenylalanine, threonine, and tryptophan drop-out diets, egg laying declined at a similar rate as it did on the diet without any amino acids (Figure 2A). As a result, over the examined nine days these females laid similar numbers of eggs as those on the diet without amino acids (Figure 2B). However, for the histidine, methionine, and valine drop-out diets, egg laying declined more gradually with time (Figure 2A), resulting in significantly higher numbers of eggs laid over the nine-day period (Figure 2B, GLM: $\chi^2_{114,119} = 175$, $p < 0.001$).

To test if egg laying rates differed in response to amino acid drop-out diets, we grouped these diets into two categories: those that induced a "fast-decline" in egg laying (including arginine, isoleucine, leucine, lysine, phenylalanine, threonine, tryptophan drop-out diets, and No AA diets), and those that induced a "slow decline" in egg laying (including methionine, histidine, and valine drop-out diets). Because the shapes of these curves were notably non-linear, we fit them with either linear, second order polynomial, third order polynomial, or 3-paramenter logistic curves. The logistic curves significantly improved model fit to the data for the No AA when compared to all other models, indicated by a dramatic reduction in AIC and BIC values (Table S1).

Table 1 - Comparison of egg laying dynamics coefficients between fast decline and slow decline diets. Diets were compared using a logistic model, $y = \frac{asymptote}{1 + e^{\frac{midpoint - x}{slope}}}$, where y is the number of eggs laid per female and x is day.

	Upper Asymptote	**Midpoint**	**Slope**
Grouped	41.5	-0.11	-1.69
Slow Decline Diets	28.980	1.90	-2.38
Fast Decline Diets	21.57	1.49	-0.65
	F-value	**df**	**p-value**
Grouped vs (Slow Decline Diets | Fast Decline Diets)	547.04	2	**<0.001 *****

We then determined whether the fast and slow decline diets differed in their egg laying dynamics by asking whether fitting coefficients specific to each diet group significantly improved the fit of the logistic model when compared to a model with shared coefficients. We found that fitting the data with separate coefficients for fast and slow decline diets significantly improved the fit of our logistic regression (Table 1), suggesting that the histidine,

methionine, and valine drop-out diets induce slower declines in egg production relative to all other drop-out diets and to the diet with no amino acids (Figure 2A). This in turn suggests that the decline in egg laying is not equally as sensitive to the absence of each amino acid in the diet.

2.4.2 | TOR, but not GCN2, signalling mediates the difference in response to amino acid drop-out diets

Because amino acids are sensed within the cell by the GCN2 and TOR intracellular signalling pathways, we hypothesized that the difference in the rate of decline in egg laying between slow and fast-decline diets arises from differences in the sensitivity of either of these pathways to individual amino acids. To determine which of these two pathways was responsible to the phenotypic differences in sensitivity to individual amino acids, we reduced either GCN2 or TOR signalling. We then compared egg laying when these females were fed on one of eight experimental diets: a diet containing all amino acids, a diet without amino acids, one of three diets that induced a fast decline in egg production (arginine, leucine, or phenylalanine drop-out diets), or one of three diets that induced a slow decline in egg production (histidine, methionine, or valine drop-out diets). The three fast-decline diets were chosen because they showed biochemical similarities with histidine, methionine, and valine: histidine and arginine are cationic amino acids, methionine and phenylalanine are hydrophobic amino acids, and valine and leucine are branched chain amino acids. If the differences in the rates of decline in egg production were due to differences in sensitivity of either GCN2 or TOR signalling to those amino acids, we would predict that reducing the signalling of these pathways would result in similar rates of decline across all amino acid drop out diets.

To alter GCN2 signalling, we compared egg production in females that carry a null mutation for GCN2 (GCN2Δ) to wild type females with the same genetic background, wDah. When comparing the response of egg laying across diets in this experiment, we found that the valine drop-out diet no longer induced a slow decline in egg laying for either genotype (Figure S1, $F_3 = 1.48$, p value = 0.22). For this reason, we eliminated the valine and leucine drop-out diets from our analysis and from subsequent experiments.

To ensure that any differences in egg laying dynamics between genotypes did not result from differences in ovary size, we first dissected out the ovaries of GCN2D and wDah females and counted the number of ovarioles, a commonly used measure of ovary size (Hodin & Riddiford, 2000; Sarikaya et al., 2012; Mendes et al., 2016). The number of

ovarioles per ovary of GCN2Δ females did not differ from wDah females (Figure S2, c^2_1 = 0.44, p value = 0.51). However, the number of eggs laid by GCN2Δ females on all diets decreased faster than the wDah controls (Figure 3A, Table 2), resulting in fewer eggs produced over the 8 days of assay (Figure 3B, Table S2). Even so, GCN2Δ females on the methionine and histidine diets still exhibited a slower rate of decline than those on the arginine and histidine drop-out diets (Figure 3A, Table 2). As a result, within the same genotype both the control and GCN2Δ flies laid significantly more eggs on the methionine

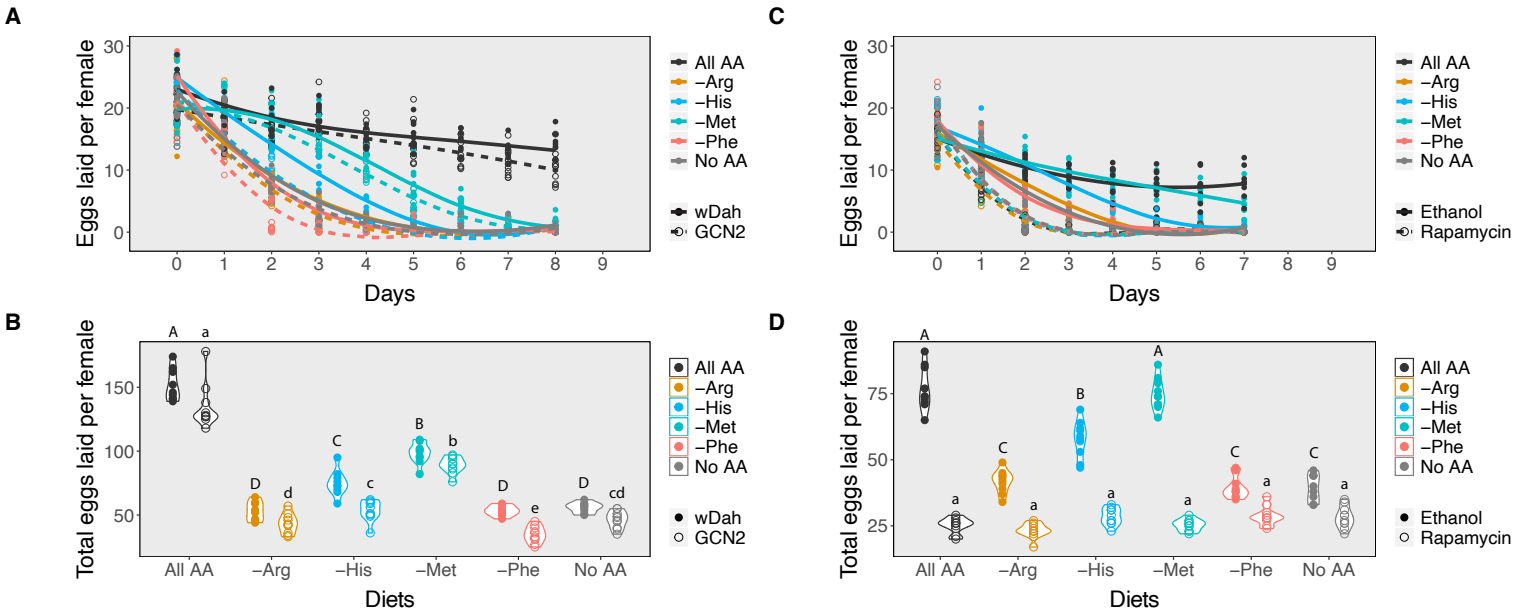

Figure 3: Inhibiting either the GCN2 or Target of Rapamycin pathways alters the impact of diet on egg laying. A) Changes in the rate of decline in egg laying in response to amino acid availability in the diet between control (wDah, solid circles and lines) and GCN2Δ flies (open circles and dashed lines). B) Mean number of eggs laid per female in response to amino acid availability between control (wDah, closed circles) and GCN2Δ flies (open circles). C) Changes in the rate of decline in egg production in response to amino acid availability in the diet between ethanol-treated Red Dahomey flies (solid circles and lines) and rapamycin-treated Red Dahomey flies (open circles and dashed lines). D) Mean number of eggs laid per female in response to amino acid availability between ethanol-treated Red Dahomey flies (solid circles) and rapamycin-treated flies (open circles). In B) and D), diets that have differing letters are significantly different within a genotype or treatment, as determined from post hoc pairwise comparisons derived from a generalised linear model assuming a Poisson distribution (Table S2). Capital letters indicate significance groups across diets for the control flies. Lower cases letters indicate significance groups for the GNC2 mutant (B) or rapamycin-treated (D) flies across diets.

and histidine drop-out diets than on the arginine and leucine drop out diets (Figure 3B). This suggests that GCN2 is required for normal rates of egg laying, but that it is not required to set the differences in rates of decline in egg laying among amino acid drop-out diets.

Table 1: Comparison of egg laying dynamics coefficients between White Dahomey and GCN2Δ flies. Diets were compared through a self-starting logistics model.

	WDAH			GCN2Δ		
	Asymptote	**Midpoint**	**Slope**	**Asymptote**	**Midpoint**	**Slope**
Grouped	30.83	1.38	-1.43	24.85	1.46	-0.95
SLOW DECLINE DIETS	24.76	3.01	-1.34	22.88	2.62	-1.04
FAST DECLINE DIETS	24.14	1.56	-0.72	21.12	1.38	-0.51
	F-value	**df**	**p-value**	**F-value**	**df**	**p-value**
Grouped vs (Slow Decline Diets | Fast Decline Diets)	107.57	3	**<0.001**	107.42	3	**<0.001**

Table 2 – Comparison of egg laying dynamics coefficients between Ethanol and Rapamycin treated flies. Diets were compared through a self-starting logistics model.

	ETHANOL			RAPAMYCIN		
	Asymptote	**Midpoint**	**Slope**	**Asymptote**	**Midpoint**	**Slope**
Grouped	28.49	0.55	-1.72	17.35	1.03	-0.30
SLOW DECLINE DIETS	19.34	2.74	-2.00	16.82	1.06	-0.29
FAST DECLINE DIETS	19.21	1.48	-0.78	17.58	1.02	-0.30
	F-value	**df**	**p-value**	**F-value**	**df**	**p-value**
Grouped vs (Slow Decline Diets | Fast Decline Diets)	128.25	3	**<0.001**	2.37	3	0.07

Next, we assessed the effects of reducing TOR signalling on egg production on our six experimental diets. Since mutants for this pathway are usually developmentally lethal, we added rapamycin, a drug that inhibits this pathway, to the adult diets only. For our controls, we added the same volume of the carrier, ethanol, to the diets. We observed that when rapamycin was added to the diets, egg production declined at the same fast rate

across all diets tested (Figure 3C, Table 3). Indeed, the decline in egg production in females treated with rapamycin was even faster than in females treated with ethanol on the no amino acid, arginine, and phenylalanine diets. This resulted in similarly low mean egg production per female across all diets (Figure 3D, Table S2), and suggests that the differences in decline in egg production across amino acid drop-out diets result from differences in the extent to which the absence of an amino acid can reduce TOR signalling.

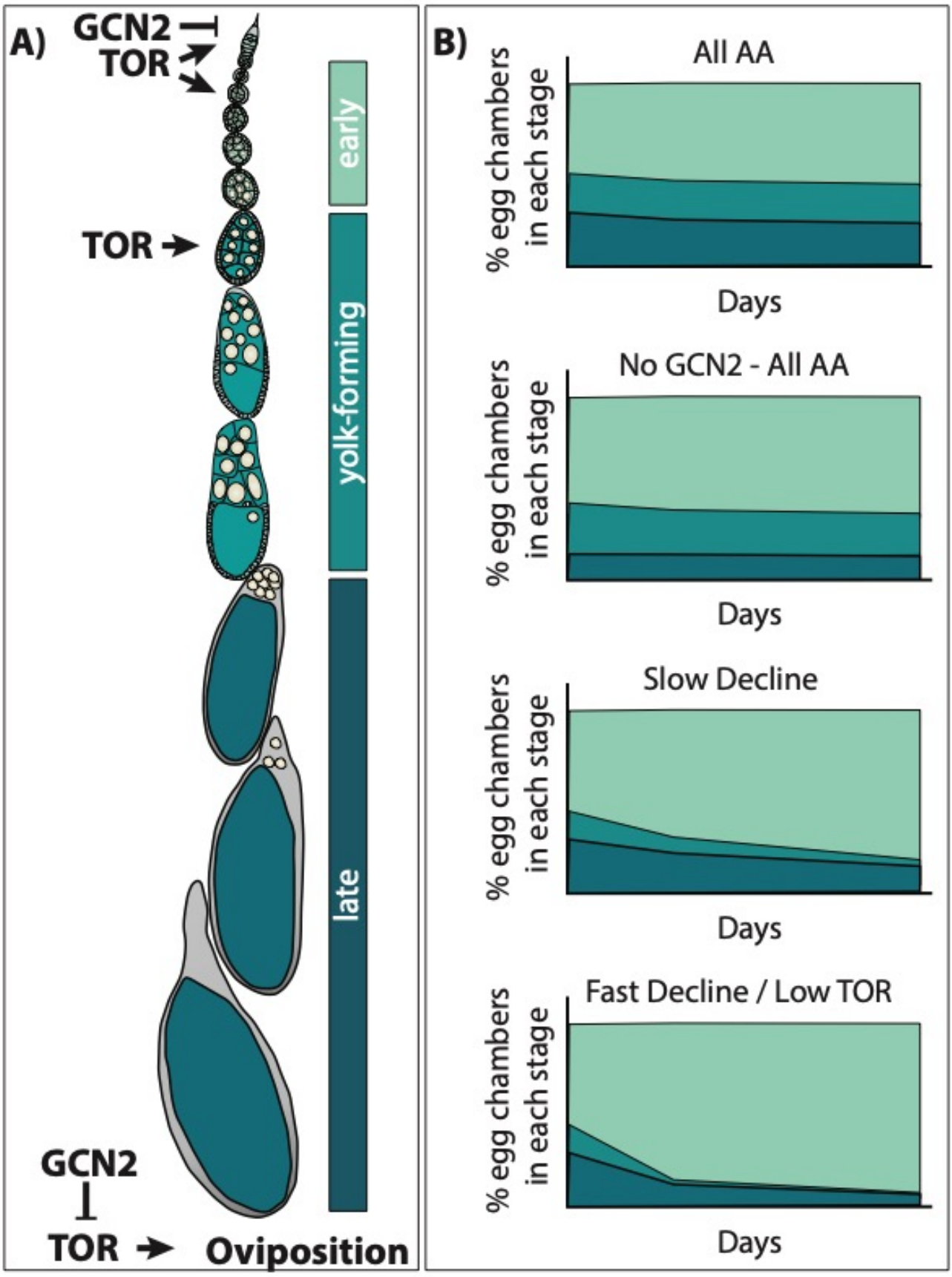
A)
GCN2
TOR
TOR
early
yolk-forming
late
GCN2
TOR
Oviposition
B)
All AA
% egg chambers in each stage
Days
No GCN2 - All AA
% egg chambers in each stage
Days
Slow Decline
% egg chambers in each stage
Days
Fast Decline / Low TOR
% egg chambers in each stage
Days

Figure 4: Predicting how diets that induce a slow versus fast decline impact egg laying via the TOR pathway. A) Egg chamber development can be broadly divided into three stages: 1) early stages (stages 1-7), yolk-forming stages (stages 8-10), and late stages (stages 11-14). TOR signalling promotes germline stem cell maintenance, egg chamber growth, and the transition into yolk-forming stages (Lafever et al., 2010). Both TOR and GCN2 regulate oviposition; TOR signalling promotes oviposition while GCN2 signalling inhibits TOR to suppress oviposition (Armstrong et al., 2014). B) GCN2 mutant females (No GCN2 – All AA) should have normal stem cell numbers and should not show repression of oviposition. Relative to wild type females on diets containing all amino acids (All AA), this might result in faster rates of oviposition, reducing the percentage of late stage egg chambers. We would expect ovaries from females on amino acid drop out diets (Slow and Fast Decline) to have reduced percentages of egg chambers in the yolk-forming and late stages relative to ovaries from females on All AA. If this difference is due to differences in the sensitivity of the TOR pathway across amino acids, then we would predict that on the slow decline diets we would see a more gradual decline in the percentage of yolk-forming egg chambers than on the diets that induce fast decline in egg laying. Treatment with rapamycin (low TOR) should reduce or eliminate the differences between slow and fast decline diets.

2.4.3 | Interactions between cellular amino acid sensing pathways and egg chamber development

We can use the differences in the way GCN2 and TOR have been reported to affect egg chamber development to predict what we would expect to happen to the percentage of eggs in early, yolk-forming, and late-stages of egg development if the response to slow versus fast decline diets were due to TOR, but not GCN2, signalling. GCN2 signalling in the adult adipocytes represses both germline stem cell maintenance and ovulation (Armstrong et al., 2014, Figure 4A). Relative to control flies on diets containing all amino acids, we would predict that GCN2 null females could potentially exhibit a decrease in the percentage of egg chambers in the late stage of egg development on amino acid depleted diets (Figure 4B). This could occur since without GCN2, the stress response to uncharged tRNAs would not be induced. This would continue to trigger ovulation and deplete late egg chambers stages. TOR signalling is necessary for germline stem cell maintenance, for egg chamber growth,

and for egg chambers to progress to the yolk-forming stages (Lafever et al 2010, Figure 4A). If TOR signalling differed in sensitivity to individual amino acids, we would predict that the percentage of egg chambers in yolk forming stages, and by association late stages, would decline more slowly in females offered the diets that induce a slow decline in egg laying when compared to females offered diets that induce a fast decline (Figure 4B). Rapamycin treatment would eliminate the differences between diets, and so, would result in an equal loss of yolk forming stages across all diets.

To test our predictions, we compared the developmental stages of egg chambers when females were subjected to one of six diets, all AA, no AA, or drop-out diets for arginine,

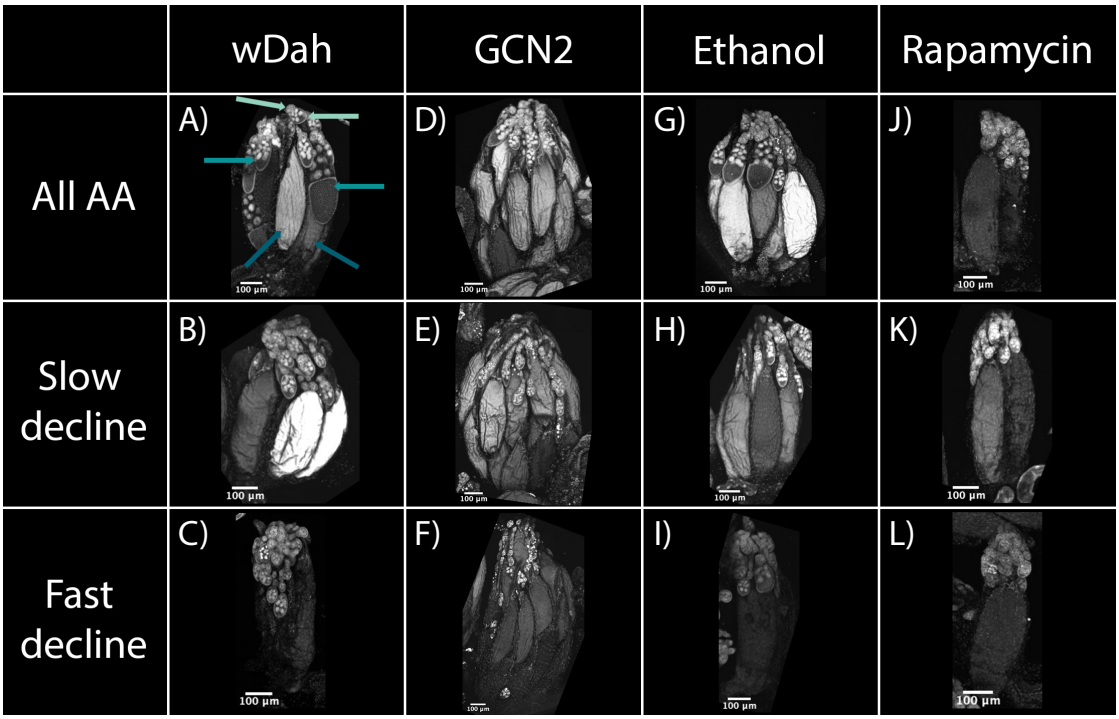

Figure 5: Egg chamber development changes with diet and with reductions in GCN2 and TOR signalling. Ovaries from white Dahomey (wDah, A-C), GNC2 mutant (D-F), red Dahomey fed diets laced with ethanol (Ethanol, G-I), and red Dahomey fed diets laced with rapamycin (Rapamycin, J-L) females. Females were fed either a diet consisting of all amino acids (All AA, A, D, G, and J), diets that induced a slow decline in egg laying (no histidine, B, E, H, and K), or diets that induced a fast decline in egg laying (no arginine, C, F, I, and L). All samples are stained with DAPI to visualise nuclei. In A), the light teal arrows point to early, the teal arrows point to yolk-forming, and the dark teal arrows point to late stage egg chambers. All pictures were taken after 7 days of diet exposure.

phenylalanine, methionine, or histidine. We further reduced amino acid signalling either by using the GCN2Δ mutant flies or by adding rapamycin to the diet. Ovaries were imaged and egg chambers were then staged and allocated into one of the three groups, early, yolk-forming, and late stages.

