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The role of nutrition in mediating responses to climate change

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Table of contents

ABSTRACT

Ongoing climate change is affecting multiple aspects of the habitats of terrestrial organisms. To persist in their altered environment, animals must adjust through phenotypic plasticity or adapt through evolved changes. Temperature and nutrition are amongst the most common environmental challenges for small ectotherms and will become increasingly so under projected climate change. While plastic and evolved responses to thermal and nutritional stress during development have been often studied in isolation, not much is known about their combined effects and how these might change across life stages. Additionally, studies investigating the response of life history traits to the larval thermal and nutritional environment have used a limited number of diets that do not fully encompass the range of diets that insects can experience in nature. Furthermore, very little is known about adaptation to thermal and nutritional stress in combination, despite its importance for surviving climate change, and even less well understood is how evolution under thermal and nutritional stress can affect the plasticity of individuals. Given the importance of both plasticity and adaptation in determining a species ability to persist under climate change, enhancing our understanding of how animals respond to combined stress is of utmost importance.

The goal of this thesis was twofold: first, to characterize plastic changes of key life history and adult stress resistance traits in response to the combined effects of larval diet and developmental temperature; and second, to explore the evolutionary response of key life history traits after selection to suboptimal larval diet and high developmental temperature. I found that increased developmental temperature reduced the number of diets that optimized viability, development time, and size in *Drosophila melanogaster* and resulted in harsher trade-offs between traits. Additionally, I showed that, while the larval environment had carry-over effects on adult stress resistance, these effects were small. Changes in the energy stores of individuals between the time of eclosion and the stress assays indicated that the adult environment could alleviate the effects of combined thermal nutritional stress experienced in larval stages. When investigating evolutionary responses, I showed that the effect of adapting to elevated temperatures and poor diets simultaneously cannot be predicted from the results of adaptation to each stressor singly, and that, in some cases, co-adaptation can increase the response to selection. Additionally, I found that the effect of adaptation on plasticity depended on the trait studied, the selective pressure applied, and the rearing conditions. Finally, my results suggest a cross-over between genes involved in thermal and nutritional adaptation and genes controlling the plastic response to diet and temperature respectively.

The results from this thesis show that, in *D. melanogaster,* both plasticity and evolution have the potential to mitigate the negative impacts of thermal and nutritional stress. This thesis, while adding to the effort to understand responses to environmental stress, highlights the intricacy of the effects of stressful developmental conditions in insects, and emphasises that much more research is needed if we are to understand how species will cope with ongoing global change.

Publications during enrolment

Kutz, T. C., Sgrò, C. M. and Mirth, C. K. (2019) 'Interacting with change: Diet mediates how larvae respond to their thermal environment', *Functional Ecology*. doi: 10.1111/1365-2435.13414.

Alton, L. A., Kutz, T. C., Bywater, C. L., Beaman, J. E., Arnold, P. A., Mirth, C. K., Sgrò, C.M. and White C. R. (2020) 'Developmental nutrition modulates metabolic responses to projected climate change', *Functional Ecology*. doi: 10.1111/1365-2435.13663.

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 one original paper published in a peer reviewed journal and one submitted publication. The core theme of the thesis is evolutionary ecology. The ideas, development, and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the School of Biological Sciences under the supervision of Prof. Carla Sgrò and Dr. Christen Mirth.

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 chapter 2 and 3 my contribution to the work involved the following:

Thesis Chapter	Publication Title	Status	Nature and % of student contribution	Co-author name(s) Nature and % of Co-author's contribution*	Co-author(s), Monash student Y/N*
2	Interacting with change: Diet mediates how larvae respond to their thermal environment	Published	70%. Concept, data collection and analysis, and first manuscript draft.	Carla Sgrò, 15%: concept, edits to manuscript. Christen Mirth, 15%: concept, edits to manuscript.	No No
3	Resetting stress responses: the combined impacts of temperature and nutrition during larval development show only minimal carry over on adult stress resistance	Submitted	60%. Concept, data collection and analysis, and first manuscript draft.	Carla Sgrò, 20%: concept, edits to manuscript. Christen Mirth, 20%: concept, edits to manuscript.	No No

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

Student signature:  Date: 25/07/2021

The undersigned 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 signature:  Date: 25/07/2021

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Chapter 1 | General Introduction

1.1 | Impacts of climate change

The rapid warming of the atmosphere and the oceans since 1950 is causing environmental change at unprecedented rates. The latest projections predict an increase in global surface temperature of 3^{o}C for many scenarios by the end of the 21st century accompanied by an increased frequency of extreme weather events and altered precipitation patterns (IPCC, 2018; IPBES, 2019). Additionally, rapid environmental change is affecting most biological and ecological processes on Earth, including population distributions and dynamics, community structure, and ecosystem functioning (Scheffers et al., 2016). Species shifts tracking optimal habitat conditions both latitudinally (Vergés et al., 2014; Fossheim et al., 2015; Lehikoinen and Virkkala, 2016; Poloczanska et al., 2016) and altitudinally (Chen et al., 2009; Comte and Grenouillet, 2013; Freeman and Class Freeman, 2014; Wolf et al., 2016) are well documented for marine, freshwater, and terrestrial species. Changes in species distributions lead to high community turnover (Tingley and Beissinger, 2013; Sgardeli, Zografou and Halley, 2016) resulting in new ecosystem dynamics (Jankowski, Robinson and Levey, 2010; Garciá Molinos et al., 2016). In turn, altered species interactions can lead to trophic disruptions. For example, changes in species distributions led to the phenological mismatch between butterflies and their hostplants (Parmesan *et al.*, 2013). Disrupted trophic interactions are one of the main climate-driven threats to species and lead to species declines (Cahill *et al.*, 2012). For instance, the population of tawny owl from the Kielder Forest (UK) is declining because its main pray, the field vole, suffers from climate-driven dampening of population cycles (Millon *et al.*, 2014), and the seabird Cassin's auklet population, inhabiting the California Current Ecosystem, is declining because climate change is affecting coastal upwelling, thereby reducing habitat productivity and limiting Cassin's auklet's food resources (Wolf et al., 2010). Therefore, under climate change, organisms face challenging abiotic

(increased average temperatures, altered rain fall, floods, heat waves, increased frequency of extreme weather events, higher atmospheric CO_2 concentration, etc.) and biotic (altered ecosystem productivity, altered predator prey interactions, phenological mismatches, etc.) changes in their habitat which threaten their persistence.

Part of the reason that climate change is so problematic is that it affects all levels of the trophic cascade, and impacts multiple aspects of an organism's life history. Impacts range from affecting the quality and distribution of food resources, to altering life history traits relevant to fitness, to impacting stress resistance and the ability of populations to persist. To be able to persist in their environments, species must either adjust their phenotypes to the environment via plasticity and/or adapt to the new conditions through evolved changes (Hoffman and Parsons, 1991). Below I discuss i) the effects of climate change on plants and how those changes will affect primary consumers and the trophic web, ii) how changes in food resources will interact with increased temperatures to affect animals, and insects in particular, across life stages, and iii) how animals can respond to those changes via plasticity and/or evolution.

1.1.1 | Food availability and composition

Plant communities are vulnerable to climate change as temperature, water availability, drought, and CO_2 levels (which are key factors in plant growth, phenology, and productivity) are being rapidly modified by climate change (Morison and Morecroft, 2007). Climate change affects plant phenology, range shifts, altered species interactions, and overall system productivity (Pecl *et al.*, 2017). In addition, extreme or catastrophic weather events can abruptly and permanently change ecological systems, and are becoming increasingly frequent (Iglesias and Whitlock, 2020; Turner *et al.*, 2020). Together, these alterations have the potential to impact the quantity and quality of food available to primary consumers and affect energy transfer in the ecosystem (Gilbert *et al.*, 2014).

In addition to plant abundance, plant macronutrient composition will also be affected by climate change. In their review, DaMatta *et al.*, (2010) concluded that decreased protein and mineral content and altered lipid composition of crops are expected under elevated CO_2 conditions and warmer temperatures. In a more recent review, Rosenblatt and Schmitz, (2016) pointed out that CO_2 levels, temperature, and water availability can affect nutrient content in plants, which can in turn affect higher trophic levels in food webs. Additionally, a 2017 meta-analysis (Sardans *et al.*, 2017) found that increased CO_2 concentration decreased nitrogen content (which is an proxy for protein concentration; Lenhart, 2017) of leaves and roots of plants across many vegetation types, while drought increased nitrogen concentration. Another more recent meta-analysis found that non-structural carbohydrates in plants increased with elevated CO_2 concentration, but were reduced with drought and warming (Du, Lu and Xia, 2020). These findings suggest that the protein to carbohydrate (P:C) ratio of plants will likely decrease under climate change.

However, as Rosenblatt and Schmitz (2016) point out, climate variables often interact. For example, high CO_2 increased plant carbohydrate content, but, when temperatures increased, this effect disappeared (Zvereva and Kozlov, 2006). And in gymnosperms, protein content was not affected by either increased CO_2 or temperature on their own, but decreased significantly when plants were exposed to both factors simultaneously (Zvereva and Kozlov, 2006). Additionally, as drought stress increases, the relative water content of leaves usually decreases (English-Loeb, Stout and Duffey, 1997), potentially limiting the ability of herbivores to exploit increased foliar protein (Scriber, 1977; Huberty and Denno, 2004). Finally, all of these effects can be nonlinear and are not universal; they can vary in sign and magnitude depending on species, ecosystem type, tissue type, growth stage, and severity and duration of the environmental stress (Rosenblatt and Schmitz, 2016).

Despite how case-specific the effect of environmental variables on primary producers may be, it seems that under higher CO_2 and warmer temperatures plant composition will generally tend towards lower protein to carbohydrate ratios in many environments (Myers *et al.*, 2014;

Rosenblatt and Schmitz, 2016; Sardans *et al.*, 2017; Zhu *et al.*, 2018; Asseng *et al.*, 2019; Du, Lu and Xia, 2020). For example, under elevated temperatures and reduced water, amino acid content of fruits and other above-ground plant organs is predicted to decrease (Wu *et al.*, 2019; Gutiérrez-Gamboa *et al.*, 2020), while carbohydrate content is predicted to increase (Moretti *et al.*, 2010; Rosenblatt and Schmitz, 2016). This would result in changes to the abundance and composition of the yeast communities growing on them, because amino acids in fruits are the primary source of nitrogen for yeast (Wu et al 2019; Gutierrez-Gambo et al 2021). As a result, herbivorous and frugivorous animals will be exposed to higher carbohydrate concentrations in their diet, possibly impacting their abundance and hence affecting higher trophic levels.

Indeed, shifts in plant nutrient composition can have far-reaching impacts on food webs by altering herbivore feeding rates, assimilation efficiencies, and growth rates (Koricheva, Larsson and Haukioja, 1998; Zvereva and Kozlov, 2006; Dijkstra *et al.*, 2012). These changes can permeate up the trophic cascade in the form of altered energy transfer and altered trophic interactions, and result in the de-stabilisation of the food web (Gilbert *et al.*, 2014). This makes it all the more important to consider how climate change will alter plant nutrient composition when trying to understand animals' responses to climate change.

1.1.2 | Species vulnerability to combinations of environmental stressors

Because increasing temperature is one of the main effects of climate change, assessments of vulnerability to climate change often focus on the thermal limits of animals, or their ability to tolerate thermal extremes (Deutsch *et al.*, 2008; Somero, 2010; Bush *et al.*, 2016). However, thermal tolerance will also be impacted by other environmental stressors, such as nutritional stress (Andersen et al., 2010; Sisodia and Singh, 2012; Kristensen et al., 2016). Because environmental stressors can interact to shape animal responses (e.g., Crain, Kroeker and Halpern, 2008; Hecky *et al.*, 2010; Holmstrup *et al.*, 2010; Gunderson, Armstrong and Stillman, 2016; Alton and Franklin, 2017), it is necessary to consider them in combination.

In aquatic environments, stressor interactions have been studied systematically (Crain, Kroeker and Halpern, 2008; Deutsch *et al.*, 2015; Boersma *et al.*, 2016) and have often been found to interact synergistically on animal fitness. For instance, the growth of the copepod *Acartia tonsa* depended on the interactive effects of temperature and food quality such that, at higher temperatures, higher carbon to phosphorus ratio in food was necessary to meet the metabolic demands of the individuals (Malzahn, Doerfler and Boersma, 2016). Studies of stressor combinations in terrestrial systems is starting to be explored; however, their effects are still poorly understood (Kaunisto, Ferguson and Sinclair, 2016).

Food limitation is the most common stressor for animals in nature (Raubenheimer, Simpson and Tait, 2012; Cross *et al.*, 2015) and with climate change altering the abundance and composition of plants (Rosenblatt and Schmitz, 2016) it is likely that food availability and quality will become increasingly relevant under climate change and will interact with other climatic stressors such as thermal stress or water limitation. Therefore, it is important to understand how animals will respond to simultaneous changes in their thermal and nutritional environment in order to better assess the impacts of climate change on species.

1.1.3 | Impacts of thermal stress on insects

While interactions between elevated temperature and other climate change related stressors in terrestrial systems have only recently gained interest, literature on the individual effects of thermal stress on animal performance is abundant (e.g., Seebacher, 2005; Polsky and von Keyserlingk, 2017; Gangloff and Telemeco, 2018), especially for insects (e.g., Loeschcke, Krebs and Barker, 1994; Malmendal *et al.*, 2006; Fischer and Karl, 2010; Zizzari and Ellers, 2011; Piyaphongkul, Pritchard and Bale, 2012; Roitberg and Mangel, 2016).

Insects account for the majority of animal biodiversity, and fulfil many ecosystem functions such as nutrient cycling, pollination, seed dispersal, maintenance of soil structure and fertility, population control of other organisms, and they provide a major food source for other taxa (Scudder, 2017). As

small ectotherms, insects have low thermal inertia and limited capacity to thermoregulate (Huey *et al.*, 1999); thus, they are at greatest risk of changes in temperature. Ectotherm populations display upper and lower thermal limits that correlate with the latitude of their place of origin (Hoffmann, Sørensen and Loeschcke, 2003) indicating that their tolerance of heat and cold can limit their distribution. The means by which heat stress can cause damage in ectotherms are varied, including damage to mitochondria (Pörtner, 2010), neuronal circuit failures (Robertson & Money, 2012), reduced antioxidant enzyme activity (Mujahid et al., 2007), or compromised immune function (e.g. Karl et al., 2011). Further, the effects of thermal stress can be long-lasting; for example, bees can take days to recover sperm viability (Stürrup et al., 2013), and heat shock can have transgenerational effects on the red flour beetle *Tribolium castaneum*, (Eggert et al., 2015). Therefore, because insects are especially sensitive to heat stress, climate change can represent a threat to insect species by elevating average temperatures and increasing the frequency of heat waves.

Additionally, insects have complex life cycles and often juvenile stages occupy separate niches from the adults (e.g. Kingsolver *et al.*, 2011). For holometabolous insects, the larval stages are particularly susceptible to changes in temperature because behavioural avoidance of thermal stress is limited (Medina-Muñoz and Godoy-Herrera, 2005), and growth and survival depend greatly on the thermal conditions experienced during larval development (Kingsolver and Huey, 2008; Mirth and Shingleton, 2012). Further, the responses of insects to many environmental variables are stage dependent, meaning that the responses of larvae and adults to the same variable can differ (e.g., Frommel *et al.*, 2014) including the response to food limitation (Boggs and Freeman, 2005) and thermotolerance (Crill, Huey and Gilchrist, 1996; Zhang *et al.*, 2003; Galarza *et al.*, 2019; Moghadam *et al.*, 2019). Understanding the difference in the response to stress between different life stages is key for management strategies which should concentrate on protecting the most vulnerable life stage (Hodgson, Essington and Kaplan, 2016).