In the ovaries of control white Dahomey females fed on a diet containing all amino acids, we found that 62% of the ovaries were in early stages, 22% were in yolk-forming stages, and 16% were in the late stages of egg chamber development (Figure 5A, Figure 6). These percentages remained relatively constant over the 7-day period examined. When these females were maintained on a diet that induced a slow decline in egg laying, either a diet lacking histidine or methionine, the percentage of yolk-forming and late-stage egg chambers declined with time (Figure 5B, Figure 6, Table 4). This resulted in a significant difference in the mean percentage of egg chambers in the late stages in comparison with ovaries from females fed a complete diet. Ovaries from females on the diets that induced a fast decline in egg laying, including diets lacking arginine, phenylalanine, or all amino acids, showed a similar rate of decrease in yolk-forming egg chambers, but a steeper decrease in late-stage egg chambers (Figure 5C, Figure 6, Table 4). The mean percentage of egg chambers in the yolk forming and late stages was significantly different to ovaries from females fed on diets with all amino acids or on diets that induced slow decline in egg laying. These data support our prediction that diets that induce a slow decline in egg production will also induce a slower decrease in the percentage of yolk-forming and late stage egg chambers than for flies on the amino acid dropouts that produce a rapid decline in egg laying (Figure 4B).

Egg chamber development in the ovaries of GCN2 mutant females differed from controls even on the diet that contained all amino acids. In these females, 57% of egg chambers were in early stages, 18% in yolk-forming stages, and 25% in late stages of development (Figure 5D, Figure 6). Ovaries with a higher proportion of late stage egg chambers are larger, resulting in larger sized GCN2 ovaries when compared with wDah flies across the different treatments. The increase in late stages of egg chamber development appear to reflect a block in ovulation (Figure 5D-F). Nevertheless, diets that induced fast decline still showed significant differences in the mean percentage and the rate of decline in both yolk-forming and late stages of egg chamber development from diets that induced a slow decline in egg laying (Figure 5E and F, Figure 6, Table 5). This suggests that while the mutation in GCN2 does appear to increase the percentage of eggs in the late stages, it is not responsible for the differences in rates of decrease across amino acid drop-out diets.

Table 4: Drop-out diets that induce either a slow or fast decline (diet group) in egg laying differ in how they affect the percentage of egg chambers in each category over time in White Dahomey (wDah) control flies. Categories include early stages (egg chamber stage 1-7), yolk-forming stages (stages 8-11), and late stages (12-14). Diet groups that differ in the mean or slope for each category are indicated with different letters.

	wDah		
	Chi-square	df	p-value
Day	0	1	1
Category	**9724**	**2**	**<0.001**
Diet Group	0	2	1
Day × Category	**367**	**2**	**<0.001**
Day × Diet Group	0.3	2	0.74
Category × Diet Group	**176**	**4**	**<0.001**
Day × Category × Diet Group	**108**	**4**	**<0.001**

Significance Groups Across Diet Groups						
	Early		**Yolk-Forming**		**Late**	
	Mean	**Slope**	**Mean**	**Slope**	**Mean**	**Slope**
All AA	A	A	A	A	A	A
Slow Decline Diets	B	B	A	B	B	B
Fast Decline Diets	C	B	B	B	C	C

Table 5: Manipulating both diet and either GCN2 or Target of Rapamycin signalling alters the percentage of egg chambers in each category over time. Categories include early stages (egg chamber stage 1-7), yolk-forming stages (stages 8-11), and late stages (12-14)

	wDah & GCN2			Ethanol & Rapamycin		
	Chi-square	df	p-value	Chi-square	df	p-value
Day	0	1	1	0	1	1
Category	**16083**	**2**	**<0.001**	**25606**	**2**	**<0.001**
Genotype	0	1	1	0	1	0.98
Diet Group	0	2	1	0	2	0.99
Day × Category	**450**	**2**	**<0.001**	**1076**	**2**	**<0.001**
Day × Genotype	0	1	0.89	1.5	1	0.22
Category × Genotype	**492**	**2**	**<0.001**	**392**	**2**	**<0.001**
Day × Diet Group	0.3	2	0.87	0.9	2	0.66
Category × Diet Group	**297**	**4**	**<0.001**	**154**	**4**	**<0.001**
Genotype × Diet Group	0.2	2	0.89	0	2	1
Day × Category × Genotype	**96**	**2**	**<0.001**	**421**	**2**	**<0.001**
Day × Category × Diet Group	**169**	**4**	**<0.001**	**101**	**4**	**<0.001**
Day × Genotype × Diet Group	0.2	2	0.88	0.4	2	0.82
Category × Genotype × Diet Group	**29**	**4**	**<0.001**	**88**	**4**	**<0.001**
Day × Category × Genotype × Diet Group	**39**	**4**	**<0.001**	**57**	**4**	**<0.001**

Significance Groups Across Diet Groups

	wDah		**GCN2**		**Ethanol**		**Rapamycin**	
	Mean	**Slope**	**Mean**	**Slope**	**Mean**	**Slope**	**Mean**	**Slope**
Early Stages								
All AA	A	A	A	A	A	A	A	A
Slow Decline Diets	B	B	A	A	B	B	AB	A
Fast Decline Diets	C	B	B	B	C	C	B	B
Yolk-forming Stages								
All AA	A	A	A	A	A	A	A	A
Slow Decline Diets	A	B	A	A	A	A	B	B
Fast Decline Diets	B	B	B	B	B	B	B	B
Late Stages								
All AA	A	A	A	A	A	A	A	AB
Slow Decline Diets	B	B	A	A	B	B	A	A
Fast Decline Diets	C	C	B	B	C	C	A	B

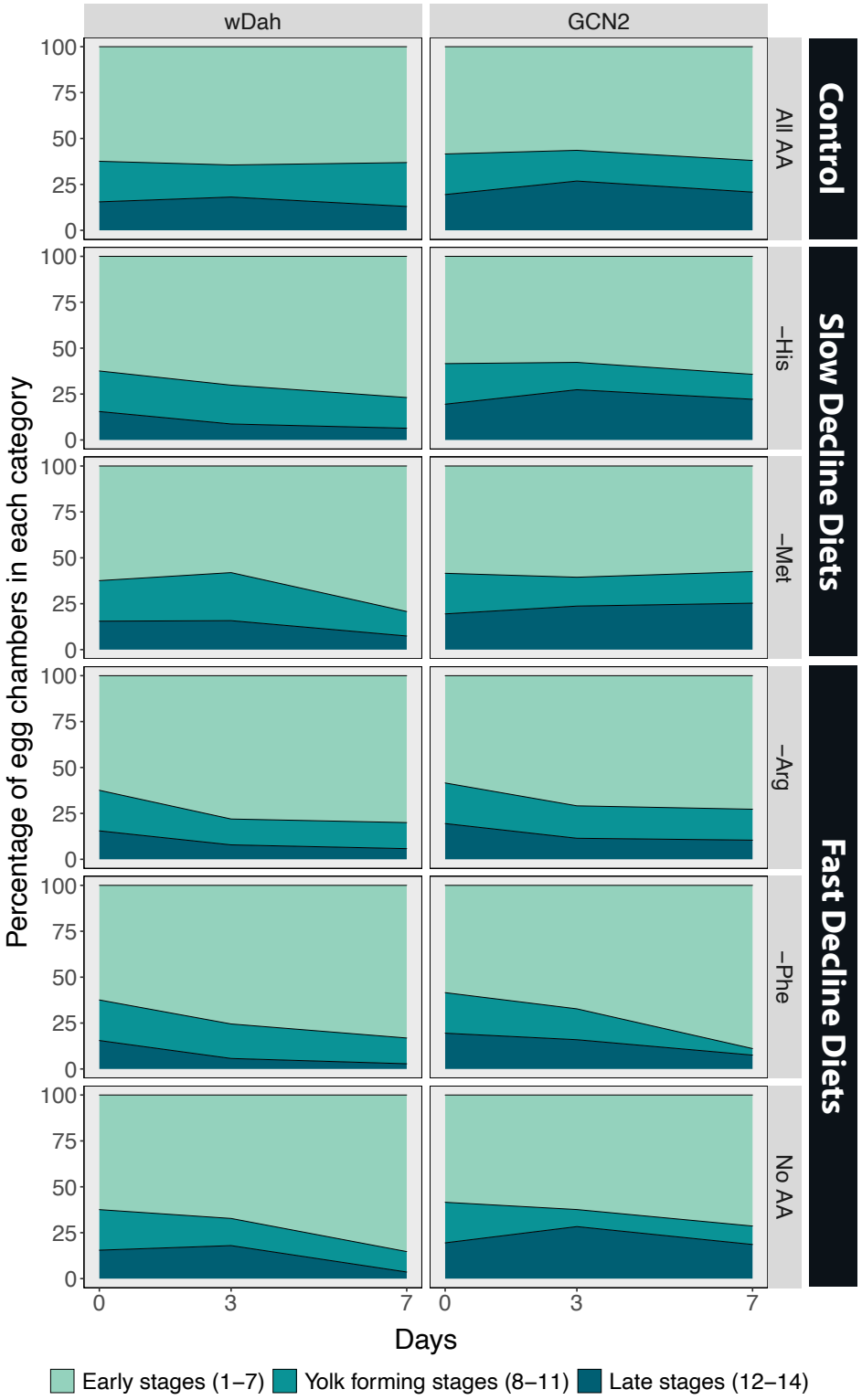

Figure 6: The percentage of egg chambers in each category is affected by diet, and these effects are partially mediated by GCN2 signalling. Comparison between the percentage of egg chambers between white Dahomey (wDah) and GCN2Δ flies.

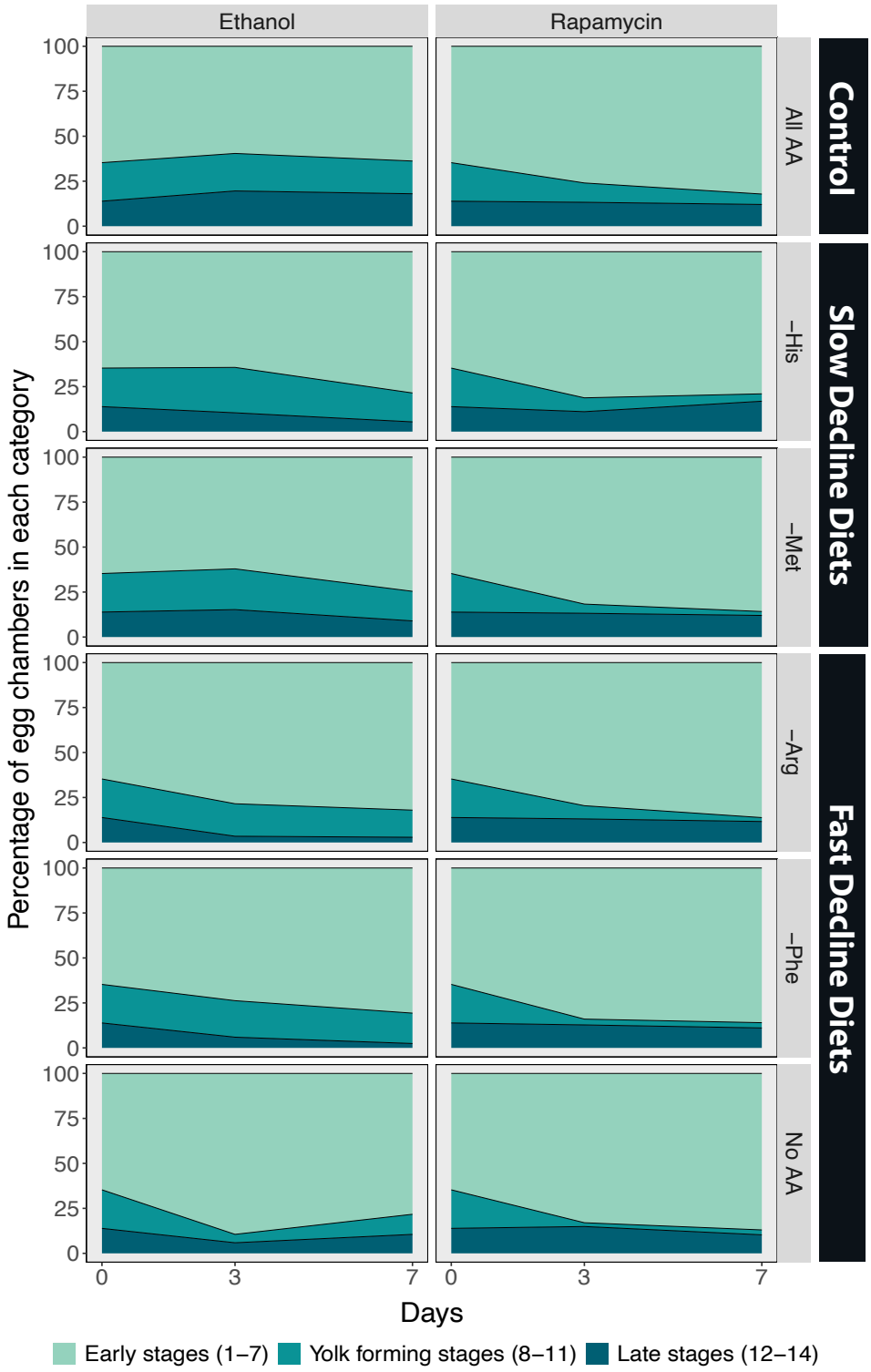

Figure 7: The percentage of egg chambers in each category differs among amino acid drop-out diets, and these effects are eliminated when Target of Rapamycin signalling is inhibited using the drug rapamycin. Comparison between the percentage of egg chambers between red Dahomey females treated with ethanol and treated with rapamycin (30μM).

We next examined the effects of inhibiting TOR signalling on egg chamber development by adding rapamycin to the diet. As a control, we added the same volume of ethanol to the diets of red Dahomey females. We observed similar changes in the percentages of egg chambers in early, yolk-forming, or late stages when ethanol was added to the food of red Dahomey females as we did for the wDah females (GCN2D controls) (Figure 5G-I, Figure 7, Table 5).

Adding rapamycin caused rapid declines in the percentage of egg chambers in the yolk-forming stages in all diets (Figure 5J-L, Figure 7, Table 5). In addition, on diet without any amino acids the ovaries from rapamycin-treated flies had a higher percentage of late stage egg chambers (15%) relative to the ethanol treated controls (11%), indicative of a block in ovulation (Figure 5J-L, Figure 7). Finally, rapamycin treatment eliminated most of the differences in percentage of yolk-forming and late stage egg chambers between diets that induced a slow versus fast decline in egg laying (Figure 7, Table 5). Taken together, our data supports our predictions that the difference in decline in egg laying between slow and fast decline diets is mediated by TOR signalling. This provides further evidence that TOR signalling differs in its sensitivity across amino acids.

2.5 | Discussion

Accurately sensing the composition of the diet allows life-history traits to adjust with food quality and availability. How cellular signalling pathways can coordinate different sensitivities to amino acid availability and impact life-history traits is still unknown. Here, we used the ovaries of *D. melanogaster* to understand how diet and the signalling pathways that regulate responses to amino acids impact a key life-history trait, fecundity. By manipulating individual essential amino acids of the diet, we found that egg laying shows amino acid-specific responses. Furthermore, we provide evidence that the TOR pathway is mediating difference in the response of egg laying to essential amino acids. These studies will provide insight into what limits the extent to which nutrient availability can be reliably conveyed to the organs of the body to regulate phenotypic responses.

Whether amino-acid sensing pathways are required at all for differences in sensitivity to the depletion of amino acids in whole animals is unclear. Previous studies have shown that removal of methionine from the culture medium of HEK293T cells blocks translation (Mazor et al., 2018). However, the block in translation does not appear to require either GCN2 or TOR pathways (Mazor et al., 2018). In the absence of methionine, GCN2 is not activated and TOR fails to inactivate. Instead, translation is blocked in these cells due to a

lack of methionine-charged tRNAs necessary to initiate the process (Mazor et al., 2018). In addition, the absence of methionine results in an increase in DNA and histone methylation, which also inhibits translation. These studies suggest that at least in cell culture, the absence of methionine is not sensed. In a whole animal, this would mean that differences in phenotypic responses to methionine depletion would not be affected by altering either GCN2 or TOR signalling.

Our study suggests that these findings from cell culture studies do not apply to egg laying responses to amino acid depletion. While altering GCN2 signalling does not change the way egg laying responds to the depletion of single amino acids, we find evidence that the TOR pathway is important for the differences in response to methionine and histidine. This further suggests that TOR signalling itself might differ in sensitivity to individual amino acids, potentially driving a mismatch between the output of a nutrient sensing pathway and the true nutritional status of an organism.

Drosophila flies obtain amino acids primarily from the yeast that grows on the decomposing matter on which they live. Relative to the amino acid requirements encoded within the fly's genome, methionine and histidine are the two most limiting amino acids in yeast (Piper et al., 2014; Gómez Ortega et al., 2021 (BioRxiv)). Given that these two amino acids are present in lower abundance than the fly requires, potentially TOR signalling has adapted to be more tolerant of methionine or histidine deficiency via reduced sensitivity to these amino acids. The mechanisms that confer differences in sensitivity across amino acids are not yet understood.

TOR signalling acts on a number of cell types within the ovary to control egg chamber development (LaFever et al., 2010). In addition, TOR signalling acts in the fat body to regulate oviposition and is likely to also control the production of hormones, like the insulin-like peptides, ecdysone, and juvenile hormone, necessary for eggs to develop (Armstrong et al., 2014, Layalle et al., 2008, Hatem et al., 2015). Future studies manipulating TOR signalling in cell types known to be important for yolk-forming stages of egg development will highlight the cells responsible for differences in egg laying rates across diets.

While the GCN2 pathway did not drive the differences in egg laying across diets, flies mutant for GCN2 did show faster rates of decline in egg laying than wDah controls. These reduced egg laying rates appear to be caused by reduced oviposition frequencies in GCN2 mutants. This was surprising, as reducing GCN2 signalling in the fat body has previously been shown to relieve the impacts of amino acid depletion, which we anticipated would result in a reduction of egg production in the absence of essential amino acids (Armstrong et al., 2014). Our results suggest that GCN2 has a more complex role in egg laying than was

previously thought. Whether this occurs via GCN2 activity in the fat body or in other cell types remains to be seen.

Finally, numerous studies have demonstrated cross talk between the TOR and GCN2 pathways (Rousakis et al., 2013; Yuan et al., 2017). Given this, it might seem surprising that they differ in the way they respond to the depletion of individual amino acids. However, TOR and GCN2 diverge in the pathways with which they interact. For example, TOR also responds to insulin signalling and carbohydrate levels (Jacinto and Hall, 2003). Moreover, null mutations in GCN2 are viable while null mutations in TOR are lethal. Future work will uncover the extent of overlap between these two pathways, and the contexts in which they diverge.

In this study, we aimed to understand whether amino acids have distinct functions in regulating amino acid signalling within cells using egg laying as an output. We observed that egg laying suppression was less sensitive to the absence of methionine and histidine than to the absence of other essential amino acids. Further, we found these effects could be attenuated by inhibiting the TOR, but not the GCN2, pathway. This difference in sensitivity to depleting individual amino acids appears to primarily affect the yolk-forming stages of egg development, further highlighting that these effects might be specific to a cell type or stage of development. Finally, this study shows that precise dietary manipulations can expose how nutrient sensing pathways can be partially uncoupled from nutrient availability, providing a glimpse into the cues organisms use to regulate their phenotypes in response to diet.

2.6 | Acknowledgements

We would like to thank members of the Mirth and Piper labs, past and present, for their help throughout this project. In particular, we would like to thank Amy Dedman and Tahlia Fulton for their help in ovary collections. We would also like to thank Linda Partridge and Sebastian Grönke for sharing their unpublished GCN2 null stock. This research was funded by ARC (DP180103725) for CMS, ARC (FT170100259) for CKM, and ARC (FT150100237) and NHMRC (APP1182330) for MDP.

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2.8 | Supplementary Figures & Tables

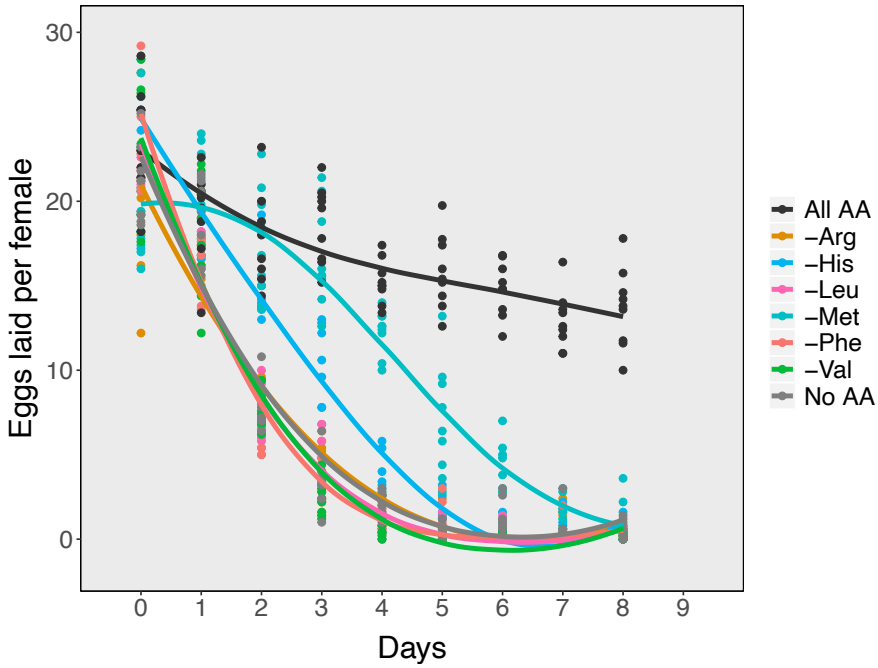

Figure S1: In the ovaries of white Dahomey females, a diet missing valine shows declines in egg laying with time that are indistinguishable with other diets that induce a fast decline in egg laying.

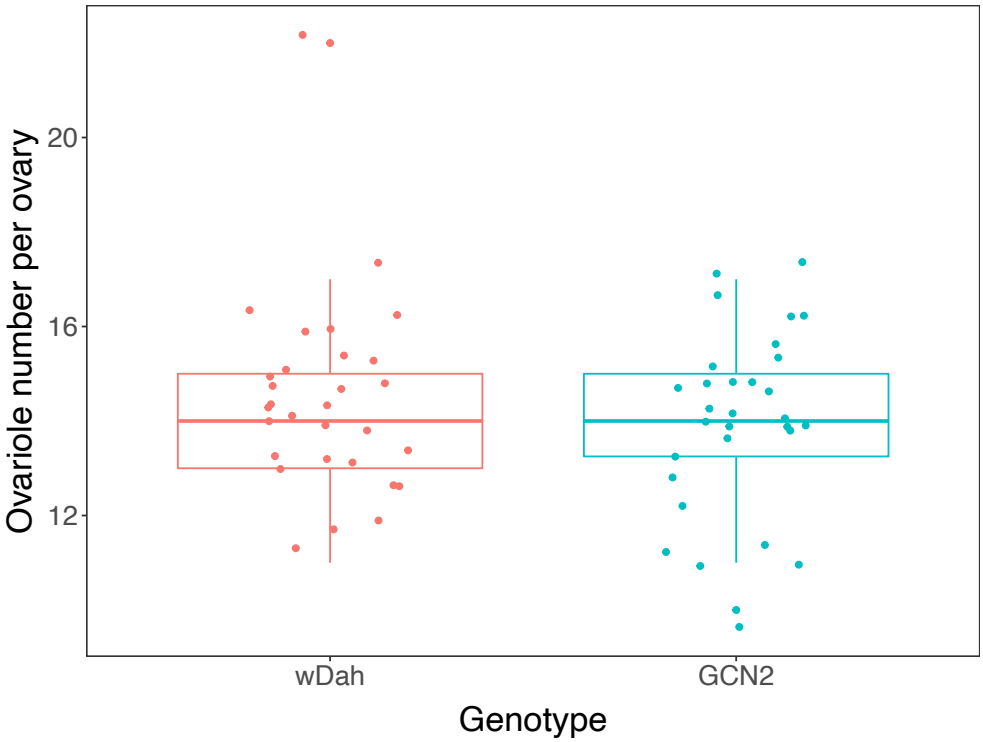

Figure S2: Ovariole number does not differ between white Dahomey and GCN2 mutant females.

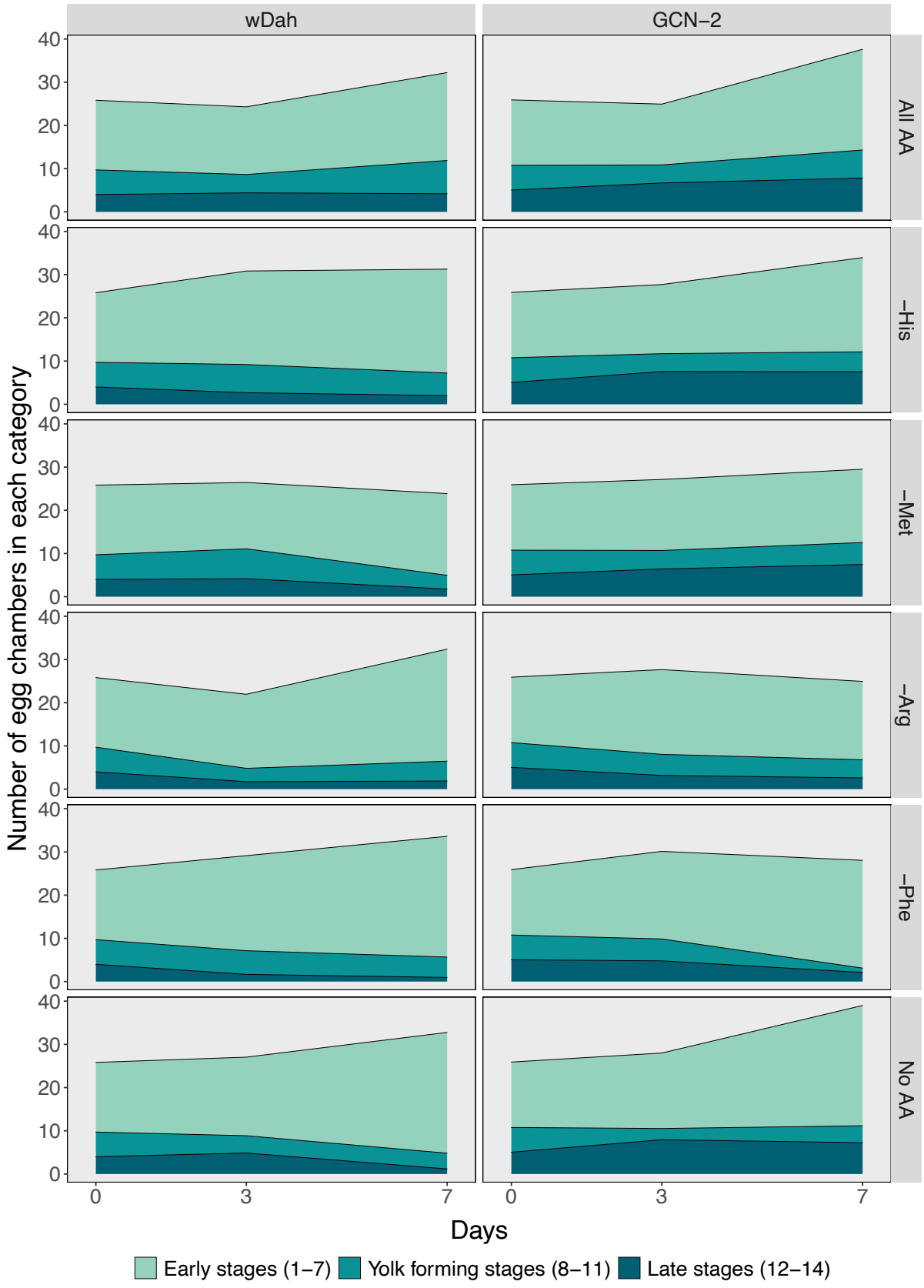

Figure S3: The total number of egg chambers in each category over time in white Dahomey and GCN2 mutant females.

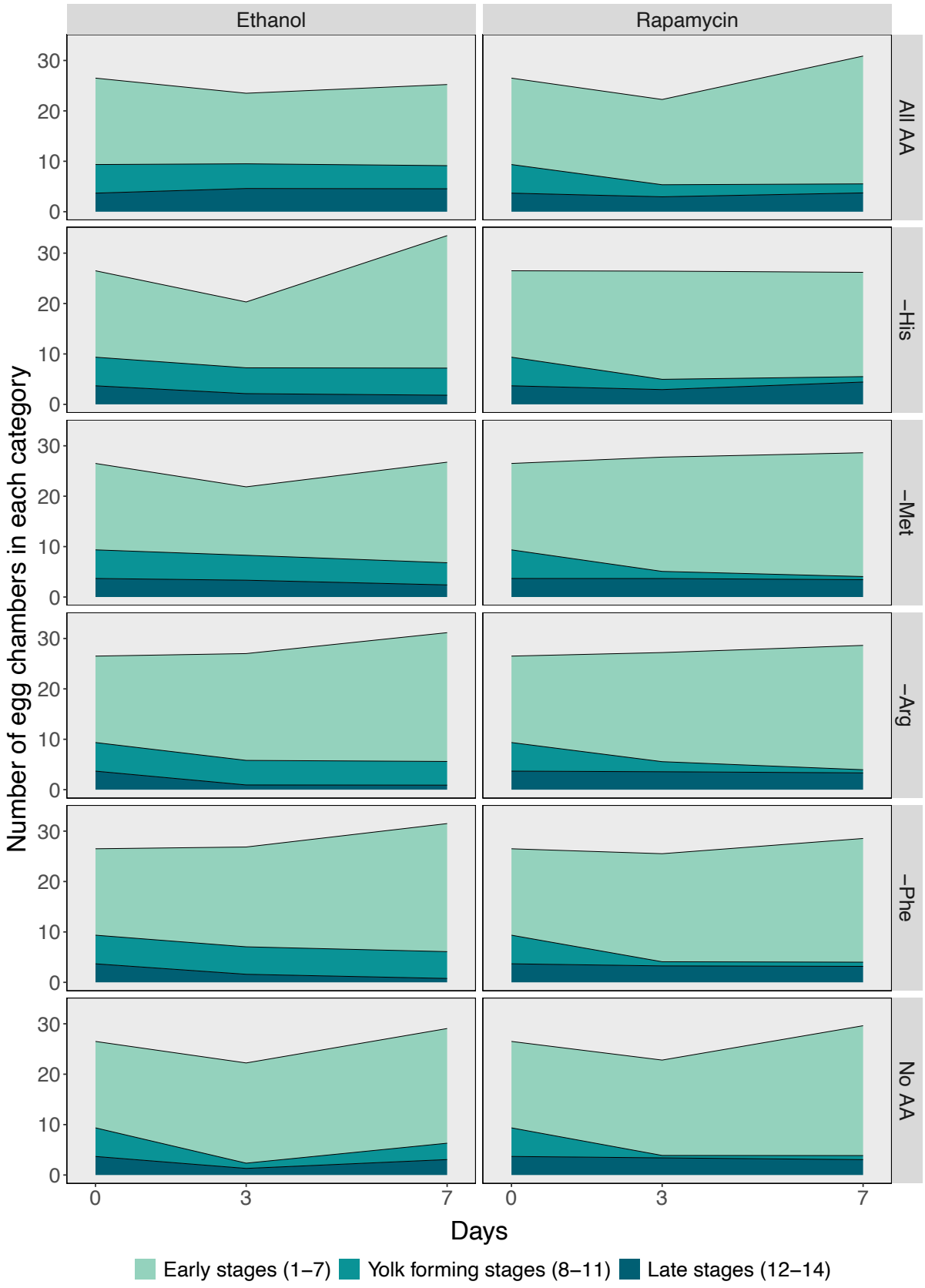

Figure S4: The total number of egg chambers in each category over time in red Dahomey females treated with ethanol or rapamycin.

Table S1 – Comparison of model fit for the No AA diet. Df: degrees of freedom, AIC: Akaike's Information Criteria, BIC: Bayes Information Criteria

	df	AIC	BIC
Linear	3	571	579
Second Order Polynomial	4	472	482
Third Order Polynomial	5	464	477
Logistic	4	408	419

Table S2: The total number of eggs laid across diets differs between wDah and GCN2 genotypes, and between ethanol and rapamycin treatments. Data are fit with generalised linear models assuming a Poisson distribution.

	Total eggs laid		
	Chi-square	df	p-value
wDah & GCN2			
Diet	**1669**	**5**	**<0.001**
Genotype	**76**	**1**	**<0.001**
Diet * Genotype	**25**	**5**	**<0.001**
Ethanol & Rapamycin			
Diet	**175**	**5**	**<0.001**
Genotype	**607**	**1**	**<0.001**
Diet * Genotype	**112**	**5**	**<0.001**

Chapter 3 | Plasticity in fecundity does not evolve despite the existence of G×E interactions in a wild population of Drosophila melanogaster

“And above all, watch with glittering eyes the whole world around you because the greatest secrets are always hidden in the most unlikely places”

By Roald Dahl

André Nogueira Alves, Avishikta Chakraborty, Greg M. Walter, Matthew D.W. Piper, Christen K. Mirth*, Carla M. Sgrò*

School of Biological Sciences, Monash University, Melbourne, VIC 3800, Australia

*Equal supervision of the research. Order decided by coin toss.

Keywords: Relative fecundity, genetic correlation, experimental evolution, isogenic panel, nutritional plasticity, reaction norm

3.1 | Abstract

Climate change is increasing the exposure of organisms to environmental stress. With climate change, nutritional stress is predicted to increase as access to sufficient quantities and qualities of nutritional resources changes. One strategy that organisms use to buffer from the effects of this stress is plasticity. How plasticity varies within a population is of great importance since this variation underpins the response of a population to selection. Not much is known about how variation in plasticity contributes to a population's sensitivity to nutritional stress for egg production and how that variation shapes the response to selection. To address this, we created two genetic resources from the same wild-caught population: a panel of 81 isogenic lines and a panel of 10 experimental evolution lines adapted for 30 generations to either control or low calorie diets. We subjected each of these panels to three diets varying in yeast content, the main protein source for *D. melanogaster,* and assessed the number of eggs laid over seven days. With our isogenic lines, we find that our wild-caught population harbours significant genetic variation in plasticity for fecundity, resulting from differences in genetic correlations between diets. However, we found that plasticity for number of eggs laid did not evolve when populations were adapted to a low calorie diet. Our results show that even if there is significant genetic variation in nutritional plasticity within a population, plasticity does not always evolve in response to nutritional stress.

3.2 | Introduction

Climate change is increasingly exposing natural populations to environmental stress, which is influencing the persistence, abundance, and distribution of many species (Scheffers et al., 2016; Houghton et al., 1990; Dawson et al., 2011; Thomas et al., 2004; Foden et al., 2013). Beyond increasing temperatures, climate change is impacting CO_2 levels and precipitation, which is affecting the distribution of primary producers and the timing and abundance of food resources for herbivorous and frugivorous animals (Rosenblatt & Schmitz, 2016). In addition, the nutritional content of plants may vary with increasing temperatures projected with climate change (DaMatta et al., 2010; Sardans et al., 2017; Lenhart, 2017). Nutritional stress, which is already one of the most common stressors in nature (Long et al., 2014), is therefore becoming increasingly prevalent under climate change. Understanding how populations can respond to nutritional stress is crucial to better understand and predict population persistence in response to ongoing climate change.

Plasticity will underpin species' persistence in the face of nutritional stress resulting from ongoing environmental change (Lynch & Walsh, 1998; Hoffmann & Merilä, 1999; Franks et al., 2007; Geerts et al., 2015). This is because phenotypic plasticity, the capacity of a single genotype to produce multiple phenotypes when exposed to environmental variation (Gause, 1947; Bradshaw, 1965; Ørsted et al., 2017), is crucial for determining immediate responses to environmental change (Sgrò et al 2016; Chown et al., 2007). Traits closely linked to fitness, such as lifespan and reproduction, have been shown to be particularly sensitive to nutrition (Zajitschek et al., 2018; King et al., 2011), which highlights the importance of nutritional plasticity for responding to changes in food availability and quality (Sørensen et al. 2016).

Genetic variation in plasticity, which is quantified as genotype-by-environment (G×E) interactions (Schlichting and Pigliucci, 1998; Lande, 2009), can provide the potential for selection to change patterns of plasticity (Narum et al., 2013; Chevin & Hoffmann, 2016; Chakraborty et al., 2020). Populations from along environmental gradients have been shown to respond differently to nutritional stress, suggesting that genetic variation in nutritional plasticity can evolve (Chakraborty et al., 2020, Klepsatel et al., 2020).

Genotypes can also vary in the extent of their plastic responses, resulting in G×E that represents genetic variation in plasticity within a population (Schlichting and Pigliucci 1998; Lande 2009). To better understand how and why nutritional plasticity evolves among populations, we need to quantify genetic variance within a population and identify the

potential for plasticity to respond to selection (Chevin et al., 2010; Dreyer et al., 2016; Frankino et al., 2019).

One way to study genetic variance in plasticity within a population is to use panels of highly inbred lines that are exposed to a gradient of nutritional stress. This approach has been used with panels of isogenic lines, such as the *Drosophila melanogaster* Genetic Reference Panel (DGRP) and the *Drosophila* Synthetic Population Resource (DSRP) (Mackay et al., 2012; King et al., 2012). The advantage of using such isogenic lines is that differences in responses to environmental variation between lines can be associated with genetic differences in plasticity. Studies have revealed genetic variation in plasticity in response to temperature or nutrition for a range of traits such as body size, olfactory behaviour, and wing-body scaling relationships (Lafuente et al., 2018; Sambandan et al., 2008; Frankino et al., 2019).