1.2 | Plastic responses to combinations of nutritional and thermal stress

While insects will face changes in their thermal and nutritional environment under climate change, they can respond to those changes by adjusting their phenotype through plasticity. Insects display plasticity for many traits, including life history (Bakker, 1959; Atkinson, 1994; Angilletta, Jr. *et al.*, 2004; Kozłowski, Czarnołęski and Dańko, 2004; Simpson *et al.*, 2004; Bradshaw and Holzapfel, 2008; Roeder and Behmer, 2014; Matavelli *et al.*, 2015; Gray, Simpson and Polak, 2018) and stress resistance traits (Crill, Huey and Gilchrist, 1996; Hoffmann, Sørensen and Loeschcke, 2003; Andersen *et al.*, 2010; Sobek *et al.*, 2011; Sisodia and Singh, 2012; Bauerfeind *et al.*, 2014; Luo *et al.*, 2015; Kristensen *et al.*, 2016; MacLean *et al.*, 2017; Henry, Overgaard and Colinet, 2020). The first part of my PhD explores the plastic responses of key life history traits (Chapter 2) and adult stress resistance traits (Chapter 3) to combinations of thermal and nutritional stress experienced during development.

1.2.1 | Plastic responses of key life history traits to changes in the larval thermal and nutritional environment

Plastic responses of important life history traits to increased temperature during development in insects have been well studied. For instance, development time decreases with increasing temperature (Atkinson, 1994; Atkinson and Sibly, 1997; Miller *et al.*, 2009), but in a non-linear manner, such that, temperatures close to the animals' critical thermal maximum prolong development time (Petavy *et al.*, 2001). In addition, elevated temperatures lead to lower larval viability (Angilletta, Jr. *et al.*, 2004; Kozłowski, Czarnołęski and Dańko, 2004), and smaller adult sizes (Atkinson, 1994; Angilletta, Jr., and Dunham, 2003).

In addition to the impacts of elevated temperature, insects will be faced with altered nutritional environments caused by climate change. Larval nutrition is also known to elicit plastic responses in life history traits in insects. For instance, low P:C ratios generally result in lower pre-adult viability, longer development time, and smaller body sizes in insects, including caterpillars (Simpson *et al.*,

2004; Roeder and Behmer, 2014) and Drosophilid fruit flies (Bakker, 1959; Bradshaw and Holzapfel, 2008; Matavelli *et al.*, 2015; Rodrigues *et al.*, 2015; Kristensen *et al.*, 2016; Silva-Soares *et al.*, 2017; Gray, Simpson and Polak, 2018).

Increasingly more attention is being given to the combined effects of temperature and nutrition on larval life history traits in insects (Kingsolver *et al.*, 2006; Lee and Roh, 2010; Lee *et al.*, 2015; Lemoine and Shantz, 2016; Kim, Jang and Lee, 2020). Research has shown that changes in larval nutrition can alter thermal sensitivity of insect growth and development. For example, for the caterpillar *Spodoptera exigua*, growth rate increased with increasing temperature but the difference in growth rate caused by temperature was more pronounced at P:C ratios around 2:1 or 1:1 than at higher or lower P:C ratios (Lee and Roh, 2010; Lemoine and Shantz, 2016). Further, mean larval growth rate in cabbage white butterfly was greater on a natural diet compared to an artificial diet when larvae were exposed to temperatures between 11-35^{o}C, but this was reversed at 40^{o}C (Kingsolver et al., 2006). More recently Kim, Jang and Lee, (2020) explored trait responses across a range of 6 temperatures and 8 diets varying in their P:C ratios, creating a large temperature-nutrient space for several larval and adult life history traits. They found significant interactions between temperature and nutrition for six of the eight traits studied, showing that the response to nutrition cannot be predicted without considering temperature and vice versa.

Larval nutrition can also change the temperature-size rule, which predicts that ectotherms should grow to be larger in colder environments. For instance, while the tobacco hornworm reached larger size at cooler rearing temperatures when fed their preferred host plant (high-quality host), following the temperature-size rule, this was reversed when fed a suboptimal host plant (low-quality host): caterpillars were largest at warmer developmental temperatures (Diamond *et al.*, 2010). In contrast, *Spodoptera exigua* larvae followed the temperature-size rule when fed low P:C ratio diets, but when fed balanced food, temperature had no effect on the dry pupal mass of this insect (Lee *et al.*, 2015).

The above studies indicate that temperature and nutrition interact to shape life history variation in insects; however, they used a limited number of diets and ignored the fact that diet can vary not just in macronutrient composition but also in the total amount of calories ingested. Thus, their interpretations are restricted to a small region of the nutrient space. Using a broader range of diets in combination with different temperatures is important to better understand the interactive effects of temperature and nutrition on life history traits because trait optima can vary significantly across the nutrient space (e.g., Lee *et al.*, 2008; Rodrigues *et al.*, 2015; Silva-Soares *et al.*, 2017). It is especially important to study a broad nutrient space given the uncertainty regarding changes in food availability and quality under climate change (Rosenblatt and Schmitz, 2016).

1.2.2 | Plastic responses of adult stress resistance traits to changes in the larval thermal and nutritional environment

In addition to shaping important life history traits, the thermal environment during insect development influences adult thermal stress resistance through a process known as acclimation (Hoffmann, Sørensen and Loeschcke, 2003). Exposing larvae to increased temperatures during development can carry over to the adult stage resulting in plastic increases in adult heat resistance (e.g. Crill, Huey and Gilchrist, 1996; van Heerwaarden, Kellermann and Sgrò, 2016; Kellermann, van Heerwaardenf and Sgrò, 2017; MacLean *et al.*, 2017; Zheng *et al.*, 2017) and exposure to cooler developmental temperatures can result in plastic increases in adult cold resistance (e.g. Bauerfeind *et al.*, 2014; MacLean *et al.*, 2017). There is little evidence of developmental temperature affecting other adult stress resistance traits. Development temperature seems to have non-linear effects on adult starvation resistance with intermediate temperatures leading to highest starvation resistance (Jang and Lee, 2018), and cooler developmental temperatures do not seem to influence adult desiccation resistance (Bauerfeind *et al.*, 2014; Krupp *et al.*, 2020).

The nutritional environment during insect development also shapes adult stress resistance traits (Andersen et al., 2010; Sisodia and Singh, 2012; Kristensen et al., 2016; Henry, Overgaard and Colinet, 2020). For example, when larvae are reared on low P:C diets, both starvation resistance

and cold resistance in adults increase (Andersen et al., 2010; Sisodia and Singh, 2012; Lee and Jang, 2014; Kristensen et al., 2016; Henry, Overgaard and Colinet, 2020). In contrast, adult heat resistance increases if larvae are fed a high P:C diet (Andersen et al., 2010; Sisodia and Singh, 2012; Kristensen et al., 2016). The effect of larval diet on adult dessication resistance is less clear. While some studies found that adult desiccation resistance increased when larvae developed on high P:C diets in *D. melanogaster* (Andersen et al., 2010; Kristensen et al., 2016) and *D. ananassae* (Sisodia and Singh, 2012), a more recent study (Henry, Overgaard and Colinet, 2020) found that adult desiccation resistance in *D. melanogaster* was reduced by high protein content in the larval diet. These conflicting results may be due, at least in part, to the limited number of diets used in these studies, as they may have sampled different parts of the nutrient space.

While many studies have focused on the individual effects of temperature and nutrition within and across life stages of insects, the two factors are tightly linked through an animal's metabolic rate and energy requirements, as relatively more energy is used to fuel growth with increasing temperature (Clissold and Simpson, 2015; Cross et al., 2015; Alton et al., 2020). As discussed in the previous section, several studies have shown that life history traits of insect larvae (Kingsolver and Woods, 1998; Kingsolver *et al.*, 2006; Stillwell *et al.*, 2007; Diamond *et al.*, 2010; Lee *et al.*, 2015; Kutz, Sgrò and Mirth, 2019; Chakraborty, Sgrò and Mirth, 2020) and adults (Kim et al., 2020) are affected by the interactive effect of temperature and nutrition. However, only one study (Jang and Lee, 2018) investigated the combined effects of temperature and nutrition on adult stress resistance traits. Jang and Lee (2018) were interested in the effects of the adult thermal and nutritional environment on starvation resistance and the larvae were reared on standard food at a constant temperature. They found that adult starvation resistance increased when adults were fed low P:C diets. Additionally, when adults fed low P:C diets experienced high temperature during feeding, they showed higher starvation resistance than adults fed low P:C diets at low temperature. However, temperature had almost no effect on starvation resistance for those adults fed on a high P:C diet (Jang and Lee, 2018). This study shows that diet and temperature during the adult stage

interact to shape starvation resistance. However, they did not go on to explore how temperature and diet in the larval stages might affect adult starvation resistance.

While we know that temperature and nutrition interact to shape important life history traits, we know little about how developmental temperature might interact with larval nutrition to affect, not just adult starvation resistance, but other adult stress resistance traits important for climatic adaptation such desiccation resistance, cold resistance, and heat resistance (Lasne et al., 2018).

1.3 | Evolutionary responses of key life history traits to larval thermal and nutritional stress

While plasticity allows organisms to respond quickly to new conditions, evolutionary adaptation allows species to optimise their fitness in a given environment via genetic changes in the population (Franks and Hoffmann, 2012). Under current global change, the extent and velocity of environmental change expected could exceed the capacity of many species to adapt (Etterson and Shaw, 2001; Quintero and Wiens, 2013) instigating a debate about what evolution can contribute to species survival under climate change (Kellermann and van Heerwaarden, 2019; González-Tokman et al., 2020). However, rapid adaptation has already been observed in many taxa (Franks, Sim and Weis, 2007; Skelly *et al.*, 2007; Sinclair, Williams and Terblanche, 2012; Krehenwinkel, Rödder and Tautz, 2015; Bi *et al.*, 2019). For example, the annual plant, *Brassica rapa*, when faced with a multiyear drought, adapted by flowering early (Franks, Sim and Weis, 2007). Franks, Sim and Weis (2007) used pre-drought and post-drought genotypes recovered from stored seed to show that summer drought selected for early flowering, that flowering time was heritable, and that selection intensities in the field were more than sufficient to account for the observed evolutionary change. Other examples of adaptive responses to climate change in different taxa include the adaptation of invasive European wasp spiders when colonising new habitats (Krehenwinkel, Rödder and Tautz, 2015) and the adaptation of chipmunks as a response to range contractions (Bi

et al., 2019). These examples imply that adaptation could be a viable option for overcoming the challenges posed by climate change, at least for some species.

Evolutionary adaptation involves genetic changes in a population across generations and results in increased fitness in an altered environment. Usually, evolution is thought to operate by directionally selecting a desired trait mean, and evolution of the trait mean can be essential for survival in an altered environment; for instance the abbreviated growing seasons caused by drought required an earlier onset of flowering in *Brassica rapa* for species persistence (Franks, Sim and Weis, 2007). However, adaptation can also affect the plasticity of a trait, either by selection directly targeting plasticity (Bradshaw, 1965; de Jong, 1995), or as a correlated response to changes in trait mean (Falconer, 1952; Via and Lande, 1985; Via, 1993). Because organisms often experience environmental fluctuations in their habitat and extreme weather events are expected to become more frequent in the future (IPCC, 2014; Stott, 2016; Chevin and Hoffmann, 2017), phenotypic plasticity is important for species' survival. Therefore, when investigating adaptation in response to climate change, it is essential to consider not just changes in the mean of the trait under selection but also evolved changes in the plasticity of that trait.

1.3.1 | Changes in trait mean in response to thermal and nutritional adaptation

One way of investigating animals' adaptive potential to future environmental conditions is to use laboratory natural selection or experimental evolution. In experimental evolution, animals are placed under controlled conditions and subjected to selection pressures in the lab to investigate how they adapt to these pressures.

Many studies have used experimental evolution to investigate species' ability to adapt to different thermal environments. In *Drosophila* species, adaptation to cooler temperatures usually results in faster development (Partridge *et al.*, 1994; James and Partridge, 1995), larger size (Anderson, 1973; Cavicchi *et al.*, 1985), and lower heat resistance (Cavicchi *et al.*, 1995). In addition, adaptation to warmer temperatures has often resulted in smaller size (Anderson, 1973; Cavicchi

et al., 1985), slower development (Cavicchi *et al.*, 1995), higher heat tolerance (Cavicchi *et al.*, 1995; Gilchrist, Huey and Partridge, 1997), higher fecundity, higher metabolic rate, and lower body fat (Barghi *et al.*, 2019).

However, there are exceptions to these general patterns. For instance, James and Partridge, (1995) found no difference in the development time of 25°C and 29°C selected lines; Huey, Patridge and Fowler, (1991) found that cold selected lines had longer development time when reared at 25°C; Schou *et al.* (2014) found lower heat tolerance and unchanged fecundity for warm-adapted *D. melanogaster* lines; and Tobler, Hermisson and Schlötterer, (2015) found that both warm- and cold-adapted populations had increased tolerance to both heat and cold. The discrepancies in the observed response to selection across studies could be due to differences in the duration of selection (Langmüller and Schlötterer, 2020), to differences in the standing genetic variation of the founder population (Blows and Hoffmann, 2005), or to differences in the timing and intensity of the selection pressures applied. Therefore, while *Drosophila* species can show adaptive potential in response to changes in the thermal environment for life history and stress resistance traits, evolved responses can be context dependent. Hence, more research is needed to better understand how the conditions of selection and the testing environment influence the observed responses to selection.

Experimental evolution has also been used to study adaptation to challenging nutritional environments in insects. The nutritional stress most commonly used was calorie restriction. Studies investigating adaptation to low-calorie diets found that adaptation led to lower adult weight (Kolss *et al.*, 2009; May *et al.*, 2019), smaller wing size (Vijendravarma, Narasimha and Kawecki, 2011), and lower early life fecundity (Kolss *et al.*, 2009) when flies were tested on standard diet. In addition, when individuals were reared on low-calorie food, adaptation to low-calorie food resulted in faster development (Kolss *et al.*, 2009; May *et al.*, 2019) and higher viability (Kolss *et al.*, 2009) when compared to control lines reared on the same low-calorie food. Further, adaptation to low-calorie food led to reduced adult starvation tolerance (Kolss *et al.*, 2009;

Kawecki *et al.*, 2020), higher susceptibility to intestinal infection (Vijendravarma *et al.*, 2015), lower dependence on gut microbiota for nutrient digestion (Erkosar *et al.*, 2017), increased nutrient assimilation (Cavigliasso *et al.*, 2020), genomic enrichment of genes implicated in hormone, carbohydrate, and lipid metabolism (Kawecki *et al.*, 2020), and changes in expression levels of dFOXO transcription factor (Erkosar *et al.*, 2017). These studies illustrate the wide range of outcomes of adaptation to a low-calorie diet.