While many studies of genetic variation in plasticity within populations have focused on developmental, morphological, behavioural, or stress resistance traits (Van Buskirk & Steiner, 2009; Ghalambor et al., 2007, LaFuente et al., 2018; Cunningham et al., 2020), few studies have examined genetic variation directly in fitness across environments. This is largely because fitness is difficult to measure, and so studies often focus on life-history traits that represent close proxies of fitness, such as lifespan and fecundity (Fisher, 1930). Fecundity is tightly correlated with fitness and is sensitive to changes in nutritional availability and quality (Wallin et al., 1992, Behmer et al., 2001, Lee et al., 2008; Piper et al., 2014). Genetic variation in nutritional plasticity within populations has been documented for fecundity in *D. melanogaster* in the form of egg production (Ng'oma et al., 2018; King et al., 2011; Camus et al., 2017). Furthermore, studies have revealed within population G×E for ovariole number, a good predictor of fecundity in *D. melanogaster*, in response to protein restriction (Bergland et al., 2008). However, little is known about the long-term consequences of genetic variation in plasticity during adaptation to changed nutritional environments.

Several studies have used experimental evolution to adapt flies to nutritionally poor larval diets (Zajitschek et al., 2017). This adaptation either does not influence (Zajitschek et al., 2017) or reduces adult fecundity (Kolss et al., 2009) when measured on a standard diet. While it appears that in some cases fecundity might evolve in response to larval diet, it is unclear whether plasticity in fecundity changes. Furthermore, because these studies did not consider the distribution of genetic variation in plasticity prior to selection, this makes it difficult to discern the role of genetic variation in plasticity in source population in the adaptive response.

To understand how the fitness of a population responds to changes in nutrition, we need to: 1) quantify genetic variance in plasticity for fecundity, and 2) identify the potential for that same genetic variation in plasticity to respond to selection and shape the evolution of plasticity for the population. To address these knowledge gaps, we used a newly-derived set of isogenic lines of *D. melanogaster* to quantify within-population genetic variance for nutritional plasticity. We then used experimental evolution to examine the extent to which nutritional plasticity in fecundity can evolve in response to different diets. Our study provides insight into how within-population genetic variation in plasticity might contribute to the long-term responses to climate change, which is overlooked when only examining variation in plasticity at a population level.

3.3 | Methods

3.3.1 | Field collections and establishing experimental lines

We collected 200 field-inseminated *Drosophila melanogaster* females from a banana plantation and Tropical Fruit World in Duranbah, in the east coast of Australia in January 2018 (28.3° S, 153.5° E). We generated two independent isofemale lines from each of the wild-caught females resulting in 400 lines in total. All lines were treated with tetracycline to remove *Wolbachia* and reared for two generations prior to 20 generations of inbreeding (described below). Flies were maintained on standard yeast-potato-dextrose medium (potato flakes 20 g/L; dextrose 30 g/L; Brewer's yeast 40 g/L; agar 7 g/L, Nipagin 6 mL/L; and propionic acid 2.5 mL/L) at 25 ^{o}C, on a 12h light/dark cycle.

We also used the isofemale lines established from the field collection to initiate a mass bred population that was then used as the base population for the experimental evolution experiment described below. To establish the base population, we collected 5 virgin females and 5 males from each isofemale line (after two generations of lab culture), which we pooled together and then expanded into 60 bottles, each containing 750 to 1000 flies. We maintained the mass-bred population on the standard yeast-potato-dextrose medium at 25 ^{o}C, on a 12h light/dark cycle for 2 generations before we started experimental evolution, which is described in detail below.

3.3.2 | Isogenic lines

Inbreeding was applied to each of the 400 isofemale lines through single pair brother-sister mating, for a minimum of 20 generations, resulting in a panel of 81 fertile isogenic lines used for this study. This panel of isogenic lines resulted in an inbreeding coefficient of F=0.986 (Mackay et al., 2012, Reddiex et al., 2018). All lines were maintained at a constant temperature of 18 °C, under a 12h light/dark cycle, and fed yeast-potato-dextrose medium.

3.3.3 | Experimental evolution lines

We used experimental evolution lines, established previously in the laboratory (Kutz, 2021), to examine whether the mass-bred population shifted fecundity in response to variation in diet. To initiate experimental evolution, females from the mass bred population were placed in laying pots with yeast-potato-dextrose media and allowed to lay for three days. Eggs from these laying pots were then allocated into two selection regimes with different diets: a standard yeast-potato-dextrose diet and a low-calorie diet, consisting of the same macronutrient proportions, but with 25% of the calories (potato flakes 5 g/L; dextrose 7.5 g/L; Brewer's yeast 10 g/L). This low-calorie diet was chosen to reflect a decrease in food abundance predicted to occur with climate change (Raubenheimer et al., 2012; Rosenblatt & Schmitz, 2016).

To conduct experimental evolution, we established five replicate lines for each selection treatment, with each line containing four 50 ml bottles each containing 400 to 500 eggs (Sgrò and Blows 2004). In each generation, to avoid selection for fast development, we collected adults daily until all adults eclosed. Adult flies from all four bottles of each replicate line were then mixed and left to mate under control conditions (25 °C and standard medium) for three days to maintain as large a population as possible and avoid any bottleneck effects. After this three-day mating period, eggs were collected and allocated to their respective treatment to initiate the next generation of selection. To equalise density among treatments and avoid larval competition in subsequent generations, 200-250 eggs were allocated per bottle for each of the four bottles per replicate line. Selection lines were maintained at 25 °C. At the time of the experiments, the experimental evolution lines had undergone 30 generations of selection. These experimental evolution lines were derived from the same base population as the isogenic lines and have genetically diverged for nutritional plasticity in body size and development time which indicates these lines have evolved (Kutz, 2021).

3.3.4 | Fecundity assays

For both the isogenic and experiment evolution lines, we focussed on fecundity because we were interested in quantifying the plastic response of a trait that was closely linked to fitness. To assess fecundity, flies were maintained for two generations in a common environment of standard yeast-dextrose-potato medium at 25 ºC to reduce effects of maternal or grand-maternal environment. To control for larval rearing density and to synchronize adult emergence time, eggs from each isogenic line / replicate experimental evolution line were collected by allowing adults to lay in embryo collection cages (Genesee Scientific) on 60 mm petri dishes half-filled with apple juice/agar medium, as described in Linford et al., (2013), for 24 h at 25ºC. We then distributed 50 eggs from each line into each of 20 vials containing standard yeast-dextrose-potato food at 25ºC. The adult flies that emerged from these vials were collected over a 48 h period, transferred to new bottles containing yeast-dextrose-potato medium, and left to mate with males from their corresponding lines for 48 h prior to the assessments of fecundity described below.

After 48 h of mating, females were separated from males, and five females from each isogenic and replicate experimental evolution line were transferred into vials that contained one of three diets to assess the nutritional plasticity of fecundity. To test for changes in fecundity due to diet, we compared a 100% standard diet (yeast-dextrose-potato) with two other diets that contained 5% or 50% of yeast (relative to the 100% diet), but the same amount of dextrose and potato. We chose these diets because yeast is the main source of protein for *D. melanogaster*, and protein concentration correlates tightly with egg production (Piper et al., 2014; Ortega et al., 2021; Chapter 2, Alves et al. (accepted)). The low protein diets chosen for this study simulate not only variation in diet that animals already experience (Markow & O'Grady, 2008; Matavelli et al., 2015; Silva-Soares et al., 2016), but also the predicted reductions in nutrition expected under climate change (DaMatta et al., 2010; Sardans et al., 2017). To quantify changes in fecundity for the three diets, we established ten replicate vials, each containing five females from each isogenic/replicate experimental evolution line for each diet/line combination. Females were transferred to fresh food every 24 h. Eggs laid on days 5, 6, and 7 of the assays were counted since this interval is known to capture the peak period in fecundity for *D. melanogaster* and is often used as a measurement for fecundity (Novoseltsev et al., 2003).

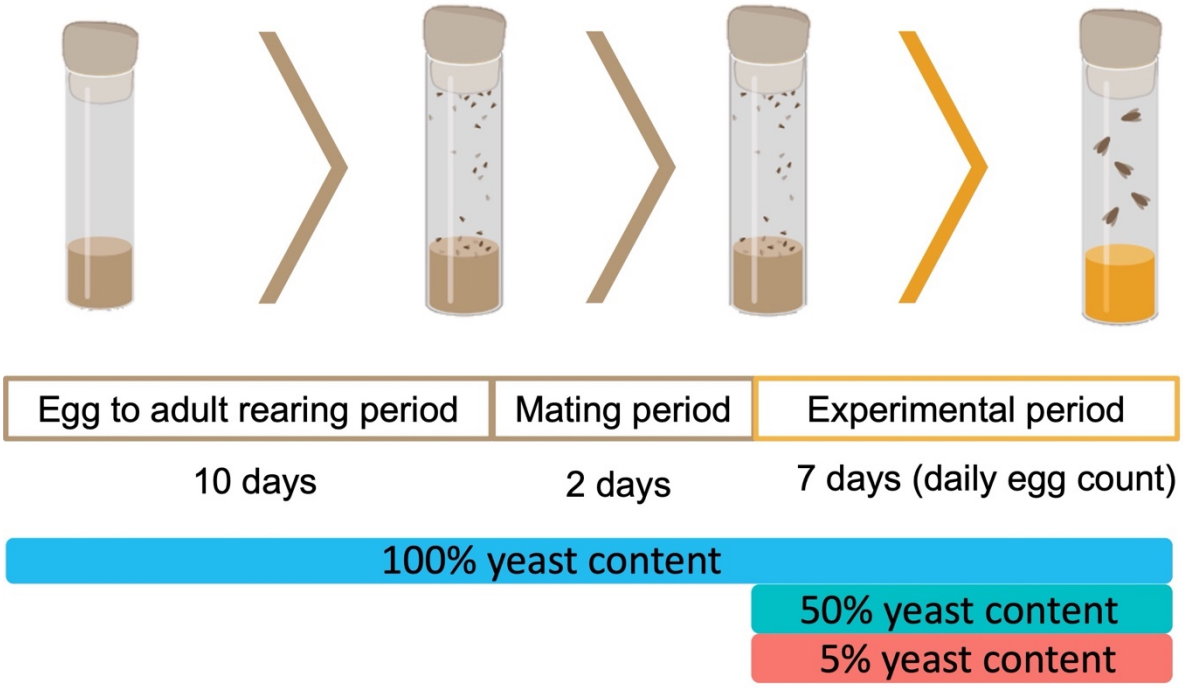

Figure 1: Illustration of the protocol used to assess fecundity in isogenic and experimentally evolved lines. Flies were reared in a standard, 100% yeast content diet, from egg to adult. Once eclosed, flies were put in a new vial with fresh food and allowed to mate for 48 h. After 48h, 5 females from each line per replicate were placed in a new vial containing one of three diets: 100%, 50% or 5% yeast content diets to a total of 10 replicates per line and per diet. Flies were transferred to fresh food every 24 h and eggs laid in the previous 24 h were counted manually for seven consecutive days.

3.3.5 | Statistical analysis

3.3.5.1 | Isogenic lines

3.3.5.1.1 | Testing for significant interactions for absolute fecundity

We first tested for significant effects for fecundity quantified for all isogenic lines exposed to three diets. An ANOVA was applied to the model with the best fit, using absolute number of eggs as a Poisson-distributed response variable. Diet, genotype, and their interaction were included as fixed effects to assess the effect of diet and genotype on absolute fecundity of the isogenic lines. Experimental block and replicate vial were included as random effects.

3.3.5.1.2 | Testing for overall genotype-by-environment interactions for relative fecundity

To test for significant genotype-by-environment interactions for relative fecundity, we applied a linear mixed-effects model using a normal distribution with relative fecundity (the sum of all the eggs laid from days 5 to 7 for each line, for each diet, standardised to the average number of eggs laid across all lines for each diet) as the response variable. We used relative fecundity (rather than absolute fecundity) to equalize differences in the mean number of eggs laid across environments and compared the extent of differences among lines relative to the mean fecundity in each diet. To apply the linear model, we used the lme4 package in R. We fit sequential models (Table 1) with diet as a fixed effect. The first model included experimental block, and replicate vial as random effects (model 1.1, Table 1). Isogenic line was included as a random effect in model 1.2 and a likelihood ratio test used to test for improved model fit (model 1.2, Table 1). To test for genotype-by-environment interactions, the interaction between isogenic line and diet was then included as an additional random effect (model 1.3, Table 1) and a likelihood ratio test was used to test for improved model fit over the model that did not include this interaction (model 1.2 vs model 1.3, Table 1).

Model 1.1: Relative # Eggs ~ Diet + (1|Block) + (1|Replicate)

Model 1.3: Relative # Eggs ~ Diet + (1|Genotype) + (1|Diet:Genotype) + (1|Block) + (1|Replicate)

3.3.5.1.3 | Quantifying genetic variance in fecundity

We then explored whether G×E across diets was the result of changes in genetic variation for fecundity across diets or due to genetic correlations across diets of less than 1 (Falconer 1952; Via and Lande 1985). We used Bayesian models to estimate genetic variance within environments, and genetic correlations in relative fecundity between diets using a generalized linear mixed model with the R package ‘MCMCglmm’ (Hadfield, 2010):

$$\text{Eq 1: } y_{ijkl} = D_i + g_{j(i)} + b_{g(kij)} + e_{m(ijkg)}$$

where relative fecundity was included as a univariate Poisson-distributed response variable (y_{ijkl}). The *i*th diet was included as the only fixed effect (D_i). The *j*th isogenic line ($g_{j(i)}$) and experimental block ($b_{l(kij)}$) were included as random effects to account for variation among lines within each diet, and among experimental blocks. Residual variance is represented by $e_{m(ijkg)}$. The model was implemented with 1.1 million iterations, a burn-in of 100 thousand iterations, and a thinning interval of 500 iterations. We checked autocorrelation for model convergence by confirming that the effective sample size exceeded 85% of the number of samples that were saved. For each diet we estimated random intercepts and slopes, which calculated among-genotype genetic variance within each diet as well as the genetic covariance between diets, resulting in a 3×3 genetic covariance matrix.

To quantify the genetic correlation between relative fecundity across environments, a correlation matrix was calculated from the covariance matrix of relative fecundity. To take into account the uncertainty in our estimates of genetic variance and genetic correlations between treatments, we calculated the 95% Highest Posterior Density (HPD) credible intervals. Density distributions of genetic variance and genetic correlations were created by extracting 2,000 samples from the model.

3.3.5.2 | Experimental Evolution

To test for significant effects of diet and selection regime, an ANOVA was applied to the model with best fit, using absolute number of eggs as a Poisson-distributed response variable to assess the effect of diet, selection regime, and their interaction on absolute fecundity for the experimental evolution lines. Replicate selection line and replicate vial were included as random effects.

For the experimental evolution experiment, we used a linear mixed-effects model with the R package lme4 to test for a shift in plasticity of the evolved lines (i.e.G×E). Relative fecundity (the sum of eggs laid from day 5 to 7 standardised to the average in each diet) was included as a response variable, while diet and selection regime were included as fixed effects, with replicate vial and replicate selection line included as random effects (model 2.1, Table 2). To test for significant differences in plasticity between the selection regimes (i.e. G×E), a model with an interaction between diet, selection line, and selection regime as a random effect was created (model 2.2) and compared to the previous model (model 2.1) through the use of a likelihood ratio test.

Model 2.1: Relative # Eggs ~ Diet*Regime + (1|Replicate) + (1|Line)

Model 2.2: Relative # Eggs ~ Diet * Regime + (1|Line) + (1|Replicate) + (1|Diet:Regime:Line)

All statistical analyses were performed in R (version 3.4.1). The packages used for statistical tests were *lme4*, *MCMCglmm*, and *lrt*. All data and scripts are available in Figshare (DOI: 10.26180/18515243).

3.4 | Results

3.4.1 | Testing for within-population genotype-by-environment interactions for fecundity

To test for overall genetic variance in the plastic response of fecundity to nutrition, each isogenic line was placed onto diets containing either 5%, 50%, or 100% yeast and allowed to lay eggs for seven days. We found that total fecundity increased with yeast content in the diet, and that genotypes differed in the total number of eggs laid across all diets (Figure 2A, Supplementary Table 1). Furthermore, genotypes differed in their response to diet, resulting in a significant diet by genotype interaction.

To equalise differences in mean across environments we assessed genotype-by-environment interactions using relative fecundity. We calculated relative fecundity across these diets, by calculating the sum of eggs laid between days 5 and 7 for each line in each diet and standardising it by the mean number of eggs laid across all lines in each diet. When analysing the response of relative fecundity to diet, including genotype as a random effect significantly improved the model fit, as indicated by a significant likelihood ratio test and a lower AIC score (Table 1, model 1.1 and 1.2). As evidence of significant G×E, the model that included the genotype by diet interaction as a random effect further improved the model fit, with a significant likelihood ratio test and lower Akaike Information Criterion (AIC) value, which determines which of multiple models has the least predicted error, and is, therefore, the most likely to be the best model taking into account the data given (Stoica & Selen, 2004) (Figure 2, Table 1, model 1.2 and 1.3).

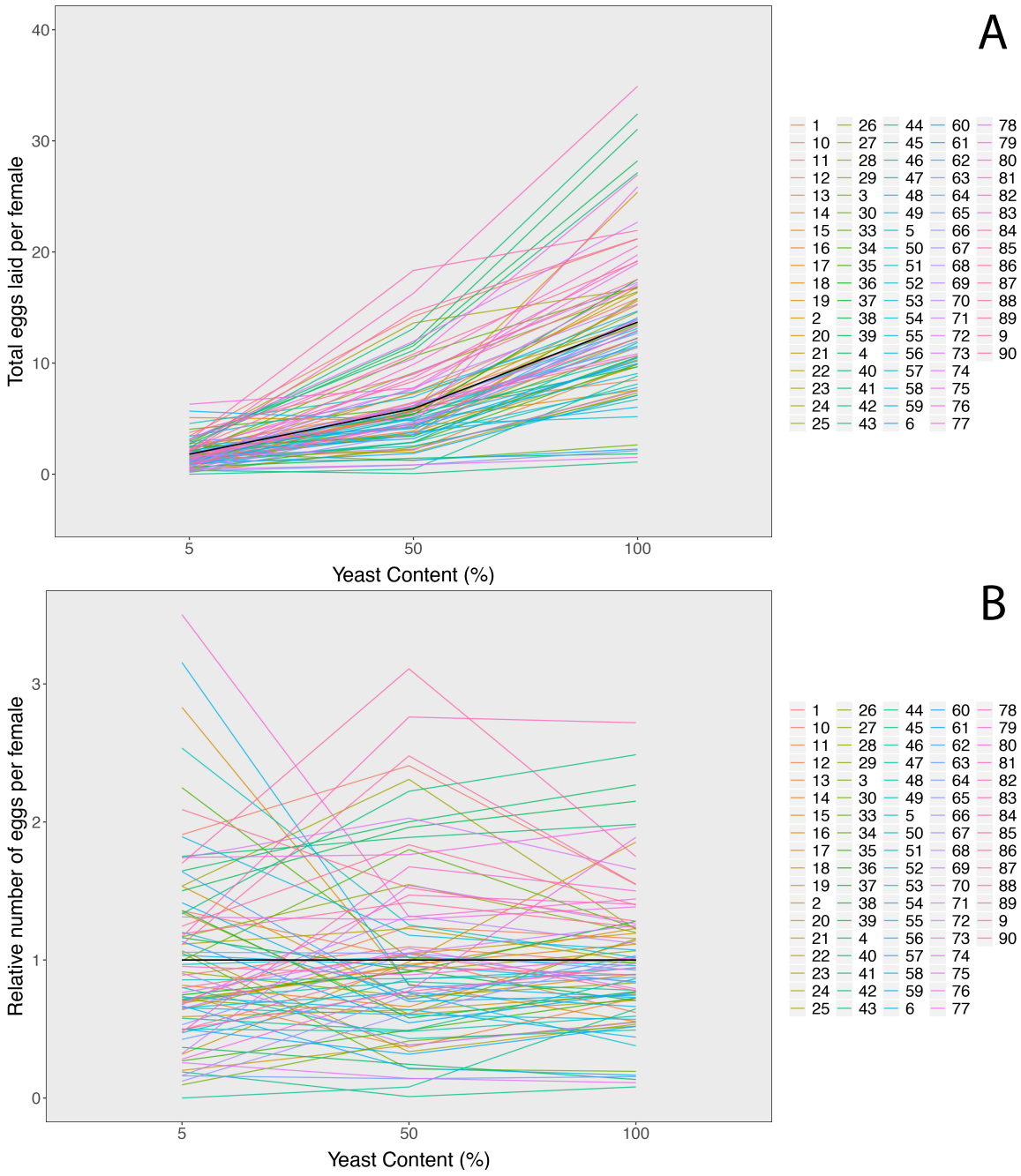

Figure 2: Phenotypic reaction norms of fecundity for each isogenic line across diets with 5, 50 and 100% yeast content. A) The sum of eggs laid between days 5 to 7 for each female were counted in each diet for each isogenic line. B) Relative fecundity, i.e., the number of eggs standardized to the mean of each diet, were counted for each isogenic line. Each colour represents a different isogenic line. The mean reaction norm of the population is represented by the black line.