Two studies investigated adaptation to macronutrient imbalance (Warbrick-Smith *et al.*, 2006; Zajitschek *et al.*, 2019). Zajitschek *et al.*, (2019) found that adaptation to high-protein foods led to increased fecundity, but low survival when individuals were tested on standard and low-protein diets, while evolution on low-protein foods reduced survival without increasing fecundity in *D. melanogaster*. In addition, after adaptation to carbohydrate-rich food, the caterpillar *Plutella xylostella* could eat excess carbohydrate without laying it down as fat, while caterpillars adapted to carbohydrate-poor food stored more fat (Warbrick-Smith *et al.*, 2006). All in all, dietary adaptation affects a wide range of life history and fitness traits, post-ingestive mechanisms, and many genes involved in nutrient processing and metabolism.

Because animals in nature will face a range of environmental stressors under climate change, it is imperative to understand how animals will deal with combinations of stressors. Very few studies have investigated the outcomes of adapting to two environmental stressors simultaneously and how that differs from adapting to each stressor independently. Sgrò and Hoffmann (1998) found increased additive genetic variance and adaptive capacity for fecundity when larvae were exposed to cold shock and low-protein diet during development. They suggested that thermal and nutritional stresses in combination could increase the expression of adaptive genetic variation and facilitate adaptation.

Bochdanovits and Jong (2003) empirically tested this hypothesis by investigating shifts in development time, viability, and size caused by evolutionary adaptation to either a warm (27.5°C) or cold environment (17.5°C) combined with a low-calorie diet or a standard diet. They found

unexpected synergistic effects of simultaneous adaptation to low-calorie food and temperature on size and viability driven by evolved changes in plasticity. For instance, the increase in size (regardless of diet) resulting from adaptation to cold conditions was apparent only when flies were reared under warm, but not cold, conditions. This was because the warm-adapted flies were more plastic for size than the cold-adapted flies. These results indicate that stressors can interact to shape adaptive responses and that selection can have correlated adaptive responses that affect plasticity. However, more research is needed to understand how different types of stress might interact to affect adaptation and under what circumstances plasticity is more likely to change with evolution.

1.3.2 | Changes in plasticity after experimental evolution

Experimental evolution studies also illustrate how plasticity might change during adaptation to environmental stress. Plasticity is expected to increase or decrease with selection under a constant environment depending on whether genes controlling the phenotypic evolutionary response also regulate the plastic response to a given environmental factor (Falconer, 1952; Via and Lande, 1985; Via, 1993). Both thermal and nutritional adaptation have led to evolved changes in plasticity in experimental evolution studies.

Best understood are changes in thermal plasticity after adaptation to cooler or warmer environments. For instance, Cavicchi et al. (1995) showed that, while flies evolved at 18^{o}C could increase their thermal tolerance through acclimation (i.e. plasticity), lines adapted to 28^{o}C had lost their acclimation ability, suggesting a loss of thermal plasticity after adaptation to increased temperature. More recently, Stazione et al. (2020) showed that *D. buzzatii* lines selected for mating success at high temperature could increase their heat resistance after a short heat pre-treatment (i.e. heat hardening) while control lines could not. Both the Stazione et al. (2020) and Cavicchi et al. (1995) studies illustrate how adaptation to warmer environments can result in correlated shifts in the plasticity of upper thermal limits. They suggested that the observed

changes in plasticity could be linked to the involvement of heat shock proteins in both thermal adaptation and the hardening response (Cavicchi et al., 1995; Stazione et al., 2020).

Adaptation to temperature has also been found to result in shifts in the plasticity of life history traits. James and Partridge, (1995) found that flies selected at 25°C or 28°C had longer development times than flies selected at 16.5°C. However, this effect was larger when selected lines were tested at 16.5°C than when they were tested at 25°C or 28°C because 16.5°C selected lines were less plastic than 25°C or 28°C selected lines. Interestingly, 25°C and 28°C selected lines showed no differences in development time at any rearing temperature, meaning that the effects of selection to temperature on development time are non-linear and that the changes in plasticity after thermal evolution depend on the selection temperature. Tobler, Hermisson and Schlötterer (2015) found that populations adapted to both cyclic cold and hot thermal regimes showed increased fitness in both environments, but that selected lines were less plastic than controls in their fitness. These studies show that temperature adaptation can also affect the plasticity of traits not directly related to temperature tolerance such as development time and fitness (James and Partridge, 1995; Tobler, Hermisson and Schlötterer, 2015).

Finally, Kolss et al. (2009) found differences in the plasticity of size and development time after adaptation to low-calorie food. They found a reduction in size and development time after adaptation to a low-calorie diet. However, these effects were larger when flies were tested on low-calorie food compared to standard food. The interaction between selection treatment and testing environment was caused by control lines being more plastic than the low-calorie selected lines and it indicates that nutritional adaptation can also lead to correlated evolved changes in the plasticity of size and development time.

Considering the amount of work that addresses adaptation to thermal and nutritional environments singly, our current understanding of adaptation to these variables in combination is extremely limited, as is the parallel evolution of plasticity when adapting to different environments. Given current climate change, there is a pressing need to develop a better understanding of the

adaptive potential of animals to multiple stressors, and how evolution might change organisms' plasticity. This understanding will be important to better predict how animals in nature will deal with changes in their environment imposed by climate change.

1.6 | Aims

The main goal of this PhD thesis was to better understand plastic and evolved responses to combinations of thermal and nutritional stress using *Drosophila melanogaster* as a model system. To do this, I first focused on the plastic responses of key life history traits established in the larval stages to combinations of thermal and nutritional stress (Chapter 2). Next, I explored how combined thermal and nutritional stress during larval development impacted adult stress resistance traits (Chapter 3). Finally, I used experimental evolution to examine how adaptation to combined thermal and nutritional stress during development shaped evolved and plastic responses of life history traits (Chapter 4).

1.7 | Thesis organisation

This thesis is presented as a "thesis including published works" consisting of a general introduction (Chapter 1), one peer-reviewed and published paper (Chapter 2), one manuscript submitted to a peer-reviewed journal (Chapter 3), one thesis chapter which will be part of a larger publication in the future (Chapter 4), and a general discussion (Chapter 5). Chapter 2 is published in Functional Ecology and Chapter 3 has been resubmitted to Functional Ecology.

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Chapter 2 | Interacting with change: Diet mediates how larvae respond to their thermal environment

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RESEARCH ARTICLE

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Interacting with change: Diet mediates how larvae respond to their thermal environment

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Abstract

1. Temperature and nutrition are amongst the most common environmental challenges faced by organisms and will become increasingly so with ongoing climate change. While we have learnt a great deal about how temperature and nutrition affect life-history traits on their own, we know very little about their combined effect on animal performance. Given that animals in the wild are likely to experience changes in both their thermal and nutritional conditions, we need to understand how interactions between these conditions shape an animal's response if we hope to mitigate the effects of environmental change.
2. In the present research, we investigated the combined effects of nutrition and temperature on key life-history traits in *Drosophila melanogaster*. Using nutritional geometry, developing larvae were exposed to a range of diets varying in their protein and carbohydrate content and to one of two developmental temperature regimes (25°C and 28°C). We then examined key life-history traits: development time, viability, and two estimates of body size—wing and femur size.
3. We found that developmental temperature significantly changed the response to nutrition for all traits. Increased temperature led to more restricted trait optima for all traits and exacerbated the negative effects of carbohydrate-rich diets, resulting in harsher trade-offs between life-history traits. For example, at 25°C there were more diets that led to high viability, fast development and large body size than at 28°C. However, for the diets that produced the best outcomes for each trait, temperature had less of an effect.
4. These findings highlight the importance of studying the effects of combined stressors when assessing animals' responses to changing environmental conditions.

KEYWORDS
development, life-history traits, nutrition, nutritional geometry, plasticity, temperature

1 | INTRODUCTION

Rapid ongoing climate change will impact species' fitness by altering multiple aspects of their environment (Dawson, Jackson, House, Prentice, & Mace, 2011; Foden et al., 2013; Thomas et al., 2004). As a consequence of this, researchers have placed considerable effort into predicting how species will respond to changing environmental conditions. For the most part, studies have focussed on how species respond to thermal extremes, as this is expected to be the main driver of how species respond to climate change (Bush et al.,

Carla M. Sgrò and Christen K. Mirth have equal supervision of the research. Author order was determined by coin toss.

2016; Deutsch et al., 2008; Somero, 2010, 2011). However, rising temperatures will affect other features of a species habitat, such as timing and abundance of food resources. Further, ongoing changes in temperature, CO_2 levels and water availability will also affect the macronutrient composition of plant organs upon which animals feed (Rosenblatt & Schmitz, 2016). Because food availability and quality are amongst the most common stressors faced by animals in nature (Cross, Hood, Benstead, Huryn, & Nelson, 2015; Raubenheimer, Simpson, & Tait, 2012), the ability of species to respond to simultaneous changes in nutrition and temperature will be key for their persistence.

In addition to focussing on responses to single stressors, current assessments of vulnerability to climate change have mostly focused on the response of adult traits to environmental change (Bush et al., 2016; Deutsch, Ferrel, Seibel, Portner, & Huey, 2015; Kellermann, van Heerwaarden, Sgro, & Hoffmann, 2009). However, changing the environmental conditions of juvenile animals often has significant effects on an animal's fitness, altering the ability for the animal to survive to adulthood as well as affecting numerous fitness-related traits like body size and reproductive capacity (Aguila, Hoshizaki, & Gibbs, 2012; Van Heerwaarden & Sgrò, 2011; Rodrigues et al., 2015; Sørensen & Loeschcke, 2001; Tu & Tatar, 2003).

We understand the most about how changing environmental conditions affect juvenile stages from studies in insects (Clissold & Simpson, 2015; Flatt, 2005; Mirth & Shingleton, 2012). Insects are the most abundant class of animal on the planet (Erwin, 1983), fulfilling countless ecosystem functions, and serving as the main food source for many animals (Hallmann et al., 2017). As ectotherms, insects are particularly vulnerable to changes in temperature. This is especially true in the larval stage where growth and survival are highly dependent on the thermal and nutritional conditions experienced (Kingsolver & Huey, 2008; Mirth & Shingleton, 2012), and where behavioural avoidance of thermal stress is limited (Medina-Muñoz & Godoy-Herrera, 2005). Because many fitness-related traits, such as body size, reproductive capacity, and survival to adulthood, are established in the larval stage of insects, it is imperative to understand both pre-adult and adult responses to changing environmental conditions.

The individual effects of either diet or temperature on growth and life-history traits have been studied extensively. For instance, higher temperatures result in shorter development time (Atkinson, 1994; Miller, Clissold, Mayntz, & Simpson, 2009), increased larval growth rates (Atkinson & Sibly, 1997), lower larval viability (Angilletta, 2004; Kozlowski, Czarnoleski, & Danko, 2004) and smaller adult sizes (Angilletta & Dunham, 2003; Atkinson, 1994), which reduce adult reproductive success and fitness. Nutrition itself affects key life-history traits, and many studies have shown that caloric content, macronutrient composition and the relative ratios between macronutrients all have significant effects. For instance, low protein to carbohydrate (P:C) ratios generally result in lower pre-adult viability and longer development time and smaller body sizes in a range of insects, including caterpillars (Roeder & Behmer, 2014; Simpson, Sibly, Lee, Behmer, & Raubenheimer, 2004) and Drosophilid fruit flies (Bakker, 1959; Bradshaw & Holzapfel, 2008; Gray, Simpson, & Polak, 2018; Kristensen et al., 2016; Matavelli, Carvalho, Martins, & Mirth, 2015; Rodrigues et al., 2015; Silva-Soares, Nogueira-Alves, Beldade, & Mirth, 2017). Further, nutritional optima vary depending on the trait and species under study (Gray et al., 2018; Matavelli et al., 2015; Rodrigues et al., 2015).

The few studies that have explored the combined effects of temperature and nutrition on key life-history traits have found that the thermal environment changes the way animals respond to nutrition. In caterpillars, growth rate increased with increasing protein to carbohydrate (P:C) ratio or increasing temperature; however, temperature and nutrition interacted such that the increase in growth rate with temperature was more pronounced as P:C ratio increased (Lee & Roh, 2010; Lemoine & Shantz, 2016). Similar interactions between macronutrient ratio and temperature were found for viability and pupal mass of the caterpillar *Spodoptera litura* (Lee, Jang, Ravzanaadii, & Rho, 2015). These findings indicate that temperature can alter the response of at least some traits to nutrition, making predictions of climate change risk based on single stressors inaccurate.

While the above studies suggest that interactions between temperature and nutrition may generally affect animal responses to changing environmental conditions, their interpretations are restricted to a small region of nutrient space because they used a limited number of diets. Because trait optima can change considerably across nutrient space (e.g. Rodrigues et al., 2015; Silva-Soares et al., 2017), investigating the effects of temperature on life-history traits across a broader range of diets will provide more accurate insight into the conditions under which temperature and nutrition will interact to affect fitness. This is especially important given the uncertainty associated with predictions of nutritional changes under climate change (Rosenblatt & Schmitz, 2016).

Here, we examine the combined effect of thermal and nutritional conditions during larval development in *Drosophila melanogaster*. We assess four traits known to depend on larval rearing conditions: egg-to-adult development time, viability, and two adult size traits, wing centroid size and femur length. To account for effects of macronutrient balance as well as calorie content of diet, we used nutritional geometry (Simpson & Raubenheimer, 1993, 1999, 2012; Simpson et al., 2004) to generate a nutrient space composed of 36 diets of varying P:C ratio and calorie content. We explicitly tested for an interaction between nutrition and temperature during development by rearing all flies on these 36 diets at either 25 or 28°C. We found that temperature modified the response to nutrition for all traits examined. Our study emphasizes why studying environmental stressors in combination, rather than independently, will provide more insight into the complex nature of animal responses to climate change.

2 | MATERIALS AND METHODS

2.1 | Fly stocks

We used an outbred population of *D. melanogaster* from Ballina, Australia, initiated from wild-caught females collected in April 2016

(Lasne, Hangartner, Connallon, & Sgrò, 2018). Flies were maintained at 25°C and 12-hr light (L):12-hr dark (D) photoperiod on yeast–dextrose–potato medium (potato flakes 20 g/L; dextrose 30 g/L; Brewer's yeast 40 g/L; agar 7 g/L; nipagen 6 ml/L; and propionic acid 2.5 ml/L). They were maintained as a mass-bred population at a census size of approximately 2,000 individuals for approximately 60 generations before the experiments described below.

2.2 | Nutritional geometry

We created 36 experimental diets varying in their protein to carbohydrate (P:C) ratios and calorie concentrations. We produced these diets following a protocol similar to Rodrigues et al. (2015). Briefly, six different P:C ratios (1:16, 1:8, 1:4, 1:3, 2:3, and 3:2) were created by varying the quantities of inactive yeast, dextrose and potato flakes. Each ratio was prepared at a concentration of about 620 g of dry nutrient mass per litre and was then diluted sequentially by 50% in each step to make six different concentrations per ratio, giving a total of 36 diets. The low viability of individuals on the lowest P:C ratio and lowest calorie concentration meant that the sample sizes for body size measures would be highly imbalanced, and therefore, these diets were not included in the size analysis (wing centroid size and femur length), meaning that adult body size was measured across 25 diets rather than 36. Importantly, the chosen nutrient space extends across the range of P:C ratios and caloric concentrations found in rotting fruit (Matavelli et al., 2015; Silva-Soares et al., 2017), ensuring that our results are relevant to the feeding ecology of the fruit feeding *D. melanogaster* in the wild.