Table 1: Linear mixed model fit by maximum likelihood for isogenic lines and the effect of diet on egg production. lmer was performed to assess if diet had any effect on relative egg production taking into account the variation within each block, within replicates, within genotypes, and the interaction between genotype and diet. Models were compared using a likelihood ratio test and AIC to determine best fit. The summary of the model with the best fit is presented below.

Model Number	Model	AIC	Likelihood ratio test (p-value)
1.1	Relative # Eggs ~ Diet + (1|Block) + (1|Replicate)	5790.585	-
1.2	Relative # Eggs ~ Diet + (1|Block) + (1|Replicate) + (1|Genotype)	5276.211	1.2 to 1.1: <2.2e-16 ***
1.3	Relative # Eggs ~ Diet + (1|Block) + (1|Replicate) + (1|Genotype) + (1|Genotype:Diet)	4900.179	1.3 to 1.2: <2.2e-16 ***

3.4.2 | Quantifying genetic variance in the plastic response of fecundity to diet

Having detected significant genotype-by-environment interactions for relative fecundity, we next determined the origin of this variation. G x E can be due to increased variation in relative fecundity on specific diets across genotypes (Walter et al., 2021; Sheth et al., 2018). Alternatively, G x E can result from differences in the response of genotypes to diets, resulting in genetic correlation of less than 1 across diets.

While we found significant genetic variance for relative fecundity on all three diets, we did not detect a significant difference in the expression of genetic variance across diets, as indicated by the overlapping credible intervals and density distributions (Figure 3A, Supplementary Figure 1). Thus, the significant G × E interaction for relative fecundity is not explained by changes in the expression of genetic variance across diets.

Genetic correlations in fecundity among diets were all strong and positive (Figure 3B, Supplementary Figure 2). Interestingly, the genetic correlation between the 5% and 100% yeast diets was significantly greater than the remaining genetic correlations, as indicated by greater and non-overlapping credible intervals and density distributions (Figure 3B, Supp Fig 2). The fact that genetic correlations across all diet pairs were all less than one indicates that there is some genetic independence in relative fecundity across diets. This means that the significant genotype-by-environment interaction for relative fecundity results from the

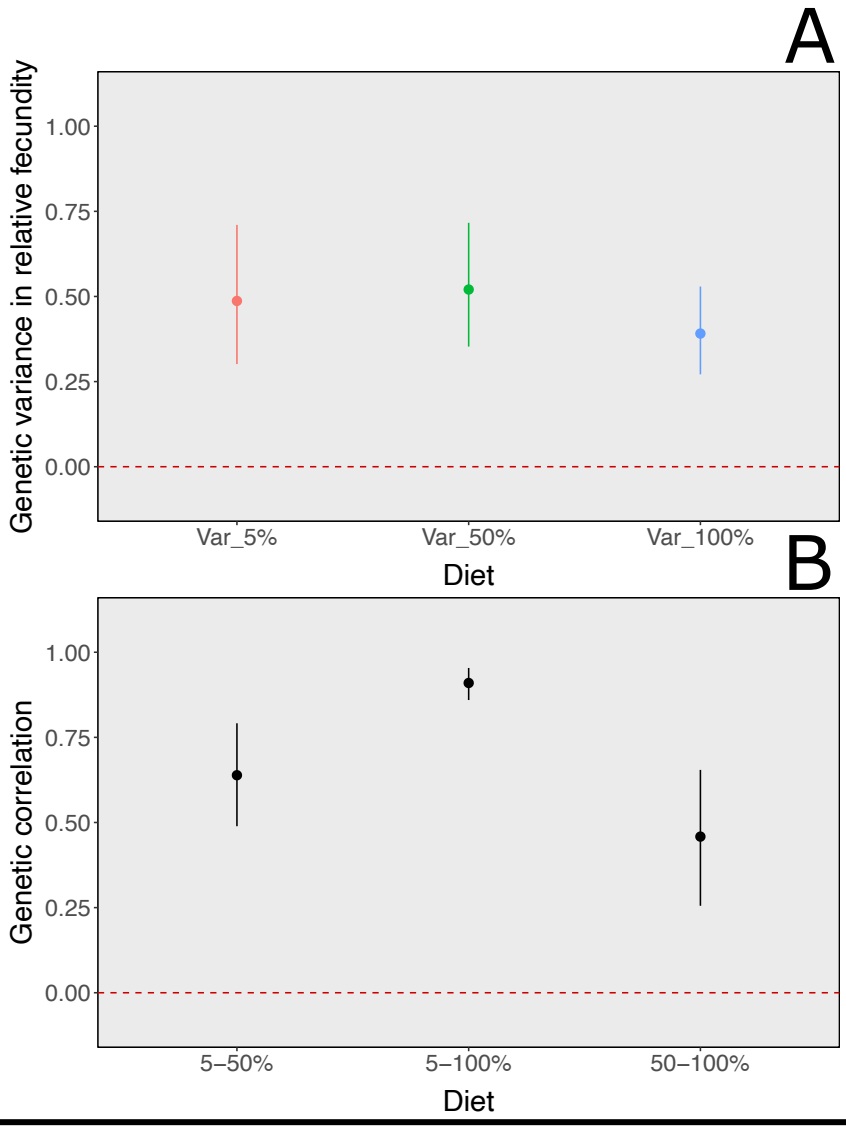

Figure 3: Estimates of genetic variance in relative fitness and genetic correlation across diets. A) Estimate of genetic variance in relative fitness and respective 95% credible interval for each diet. The dashed line represents 0 genetic variance, i.e. no variation within that diet. B) Estimates of genetic correlation and respective 95% credible intervals. The dashed line represents 0 genetic correlation, i.e. no genetic correlation between diets.

fact that the isogenic lines differ in at least some of the alleles that underpin nutritional plasticity for this trait.

3.4.3 | Plastic changes in response to adaptation to diet

To understand how plasticity in fecundity evolves as a consequence of adaptation to changes in the nutritional environment, we assessed plasticity in fecundity for experimental evolution lines after more than 30 generations of adaptation to standard diet (100%) and a diet with only 20% of the calories of the standard diet. We predicted that the presence of significant genetic variance in plasticity for relative fecundity in the original population, as indicated by the results described above, might result in changes in plasticity for this trait in response to adaptation to the different diet treatments.

As observed in the isogenic panel, diets with higher yeast content resulted in higher absolute number of eggs laid (Figure 4A, Supplementary Table 2). However, we found no difference in the response of the selection regimes to diet either for absolute fecundity (Figure 4A, Table 2, Supplementary Table 2) or for relative fecundity (Figure 4B, Table 2). Interestingly, there are no significant G × E interactions observed for these evolution lines (Table 2). This indicates that there are no differences in the expression of genetic variance across the selection regimes and that genetic correlations between diets are all possibly 1.

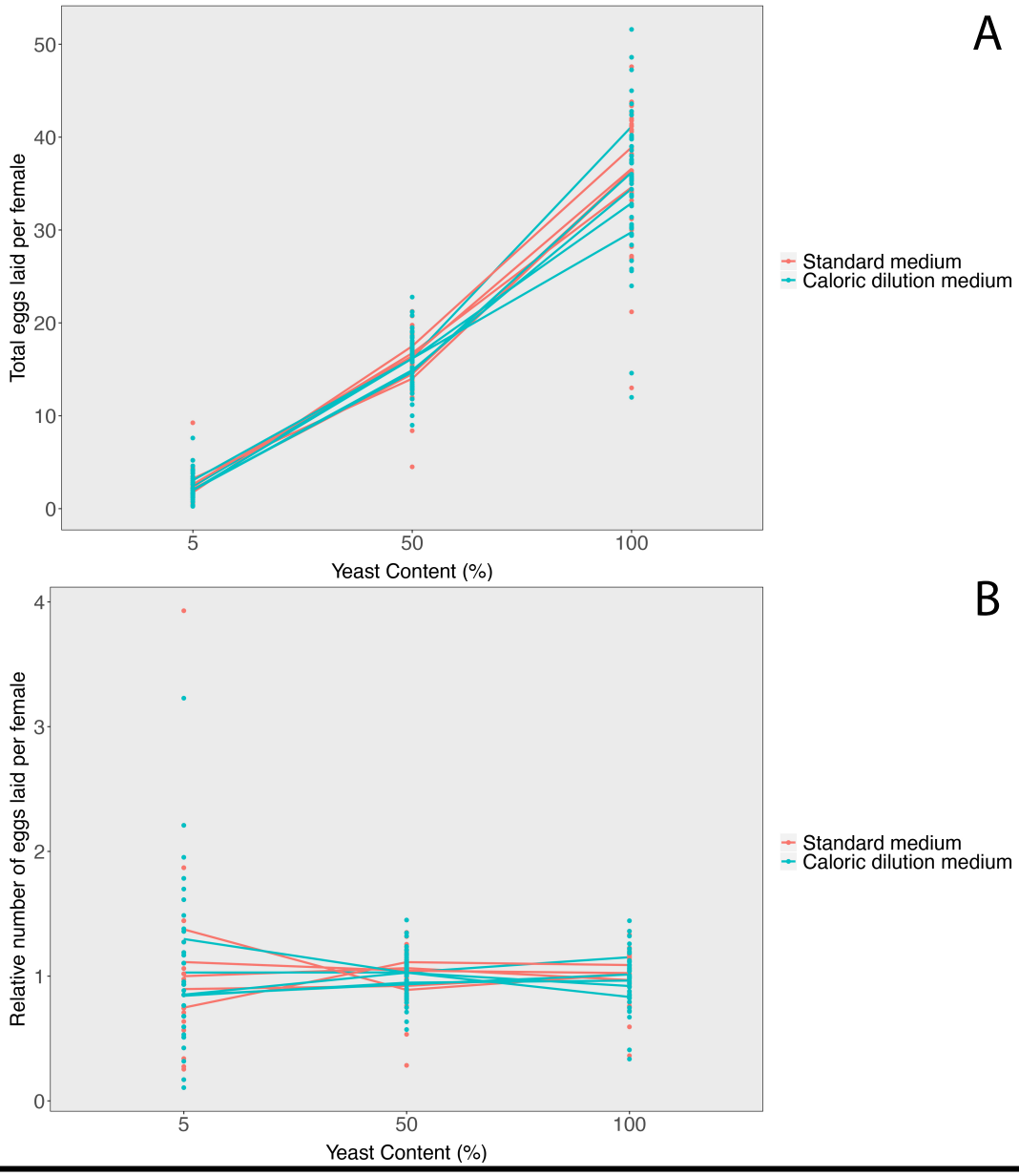

Figure 4: Phenotypic reaction norms for experimental evolution lines for the 5, 50 and 100% yeast content diets. A) Total number of eggs counted for each female in each diet. B) Relative fecundity, i.e., number of eggs standardized to each diet were counted for each line. Control lines evolved in standard diet represented in red, and lines evolved in a 20% caloric diluted diet are represented in blue.

Table 2: Linear mixed effect model for the effect of adaptation to control versus low calorie diets on egg production. lmer was performed to assess if diet and selection regime had any effect on relative egg production taking into account the variation between each line, replicate, and selection regime, and the interaction between selection regime and diet. Models were compared using a likelihood ratio test and AIC.

Model Number	Model	AIC	Likelihood ratio test (p-value)
2.1	Relative # Eggs ~ Diet*Regime + (1|Replicate) + (1|Line)	283.0487	-
2.2	Relative # Eggs ~ Diet*Regime + (1|Replicate) + (1|Line) + (1|Line:Diet:Regime)	282.3635	2.2 to 2.1: 0.1013

3.5 | Discussion

Nutritional stress is one of the most common stressors faced by animals in nature and will become increasingly so with climate change (Scheffers et al., 2016; Houghton et al., 1990; Dawson et al., 2011; Thomas et al., 2004; Foden et al., 2013). Phenotypic plasticity will play a key role in shaping how animals will respond to climate change, at least in the short to mid-term (Lynch & Walsh, 1998; Hoffmann & Merilä, 1999; Franks et al., 2007; Geerts et al., 2015). Genetic variation in plasticity exists both across (Chakraborty et al., 2020, Klepsatel et al., 2020) and within (Schlichting and Pigliucci 1998; Lande 2009) populations. While between population variation in plasticity can reveal broad patterns of evolved differences in plasticity (Chakraborty et al., 2020), within-population genetic variation in plasticity may shape the capacity of a population to adapt to on-going change (Dreyer et al., 2016; Frankino et al., 2019).

3.5.1 | The source of genotype-by-environment interactions in isogenic lines

Numerous studies have revealed within-population genetic variation for plasticity in a range of life history traits (Van Buskirk & Steiner, 2009; Ghalambor et al., 2007, LaFuente et al., 2018; Cunningham et al., 2020); fewer have assessed plasticity for fecundity which serves as a proxy for fitness (Ng'oma et al., 2018; King et al., 2011; Camus et al., 2017). Such studies emphasise the fact that the distribution of plastic responses for individual

genotypes can vary markedly from the population-level response. The current study adds to this body of work by revealing that individual genotypes within a population can vary in the distribution of plastic responses, and that this variation is not reflected by the population-level plastic response.

We then went on to reveal the cause of this within-population G×E interactions. Significant G×E interactions can arise as the result of differences in the expression of genetic variation across environments and/or cross-environment genetic correlations that are less than one (Falconer 1952; Via and Lande 1985; Walter et al., 2021; Sheth et al., 2018). While some studies have found that exposure to environmental stress may increase the expression of genetic variance for life-history traits, including fecundity (Service & Rose, 1985; Van Noordwijk et al., 1988; Etges, 1993; Holloway et al., 1990; Larsson et al., 1997; Jenkins et al., 1997; Sgrò & Hoffmann, 1998), other studies suggest that this might depend on the trait and the environmental condition studied. We found significant levels of genetic variance in relative fitness in all three nutritional environments, however the expression of genetic variance did not differ across environments. Changes in genetic variance in relative fecundity across environments, therefore, do not contribute to the significant G×E for relative fecundity.

Although several theoretical and experimental studies have shown that genetic correlations can change in sign and magnitude when individuals are exposed to new or stressful environments (Krebs & Loeschcke, 1994; Norry & Loeschcke, 2002; Sgrò & Hoffmann, 2004), we found that all three cross-diet genetic correlations were large and positive, which is consistent with other studies that show positive genetic correlations across environments (Ebert et al., 1993, Etges, 1993, Windig, 1994). The significant G×E we found for fecundity can be explained by the fact that these correlations were less than one, indicating that the alleles that contribute to relative fecundity are in part independent across environments.

Theory suggests that genetic correlations should have higher positive values between similar environments (Sgrò & Hoffmann, 2004). Thus, we expected that the genetic correlation between the standard 100% and the 50% yeast diet would be significantly larger than the correlation between the 100% and 5% yeast diets. However, we found the opposite to be true; the genetic correlation between the standard 100% and the 5% yeast diets was significantly higher than the correlations between the 100%-50% and 50%-5% yeast diets. Usually deleterious mutations expressed in common environments are quickly removed by selection, however mutations that are only deleterious in rare environments may persist and disrupt existing genetic correlations, which results in reduced genetic correlations between

the rare and the common environment (Schmalhausen, 1949; Kawecki et al., 1997; Holt & Gaines, 1992; Hoffmann & Merilä, 1999; Szafraniec et al., 2001). Previous studies have measured the protein to carbohydrate ratio of fruits throughout the rotting process, and these suggest that the range of yeast dilutions used in this study is within the range of conditions *D. melanogaster* would face in nature (Matavelli et al., 2015; Silva-Soares et al., 2016). One possible explanation as to why genetic correlations were lowest when the 100% and 5% diets were compared to the 50% diet is that the 50% yeast diet used in this study could represents a rare dietary environment. This could be because yeast grows exponentially as the fruit rots, thus this yeast concentration might represent a highly transient state. To understand more about genetic correlations between different protein content diets, comprehensive field studies would be needed to analyse the dietary composition of *D. melanogaster*'s food sources.

3.5.2 | Adaptation to diet and changes in plastic responses

Past studies have shown that adaptation to diet can alter mean fecundity (Kolss et al., 2019), specifically, mean fecundity decreased when larvae were reared in poor larval food. However, we found that adaptation to a low-calorie diet for over 30 generations did not result in changes in overall fecundity, relative fecundity, or shifts in nutritional plasticity for relative fecundity, even though the base population harboured genetic variation in plasticity for this trait. A key difference between these two investigations was that our flies were adapted to a low-calorie diet for approximately 30 generations, whereas Kolss and colleagues (2009) imposed selection for 67 generations. It is possible that we would observe differences between lines adapted to either control or poor diet with more generations of selection. Furthermore, the base population differed between studies. Further research would be necessary to understand if the differences observed across studies was due to differences in genetic variation in the based population, or the duration of selection. Finally, the type of selection used in this research was constant but applied in the larval stages, and all adults were reared in the same conditions after emergence. Previous studies have shown that adult adaptation to diet has capacity to change plasticity in fecundity (Zajutschek et al., 2018). Perhaps the selective pressures imposed by the low-calorie diet in larval stages did not impose any selection on relative fecundity in the adult stages, even though previous studies have shown that ovariole number is regulated by larval diet (David, 1970; Mendes & Mirth, 2016). Thus, even when genetic variation in plasticity for relative fecundity is

present, selection does not necessarily result in an evolved shift in plasticity (Dreyer et al., 2016).

In summary, we revealed significant levels of within-population genetic variation for nutritional plasticity in fecundity that was not evident from the population-level reaction norm. Despite this, we found that nutritional plasticity for fecundity did not evolve as a result of adaptation to a low-calorie diet. Further studies are needed to understand the contexts under which plasticity is expected to evolve if we ever hope to be able to predict species' responses to climate change. Furthermore, reproduction and longevity have been considered deeply intertwined traits and subject to one of the biggest trade-offs observed in nature. It would be of special interest to observe the how longevity fares in these conditions and if it correlates with fecundity in these isogenic and experimental evolved lines.

3.6 | Acknowledgements

We would like to thank members of the Mirth and Sgrò labs, past and present, for their help throughout this project. In particular, we would like to thank Fiona Cockerell and for the help in creating the isogenic panel. We would also like to thank Teresa Kutz for creating and sharing the experimental evolution lines used in this study. This research was funded by ARC (DP180103725) for CMS, ARC (FT170100259) for CKM, and ARC (FT150100237) and NHMRC (APP1182330) for MDP.