To obtain focal flies for the experiments described below, parental flies were placed in egg-laying chambers and left to oviposit overnight on food medium made from the same ingredients as our standard food but with the addition of blue food colourant and with twice the amount of agar (14 g/L). We transferred 20 eggs into vials containing 7 ml of treatment food, with ten vials per diet. Five of the diet replicate vials were placed at 25°C, and the other five were placed at 28°C in controlled-temperature cabinets with a 12-hr L:12-hr D photoperiod. Vials were relocated within the cabinet twice a day to avoid temperature and light gradient biases. Developing larvae were left to feed ad libitum until adult eclosion.

2.3 | Developmental temperature

Experimental flies were reared at each of two constant rearing temperatures: 25°C and 28°C. These temperatures were chosen because they represent the average summer temperate currently experienced in south-eastern Australia (25°C treatment) and the 3°C increase in temperature (28°C treatment) projected under climate change for the same region (www.bom.gov.au).

2.4 | Egg-to-adult development time and viability

To assess egg-to-adult development time, vials were checked twice a day at 9 a.m. and at 6 p.m. for eclosing adults. Once counted, eclosed adults were removed from their vial. For each vial, the assay ended when four consecutive time periods yielded no flies, at which point vials were discarded. Each fly received a development time score counted as the hours to eclosion from the mid-point of egg laying. Egg-to-adult viability was measured as number of adults eclosed relative to the 20 eggs transferred into each vial.

2.5 | Adult body size

To account for differences in scaling of different body parts in response to temperature and nutrition (Shingleton et al., 2009), body size was estimated using wing centroid size and femur length. Wings and frontal legs of 30 females per treatment were collected from adults that eclosed in the development time vials because previous studies show that such a sample size is sufficient to detect treatment effects (Lasne et al., 2018), and it allowed us to ensure a balanced design. Although size is a sexually dimorphic trait, understanding sex-specific responses to the combined effects of nutrition and temperature was not the focus of the present study, and so, only females were used. The left wing and left front leg of females were placed on a microscope slide and photographed (Leica M80 stereo microscope; Leica). Wing centroid size was calculated following Clemson, Sgrò, and Telonis-Scott, (2016). Briefly, eight wing vein landmark positions were obtained and their *x* and *y* coordinates determined using the software TPSDIG (Rohlf, 2008) version 2.17; wing area was then measured as centroid size (the square root of the sum of the squared distance from each landmark to the centroid) using COORDGEN8 (Sheets, 2003). Femur length was measured in IMAGEJ (Schindelin et al., 2012).

2.6 | Statistical analysis

The effect of protein and carbohydrate content of the food on all traits studied was analysed following the methods described by Lee et al. (2008). For viability, a generalized linear model (GLM) was fit to the data using a binomial family. Development time was rank-transformed to fit a Gaussian distribution, since transformation did not improve the fit of the data to any family of distributions. Then, a linear mixed-effects model was fit, including replicate vial as a random effect. Wing centroid size and femur length were analysed using linear models (LM). Model fit was validated by visual inspection of the residuals.

The full models included the linear and quadratic components of protein and carbohydrate and their cross-product, temperature and its cross-product with both the linear and quadratic components of carbohydrate and protein, and the three-way interaction between temperature and the linear components of protein and carbohydrate (similar to methods in Lee et al., 2008). This model allowed us to identify which, if any, macronutrient(s) drove the differences in response to temperature.

Additionally, we used response surface comparison analysis following Silva-Soares et al. (2017) to compare response shapes between temperatures for each trait. Specifically, we compared the full

model described above to a model that retained all terms except for the interactions between temperature, carbohydrate and protein. These two models were compared using partial *F* tests.

If significant interactions between protein and/or carbohydrate and temperature were found for a trait, the data were subset by temperature. Within each subset, the linear and quadratic effects of protein, carbohydrate and their cross-product were examined using the models described above but excluding temperature.

The response of each trait to the nutrient space at both temperatures was visualized using nonparametric thin-plate splines (TPS) (Blows & Brooks, 2003) using the fields package in R (version 3.4.1, R Development Core Team, 2017, https://www.r-project.org/). To better understand how the response surface differed between thermal regimes, we visualized how the increase in temperature affected the trait at each point of the nutrient space. To do so, temperature effect plots were generated by subtracting the TPS predictions for the 28°C subset from the TPS predictions for the 25°C subset. This new dataset was visualized in the same way as the trait response surfaces, using nonparametric TPS. All statistical analyses were done in R (version 3.4.1, R Development Core Team, 2017, https://www.r-project.org/).

3 | RESULTS

Our primary aim was to determine whether temperature changed the effects of larval nutrition on key life-history traits. To address this, we measured egg-to-adult viability, development time, wing centroid size and femur length of *D. melanogaster* on an array of diets (36 for viability and development time and 25 for the size traits) varying in protein, carbohydrate and caloric content at two temperatures (25°C and 28°C).

3.1 | Egg-to-adult viability

Our results revealed a significant interaction between temperature and both macronutrients (Table 1), as well as an overall difference in the shape of the nutrient response surfaces between temperatures (Table 2, Figure 1a-c). This means that temperature changed how egg-to-adult viability responded to nutrition.

To further probe how temperature alters how viability responds to diet, we analysed the viability across diets for each temperature individually. We found that the linear and quadratic components of both carbohydrate and protein, as well as their interaction, had a significant effect on egg-to-adult viability at both temperatures (Table 3). The conditions that generated the highest viability were similar across temperatures and occurred in the diets with the highest protein and calorie concentrations (Figure 1a-c). Viability decreased with decreasing protein and calorie concentrations in both thermal conditions (Figure 1a-c).

Although temperature did not change which diets generated the highest viability, it did alter the slope of the response of viability to macronutrient concentration. This leads to a higher viability at 28°C

TABLE 1 Effects of carbohydrate (C), protein (P), temperature (T) and their squares and products in the larval diet on four life-history characters: egg-to-adult viability, development time, wing centroid size and femur size

Life-history trait	T	P	C	P^2	C^2	P × C	T × P	T × C	$T \times P^2$	$T \times C^2$	T × P × C	Adjusted R^2
Viability												
β	0.0761	**0.006**	**0.00870**	**−0.29E−06**	**−1.79E−05**	**1.56E−05**	**−0.009**	0.00123	3.06E−06	**−5.75E−06**	**3.52E−05**	–
z value	0.784	**2.687****	**8.266*****	**−4.508*****	**−10.248*****	**2.544***	**−2.845****	0.828	0.322	**−2.296****	**3.774*****	
Development time												
β	**−4.85E−01**	**−2.45E−02**	**7.88E−04**	**5.48E−05**	**4.64E−06**	**1.61E−05**	**−1.11E−02**	4.78E−04	**1.85E−05**	−9.59E−07	**1.40E−05**	.630
t value	**−14.505*****	**−37.775*****	**2.678*****	**31.109*****	**8.387*****	**9.151*****	**−11.794*****	1.133	**7.357*****	−1.171	**5.412*****	
Wing centroid size												
β	**−0.086**	**4.04E−04**	**2.97E−04**	**−1.45E−06**	**−7.03E−07**	3.60E−07	**2.98E−04**	8.81E−05	−3.64E−07	−1.74E−07	−7.41E−07	.402
t value	**−12.723*****	**3.867*****	**5.103*****	**−5.203*****	**−6.820*****	1.196	**2.012***	1.070	−0.919	−1.189	−1.735	
Femur size												
β	**−0.0150**	**1.84E−04**	**1.26E−04**	**−6.74E−07**	**−3.12E−07**	1.93E−07	**1.65E−04**	−5.73E−08	−2.27E−07	−2.72E−08	−3.32E−07	.238
t value	**−5.371*****	**4.244*****	**5.222*****	**−5.807*****	**−7.275*****	1.541	**2.674****	−0.002	−1.376	−0.447	−1.864	

Notes: For all traits with the exception of viability, linear models were fitted to the data. Viability was analysed with a generalized linear model with binomial family and a logit link. Development time was rank normalized to meet the assumptions with a mean of 0 and a standard deviation of 1. Significant coefficients are in bold. Significance level codes: $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$.

TABLE 2 Comparisons between the response surfaces of flies reared at 25°C and flies reared at 28°C for the four measured traits

Trait	Degrees of freedom	*F*-value/deviance	*p*-value
Development time	5	34.897	<.001***
Viability	3	14.402	.00241**
Wing centroid size	5	3.565	.00330**
Femur length	5	5.320	<.001***

Notes: All response surfaces were compared using partial *F* tests, except for viability for which, due to its binomial distribution, response surfaces were compared using a chi-square test. Significance level codes: $^{**}p$ <.01, $^{***}p$ < .001.

wcompared to 25°C for intermediate P:C ratios (between 1:4 and 2:3 P:C), but up to a 15% decrease in survival at 28°C compared to 25°C on either high calorie and low P:C ratio diets, or on intermediate calorie and high P:C ratio diets (Figure 1c). Thus, higher temperature can have a positive effect on viability on some diets but a negative effect on others.

3.2 | Egg-to-adult development time

We found a significant difference in how development time responded to nutrition in larvae reared at 25°C when compared to larvae reared at 28°C, as shown by the significant interaction between temperature and both the linear and quadratic components of protein and the three-way interaction between temperature, protein and carbohydrate (Table 1). Further, when we compared response surfaces between temperatures we also found they were significantly different in shape (Table 2, Figure 1d-f). This indicates that the response of development time to the macronutrient composition of the diet depends on the temperature experienced throughout development.

At both 25°C and 28°C, we found that the linear and quadratic components of both carbohydrate and protein as well as their interaction had significant effects on egg-to-adult development time (Table 3). At both rearing temperatures, flies developed fastest at intermediate calorie concentrations (160–320 g/L). However, at 25°C flies developed fastest at intermediate P:C ratios (1:3), while at 28°C flies developed fastest at the highest P:C ratio (3:2; Figure 1d,e). Thus, temperature shifted the dietary conditions that result in the fastest development times.

Temperature also affected the severity of the delay caused by diet (Table 2, Figure 1c): Development time was delayed as P:C ratio decreased, but this delay was more marked at 25°C compared to 28°C (e.g. we found that the highest carbohydrate and lowest protein diet delayed development by 15 days at 25°C but just by 9 days at 28°C). The difference in development time due to temperature was minimized at the intermediate caloric concentrations and intermediate P:C ratios (1:3 and 1:4; Figure 1f). Taken together, temperature affected the way development time responded to diet by shifting the diets that gave rise to the fastest development time and by exacerbating the effects of the diets that produced the longest development time.

3.3 | Female wing centroid size and femur length at eclosion

We used two different proxies to account for differences in body size, wing and femur size, since size traits have been shown to differ in their response to nutrition in females (Shingleton, Masandika, Thorsen, Zhu, & Mirth, 2017). For both wing and femur size, we found that temperature significantly affected the way trait size responded to diet (Tables 1 and 2, Figure 2). The interaction was driven by protein, as we found no significant interaction between temperature and any component of carbohydrate (Table 1).

At both temperatures, trait size (be it wing or femur size) was significantly affected by the linear and quadratic components of both carbohydrate and protein (Table 3), but in different ways. For individuals reared at 25°C, the response surfaces of trait size for both wing and femur showed a maximum size at intermediate-high P:C ratios (1:3–2:3) and intermediate-high caloric concentrations, with size decreasing towards the extremes (Figure 2a,d). For individuals reared at 28°C, the maximum size (both for wing and femur) was shifted towards lower caloric values and higher P:C ratios (Figure 2b,e) leading to a significant difference in the response surface between the 25°C and the 28°C rearing temperature for both traits (Table 2, Figure 2c,f).

Diets of lowest P:C ratio and highest calorie concentration generated the smallest flies, and the magnitude of this response was further mediated by temperature, resulting in more extreme effects on body size at 28°C than at 25°C. On the other hand, the effects of high temperature on size were reduced at intermediate to high (1:3 and 2:3) P:C ratios at intermediate calorie concentrations (160–320 g/L). Additionally, our data suggest that size across diets varies more at 28°C than at 25°C. This was true for both size traits, but more markedly so for femur length (Figure 2), which showed a decrease in size of about 3% between the largest and smallest flies at 25°C but a decrease of about 8% at 28°C. Thus, we observed that temperature changed the way size traits responded to the dietary composition both by shifting the peaks that generate the largest trait sizes and by modulating the magnitude of the effects of diets that lead to the smallest trait sizes.

4 | DISCUSSION

Most risk assessments for climate change have focused solely on the effects of thermal stress (Bush et al., 2016; Kellermann et al., 2009; Sinervo et al., 2010). However, we know that ongoing environmental change will lead to changes in the nutrient composition of the primary producers upon which herbivores feed (Rosenblatt & Schmitz, 2016), which in turn have the potential to alter an animal's vulnerability to a changing thermal environment. It is therefore likely that temperature

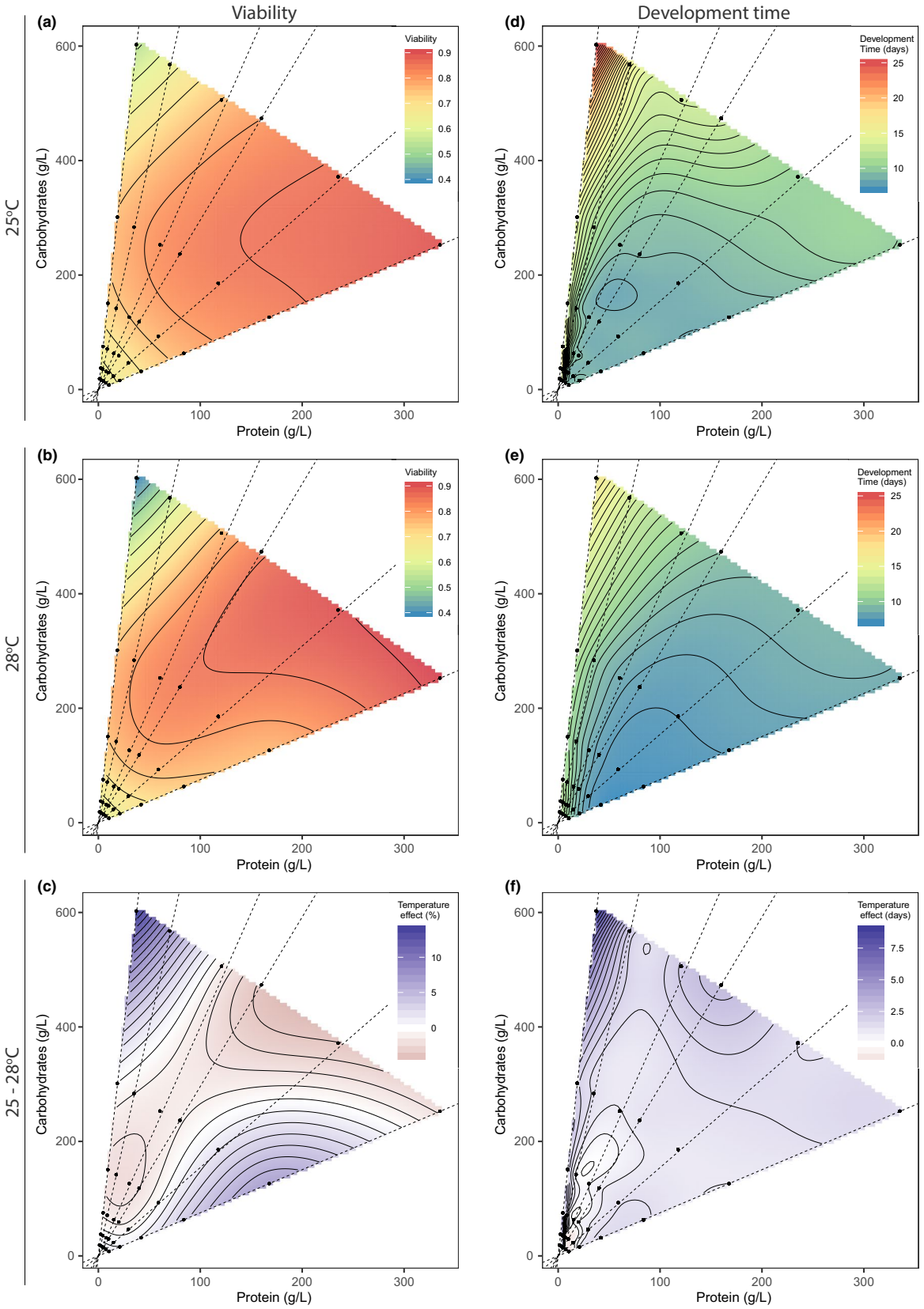

FIGURE 1 The effects of protein and carbohydrate content of the larval diet, represented as the fitted response surfaces of the effects of 36 different diets varying in protein and carbohydrate content. *Dashed black lines* represent P:C ratios. *Filled black circles* represent the respective nutritional coordinates of each of the 36 diets used. *First column (a–c)*: Egg-to-adult viability; *second column (d–f)*: Egg-to-adult development time. *Fist row*: 25°C rearing temperature; *second row*: 28°C rearing temperature; *third row*: the difference in the response between flies reared at 25°C and flies reared at 28°C