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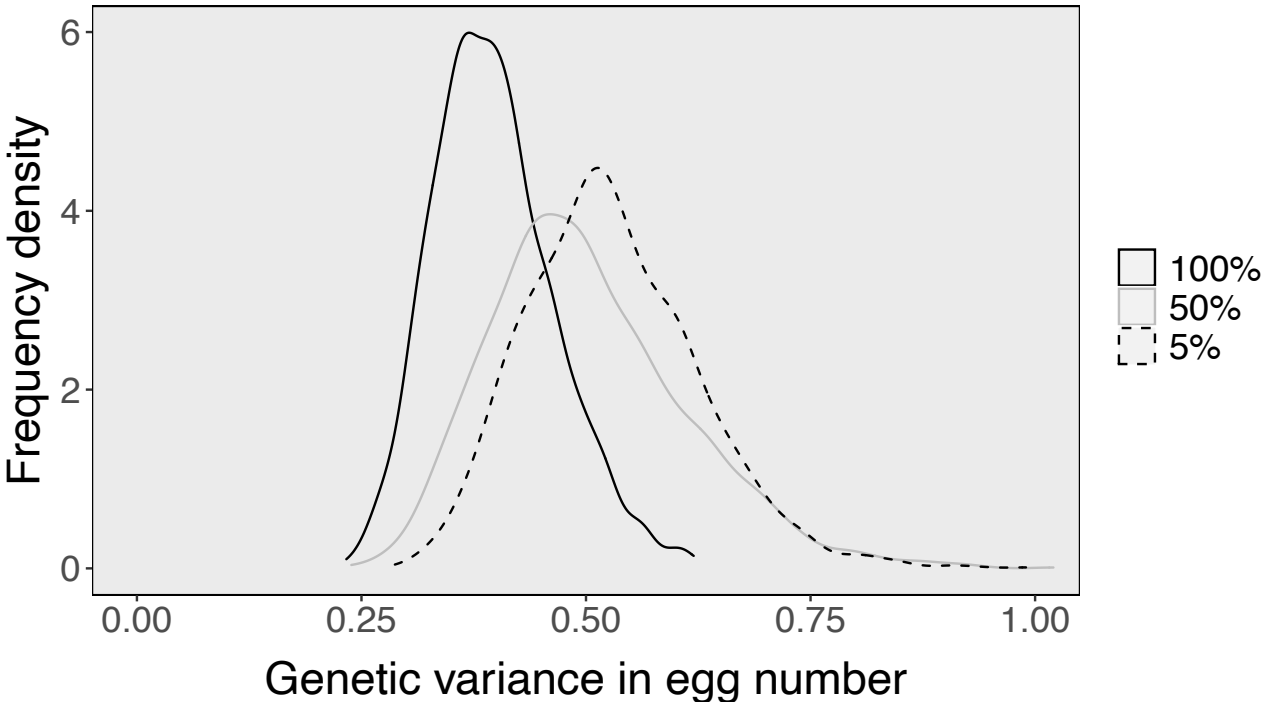
3.8 | Supplementary Figures & Tables

Supplementary Table 1: Effect of diet on absolute fecundity. ANOVA was performed on model 1.3 but with absolute fecundity as a response variable for the isogenic lines to observe the effect of diet, genotype and their interaction.

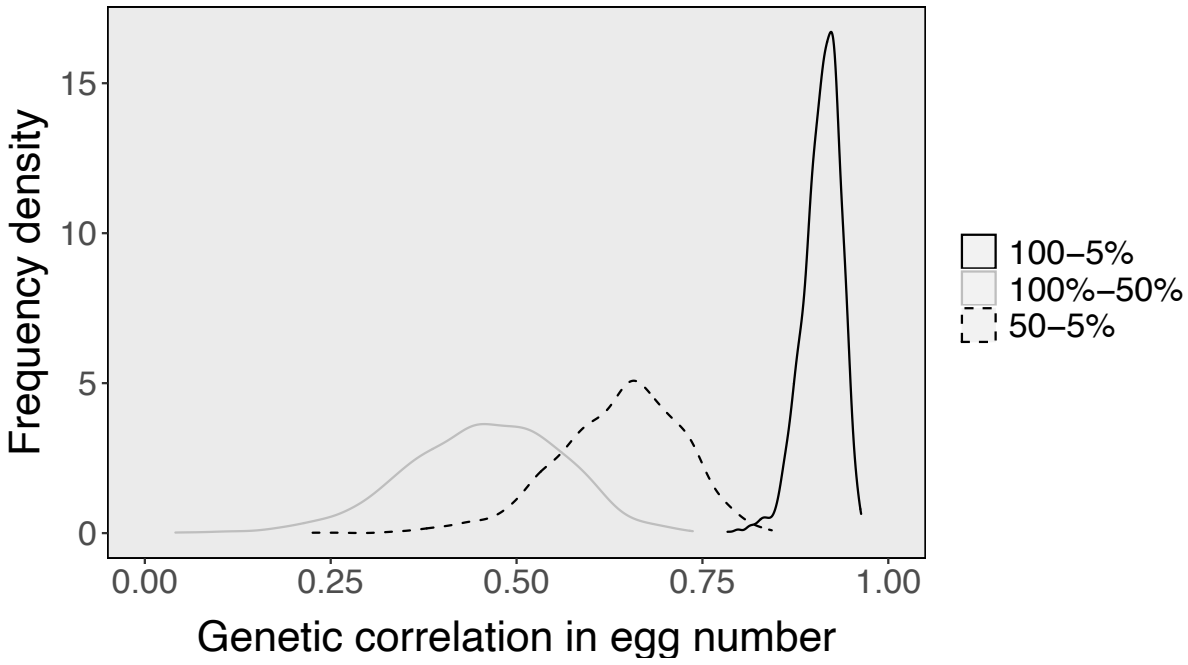
	Chi-square	Df	P-value
Diet	7074.4	2	<2e-16 ***
Genotype	2271.1	85	<2e-16 ***
Diet:Genotype	2417.7	170	<2e-16 ***

Supplementary Table 3: Effect of diet and selection regime on absolute fecundity. ANOVA was performed on model 2.1 but with absolute fecundity as a response variable for the experimental evolution lines to observe the effect of diet and selection regime.

	Chi-square	Df	P-value
Diet	2129.5007	2	<2e-16 ***
Regime	1.0749	1	0.2998
Diet:Regime	0.3845	2	0.8251



Supplementary Figure 1 – Density distributions of genetic variance in egg number. Each line represents the density distribution of genetic variance in egg number for a particular diet. Solid black corresponds to the control diet, 100% yeast content, solid grey corresponds to 50% yeast content and dashed black line corresponds to the 5% yeast content diet.



Supplementary Figure 2 – Density distributions of genetic correlations in egg number. Each line represents the density distribution of genetic correlation in egg number for a particular pair of diets. Solid black corresponds to the comparison between control diet, 100% yeast content and 5% yeast content, solid grey corresponds to the comparison between 100% and 50% yeast content and dashed black line corresponds to the comparison between 50% and 5% yeast content

Chapter 4 | Proximate mechanisms as generators of variation in nutritional plasticity for fecundity in Drosophila melanogaster

“Write what you know. That should leave you with a lot of free time.”

By Howard Nemerov

André Nogueira Alves, Mia Wansbrough, Carla M. Sgrò, Matthew D. Piper, Christen K. Mirth

School of Biological Sciences, Monash University, Melbourne, VIC 3800, Australia

Keywords: phenotypic plasticity, genotype by environment interactions, isogenic panel, ovariole number, capillary feeding assay, food intake, nutrient assimilation,

4.1 | Abstract

The amount and quality of nutrition available in an animal's diet is an important determinant of its survival and fitness. Phenotypic plasticity allows a genotype to adjust life history traits to changes in its nutritional environment. Plasticity also varies among individuals, with some genotypes showing higher plasticity for a given trait across nutritional gradients than others. The origin of this variation comes from differences in proximate mechanisms regulating trait expression, such as feeding behaviours, morphological differences, or changes in nutrient absorption. To understand how variation in plasticity is achieved, we made use of a *Drosophila melanogaster* isogenic panel to characterize nutritional plasticity for fecundity by feeding flies diets differing in their yeast content and counting number of eggs produced. With the highest and lowest plasticity lines, we dissected the proximate mechanisms that could be responsible for these differences in plasticity, such as ovariole number, food intake, and food-to-egg conversion. Our results suggest that variation in plasticity is not due to differences in ovariole number, but due to both increased food intake when the amount of protein available is low and higher efficiency at converting food to eggs in the high plasticity lines. Our results highlight the importance of understanding the mechanisms underlying shifts in plastic responses, and set the stage for future studies aiming to understand how genetic variation contributes to whether populations will persist when faced with changes in their nutritional environment.

4.2 | Introduction

Diet is vital for all organisms. It impacts life-history traits such as lifespan, growth rate, and reproduction (Simpson and Raubenheimer, 2012). Because of the wide-ranging effects of nutrition, researchers have worked extensively to understand the mechanisms that regulate the way nutrients impact life-history traits (Mirth & Piper, 2017, Mirth et al., 2018). However, little is known about the mechanisms through which nutritional plasticity can be regulated, and consequently regulate life-history traits when exposed to different nutritional environments.

The ability of a genotype to adjust its phenotypes in response to environmental conditions, known as phenotypic plasticity, underpins population and species persistence in the face of ongoing environmental changes (Lynch & Walsh, 1998; Hoffmann & Merilä, 1999; Gause, 1947; Bradshaw, 1965; Ørsted et al., 2017). Many traits exhibit plastic responses to environmental conditions, including lifespan, metabolism, and reproductive output (Bozinovic et al., 2011; Pavan et al., 2013; Sgrò et al., 2016). In addition, plasticity can be induced by a wide variety of environmental factors, such as temperature, photoperiod, humidity, and nutrition (Tauber et al., 1998; Andersen et al., 2010; Via & Lande, 1985). As such, plasticity is an important means by which organisms can adjust to environmental change.

The extent to which a phenotype is plastic varies among individuals, with some individuals showing higher sensitivity to environmental conditions for a given trait than others (Mackay et al., 2012; King et al., 2012; Lafuente et al., 2018; Alves et al. (accepted)). Plasticity in female fecundity is of particular interest, since this trait is closely associated with fitness (Van Buskirk & Steiner, 2009; Ghalambor et al., 2007) and is strongly influenced by nutritional availability and quality (Lee et al., 2008; Piper et al., 2014; Armstrong et al., 2014). In egg-laying animals, female fecundity is easily quantifiable, which makes it an excellent trait for understanding how plasticity of fitness-related traits is regulated. Furthermore, plasticity in egg laying displays significant levels of genetic variation, varying across genotypes within a population (Ng'oma et al., 2018; King et al., 2011, Bergland et al., 2008).

A number of proximate mechanisms have the potential to underly differences in the plastic responses of egg laying to nutrition. In insects, morphological mechanisms such as ovariole number can dictate the number of eggs an organism is able to produce at any time (David, 1970). Plasticity in ovariole number in response to nutrition has been observed (Bergland et al., 2008) and research has found that the larval stages of development are critical for determining nutritional plasticity in ovariole number (Green & Extavour, 2014;

Mendes & Mirth, 2016). Plastic variation in this trait, induced by nutrition during larval development, results in differences in the number of eggs females lay (David, 1970; Mendes & Mirth, 2016), ultimately impacting their fitness.

Differences in rates of food intake across genotypes can buffer the extent to which individuals will experience nutritional stress on poor diets. Food intake is carefully regulated to ensure animals reach their nutritional requirements to maximise life-history traits (Simpson & Raubenheimer, 2009; Simpson & Raubenheimer, 1995). Plasticity in food intake and other feeding behaviours occurs when organisms have to make choices between food sources with different nutritional content (Carvalho and Mirth, 2017; Rodrigues et al., 2015; Silva-Soares et al., 2017; Diegelmann et al., 2017; Fuhita & Tanimura, 2011) or change their intake strategy when forced to eat sub-optimal diets. As a result, variation in what animals eat alters whole body physiology and generates plasticity in other traits.

Genetic differences in the efficiency with which animals absorb and assimilate the nutrients ingested can also dictate the degree of plastic response. Traits such as body size have been shown to be regulated by nutrient assimilation (Sibly, 1981, Clissold et al., 2010). Even when organisms ingest large quantities of nutrients, they can still result in smaller adult body sizes if they are not able to absorb the nutrients efficiently or are unable to allocate them to the correct organs (Urabe and Watanabe, 1991; Neat et al., 1995). For example, when fed a fixed quantity of the same diet, populations of *Drosophila melanogaster* that are adapted to cold environments reach larger body sizes than those adapted to warm environments (James and Partridge, 1995). Potentially, genetic variation in the ability to absorb and assimilate nutrients could underlie differences in nutritional plasticity for fecundity as well.

In this study, we made use of isogenic lines to identify genotypes that showed either high or low plasticity in female fecundity in response to dietary protein. We then counted the number of ovarioles, measured food intake, and assessed protein-to-egg conversion efficiency in these lines across diets varying in protein content. This study will shed light on the proximate mechanisms that lead to genetic differences in the plastic response of fecundity to diet.

4.3 | Methods

4.3.1 | Fly husbandry and maintenance

For the isogenic panel, about 200 field-inseminated *Drosophila melanogaster* flies were collected from a banana plantation and Tropical Fruit World in Duranbah, in the east coast of Australia in January 2018 (28.3° S, 153.5° E). We generated two independent isofemale lines from each of the wild-caught females resulting in 400 lines in total. All lines were treated with tetracycline to remove *Wolbachia* and reared for two generations prior to 20 generations of inbreeding (described below). Flies were maintained on standard yeast-potato-dextrose medium at 25 ºC, on a 12h light/dark cycle. These lines were inbred through single pair brother-sister matings for a minimum of 20 generations, resulting in a panel of 81 fertile isogenic lines with an inbreeding coefficient of F=0.986 (Mackay et al., 2012). All lines were maintained at a constant temperature of 18 °C on a 12h light/dark cycle on a yeast-potato-dextrose medium (potato flakes 20 g/L; dextrose 30 g/L; Brewer's yeast 40 g/L; agar 7 g/L, Nipagin 6 mL/L; and propionic acid 2.5 mL/L).

4.3.2 | Identifying lines with high/low plasticity in fecundity

Using a dataset previously collected to calculate genetic variance in fitness (Chapter 3), we identified five lines with the highest plasticity and five with the lowest plasticity in egg laying in response to diet. In this dataset, the number of eggs laid over days five to seven were counted when isogenic lines were exposed to two different diets: a standard yeast-potato-dextrose diet (100% yeast content), and a diet containing 5% of the yeast from the standard diet. These two diets were chosen since they would better highlight variation in plasticity and the different strategies to achieve plasticity in extreme environments.

To measure genetic variance in plasticity, we calculated the average number of eggs laid across all replicates sampled. We then quantified plastic changes in fecundity across diets by standardizing the average number of eggs laid for each genotype on the 5% yeast content diet by its mean value on the standard diet (100% yeast content). We used a multivariate linear mixed effects model with the R package 'MCMCglmm' (Hadfield, 2010) (Equation 1) to quantify changes in plasticity. The standardized values for fecundity were used as a univariate response variable, and diet as a fixed effect. Genotype and experimental block were included as random effects to account for variation among lines

within each diet and among experimental blocks (Equation 1). The standardized fecundity to the standard diet was included as a univariate Poisson-distributed response variable (y_{ijkl}), and ith diet was included as the only fixed effect (D_i). The jth isogenic line ($g_{j(i)}$) and experimental block ($b_{l(kij)}$) were included as random effects to account for variation among lines within each diet, and among experimental blocks. Residual variance was accounted as $e_{m(ijkg)}$. The model was implemented with 1.1 million iterations, a burn-in of 100,000 thousand iterations, and a thinning of 500 iterations. We checked autocorrelation for model convergence by confirming that the effective sample size exceeded 85% of the number of samples that were saved.

$$\text{Eq 1: } y_{ijkl} = D_i + g_{j(i)} + b_{g(kij)} + e_{m(ijkg)}$$

We then calculated the best linear unbiased prediction (BLUP), obtained from the MCMCglmm model, between the 5 and the 100% yeast content diets for each genotype (Walter et al., 2021). These BLUPs estimate how genotypes rank relative to each other and the mean. After inspecting the distribution of reaction norms, we decided to eliminate any lines that had laid less than three eggs over the time period measured across all diets. This was done to eliminate isogenic lines that laid low numbers of eggs irrespective of the diet, presumably due to the presence of deleterious alleles. The five genotypes with the smallest BLUP post-filtering, which indicates the highest change in slope relative to the mean, were chosen as the high plastic lines, and the five lines with the largest BLUP, after filtering for lines that produced very few eggs overall, were chosen as the low plasticity lines.

4.3.3 | Ovariole dissection and counting

To assess if high and low plasticity groups for fecundity differed in number of ovarioles, flies were reared from egg to adult in control conditions of 25ºC in standard potato-yeast-dextrose medium. Once eclosed, adult flies were collected over a 48-hour period, transferred to new vials containing standard yeast-dextrose-potato medium, and left to mate for 48 h. At the end of this period, female flies were transferred from three vials per line into two microtubes, placed in dry ice and stored at -80 ºC until ready for dissection.

Ovariole number was measured on 9-11 females per replicate microtube (18-20 females per line). Flies were submerged in phosphate-buffer solution (PBS) and their ovaries removed and teased apart to count the number of ovarioles.

4.3.4 | Food intake and fecundity assays

The five lines with the highest and five lines with the lowest plasticity chosen from the analysis above were maintained for two generations in common garden settings of standard yeast-dextrose-potato medium at 25 ºC prior to all assays described below. To control for larval rearing density and to synchronize adult emergence time, we collected eggs from the parental generation by leaving them to lay in embryo collection cages (Genesee Scientific) on 60mm petri dishes half-filled with apple juice/agar medium, as described in Linford et al., 2013, for 24 h at 25ºC. Fifty eggs were then transferred into 10 food vials per line, each of which contained 6 ml of standard yeast-dextrose-potato medium at 25ºC.

The adult flies that emerged from these vials were collected over a 48-hour period, transferred to new vials containing standard yeast-dextrose-potato medium, and left to mate for 48 h. Once mated, five female flies per line were transferred into vials that contained 3 mL of apple juice/agar medium to a total of 10 vials per line and per diet.

We used the experimental setup for the capillary feeding (CAFE) assays from Diegelmann et al. 2017 to measure food intake and number of eggs laid. We pierced narrow cotton fly vial plugs (flugs) (Genesee Scientific, product code 076-49-103) three times with a sharp metal rod of the same diameter as the calibrated glass micropipettes used for the assays (5 µL, Sigma, product number P0549). Three glass micropipettes per vial were filled with liquid media, marked with a black marker at the meniscus, and inserted in the cotton flugs. The media used consisted of either 5N or 100N holidic food without agar (a synthetic diet containing the proportion of each amino acid according to an exome-matching study (Piper et al., 2014), with N representing the amount of nitrogen available, and by association, amount of protein available in the food, with 100N equaling 100mM of nitrogen available and 5N, 5mM of available nitrogen (Piper et al., 2014)). This medium was used over a standard yeast-dextrose-potato medium since the former is soluble, whereas the latter settles to the bottom of the capillary, impeding flies from feeding from it. We also added 0.1% blue food colouring to the media for contrast when measuring food intake (Queen food dye, blue, batch #118106). We established ten replicate vials per line and diet combination, each containing five female flies.

To measure number of eggs laid each day, females from the capillary feeding vials were transferred to fresh apple juice/agar vials every 24 h for seven days, after which the eggs laid over the previous 24 h period were counted manually. Capillaries were replaced, and amount of food left in the capillary from the previous 24 h measured with the same frequency to obtain food intake measurements. This was done for seven consecutive days. The CAFE setup was placed inside a plastic storage box (25 cm (H) x 48 cm (W) x 34.5 cm

(D)) containing wet paper towel and closed with a lid to avoid evaporation. The CAFE setup was placed in an incubator under a 12 h light/dark cycle kept at 25ºC and 80% humidity. Apple juice/agar vials containing media-filled capillaries, but no flies, were placed in the same boxes to measure evaporation of the media. Evaporation losses were subtracted from experimental readings to obtain true ingestion amounts (Diegelmann et al., 2017).

4.3.5 | Statistical analysis

To assess differences in ovariole number across plasticity lines, we fit the data with a linear mixed effects model using the 'lme4' package. The average number of ovarioles between ovaries of the same fly was used as a response variable, and plasticity group was used as a fixed effect. Microtube and isogenic line were accounted for as random effects.

Next we tested for significant differences in egg laying behaviour between high and low plasticity lines. A generalised mixed effects linear model was created with the sum of eggs laid throughout the experiment as a Poisson-distributed variable with a log link function. Diet and plasticity group were included as fixed effects. Block, replicate, storage box, and isogenic line were included as random effects.