TABLE 3 Effects of carbohydrate (C), protein (P) and their squares and interaction terms in the larval diet on four life-history traits: egg-to-adult development time and viability, wing centroid size and femur size for flies reared at 25°C and flies reared at 28°C

Rearing temp.	P	C	P^2	C^2	P × C	Adjusted R^2
Viability						
25°C						
β	**0.006**	**0.009**	**−2.93E−05**	**−1.79E−05**	**1.56E−05**	—
z value	**2.687****	**8.266*****	**−4.508*****	**−10.248*****	**2.544***	
28°C						
β	−0.003	**0.010**	**−2.63E−05**	**−2.36E−05**	**5.08E−05**	—
z value	−1.292	**9.449*****	**−3.811*****	**−13.154*****	**7.238*****	
Development time						
25°C						
β	**−0.025**	**7.88E−04**	**5.48E−05**	**4.64E−06**	**1.61E−05**	0.575
t value	**−42.579*****	**3.018****	**35.064*****	**9.453*****	**10.315*****	
28°C						
β	**−0.036**	**0.001**	**7.33E−05**	**3.68E−06**	**3.02E−05**	0.570
t value	**−47.164*****	**3.807*****	**36.911*****	**5.531*****	**14.442*****	
Wing size						
25°C						
β	**4.04E−04**	**2.97E−04**	**−1.45E−06**	**−7.03E−07**	3.60E−07	0.114
t value	**3.924*****	**5.179*****	**−5.281*****	**−6.921*****	1.214	
28°C						
β	**7.01E−04**	**3.85E−04**	**−1.82E−06**	**−8.77E−07**	−3.81E−07	0.203
t value	**6.597*****	**6.506*****	**−6.390*****	**−8.330*****	−1.240	
Femur length						
25°C						
β	**1.84E−04**	**1.26E−04**	**−6.74E−07**	**−3.12E−07**	1.93E−07	0.130
t value	**4.280*****	**5.266*****	**−5.856*****	**−7.337*****	1.554	
28°C						
β	**3.48E−04**	**1.26E−04**	**−9.01E−07**	**−3.39E−07**	−1.39E−07	0.232
t value	**7.901*****	**5.16*****	**−7.651*****	**−7.786*****	−1.087	

Notes: For all traits with the exception of viability, linear models were fitted to the data. Viability was analysed with a generalized linear model with binomial family and a logit link. Development time was rank normalized to meet the assumptions with a mean of 0 and a standard deviation of 1. Significant coefficients are in bold. Significance level codes: *p <.05, **p <.01, ***p <.001.

and nutrition will interact to shape the response of animals to climate change. To these ends, the aim of this study was to understand how the thermal and nutritional environment experienced during development interacts to shape three key life-history traits in *D. melanogaster*. Our results reveal that temperature alters the response of all traits to dietary conditions, and further highlight that this approach has the potential to greatly improve our predictions of the conditions in which species will be most vulnerable to climate change.

4.1 | Larval traits differ in their response to the macronutrient composition of the diet

Our results for how life-history traits respond to nutrition at 25°C are generally in agreement with previous work in *D. melanogaster* (Gray et al., 2018; Rodrigues et al., 2015). Development time was shortest at intermediate (1:3) P:C ratios and intermediate caloric content, and female wing size and femur length at eclosion were largest at high P:C ratio (2:3 and 3:2) and intermediate/high calorie concentrations.

Similarly, our findings for viability at 25°C agreed overall with those from Rodrigues et al. (2015) and Gray et al. (2018). In all cases, low P:C ratios in combination with high caloric content always led to reduced viability. However, there are also discrepancies in the nutritional response surfaces for viability between studies: we found highest viability at the highest P:C ratio (3:2 P:C) and calorie concentration (640 g/L), whereas Rodrigues et al. (2015) found highest viability at 3:2 P:C but intermediate calorie concentration (240 g/L), and Gray et al. (2018) showed two local maxima one at 1:16 P:C and

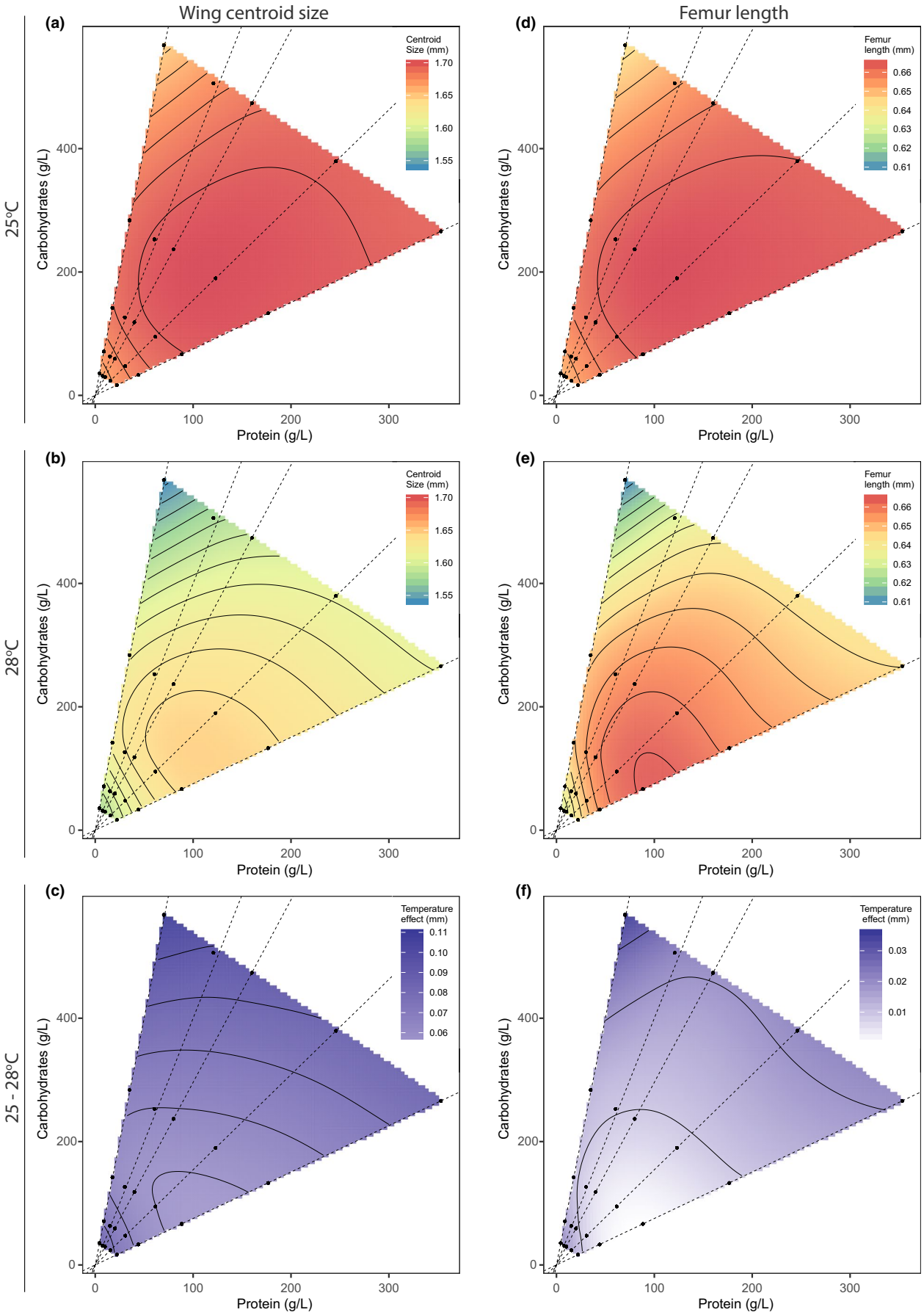
Wing centroid size
Femur length
25°C
28°C
25 - 28°C
(a)
(b)
(c)
(d)
(e)
(f)
Centroid Size (mm)
1.70
1.65
1.60
1.55
Femur length (mm)
0.66
0.65
0.64
0.63
0.62
0.61
Temperature effect (mm)
0.11
0.10
0.09
0.08
0.07
0.06
0.03
0.02
0.01
Carbohydrates (g/L)
Protein (g/L)
0
100
200
300
400

FIGURE 2 The effects of protein and carbohydrate content of the larval diet, represented as the fitted response surfaces of the effects of 25 different diets varying in protein and carbohydrate content. *Dashed black lines* represent P:C ratios. *Filled black circles* represent the respective nutritional coordinates of each of the 25 diets used. *First column (a–c)*: Female wing centroid size; *second column (d–f)*: Female femur length. *Fist row*: 25°C rearing temperature; *second row*: 28°C rearing temperature; *third row*: the difference in the response between flies reared at 25°C and flies reared at 28°C

one at 2:1 P:C. This is could be due to different origins of the fly populations used or perhaps because of differences in the ingredients used to create the diets.

Consistent with (Rodrigues et al., 2015), our data suggested the potential for a trade-off such that diets that led to highest viability did not result in the fastest development nor largest body size. However, there was a wide range of diets (i.e. intermediate to high calorie concentrations of intermediate to high P:C ratios) at 25°C that resulted in high overall performance.

4.2 | Increasing temperature modifies the way larval traits respond to diet

When temperature is increased by three degrees, we found that the number of diets that generated optimal traits values was reduced. Fastest development shifted towards more protein-rich diets, and viability on diets that optimized size and development time was lower at 28°C than it was at 25°C. The combination of fewer diets that resulted in optimal trait values and shifts in the diets that produce trait optima has the potential to result in harsher trade-offs in hotter environments.

These differences in response to nutrition due to temperature increase also mean that larvae have temperature-specific nutritional optima, presumably to accommodate metabolic differences caused by the thermal environment. Differences in nutritional optima have been shown previously between species that share food resources to reflect differences in ecological niche (Matavelli et al., 2015; Silva-Soares et al., 2017). Here we show that nutritional optima can also change for a given species due to temperature.

While the number of diets that produce the best outcomes for traits is reduced at the hotter temperature, the effects of a hotter environment on larvae within this more narrow nutritional space are minimized. For example, development time was fastest on intermediate P:C ratios and calorie concentrations at both 25 and 28°C, and on those diets our models showed virtually no difference in development time between temperatures. It seems intuitive that foods that optimize key developmental traits would help buffer animals against other types of environmental stress; however, more research is necessary to see if this finding can be generalized across traits.

On the diets that produced the poorest trait outcomes, higher temperatures made these outcomes even worse. A similar interaction between temperature and diet was found in the caterpillar *S. litura* (Lee et al., 2015) which underwent a more acute decrease in size with carbohydrate-rich food (1:5 P:C) when this diet was paired with higher temperatures, consistent with the current study.

In other studies, temperature has its most pronounced effects in the diets that produced the best values for traits (Lee & Roh, 2010; Lemoine & Shantz, 2016): caterpillar growth rate increased with increasing temperature for all diets with the largest difference in growth rate at the nutritional optimum (1:1 P:C). This contrasts with the trend we found for development time, size and viability in *D. melanogaster* larvae where the impact of temperature was less marked at the nutritional optimum. Therefore, the way temperature and diet interact is difficult to predict and the response of one trait cannot be used to predict the behaviour of another.

Given the diverse array of effects generated by interactions between the thermal and nutritional environments, our results highlight the importance of investigating a broad range of environmental contexts when making predictions about population responses to environmental change.

4.3 | Relating laboratory-based nutritional geometry to nutrient availability in the wild

Our results clearly show that the nutritional environment of animals can influence life-history responses to increased temperatures. Thus, understanding how the nutritional environment of an animal will change with climate change is an important next step. In nature, Drosophilid larvae obtain most of their protein from yeast communities growing on rotting fruit (Buser, Newcomb, Gaskett, & Goddard, 2014; Starmer & Fogleman, 1986). Yeast communities on fruit show a species-specific pattern of succession in their colonization of decaying fruits (Fogleman, Starmer, & Heed, 1981; Morais, Martins, Klaczko, Mendonça-Hagler, & Hagler, 1995) which in turn affects the macronutrient composition of the fruit (Matavelli et al., 2015). How climate change will affect the growth and composition of yeast communities growing on rotting fruit is at present unknown.

Further, the combined changes in temperature and nutrition may be met by a change in the feeding behaviour of larvae. Even though larvae are constrained to forage on a small area (Medina-Muñoz & Godoy-Herrera, 2005; Stamps, Buechner, Alexander, Davis, & Zuniga, 2005), different parts of the rotting fruit may vary in their P:C content due to the nature of the rotting process. We know that *D. melanogaster* larvae switch between foods of different macronutrient composition to achieve their P:C target (Rodrigues et al., 2015), while other species like *Drosophila suzukii* and *Drosophila biarmipes* control their nutrient intake primarily by regulating their feeding rate (Silva-Soares et al., 2017). It has also been shown that caterpillars and beetles can change their macronutrient target with increasing temperature (Lee et al., 2015; Rho & Lee, 2017). Our study suggests that *D. melanogaster* will face a more restricted nutritional space for optimal performance under increased temperature. The question of whether *D. melanogaster* will modify its feeding behaviour with

increasing temperature to match the new nutritional optimum remains open.

Finally, the current study used constant temperatures, while in nature larvae experience daily temperature fluctuations, and it is possible that the interactions between temperature and diet reported here may differ under fluctuating temperature regimes. It is also possible that, in nature, microclimatic variation and larval behaviour could play a role in mediating responses to the combined effects of thermal stress and nutrition. For example, it has been shown that locust nymphs can detect the deficiency of a specific nutrient in their food and change their preferred temperature to improve the absorption of that nutrient (Clissold, Coggan, & Simpson, 2013). *Drosophila melanogaster* larvae could potentially display a similar behaviour despite their limited mobility. Further studies would clarify the extent to which fluctuating temperatures and microclimatic variation play a role in the way larvae respond to diet and temperature. Finally, while we have focussed on pre-adult traits in the current study, it would be interesting to determine whether adult life-history-traits such, as fecundity or lifespan, are also affected by temperature and nutrition interactions, and whether such responses are sex-specific.

5 | CONCLUSION

Our study is the first to assess how animals will respond to the combined effects of temperature and nutrition across a broad nutrient space. We show that diet modulates the effects of temperature on key life-history traits in complex ways, a complexity only revealed because of the broad nutritional space examined. Importantly, we show that increasing developmental temperatures can change trait optima, generating trade-offs between life-history traits. Finally, we also show that when fly larvae feed on their optimal diets, the impact of increased temperature (i.e. smaller size, faster development and lower viability) is minimized. Our work highlights that the combined effects of both temperature and nutrition should be considered when assessing species responses to environmental change, as failure to do so may result in inaccurate predictions of risk.

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AUTHORS' CONTRIBUTIONS

T.C.K. collected all experimental data. All authors contributed to the experimental design, data analysis and final manuscript preparation.

DATA AVAILABILITY STATEMENT

All data and scripts are available on Figshare (https://doi.org/10.26180/5cfe1ddaaafac) and all materials are available upon request.

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Chapter 3 | Resetting stress responses: the combined impacts of temperature and nutrition during larval development show only minimal carry over on adult stress resistance

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Resetting stress responses: the combined impacts of thermal and nutritional stress during larval development show only minimal carry over on adult stress resistance

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ABSTRACT

1. Nutritional and thermal stress will become increasingly common stressors for animals under climate change, and all stages of their lifecycle have the potential to be impacted. While we are beginning to understand how animals respond to combinations of nutritional and thermal stress, how combined stress in one life stage impacts stress resistance in another is poorly understood.
2. To assess the impacts of the developmental environment on adult stress resistance, we examined the combined effects of larval nutrition and developmental temperature on four ecologically important adult traits in female *Drosophila melanogaster*. Using nutritional geometry, developing larvae were exposed to 25 diets varying in their protein and carbohydrate content and to one of two developmental temperature regimes (25°C or 28°C). We then examined how the developmental environment affected four adult traits known to underpin animal responses to global change, in particular starvation, desiccation, heat, and cold resistance. To identify the physiological states underlying the observed stress response, we also measured lipid and glycogen stores in adults.
3. We found that developmental temperature modulated the effect of larval diet on adult starvation, desiccation, and heat resistance, but not on cold resistance. High carbohydrate diets during development reduced adult desiccation resistance and high calorie diets reduced adult starvation resistance, but only when adults had experienced elevated developmental

temperatures. Heat resistance also decreased on diets high in carbohydrate. Developmental temperature affected all traits. Starvation and desiccation resistance were reduced on most diets when larvae developed under elevated temperatures. Cold resistance was reduced while heat resistance increased when larvae developed under elevated temperatures, independently of rearing diet. The shifts in stress resistance traits could not be explained by changes in lipid or glycogen stores in adults at the time of the stress resistance assays.

4. Our results suggest that combined stress in the larval developmental environment does affect adult stress responses, but these effects are mild when the adults themselves are not also stressed. This means that animals are able to buffer against the negative impacts of combined stress experienced earlier in the lifecycle provided these stressors are removed at later stages.

Key words: thermal resistance, starvation resistance, desiccation resistance, plasticity, development, nutritional geometry, lipids, glycogen

3.1 | Introduction

Temperature is one of the main drivers of species distributions and abundance (Cossins and Bowler, 1987; Angilletta, 2009), increasingly so with climate change (IPCC, 2018; IPBES, 2019). As a consequence, assessments of climate change risk have often focused on the ability to tolerate thermal extremes (i.e., thermal limits) (Deutsch *et al.*, 2008; Somero, 2010; Bush *et al.*, 2016). However, climate change will expose organisms to multiple stressors, including elevated CO_2 levels, shifts in precipitation, and the timing, abundance, and quality of food resources (Malhi *et al.*, 2020). Despite the potential impacts of combined stress, multi-stressor studies in terrestrial environments are rare (Kaunisto, Ferguson and Sinclair, 2016). Beyond shifts in temperature and precipitation, food limitation is one of the most common environmental challenges faced by organisms (Raubenheimer, Simpson and Tait, 2012; Cross *et al.*, 2015), and this will become increasingly so

under climate change (Rosenblatt and Schmitz, 2016). Further, many organisms have complex life cycles with different life stages that experience differences in habitats and microhabitats (Kingsolver *et al.*, 2011), resulting in differential selection and sensitivity to environmental change across life stages. While all life stages will experience changes in environmental conditions, current assessments of climate change risk are largely based on adult sensitivity to environmental change.

Insects in particular have been the focus of many studies on the impacts of climate change because of their species richness and abundance, and their critical role in ecosystem function (Hallmann *et al.*, 2017). The unique lifecycle of holometabolous insects in particular allows the juvenile stages to occupy separate niches from the adults (e.g. Kingsolver *et al.*, 2011). While this means that the niche of the adult need not be tied to that of the larva, numerous studies have demonstrated that environmental conditions experienced during the larval stages of development can carry over to shape a range adult traits such as size, stress resistance, physiology, fecundity, and lifespan (e.g. Crill, Huey and Gilchrist, 1996; Kingsolver *et al.*, 2011; Jang and Lee, 2018; Galarza *et al.*, 2019; Kutz, Sgrò and Mirth, 2019)

As small ectotherms, insects are especially sensitive to the thermal environment experienced during both the larval and adult stages. In particular, growth and survival are highly dependent on the thermal environment experienced during larval development (Kingsolver and Huey, 2008; Mirth and Shingleton, 2012), where behavioural avoidance of thermal stress is limited (Medina-Muñoz and Godoy-Herrera, 2005). This can result in direct and indirect effects within and across life stages. For example, increased temperatures during development can directly result in reduced development time (Atkinson, 1994; Miller *et al.*, 2009), which can then lead to reduced adult size (Atkinson, 1994; Crill, Huey and Gilchrist, 1996; Angilletta, Jr., and Dunham, 2003). This can in turn result in carry-over effects on other adult performance traits such as fecundity (Cook, 1961; Honěk and Honek, 1993) and lifespan (Metcalfe and Monaghan, 2003; Roark and Bjorndal, 2009). Exposing larvae to increased temperatures during development can also result in plastic increases in adult heat resistance (e.g. Crill, Huey and Gilchrist, 1996; van Heerwaarden, Kellermann and Sgrò,

2016; Kellermann, van Heerwaardenf and Sgrò, 2017; MacLean *et al.*, 2017; Zheng *et al.*, 2017), but not starvation resistance (Jang and Lee, 2018). Exposure to cooler developmental temperatures can result in plastic increases in adult cold resistance (e.g. Bauerfeind *et al.*, 2014; MacLean *et al.*, 2017), but seem to have no effect on desiccation (Krupp *et al.*, 2020 but see Bauerfeind *et al.*, 2014).

The nutritional environment also shapes larval and adult traits in insects. Indeed, food stress is likely to be a common stressor during development (De Block and Stoks, 2008) and the consequences of this can carry over to affect adult performance (reviewed in Metcalfe and Monaghan, 2001). For example, low protein to carbohydrate (P:C) ratios in larval diets can result in reduced viability, increased development time, reduced adult body size, and reduced reproductive capacity in many insects (Simpson *et al.*, 2004; Roeder and Behmer, 2014) including Drosophilids (Matavelli *et al.*, 2015; Rodrigues *et al.*, 2015; Kristensen *et al.*, 2016; Silva-Soares *et al.*, 2017; Gray, Simpson and Polak, 2018).

Importantly, adult stress resistance traits can also be affected by larval nutrition (Andersen *et al.*, 2010; Sisodia and Singh, 2012; Kristensen *et al.*, 2016; Henry, Overgaard and Colinet, 2020). For example, adult starvation and cold resistance increases when larvae are reared on low P:C diets (Andersen *et al.*, 2010; Sisodia and Singh, 2012; Lee and Jang, 2014; Kristensen *et al.*, 2016; Henry, Overgaard and Colinet, 2020). In contrast, high P:C ratios in larval diets can increase adult heat resistance (Andersen *et al.*, 2010; Sisodia and Singh, 2012; Kristensen *et al.*, 2016). The relationship between adult dessication resistance and larval diet is less clear. While some studies have found increased adult desiccation resistance when larvae developed on high P:C diets in *D. melanogaster* (Andersen *et al.*, 2010; Kristensen *et al.*, 2016) and *D. ananassae* (Sisodia and Singh, 2012), a more recent study (Henry, Overgaard and Colinet, 2020) found that a protein rich diet during development actually reduced adult desiccation resistance. These conflicting results may in part stem from the fact that these studies used a limited number of diets, and as such they are likely to have sampled different parts of the nutrient space.

While the individual effects of temperature and nutrition on insect performance within and across life stages have been extensively documented, the two factors are tightly connected through an organism's metabolism and energy requirements (Clissold and Simpson, 2015; Cross *et al.*, 2015; Alton *et al.*, 2020). This has led to an increasing interest in understanding plastic responses to the combined effects of temperature and nutrition (Stillwell *et al.*, 2007; Clissold and Simpson, 2015). Such studies reveal that interactions between nutrition and temperature can have profound effects on life history traits within and across life stages of terrestrial ectotherms (Clissold and Simpson, 2015; Lee *et al.*, 2015). In particular, changes in larval nutrition can alter thermal sensitivity of insect growth and development. For example, mean larval growth rate in cabbage white butterfly was greater on high quality (natural) diet compared to artificial (low quality) diet when larvae were exposed to temperatures between 11°-35°C, but this was reversed at 40°C (Kingsolver *et al.*, 2006). Lee and Roh (2010) showed that although larval growth rate in the lepidopteran *Spodoptera exigua* increased with increasing temperature, this association was strongly affected by the P:C ratio in the diet; growth rate was maximised at moderate P:C, but was dramatically reduced on both very low and very high P:C diets. High temperature slowed larval growth in the tobacco hornworm *Manduca sexta,* but only under food restriction (Hayes *et al.*, 2015).

Nutrition during development can also modify the relationship between temperature and body size in ectotherms. Development at cooler temperatures generally results in larger adult body size in most ectotherms, a pattern known as the temperature-size rule. However, this relationship can depend on nutritional context. For example, cooler temperatures during larval development of the tobacco horn worm resulted in larger final size on a high-quality diet, consistent with the temperature-size rule. However, this was reversed on low quality diets; largest size was achieved at warmer, not cooler, developmental temperatures (Diamond *et al.*, 2010). In contrast, Lee *et al.* (2015) found that the temperature-size rule was evident when *Spodoptera exigua* larvae were reared on diets that were high in carbohydrates, and that the relationship between developmental temperature and size disappeared on balanced diets.

More recently, we have shown that increased developmental temperature led to more restricted nutritional optima for development time, viability, and adult size, exacerbating the negative effects of carbohydrate rich diets during development in *D. melanogaster* larvae (Kutz, Sgrò and Mirth, 2019). Kim, Jang and Lee (2020) considered both direct and carry-over effects of temperature-nutrition interactions on a wider range of larval and adult life history traits in *D. melanogaster*. They found that the diets that optimised larval and adult traits differed and that these differences were dependent on developmental temperature. Thus, not only did diet and temperature interact to affect trait optima within life stages, but these effects carried over from the larval to the adult stage in ways that were difficult to predict from trait responses to temperature and diet individually.

While the above studies reveal that temperature and nutrition can interact to affect larval (Kingsolver and Woods, 1998; Kingsolver *et al.*, 2006; Stillwell *et al.*, 2007; Diamond *et al.*, 2010; Lee *et al.*, 2015; Kutz, Sgrò and Mirth, 2019) and adult (Kim *et al.*, 2020) life history traits in insects, only one study (Jang and Lee, 2018) has examined how adult stress resistance traits are affected by the combined effects of temperature and nutrition. While clearly showing that temperature can mediate the effects of nutrition on adult starvation resistance, Jang and Lee (2018) only examined how adult nutrition interacts with adult temperature to affect starvation resistance; the larvae were reared on standard food at a constant temperature. What we understand less about is how developmental temperature might interact with larval nutrition to affect not just adult starvation resistance, but other adult stress traits also known to underpin climatic adaptation, namely desiccation resistance, cold resistance, and heat resistance (Lasne *et al.*, 2018).

In nature, *D. melanogaster* larvae have been reported to feed on low P:C diets (Kristensen *et al.*, 2016) and this may become increasingly common as primary producers increase their carbohydrate content relative to protein in a warming climate (Myers *et al.*, 2014; Rosenblatt and Schmitz, 2016; Zhu *et al.*, 2018; Asseng *et al.*, 2019). For instance, amino acid content of fruits is predicted to decrease under elevated temperatures and reduced water. This would result in changes to the abundance and composition of the yeast communities living on the fruit's surface, because amino

acids in fruits are the primary source of nitrogen for yeast (Wu et al 2019; Gutierrez-Gambo *et al.*, 2021). In turn, yeast forms the primary protein source for *Drosophila* larvae and adults. The larvae and adults of fruit-feeding species of *Drosophila* often rely upon fruit for their carbohydrates (Chng *et al.*, 2017; Burke and Waddell, 2007). Elevated temperatures and reduced water will result in an increase in sugar content of fruits (Moretti *et al.*, 2010), exposing these species to higher carbohydrate concentrations. Finally, developing larvae will be increasingly exposed to elevated developmental temperatures, since larvae are often exposed to thermal stress during summer months in the field (Sgrò and Hoffmann, 1998). This will only become more common with climate change. This combination of changes in the protein and carbohydrate composition of their food, along with altered thermal environments has the potential to impact not only larval development and physiology, but also a number of adult traits.

To date, no study has examined whether the combined shifts in the thermal and nutritional environments of developing larvae expected to occur under climate change will carry over to impact adult stress resistance in *Drosophila melanogaster*. In the present study we address this knowledge gap by testing the interactive effects of dietary protein and carbohydrate and a 3°C increase in temperature during development on adult stress resistance traits. To capture the range of protein and carbohydrate found in rotting fruit in nature (Kutz *et al.*, 2019), we used nutritional geometry (Simpson and Raubenheimer, 2012) to generate a broad nutrient space of 25 diets varying in their protein, carbohydrate, and caloric composition. To explicitly test for an interaction between nutrition and temperature during development, we reared all flies on these 25 diets at either 25°C or 28°C, and assessed four stress resistance traits - starvation, desiccation, heat, and cold resistance - in adult females.