To test if the amount of ingested food differed between high and low plasticity lines, the amount of food ingested over the length of this experiment was calculated for each food/line pairing. Since the food intake data was not normally distributed, we square-root transformed the data, thereby improving the fit. The transformed food intake was used as a response variable and diet and plasticity group were included as fixed effects. Block, replicate, evaporation box, and isogenic line were included as random effects.

To test if food intake was a significant predictor of female fecundity, we used a generalised mixed effects linear model as described above. The sum of eggs laid throughout the experiment was the response variable, assuming a Poisson distribution with a log link function. Diet, plasticity group, and food intake were included as fixed effects. Block, replicate, storage box, and isogenic line were included as random effects.

All statistical analyses were performed in R (version 3.4.1). Plots were produced using ggplot2 ('tidyverse' package). All data and scripts are available in Figshare (DOI: 10.26180/18515291).

4.4 | Results

4.4.1 | Characterising plasticity across isogenic lines

To characterise differences in plasticity for female fecundity across low (5%) and high (100%) dietary protein concentrations, we measured the number of eggs laid during days five to seven of adult life across ten isogenic lines. The amount of yeast in the food has an effect on the amount of eggs laid, with increasing yeast positively correlating with amount of eggs laid (Figure 1A, Table 1). We standardised the number of eggs laid in the 5% diet to 100% diet then used these values in our linear mixed effects models. We extracted the BLUPs from our models, which estimate how genotypes rank relative to each other and the largest BLUP, after filtering for lines that produced very few eggs overall, were chosen as the low plasticity lines (Figure 1B). We then used these chosen lines to identify the proximate mechanisms underlying differences in plasticity. The five genotypes with the smallest BLUP, which indicates the highest change in slope relative to the mean, were chosen as the high plasticity lines.

Table 4: Differences in relative fecundity across diets when compared to the standard diet. Number of eggs produced standardized to the 100% diet was fit in a MCMCglmm model taking Diet into account as a fixed effect.

Diet	Mean	Lower 95% CI	Upper 95% CI	p-value
5%	0.1583	0.1271	0.1892	<5e-04 ***
100%	0.4332	0.391	0.4714	<5e-04 ***

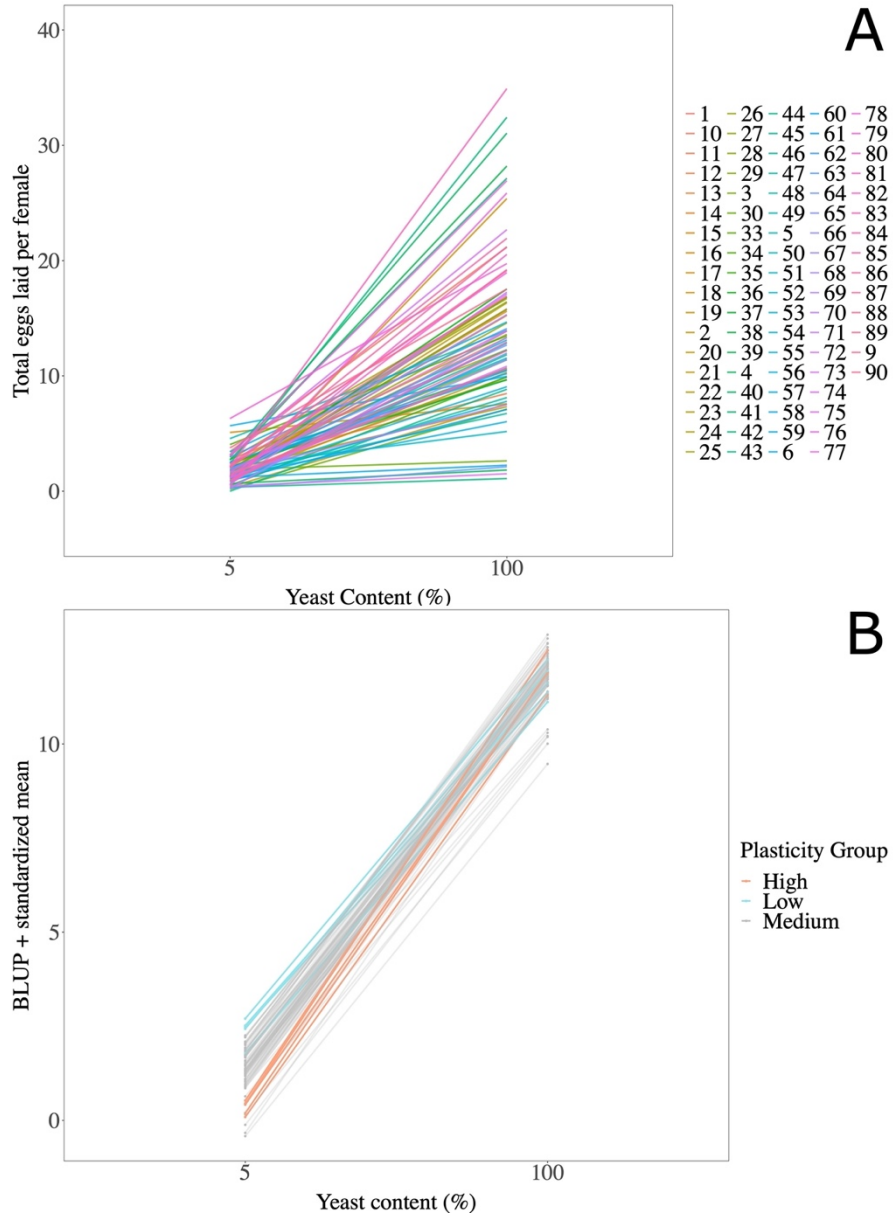


Figure 1: Isogenic lines chosen for their high or low level of plasticity for egg production between the standard and the 5% yeast content diets. A) Isogenic lines were subjected to diets with different yeast content and the number of eggs laid during days 5 to 7 were counted. Each colour represents a different isogenic line. B) After filtering out lines that laid less than three eggs across all diets, the BLUP scores plus the standardized means for each isogenic line was used to identify five isogenic lines with the highest plasticity (light salmon) and five lines with the lowest plasticity (cadet blue) for relative fecundity in response to diet. All other isogenic lines are in grey.

4.4.2 | Ovariole number does not explain differences in plasticity

We first tested whether the differences in response to diet between high and low plasticity isogenic lines resulted from differences in ovariole number. We did not find evidence for differences in the number of ovarioles between plasticity groups (Figure 2, χ^2 = 1.3248, p-value = 0.2497). This indicates that differences seen in nutritional plasticity for fecundity are not due to differences in ovariole number between isogenic lines.

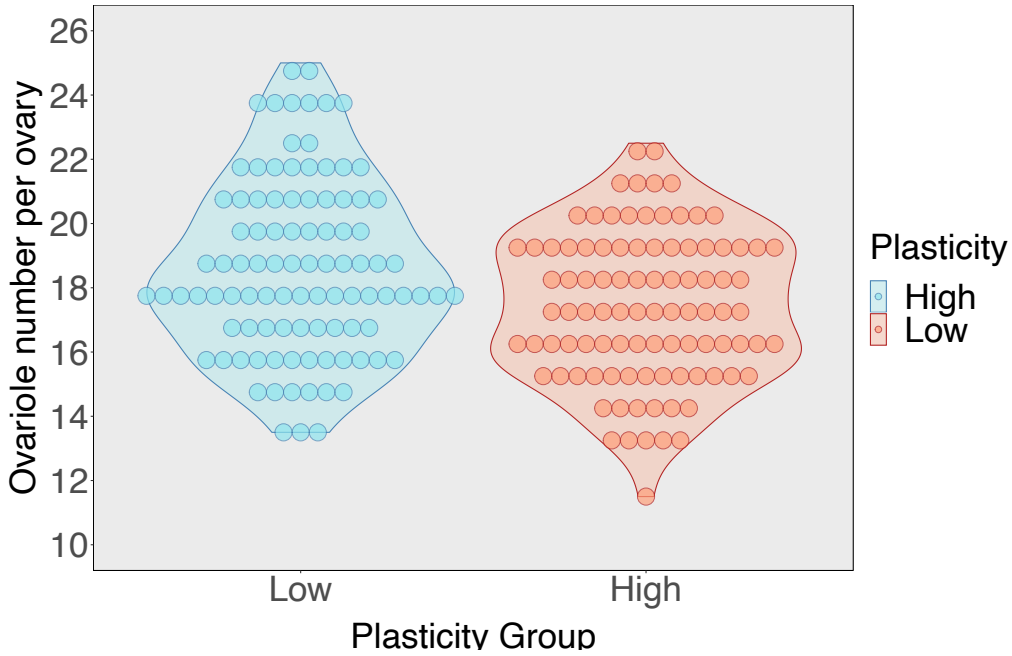


Figure 2: Ovariole number in isogenic flies with different levels of plasticity. Number of ovarioles per ovary in flies from low (cadet blue) or high (light salmon) plasticity groups. Points represent the average number of ovarioles per ovary for each female sampled.

4.4.3 | Differences in egg laying and food intake

Groups of five female flies from each of the high and low plasticity isogenic lines were placed in vials filled with apple juice/agar medium. Into the cotton flug of each vial, we inserted 3 glass microcapillaries filled with holidic medium containing either 5% or 100% of the total protein of the standard holidic diet (Piper et al., 2014). Thus, the flies' only source of protein was delivered via liquid medium in the capillaries, which could therefore be quantified. We allowed females to feed and to lay eggs for seven days.

As expected, the number of eggs laid was influenced by the protein concentration in the capillaries (Figure 3A, Table 3, Supplementary table 1), but this depended on the plasticity grouping. On average, high plasticity lines laid more eggs than the low plasticity lines across both diets. In addition, high plasticity lines showed a steeper response in the number of eggs laid in response to the protein concentration in the diet than the low plasticity lines, resulting in a significant interaction term between diet and plasticity group (Figure 3A, Table 3). Furthermore, the fact that these lines retained either high or low plasticity on the holidic medium (this experiment) suggests that this grouping method is accurate to ascertain plasticity across diet types and lines.

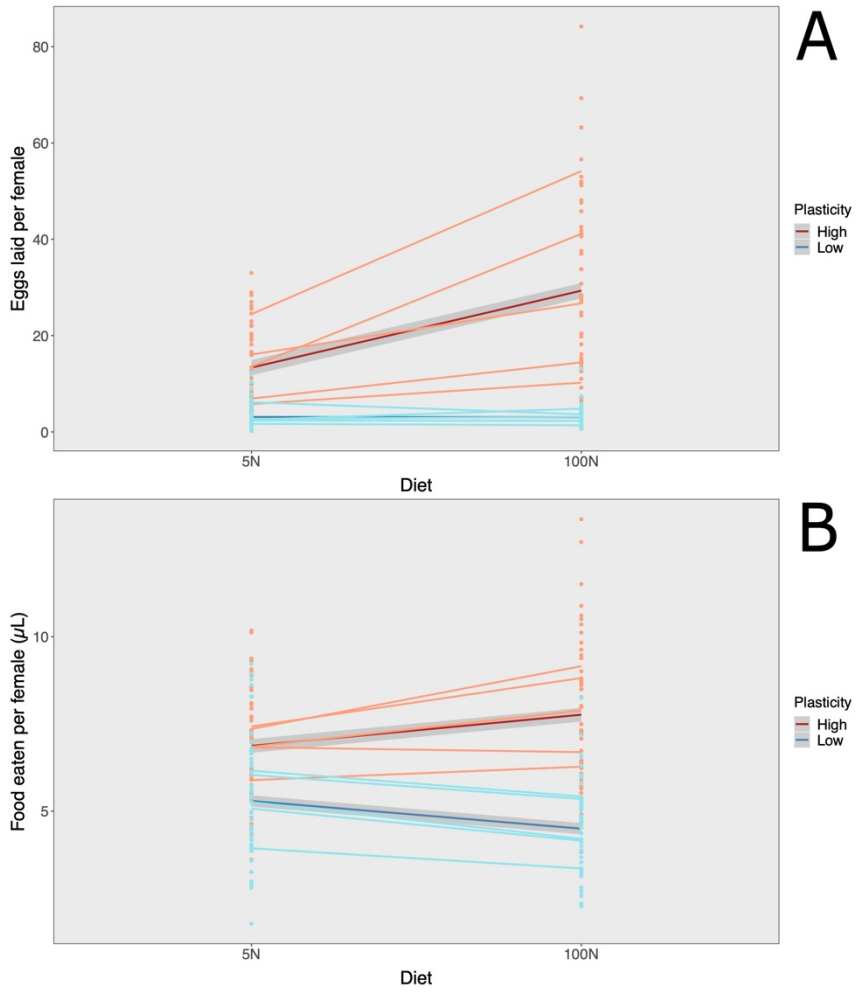


Figure 3 – Food intake and egg laying behaviour in isogenic lines with either high or low nutritional plasticity. A) Eggs laid per female in diets containing 5% (5N) or 100% (100N) protein. B) Food intake per female in diets containing either 5% (5N) or 100% (100N) protein. Points represent data from each replicate vial/diet/line. Salmon-coloured points and lines are high plasticity lines and cadet blue points and lines are low plasticity lines. Darker, bold lines with standard error represent the average for each plasticity group.

The concentration of protein in the diet also had a significant impact on food intake (Table 3). The high plasticity lines ate significantly more food than the low plasticity lines, and this difference was more apparent on the high protein diet (Figure 3B, Table 3, Supplementary table 1). Furthermore, the high plasticity lines increased their food intake with increasing protein content of the diet, while the low plasticity lines reduced their food intake with increasing dietary protein concentration, resulting in a significant interaction between diet type and plasticity group for food consumption (Figure 3B, Table 3). This suggests that high and low plasticity lines differ in the way they regulate their food intake in response to the protein content of the diet.

Table 3 – Manipulating diet across plasticity groups (high or low plasticity) alters egg laying and food intake. Egg laying data was fit with a generalized mixed effects model, whereas food intake was fit with a linear mixed effects model. * 0.05 < P < 0.01, ** 0.01< P < 0.001, *** P < 0.001

Egglay			
Term	Chi-Square	Df	P-value
Diet	1411.086	1	<2.2e-16 ***
Plasticity Group	31.255	1	2.263e-08 ***
Diet*Plasticity Group	317.383	1	<2.2e-16 ***
Model			
# of Eggs ~ Diet*Plasticity Group + (1|Block) + (1|Box) + (1|Replicate) + (1|Line)			
Ingestion			
Term	Chi-Square	Df	P-value
Diet	4.8563	1	0.02755 *
Plasticity Group	17.4365	1	2.971e-05 ***
Diet*Plasticity Group	133.0922	1	<2.2e-16 ***
Model			
Food Intake ~ Diet*Plasticity Group + (1|Block) + (1|Box) + (1|Replicate) + (1|Line)			

4.4.4 | Different strategies to achieve plasticity

To understand if variation in egg laying plasticity was due to the amount of food eaten or ability to utilise nutrients more efficiently, we next examined the relationship between amount of protein ingested and the number of eggs laid across plasticity groups. Overall, the number of eggs laid increased with protein ingested, suggesting that amount of protein ingested is a reasonable predictor of fecundity (Figure 4, Table 4). The interaction between protein ingested and plasticity group was also significant, showing that high and low plasticity lines vary in the relationship between the number of eggs laid and the amount of protein ingested (Figure 4, Table 4). The number of eggs laid increased faster with each nM of nitrogen ingested in high plasticity lines when compared to low plasticity lines for the same amount of protein ingested; this is observed by the higher slope for the high plasticity lines (Figure 4, Table 4, Supplementary Table 1). These results suggest that food intake alone

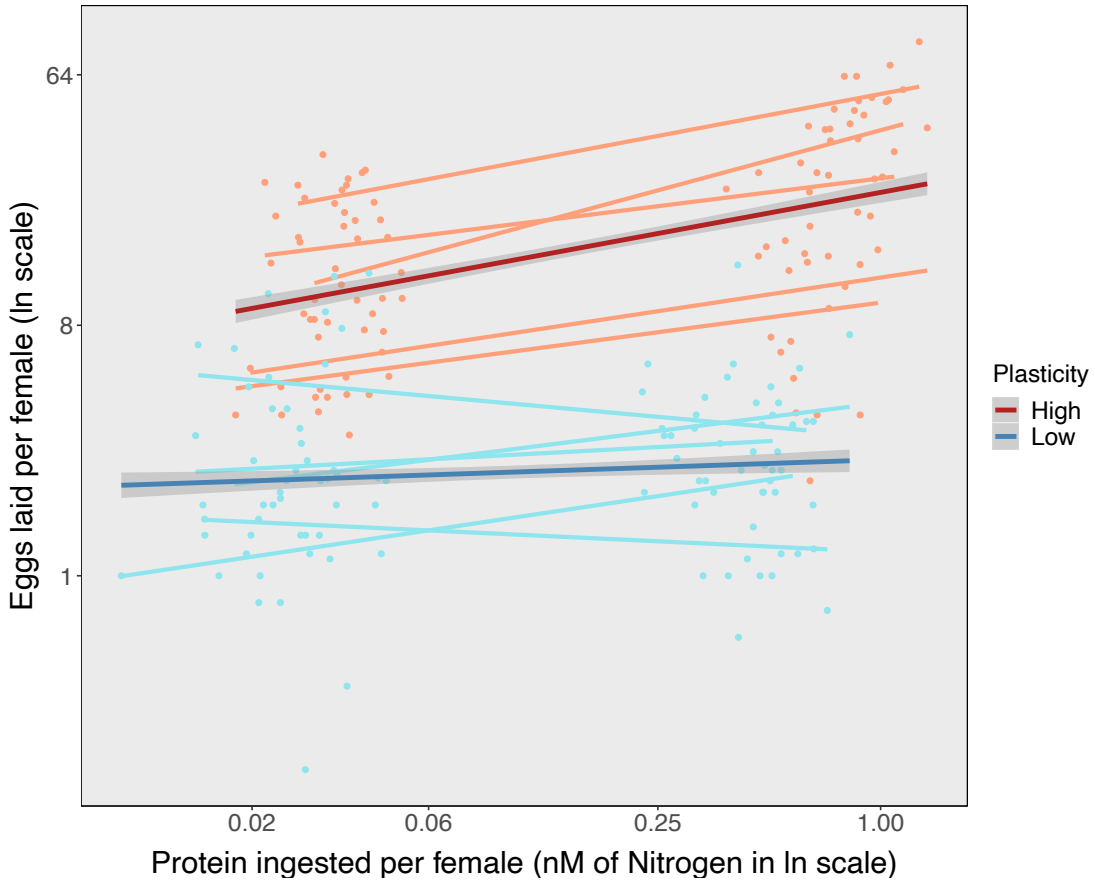


Figure 4: Relationship between number of eggs laid and amount of protein ingested. High and low plasticity lines subjected to diets differing in protein content were allowed to lay eggs for 7 days. Blue lines represent low plastic lines, orange lines represent high plastic lines. Darker lines represent the average across lines within the same plasticity group. Both axes are represented in ln scale.

cannot explain the difference in plasticity between high and low plasticity lines, and that other mechanisms such as food absorption and assimilation also contribute to the response.

Table 4 – Effect of amount of food eaten on egg laying behavior when manipulating diet and plasticity (high or low plastic lines). A generalized mixed effects model was fit to test the effect of diet, food ingestion and plasticity on egg laying. * 0.05 < P < 0.01, ** 0.01< P < 0.001, *** P < 0.001

Term	Chi-Square	Df	P-value
ln(Protein ingested)	253.621	1	<2.2e-16 ***
Plasticity Group	24.393	1	7.85e-07 ***
ln(Protein ingested) * Plasticity Group	96.198	1	<2.2e-16 ***
Model			
ln(# Eggs) ~ ln(Protein ingested) * Plasticity Group+ (1|Block) + (1|Box) + (1|Replicate) + (1|Line)			

4.5 | Discussion

Modulating the plastic response of life history traits to nutrition is vital for an organism to persist through changes in their environment. The relative contribution of variation in the proximate mechanisms that regulate plastic responses to diet is poorly understood. Here, we used isogenic lines of *D. melanogaster* that differ in their egg laying plasticity to understand how ovariole number, food intake, and protein-to-egg conversion efficiency impacted fecundity, a key trait correlated with fitness. We observed that differences in plasticity for egg laying are not due to ovariole number differences, but result from higher food intake and also a higher capacity to convert the protein into eggs in the high plasticity lines. This research helps us understand how proximate mechanisms may generate variation in plasticity for fecundity.