The larval environment is thought to impact adult stress resistance is through the accumulation of energy stores, in the form of lipids and glycogen. Larvae ingest more food than adults, as they require these energy stores for growth, but also to fuel metamorphosis and early adult stages. The amount of energy stored is known to be important to adult stress resistance. For example, adults with higher

lipid stores are more resistant to starvation (Djawdan *et al.*, 1998; Ballard, Melvin and Simpson, 2008; Lee and Jang, 2014), whereas higher glycogen stores confer higher desiccation resistance (Djawdan *et al.*, 1998; Folk and Bradley, 2004; Slocumb *et al.*, 2015). However, we hypothesized that if energy stores could be rebalanced through adult feeding, the impact of the larval environment might be transient. Thus, to understand whether any effects of diet were mediated by differences in energy reserves across treatments, we also examined glycogen and lipid content in newly enclosed and 7-day-old females.

Based on previous studies examining the effects of developmental temperature and nutrition individually, we first predicted that the warmer developmental temperature would increase adult heat resistance and reduce adult cold resistance, and that these effects would be strongest on high protein diets. Second, we predicted that cold and starvation resistance would be higher at 25°C compared to 28°C when combined with high carbohydrate/high calorie diets. Third, we expected desiccation resistance to be higher on high carbohydrate and/or high calorie diets, but unaffected by temperature.

3.2 | Methods

3.2.1 | Fly stocks

We used an outbred population of *Drosophila melanogaster* from Ballina, Australia, initiated from wild-caught females collected in April 2016 (Lasne *et al.*, 2018). Flies were maintained at 25°C with a 12-h light (L):12-h dark (D) photoperiod on yeast-dextrose-potato medium (potato flakes 20 g/L; dextrose 30 g/L; Brewer's yeast 40 g/L; agar 7 g/L; nipagen 6 mL/L; and propionic acid 2.5 mL/L). They were maintained as a mass-bred population at a census size of approximately 2000 individuals for approximately 60 generations before the experiments described below.

3.2.2 | Nutritional Geometry

We created 25 experimental diets varying in their protein to carbohydrate (P:C) ratios and calorie concentrations. We produced these diets following a protocol similar to Kutz, Sgrò and Mirth (2019). Briefly, five different P:C ratios (1:8, 1:4, 1:3, 2:3, and 3:2) were created by varying the quantities of inactive yeast, dextrose, and potato flakes. Each ratio was prepared at a concentration of about 620 g of dry nutrient mass per litre and was then diluted sequentially by 50% in each step to make five different concentrations per ratio, giving a total of 25 diets. The chosen nutrient space extended across the range of P:C ratios and caloric concentrations found in rotting fruit (Matavelli *et al.*, 2015; Silva-Soares *et al.*, 2017), ensuring that our results are relevant to the feeding ecology of the fruit-feeding *D. melanogaster* in the wild.

To obtain focal flies for the experiments described below, parental flies were placed in egg laying chambers and left to oviposit overnight on food medium made from the same ingredients as our standard food but with the addition of blue food colorant and with twice the amount of agar (14 g/L). We transferred 20 eggs into vials containing 7 mL of treatment food, with six to 20 vials per diet. Due to differences in development time associated with the range of rearing conditions, eggs were transferred every day for 12 consecutive days to ensure enough adults of the same age from every rearing condition for each day of the experimental assays. Half of the diet replicate vials were placed at 25°C and the other half were placed at 28°C in controlled-temperature cabinets with a 12-h L: 12-h D photoperiod. Vials were relocated within the cabinet twice a day to avoid temperature and light gradient biases. Developing larvae were left to feed *ad libitum* until adult eclosion.

3.2.3 | Developmental temperature

Experimental flies were reared at either constant 25°C and 28°C. These temperatures were chosen because they represent the average summer temperature currently experienced in south-eastern Australia (www.bom.gov.au; 25°C treatment) and the 3°C increase in temperature (28°C treatment) projected under many climate change scenarios.

3.2.4 | Adult maintenance

Newly-eclosed adults were collected every day for as many days as the experimental assays would last so that, when tested, adults were all the same age across all runs (i.e., 7 days old chronologically). Adults were placed on standard (yeast-dextrose-potato) medium at 25°C at a density of about 50 individuals per vial and left to mate for 4 days. Then, females were sorted under light CO_2 anaesthesia and left to recover under standard conditions for another 3 days before assays were performed. If at the time of the assay not enough 7-day-old females were available, one-day younger or older females were used to complete the required number.

3.2.5 | Stress Resistance Traits

Four stress resistance traits were measured on 7-day-old adult females: starvation resistance, desiccation resistance, cold resistance, measured as chill-coma recovery time, and heat resistance, measured as heat-shock knockdown time.

3.2.5.1 | Starvation resistance

Thirty females from each rearing diet/temperature combination were individually transferred from normal media into 40 mL plastic vials containing non-nutritive agar medium (Goenaga, Fanara and Hasson, 2010; Lasne *et al.*, 2018). To avoid loss of water or humidity, vials were covered with wet towels during the experiment. Starvation resistance was scored as mortality time, assayed at 8-h intervals until all flies died (Goenaga, Fanara and Hasson, 2010; Lasne *et al.*, 2018). Assays were performed at 25°C.

3.2.5.2 | Desiccation resistance

Thirty 7-day-old females from each rearing diet-temperature treatment were individually placed into empty 5 mL glass vials covered with fine gauze. A total of 750 vials per run were divided randomly among three glass tanks sealed with silica gel that maintained relative humidity at 1.5-3% (Hallas, Schiffer and Hoffmann, 2002; Lasne *et al.*, 2018). Desiccation resistance was scored as the time until death, assayed through hourly checks until all flies died (Hallas, Schiffer and Hoffmann,

2002; Lasne *et al.*, 2018). All flies were tested across two experimental runs, with 15 females from each diet-temperature treatment per run. Assays were performed at 25°C.

3.2.5.3 | Cold resistance

Chill-coma recovery time was measured on 30 7-day-old females from each rearing diet/temperature combination. Flies were placed individually in empty 1.5 mL Eppendorf tubes and exposed to 0°C through full immersion in a water bath containing a 10% glycol solution for 4 hours (Gibert and Huey, 2001; Lasne *et al.*, 2018). Tubes were then removed from the bath and allowed to recover at 25°C. Recovery time was recorded as the time it took for each fly to right itself. All flies were tested across 15 experimental runs, with two females from each diet/temperature treatment per run.

3.2.5.4 | Heat resistance

Heat-shock knockdown time was measured on 30 7-day-old females from each rearing diet/temperature combination. Each fly was placed in 5 mL glass vials and submerged in a water bath at 39°C. This measure of heat resistance was chosen (rather than a dynamic ramping method) because it has consistently been shown to be a good predictor of populations' distribution along a latitudinal cline for *D. melanogaster* and is therefore considered a good indicator of heat tolerance (Hoffmann, Anderson and Hallas, 2002; Sgro *et al.*, 2010). Heat-shock knockdown time was scored as the time until flies became immobilized (Clemson, Sgrò and Telonis-Scott, 2016; Lasne *et al.*, 2018). All flies were tested across 15 experimental runs, with two females from each diet/temperature treatment per run.

3.2.6 | Energy stores

To understand how larval diet and rearing temperature affected the storage and use of energy we measured lipid and glycogen content in adult females both at the time of eclosion and after 7 days in standard conditions.

3.2.6.1 | Lipid content

The method for measuring total lipid content was adapted from Parkash, Aggarwal and Kalra (2012). Flies were collected once a day as they eclosed, and samples were taken either at the time of eclosion or after 7 days in standard conditions (25°C and yeast-dextrose-potato medium). Females were snap-frozen in liquid nitrogen and stored at –80°C. Three replicates of groups of five females per diet/temperature treatment were dried, weighed, placed in a diethyl ether bath overnight, after which they were dried and weighed a second time. Lipid content was calculated as the difference in mass between the group of dried flies before and after diethyl ether treatment. Relative lipid content was calculated by dividing lipid mass by initial dry mass.

3.2.6.2 | Glycogen content

Flies were collected once a day as they eclosed, and samples were taken either at the time of eclosion or after 7 days in standard conditions (25°C and yeast-dextrose-potato medium). Females were snap-frozen in liquid nitrogen and stored at -80°C. Five replicate groups of five females per diet/temperature treatment were homogenised in PBS-Tween and 10 µl of the homogenate was incubated with starch assay reagent containing amyloglucosidase (Sigma-S9144). Homogenates with and without amyloglucosidase were then mixed with Thermo Infinity Glucose Reagent (two technical replicates each) and assayed in parallel using 96-well based colorimetric determination of glucose (Varioskan Lux Plate Reader, ThermoScientific). Total glycogen content was determined as the difference between glucose content of samples with and without amylglucosidase (protocol adapted from Musselman *et al.,* 2011). Relative glycogen content was calculated by dividing glycogen mass by initial dry mass.

3.2.7 | Statistical Analysis

The effect of protein and carbohydrate content of the food on all measured traits was analysed following the methods described by Lee *et al.* (2008). Data were analysed using linear mixed models (Bates *et al.*, 2015) with the significance of fixed effects tested using Type III Wald χ^2 tests in the *car* package (Fox and Weisberg, 2019). Models were simplified using stepwise backwards elimination

(Kuznetsova, Brockhoff and Christensen, 2017) based on Akaike's Information Criterion to arrive at a minimum adequate model. Model fit was validated by visual inspection of the residuals.

The full models included the linear and quadratic components of protein and carbohydrate and their interaction term, temperature and its interaction term with both the linear and quadratic components of carbohydrate and protein, and the three-way interaction between temperature and the linear components of protein and carbohydrate (similar to methods in Lee *et al.,* 2008). This model allowed us to identify which, if any, macronutrient(s) drove the differences in response to temperature (Table 1 and Table 2).

Random factors included in the models were as follows: tank and run for desiccation, run for heat and cold, and block for lipid content. For starvation and glycogen content, no random factors were necessary and a linear model was fit instead.

To calculate variance explained by our models (R^2) we used the function *r.squaredGLMM* from the package *lmerTest* (Kuznetsova, Brockhoff and Christensen, 2017) for the mixed models and the adjusted R^2 from the model summary for the linear models (Table 1 and Table 2).

Additionally, we used response surface comparison analysis following Silva-Soares *et al.* (2017) to compare response shapes between temperatures for each trait. Specifically, we compared the minimum adequate model described above to a model that retained the same terms except for the interactions between temperature, carbohydrate, protein, and their squares. If the minimum adequate model did not contain any temperature interactions, they were re-introduced to assess their effect in the model comparison. The two models were compared using partial F-tests (Table 3 and Table 4).

If significant interactions between protein and/or carbohydrate and temperature were found for a trait, the data were subset by temperature. Within each subset, the linear and quadratic effects of protein, carbohydrate and their cross product were examined as described for the full model (Table 5).

To compare lipid and glycogen content between newly eclosed and 7-day-old flies held under standard conditions, an additional model was used that included all the terms from the full model described above with the addition of age of the flies and all interactions between age and the factors of the full model (Table 6).

The response of each trait to the nutrient space at each rearing temperature was visualised by extracting model predictions and plotting them on a heat map of the nutrient space. For the purpose of the visualization, we extracted model predictions from the model described for the temperature subsets without model reduction. All statistical analyses were done in R version 3.6.1 (R Core Team, 2019, https://www.r-project.org/).

3.3 | Results

3.3.1 | Adult stress resistance traits

Numerous studies have shown that temperature can alter the effects of diet on larval (Kingsolver and Woods, 1998; Kingsolver *et al.*, 2006; Stillwell *et al.*, 2007; Diamond *et al.*, 2010; Lee *et al.*, 2015; Kutz, Sgrò and Mirth, 2019) and adult (Jang and Lee, 2018) life history traits in *D. melanogaster*. Other studies revealed that larval diet and thermal environment can individually impact adult stress resistance traits in insects (Crill, Huey and Gilchrist, 1996; Andersen *et al.*, 2010; Bauerfeind *et al.*, 2014; Kristensen *et al.*, 2016; MacLean *et al.*, 2017; Henry, Overgaard and Colinet, 2020), yet none have comprehensively assessed the extent to which increasing larval rearing temperatures might interact with larval nutrition to affect adult stress resistance traits.

To explicitly test the effects of the larval environment on adult stress resistance, we reared larvae on one of 25 diets, which varied in their protein and carbohydrate content, and at one of two temperatures: 25°C or 28°C. We then exposed adults to common thermal and dietary conditions (standard food and 25°C) for 7 days before measuring female starvation, desiccation, heat, and cold resistance.

3.3.1.1 | Starvation resistance

Previous literature has indicated that adults show decreased starvation resistance when reared on high P:C diets (Sisodia and Singh, 2012; Kristensen *et al.*, 2016; Henry, Overgaard and Colinet, 2020), and that increasing temperature decreases starvation resistance in adults (Da Lage, Capy and David, 1989). Given this, we predicted that the effects of high P:C larval diets on adult starvation resistance would be made worse at higher temperature, leading to a more pronounced reduction in adult starvation resistance on high P:C diets at 28°C.

Contrary to previous studies, we found that when larvae were reared at 25°C adult starvation resistance was highest in adults reared on intermediate protein, moderately low carbohydrate foods as larvae (Figure 1A, Table 1 and Table 3). The response of adult starvation resistance to diet changed significantly when larvae were reared at 28°C, as indicated by significant interactions between temperature and the linear and quadratic components of protein (Table 1) and the response surface comparison (Table 3). At 28°C, adult starvation resistance did not respond to either the protein or carbohydrate concentration of the diets (Figure 1B, Table 5). There was a significant interaction between protein and carbohydrate at 28°C, resulting in reduced starvation resistance when larvae were reared on intermediate protein, high carbohydrate diets (Figure 1B, Table 5). Taken together, our results indicate that rather than worsening the impact of high P:C diet on starvation resistance, increasing larval rearing temperature eliminates the effects of the larval diet on adult starvation resistance.

While the larval nutritional and thermal environment significantly altered adult starvation resistance, these effects were actually very small. Larval diet and temperature explained only 4.95% of the variance in starvation resistance (determined by the R^2, Table 1).

3.3.1.2 | Desiccation Resistance

Previous studies have indicated that desiccation resistance is highest on high protein diets (Andersen *et al.*, 2010; Kristensen *et al.*, 2016 but see Henry, Overgaard and Colinet (2020) which shows that high protein diets reduce desiccation resistance). Further, adults exposed to higher

temperatures show decreased desiccation resistance (Da Lage, Capy and David, 1989). Thus, we predicted that adults reared on low protein diets would show decreased desiccation resistance if they were also reared at 28°C.

Larval rearing temperature altered the way adult desiccation resistance responded to both the protein and the carbohydrate in the larval diet (Table 1 and Table 3). At 25°C, desiccation resistance was highest at intermediate to high protein concentrations and at moderately low carbohydrate concentrations (Figure 1C). For larvae reared at 28°C, only the quadratic term of carbohydrate significantly affected adult desiccation resistance, resulting in maximum desiccation resistance at intermediate carbohydrate concentrations (Figure 1D, Table 5). This was contrary to our predictions that higher larval rearing temperatures would worsen the impacts of low protein diets, and instead suggests that increasing the larval rearing temperature eliminates the effect of protein in the larval diet altogether.

While larval diet and thermal environment affected the shapes of the response surface for adult desiccation resistance, the magnitude of the effects was small. The full model only explained 3.3% of the variation in the data (Table 1).