Ovariole number is a significant predictor of fecundity in *Drosophila*, as it caps the maximum number of eggs that can be produced in optimal conditions (David, 1970). Differences in ovariole number have been studied extensively between *Drosophila* species, and it is known that environmental factors such as nutrition and temperature also regulate

ovariole number (Markow & Grady, 2008; Hodin & Riddiford, 2000; Bergland et al., 2008). Recent studies have uncovered the role of genetic mechanisms of plasticity in generating variation in ovariole number, such as differences in insulin signalling during developmental stages, induced by poor diet, result in changes in total ovariole number (Mendes & Mirth, 2016). It is possible that the impact of nutrition during development affects more than just ovariole number, or mayhap that differences in ovariole number in animals reared on standard diets can be buffered by genetic differences in other traits such as egg production rate. Our study shows, however, that genetic differences in nutritional plasticity for fecundity within a species are not achieved by differences in ovariole number.

Previous studies have shown that flies regulate their food intake by choosing to eat different diets to achieve the nutritional targets that maximise life-history traits, such as eating salt-rich diets to increase fecundity (Walker et al., 2015), by eating intermediate protein to carbohydrate ratios to minimise development time (Rodrigues et al., 2015), or even modulating survival to infection by eating lower protein to carbohydrate ratio diets (Ponton et al., 2019). However, little is known about the mechanisms through which these changes occur. Our study highlights an important role of food intake in generating variation in nutritional plasticity in fecundity.

Nevertheless, variation in food intake does not account for all of the differences in nutritional plasticity in fecundity observed across the high and low plasticity lines in the current study. Especially on the low protein diet, we found that high and low plasticity lines showed little difference in food intake, but significantly larger differences in the number of eggs laid. This suggests that other post-ingestive mechanisms, such as amino acid absorption or nitrogen retention, underlie genetic variation in this trait. Previous studies using *Pieris rapae* larvae that fed on different plants showed that larvae differ in their amino acid absorption depending on the plant on which they feed (Slansky & Feeny, 1977). Variation in the efficiency of nutrient absorption and/or assimilation can also impact growth, development, and longevity (Patt et al., 2003; Min et al., 2007). Differences in diet composition might also affect nutrient absorption and/or assimilation; for example, holidic diets have crystalline free amino acids, which are more easily absorbed by the gut then the protein content found in standard yeast-potato-dextrose medium (Piper et al., 2014). Future studies exploring nutrient absorption, assimilation, and retention as potential other sources of variation in plasticity for egg laying would provide important new insights into what generates differences in plastic responses to diet.

A complementary, but powerful, approach that could provide further insight into the proximate mechanisms that generate differences in nutritional plasticity for fecundity would

be through the use of Genotype Wide Association Studies (GWAS) in isogenic panels, such as the *Drosophila* Genetic Reference Panel (DGRP), to identify genes that contribute to differences in food intake and nutrient assimilation. This approach has been used successfully to identify loci contributing to variation in thermal plasticity in body size (LaFuente et al., 2018). Other studies have found genes associated with traits such as starvation resistance, olfactory behaviour, mean food intake, and body mass composition that increase variation in plasticity when genotypes are exposed to different diets (Nelson et al., 2016; Sambandan et al., 2008; Garlapow et al., 2015). An approach similar to this could be applied to the isogenic panel used for this study in order to uncover new genes associated with the differences in intake or nutrient assimilation observed.

To further understand nutritional plasticity for fitness-related traits, it would be of great value to examine plasticity in male fecundity in these isogenic lines. Differences in fecundity between sexes could be a by-product of sexually dimorphic gene expression. Previous studies have uncovered genetic variation in male and female dietary requirements, which leads to differences in feeding behaviour between the two sexes (Camus et al., 2017). In isogenic lines, where there is a high inbreeding coefficient and allele fixation rate, gene expression might be severely skewed towards one sex across diets, which can lead to detrimental gene expression for the other sex (Connallon & Clark, 2011). This could potentially lead to differences in plasticity, and through the use of GWAS studies, one can uncover loci associated with sexually dimorphic gene expression and also associated with variation in plasticity.

Variation in plasticity can be achieved in a number of ways. Here, we show that variation in nutritional plasticity for fecundity is not explained by differences in ovariole number, but due to differences in food intake and the ability to convert ingested nutrients into eggs. Further research into the potential loci responsible for creating these differences will provide insight into how variation in plasticity can be achieved. Furthermore, this study enhances the importance of understanding the proximate mechanisms that generate variation in plasticity, since these will regulate the fitness-related traits that will ultimately contribute to species' persistence and adaptation.

4.6 | Acknowledgements

We would like to thank members of the Mirth and Piper labs, past and present, for their help throughout this project. In particular, we would like to thank Tahlia Fulton for their help in finding the best conditions for setting up CAFE assays, and providing valuable advice

on feasible number of replicates one human can handle. We would also like to thank Brendan Houston for their help in recording data on paper over weekends. This research was funded by ARC (DP180103725) for CMS, ARC (FT170100259) for CKM, and ARC (FT150100237) and NHMRC (APP1182330) for MDP.

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4.8 | Supplementary Figures & Tables

Supplementary Table 1: Estimated marginal means for number of eggs produced and food intake, and estimated marginal trends for number of eggs produced as a function of protein ingested. The group column represents statistically significant groups that differ from each other with p-value <0.05.

Egglay				
Diet	**Plasticity Group**	**emmean**	**SE**	**group**
100N	Low	1.16	0.228	1
5N	Low	1.20	0.228	1
5N	High	2.46	0.226	2
100N	High	3.21	0.226	3

Food Intake				
Diet	**Plasticity Group**	**emmean**	**SE**	**group**
100N	Low	2.09	0.132	1
5N	Low	2.29	0.132	2
5N	High	2.62	0.132	2
100N	High	2.75	0.132	3

# Eggs ~ Protein ingested				
	Plasticity Group	**emtrend**	**SE**	**group**
	Low	0.0515	0.0138	1
	High	0.2320	0.0126	2

Chapter 5 | General Discussion

“Somewhere, something incredible is waiting to be known.”

By Carl Sagan

In this thesis, I explored how nutrition can impact female fecundity in *D. melanogaster*. I began by exploring how developmental processes that regulate fecundity can be modified by the environment to generate plastic responses to individual amino acids (Chapter 2). Next, I characterized variation in nutritional plasticity for fecundity in a natural population and observed if selection for new nutritional environments can impact the evolution of plasticity (Chapter 3). Finally, I dissected the proximate mechanisms underlying the genetic differences in nutritional plasticity for fecundity seen in natural populations (Chapter 4). In this fifth, and final chapter, I will review the main findings of this work and indicate possible future avenues of research that could improve our understanding of how plasticity can be a key instrument to generate variation to allow organisms to persist in their environments and to create the myriad forms of life seen in our world.

5.1 Genetic mechanisms generate plasticity in fecundity in response to amino acids.

Numerous studies have shown that female fecundity varies greatly in response to changes in nutritional conditions (Wallin et al., 1992, Behmer et al., 2001; Lee et al., 2008; Piper et al., 2014; Armstrong et al., 2014; Green and Extavour, 2014; Hodin and Riddiford, 2000). In particular, protein is critical for this life-history trait (Piper et al., 2014). However, research has not yet addressed which components of protein, i.e. which amino acids, affect female fecundity. Furthermore, although it is known that certain cellular signalling pathways sense amino acids (Sonenberg & Hinnebusch, 2009), it is not known if these pathways are equally sensitive to all amino acids or if their sensitivity differs among them.

The work presented in this thesis is the first to identify two different types of responses to amino acid deprivation in regulating female fecundity. The first response includes amino acids that, when absent from the diet, result in a rapid decline in egg production and greatly reduced numbers of eggs laid over time (Chapter 2). On the other hand, when the amino acids methionine and histidine are absent from the diet, this results in a slower decline in egg production and a more moderate reduction in eggs laid. Thus, the extent of plasticity in fecundity greatly depends on the dietary composition of a food source; depending on which amino acid is absent, the response in egg laying can vary greatly. This highlights the importance of individual nutrients in determining the plasticity of life-history traits.

Both the TOR and GCN2 amino acid sensing pathways are known to impact egg production in response to protein (Armstrong et al., 2014; LaFever et al., 2010). Our work

contributes to the existing literature by demonstrating that differences in the response of egg laying to single amino acid deficiencies depends on TOR signalling. This leads us to hypothesize that TOR signalling is more sensitive to amino acids that generate a fast decline in egg laying. In contrast, we propose that TOR signalling is less sensitive to amino acids that generate a slow decline in egg laying.

Exome-matching studies show that flies, and other animals, require each amino acid in differing proportions (Piper et al., 2014, Gómez Ortega et al., 2021). Moreover, the two amino acids whose deprivation caused slow decline in egg laying, histidine and methionine, are the two most limiting amino acids in yeast, the major protein source for fruit-flies (Gómez Ortega et al., 2021). In principle, the TOR pathway might show reduced sensitivity to these amino acids as an adaptation to tolerate their deficiency in yeast. Further research about whether methionine and histidine have these effects in other organs would help elucidate if pathways are equally sensitive across organs or if each trait is regulated differently. It is known that organs respond differently to hormone signalling (Koyama et al., 2013; Koyama & Mirth, 2018; Mirth & Shingleton, 2012), so it would not be surprising if this would be the case in nutrient sensing pathways.

In addition to our insights on differences in the sensitivity of TOR signalling across amino acids, we also deepened our understanding of the role for GCN2 in regulating oviposition. These findings support those of previous studies, which identified a potential role of GCN2 in regulating oviposition in response to protein (Armstrong et al., 2014). This previous study concluded that in low protein conditions, GCN2 becomes active in the fat body, which results in the inhibition of TOR signalling and consequently inhibition of ovulation (Armstrong et al., 2014). The study by Armstrong et al. (2014) identified the potential role for GCN2 to be involved in ovulation, it did not provide definitive evidence, since this effect on ovulation was a consequence of GCN2 activity increasing by the lack of amino acids in the fat body, which triggers TOR signalling to be inhibited, resulting in an inhibition of ovulation. In my study, I provided evidence that in the absence of GCN2 across the whole organism, ovulation is reduced both in normal nutritional conditions and in specific amino acid starvation conditions, independent of TOR signalling activity.

The role of amino acid sensing pathways in regulating germ stem cells in the ovary has been characterized previously (Armstrong et al., 2014; LaFever et al., 2010). However, this was only done in the presence or absence of protein as a macronutrient. The role of the GCN2 and TOR pathways at regulating other stages of egg development in response to different amino acids was not yet fully characterized. We used previous characterisations on the activity of these pathways in the ovary to propose hypotheses based on whether

GCN2 or TOR signalling were responsible for differences in sensitivity to amino acids. In this work, I uncovered the role of TOR in regulating stages characterized by protein intake into the oocyte, the vitellogenic stages, whereas GCN2's role was primarily in reducing oviposition frequencies. While these results are important, my manipulations inhibit TOR or GCN2 signalling throughout the whole body, providing little information on what tissues are necessary for this response. Further studies using tissue-specific manipulations, including manipulating TOR and GCN2 in specific cells of the ovary, would help uncover the role of amino acid sensing pathways in regulating female fecundity in response to specific amino acids.

5.2 | Plasticity in fecundity and its potential to evolve

When environments change, plasticity is the first buffer against the immediate effects of stress (Sgrò et al., 2016; Chown et al., 2007). Plasticity is thought to allow animals to delay the negative effects of stress and diminish the risk of extinction in the face of rapid environmental change, allowing for genetic adaptation to occur in the longer-term (Chown et al., 2010; Bonamour et al., 2019). Variation in plasticity is, therefore, crucial to allow a population to persist against changing environments.

This variation in plasticity is common and studied in many traits (Narum et al., 2013; Chakraborty et al., 2020). Plasticity in fecundity is especially important, since it is tightly correlated with fitness and influenced greatly by diet, one of the components of the environment subject to major changes (Lee et al., 2008; Armstrong et al., 2014). However, research has not yet addressed how variation in plasticity for fecundity within a population might contribute to longer-term responses to nutritional stress present in new environments. If a population harbours variation in plasticity for fecundity, does plasticity evolve when submitted to diet-related selective pressures?

My findings, described in Chapter 3, highlight that even when there is genetic variation in plasticity for fecundity in a population, plasticity itself does not necessarily evolve. This might be a result of the distribution of plastic responses in our population and / or the type of selection imposed. Genetic variation in plasticity within a population generates distinct distributions of reaction norms across individuals (Frankino et al., 2019; Dreyer et al., 2016). Theoretical studies suggest that these distributions of reaction norms can shape a population's response to selection, since the type of distribution determines if plasticity can be altered and evolve with environmental change (Dreyer et al., 2016). This theoretical approach to plasticity has been validated for wing-body scaling relationships (Frankino et

al., 2019). In my study, we cannot know the type of selective pressure to which our populations were adapting. However, further modelling of the distribution of reaction norms in my study would prove useful to understand if it supports the theory described in Dreyer et al., (2016).

In my study (Chapter 3), selection was applied to the larval stages only. Previous studies that used experimental evolution to test the effect of different nutritional environments during the larval stages have shown varying effects on adult fecundity, from no change to a decrease in fecundity (May and Zwaan, 2017; Zajitschek et al., 2017; Kolss et al., 2009; Klepsatel et al., 2020). While these studies did not focus on plasticity, knowing the distribution of reaction norms in the original population would be useful to ascertain if fecundity suffered little change due to the original distribution of reaction norms. Furthermore, in these studies populations adapted to poor nutritional conditions for over 60 generations, rather than the 32 generations of my study. This additional time to adapt might have resulted in evolved changes in nutritional plasticity in fecundity. Future work on adapting flies to poor diets at different stages of the life cycle would reveal the stage in which adaption to diet is most likely to result in evolved plastic responses in fecundity. Alternatively, continuing selection on the lines already established and used in my study could prove to be a good strategy to understand if the length of the selection period is important for plasticity to evolve.

Finally, given that we know the distribution of reaction norms for nutritional plasticity in female fecundity for our panel of isogenic lines, a potential research avenue could be to sequence the genome of each isogenic line, and then to use these lines to generate a new outbred population for which the source genotypes are fully known. This new outbred population could then be adapted to a range of dietary conditions that might be expected to change plasticity. This would allow for a deeper insight not only into what genetic regions are involved in the evolution of plasticity, but also how selection shapes plasticity and in turn, allows populations to persist through changing environments.

5.3 | Proximate mechanisms generating variability in plasticity

The invasive species *Drosophila suzukii*, initially discovered in 2008 and now widely distributed throughout the world, shows differences in its plasticity for behavioural and physiological traits relative to its close relatives (Little et al., 2020). *D. suzukii* is one of the few species of *Drosophila* that colonizes ripe instead of rotting fruit, matched only by its relatives *D. biarmipes* and *D. subpulchrella,* making it an important agricultural pest (Cini et

al., 2012; Durkin et al., 2021). Plasticity enables *D. suzukii* to persist across a wide range of environmental conditions, and is thought to underlie its success as an invasive species (Little et al., 2020). This species is more sensitive to volatiles produced by ripe fruits (Keesey et al., 2015), more resistant to cold-stress (Jakobs et al., 2015), and its body size is less sensitive to levels of protein in the diet (Soares et al 2017), allowing it to colonize environments that are inhospitable for other *Drosophila* species. *D. suzukii* is a crucial example of why differences in plasticity for key life history traits determine whether species can colonize and persist in new environments.

Variation in plasticity is not always achieved through changes in the same mechanisms (Beldade et al., 2011). Plasticity can be regulated by genetic mechanisms, as mentioned in section 5.1, and also by other proximate mechanisms such as morphological differences, feeding behaviour, and nutrient absorption or assimilation (Chakraborty et al., 2021, Chap 4). Changes in genetic mechanisms can result in changes in multiple proximate mechanisms, since traits like feeding or oviposition behaviour and ovary size are known to be regulated by allelic variation in genes and pathways that regulate a wide range of traits (Schlichting & Smith, 2002). Importantly, understanding how proximate mechanisms, including genetic, behavioural, morphological, and physiological mechanisms, drive the major differences in plasticity becomes vital to understanding how plasticity for fecundity can vary.

My work described in Chapter 4 focused on whether morphological differences in ovariole number, feeding behaviour, and efficiency of nutrient use generates variation in plasticity for fecundity. Ovariole number dictates the number of eggs an organism is able to produce at any time (David, 1970), which ultimately impacts an organism's fitness. This trait has been shown to be sensitive to nutrition and it constrains the upper limit of egg production such that ovaries with more ovarioles can produce more eggs (Bergland et al., 2008). Food intake is also known to have important impacts on life-history traits, and it is carefully regulated to ensure animals are able to achieve their nutritional requirements to maximise important life-history traits like development time, body size, and fecundity (Simpson & Raubenheimer, 2009, Simpson & Raubenheimer, 1995). Finally, food absorption or assimilation is also a heavily regulated trait that impacts organ function, and therefore, traits related to fitness (Sibly, 1981). Even when organisms eat the same amount of food, their ability to assimilate nutrients might differ resulting in differences in phenotypes like body size (Urabe and Watanabe, 1991; Neat et al., 1995). Which of these mechanisms contributes the most to difference in nutritional plasticity for fecundity is poorly understood.

My work compared the proximate mechanisms that contributed to variation in nutritional plasticity by examining lines which I identified as having either high or low nutritional plasticity for fecundity. On a diet poor in protein, flies from high and low plasticity lines ate similar amounts, but high plasticity lines laid more eggs. This could stem from germ cell division rates being more or less sensitive to the presence of amino acids, which could result in high plasticity lines have a higher efficiency in post-ingestive mechanisms such as nutrient absorption or assimilation (Slansky & Feeny, 1977; Zanotto et al., 1995).

A useful avenue of future research would be to measure nutrient assimilation or perhaps how nutrients are allocated amongst all organs in these lines. Using isotope-labelled amino acids in the holidic diets would allow us to observe how these isogenic lines differ in the amount of isotope-labelled amino acids that get absorbed by their guts and how much is excreted, providing a mass balance equation. In addition, it should be possible to measure how amino acids are allocated across organs such as ovaries versus fat body.

While I was unable to address this in this thesis, another approach would be to identify alleles that underlie variation in plasticity. Through the use of sequencing technologies paired with tools such as Genome Wide Association Studies (GWAS), it is possible to find genetic regions or loci that are responsible for regulating these traits and/or contribute to the variation in plasticity. These strategies have been applied before for other isogenic panels in the context of myriad traits. Several QTLs have been associated with plasticity for body size in response to temperature using this approach (LaFuente et al., 2018). Approaches using isogenic panels and GWAS have also mapped genetic loci involved in variation in responses to diet in traits such as starvation resistance, body mass composition, and olfactory behaviour (Nelson et al., 2016; Sambandan et al., 2008). Because female fecundity is so tightly correlated with fitness, this would be a key trait to further understand how variation in plasticity can be generated and what potential genetic regions can be targeted by selection.

My work has focussed on the mechanisms generating variation in plasticity in fecundity and how variation in plasticity impacts the evolution of plasticity. While the insights generated by my work are important, these findings are just one small drop in an ocean of knowledge yet to be explored. Together with the current literature on the evolution of plasticity, and genetic, developmental, and behavioural mechanisms regulating traits in response to nutrition, my results highlight that variation in nutritional plasticity for fecundity can be regulated at several levels: amino-acid sensing cellular pathways, behavioural differences in food intake, or differences in post-ingestive mechanisms that result in differences in nutrient use. Further, while several authors have proposed that the way

plasticity varies within a population will determine if adaptation to poor diet will result in extinction or persistence of the population in question, we still have much to learn about the relationship between genetic variation in plasticity and evolved plastic responses. Understanding how these mechanisms are regulated, how populations vary in their plasticity, and what environmental factors impact them could prove to be fruitful opportunities of research.

5.4 | References

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