3.3.1.3 | Heat resistance

Heat resistance is known to increase when larvae are reared at higher temperatures (e.g. Crill, Huey and Gilchrist, 1996; van Heerwaarden, Kellermann and Sgrò, 2016; Kellermann, van Heerwaardenf and Sgrò, 2017; MacLean *et al.*, 2017; Zheng *et al.*, 2017) and on diets with high P:C ratios (Andersen *et al.*, 2010; Sisodia and Singh, 2012; Kristensen *et al.*, 2016). Thus, we predicted that higher rearing temperatures might further increase heat resistance in adults reared on high P:C diets.

Similar to previous studies, we found that flies reared at 28°C had significantly higher heat resistance than flies reared at 25°C (Figure 2A&B, Table 1). Furthermore, temperature changed the way adult heat resistance responded to diet (Tables 1 and Table 3). However, these changes in the

response appear to be driven by interactions between temperature and carbohydrate, not protein as we would have predicted (Table 1). At 25°C, neither protein nor carbohydrate concentrations in the larval diet significantly affected heat resistance (Figure 2A, Table 5). However, at 28°C both the linear and quadratic terms of carbohydrate significantly affected adult heat resistance, resulting in maximum heat resistance at intermediate carbohydrate concentrations (Figure 2B, Table 5). Therefore, increasing the larval rearing temperature made adult heat resistance sensitive to the carbohydrate concentration in the larval diet.

Similar to what we found for starvation and desiccation resistance, the larval diet and thermal environment had only a small effect on adult heat resistance. Our full model explained only 5.6% of the variance in our data (Table 1), once again suggesting that while the larval environment did shape this adult resistance trait, its impacts were limited.

3.3.1.4 | Cold resistance

Cold resistance is known to decrease in larvae reared at warmer temperatures (e.g. Chown and Terblanche, 2006; Bubliy *et al.*, 2012; Bauerfeind *et al.*, 2014; van Heerwaarden, Kellermann and Sgrò, 2016; Overgaard and MacMillan, 2017). Furthermore, previous studies have found that cold resistance increases in low P:C diets (Andersen *et al.*, 2010; Sisodia and Singh, 2012; Kristensen *et al.*, 2016; Henry, Overgaard and Colinet, 2020). This would suggest that the adults reared under warmer temperatures on high P:C diets should have the lowest cold resistance.

Our results confirm previous findings, that higher larval rearing temperatures significantly decreases adult cold resistance (Figure 2C&D, Table 1). At both larval rearing temperatures, adult cold resistance decreased with increasing protein concentrations but increased with increasing carbohydrate concentrations (Figure 2C&D, Table 5). This resulted in the highest adult cold resistance in larvae reared on low protein, high carbohydrate diets. However, there were no significant interactions between temperature and the protein or carbohydrate concentration in the diet (Tables 1 and Table 3), meaning that the negative effects of high rearing temperature and high P:C larval diet on adult cold resistance were additive.

Similar to the previous stress resistance traits, we found that our full models explained only 4.6% of the variation in our data. Thus, the larval thermal and nutritional environment had minimal impacts on adult cold resistance.

3.3.2 | Energy stores

While for all cases we did see significant interactive carry-over effects of the larval thermal and nutritional environment on adult stress resistance traits, we were surprised that the effects of the developmental environment were so small. One of the mechanisms through which the larval environment is thought to impact adult stress resistance is through the accumulation of energy stores, in the form of lipids and glycogen. Larvae ingest more food than adults, as they require these energy stores for growth, but also to fuel metamorphosis and early adult stages. The amount of energy stored is known to be important to adult stress resistance. For example, adults with higher lipid stores are more resistant to starvation (Djawdan *et al.*, 1998; Ballard, Melvin and Simpson, 2008; Lee and Jang, 2014), whereas higher glycogen stores confer higher desiccation resistance (Djawdan *et al.*, 1998; Folk and Bradley, 2004; Slocumb *et al.*, 2015). However, if the adult is able to rebalance its own energy stores when provided high quality diet, the impact of the larval environment might be transient. Such changes in energy stores would explain why we observed only limited effects of the larval environment on adult stress resistance. To test how the larval environment affects energy stores in newly-eclosed versus reproductively-mature adults, we measured lipid and glycogen stores both at the time of eclosion and after 7 days on standard medium.

3.3.2.1 | Lipid content

The age of the adult fly significantly affected the response of lipid accumulation to temperature as well as to the protein and carbohydrate concentrations in the larval diet (Figure 3 A&B cf C&D, Table 6). Unlike our models for stress resistance, the model for adult lipid stores (including adult age, larval rearing temperature, and larval diet) explained 76.7% of the variation in the data (Table 6).

In newly eclosed adults, lipid content decreased with the protein concentration of the larval diet, but increased with carbohydrate concentration (Fig. 3 A&B, Table 2). Rearing temperature had no significant effect on lipid content at eclosion and did not interact with any of the diet terms (Table 2 and Table 4). This meant that adults reared on low protein, high carbohydrate diets had the highest lipid stores at both temperatures (Fig. 3 A&B).

After 7 days on standard medium, lipid content was significantly higher across all conditions when compared to newly eclosed flies (Fig. 3 C&D cf A&B, Table 6). In 7-day-old adults, protein concentration of the larval diet negatively correlated with lipid accumulation, but carbohydrate concentration no longer showed any significant effects (Fig. 3 C & D, Table 2). In addition, flies reared at 28°C had higher lipid content than flies reared at 25°C (Fig. 3 D cf C, Table 2). Rearing temperature and rearing diet did not interact (Table 2 and Table 4).

Finally, while the larval environment explained 50.8% of the variation in lipid stores in newly eclosed flies, after 7 days on standard conditions only 7% of the variation was explained by the rearing conditions (Table 2). This suggests that adult flies can compensate for suboptimal rearing conditions through adult feeding.

3.3.2.2 | Glycogen content

Similar to what we found for lipid stores, the full model including adult age, larval rearing temperature, and larval diet explained 54.8% of the variation in glycogen stores. Glycogen content of newly-eclosed flies decreased with the linear component of protein (Fig. 4 A & B, Table 2), but temperature had no effect on the glycogen content (Table 2) and it did not interact with diet (Table 2 and Table 4).

After 7 days on standard medium, adults had significantly greater glycogen stores than at eclosion (Fig. 4 C&D, Table 6). While glycogen content was still negatively correlated with protein concentration in 7-day-old flies, it also was positively correlated with carbohydrate content of the

larval diet (Table 2). Temperature had no effect on the glycogen content of 7-day-old flies (Table 2) and it did not interact with diet (Table 2 and Table 4).

Rearing temperature and diet explained less than 5% of the variation in glycogen content at either age (Table 2). This suggests that larval rearing conditions had only a small effect on glycogen stores of adults after metamorphosis, and that adults increased their glycogen stores by feeding on standard medium.

3.4 | Discussion

While the individual effects of temperature and nutrition within and across life stages have been extensively documented (e.g., Crill, Huey and Gilchrist, 1996; Andersen *et al.*, 2010; Mirth and Shingleton, 2012; Roeder and Behmer, 2014; Kellermann, van Heerwaardenf and Sgrò, 2017; MacLean *et al.*, 2017; Silva-Soares *et al.*, 2017; Gray, Simpson and Polak, 2018; Henry, Overgaard and Colinet, 2020), temperature and nutrition also interact to directly affect larval and adult life history traits (Clissold and Simpson, 2015; Cross *et al.*, 2015; Kutz, Sgrò and Mirth, 2019; Alton *et al.*, 2020). Importantly, the interactive effects of the thermal and nutritional environment experienced during development can also carry over to affect adult life history traits (Kim *et al.*, 2020). While previous work has shown that animals can compensate for the effects of exposure to short periods of nutritional stress during development, especially with respect to life history traits such as body mass (Metcalfe and Monaghan, 2001; De Block and Stoks, 2008), less is known about the extent to which exposure to combinations of thermal and nutritional stress during development will carry over to affect adult stress resistance traits. Thus, the aim of this study was to determine how the thermal and nutritional environment experienced during development interacts to shape four adult stress resistance traits and adult energy reserves in *D. melanogaster.*

3.4.1 | Carry-over effects of combined larval thermal and nutritional stress may have little impact on adult sensitivity to climate change when adults are not also stressed

Based on the fact that exposure to thermal and nutritional stress during development can result in carry-over effects on adult life history (Metcalfe and Monaghan, 2001; Kingsolver *et al.*, 2006; De Block and Stoks, 2008; Jang and Lee, 2018) and stress resistance (Crill, Huey and Gilchrist, 1996; Jang and Lee, 2018) traits individually and in combination for life history traits (Clissold and Simpson, 2015; Kutz, Sgrò and Mirth, 2019; Kim *et al.*, 2020), we expected to see significant carry-over effects of combined larval thermal and nutritional stress on adult stress resistance traits.

We detected significant, albeit small, effects of developmental diet and rearing temperature on adult resistance traits after the focal adults had been held on control food at 25°C for seven days prior to the adult stress assays. We would expect the observed effects to be exacerbated if adults had also been held on experimental diets (e.g., Kristensen *et al.*, 2016; Henry, Overgaard and Colinet, 2020) and at elevated temperatures prior to trait assessments, particularly since the same developmental diet and temperature regimes assessed here have been shown to directly affect larval performance. In particular, low P:C diets reduce developmental success under cooler (Rodrigues *et al.*, 2015; Silva-Soares *et al.*, 2017; Gray, Simpson and Polak, 2018) and warmer rearing conditions (Kutz, Sgrò and Mirth, 2019; Kim *et al.*, 2020). Importantly, we have previously shown that increased developmental temperature results in more restricted nutritional optima for egg-adult development time, egg-adult viability, and body size, and exacerbates the negative effects of carbohydrate-rich diets during development (Kutz, Sgrò and Mirth, 2019). When combined with the results of the current study, these results suggest that projections of climate change risk may be underestimated when we ignore the consequences of exposure to multiple stressors, but only when stress is experienced across life stages.

3.4.2 | Adult feeding after eclosion alleviates carry-over effects of developmental diet on energy stores and adult stress resistance

To gain insight into why larval thermal and nutritional conditions showed only minimal impacts on adult stress resistance traits, we measured lipid and glycogen content in adult flies at eclosion and after being held for 7 days on standard conditions (i.e. control food and 25°C). It is generally accepted that in *Drosophila* species higher lipid stores lead to higher starvation resistance (e.g., Djawdan *et al.*, 1998; Ballard, Melvin and Simpson, 2008; Lee and Jang, 2014), while higher glycogen content confers higher desiccation resistance (e.g., Djawdan *et al.*, 1998; Folk and Bradley, 2004; Slocumb *et al.*, 2015). However, while we did see a significant effect of larval diet on lipid stores in newly eclosed flies, with low P:C diets and high calorie diets resulting in increased lipid content at eclosion, this effect largely disappeared after the adults had been held for 7 days on control food. Rearing diet had a significant effect on glycogen content, both at eclosion and after 7 days on standard conditions, but the size of the effect of larval diet was small in comparison to the increase in glycogen content observed for all flies after 7 days on standard conditions (i.e., "age" in Table S1). This suggests that adult flies were able to compensate for differences in their larval nutritional environment by feeding to balance their lipid and glycogen stores.

The fact that lipid and glycogen reserves were similar at the time of the stress assessments (i.e., 7-day-old flies), regardless of larval environment, means that the significant effect of diet on all four stress resistance traits in this study cannot be explained by differences in energy reserves as measured here. However, we did not measure the protein reserves of the flies in this study. Previous work (Lee and Jang, 2014) showed that *Drosophila* prioritise non-lipid compounds, such as proteins and to carbohydrates, in the early stages of starvation. It is therefore possible that protein reserves differed in flies across our treatments, which could help explain the shifts in stress resistance with diet that we have observed. In addition, we only assessed total lipid reserves – it is also possible that the type of lipid stored matters as well.

Finally, it is possible that the small differences observed in stress resistance could be due to the rate at which the energy stores were utilized rather than to the total amount of lipid and glycogen, as this did not vary much between treatments. Such an effect would be reflected in differences in metabolic rate across treatments. We have recently shown (Alton *et al.*, 2020) that metabolic rate of females tested at 25°C was lower on low P:C diets and maximised at intermediate to high P:C ratios, which is consistent with Henry, Overgaard and Colinet, (2020). However, these were not the diets that also maximised starvation, desiccation, or cold resistance in the current study. In fact, the diets that maximised metabolic rate (Alton *et al.,* 2020) were those that also increased desiccation and starvation resistance, and reduced cold resistance (current study). This is inconsistent with the prediction that higher metabolic rates would lead to higher cold resistance and reduced starvation (Berrigan, 1997; Pijpe, Brakefield and Zwaan, 2007) and desiccation resistance (Henry, Overgaard and Colinet, 2020).

Many studies have examined plastic shifts in metabolic rate to increases in temperature (see Seebacher, White and Franklin, 2015). Our earlier results (Alton *et al.*, 2020) show a compensatory decrease in metabolic rate in those flies reared at 28°C when compared to those reared at 25°C, which supports previous studies (Berrigan, 1997; Pijpe, Brakefield and Zwaan, 2007; Seebacher, White and Franklin, 2015). However, this reduction in metabolic rate at warmer temperatures, while potentially reducing the energetic demands imposed by a warming world, was not associated with an increase in starvation or desiccation resistance (current study).

Overall, our earlier results (Alton *et al.,* 2020) suggest that the way in which nutrition and temperature affect the adult stress resistance traits examined in this study are independent of plastic changes in metabolism. While perhaps surprising on the one hand, this result is nonetheless consistent with the fact that the link between metabolic rate and stress resistance can be inconsistent across studies (Hoffmann and Harshman, 1999).

3.4.3 | Developmental temperature and diet interact to affect heat resistance but not cold resistance

Developmental temperature modulated the effect of larval nutrition on adult heat resistance. When flies were reared at 28°C, increasing carbohydrate content in the larval diet decreased adult heat resistance; however, when flies were reared at 25°C, diet had no effect on adult heat resistance. Our results for 28°C are consistent with previous studies (Andersen *et al.*, 2010; Sisodia and Singh, 2012; Kristensen *et al.*, 2016) showing that high P:C ratio (i.e., reduced carbohydrate content) in larval food can increase adult heat resistance. Our results for flies reared at 25°C are consistent with results of Henry, Overgaard and Colinet, (2020), who found no effect of larval diet on heat resistance.

As expected, (e.g. Hoffmann, Shirriffs and Scott, 2005; Colinet *et al.*, 2013; van Heerwaarden, Kellermann and Sgrò, 2016; MacLean *et al.*, 2017) heat resistance improved when larvae were reared at 28°C compared to 25°C independent of diet. However, this beneficial effect of developmental acclimation on adult heat resistance may be counteracted by the observed reduction in heat resistance when larvae developed on high carbohydrate diets at 28°C. While these carry-over effects on adult heat resistance were small, they may be amplified if adults were also exposed to high carbohydrate diet as could often be the case under climate change (*Moretti et al 2010;* Myers *et al.*, 2014; Rosenblatt and Schmitz, 2016; Zhu *et al.*, 2018; Asseng *et al.*, 2019).

In contrast to the results for heat resistance, developmental temperature and nutrition independently affected cold resistance. Rearing larvae on high carbohydrate, low protein diets (low P:C ratios) increased adult cold resistance at both rearing temperatures consistent with previous studies (Andersen *et al.*, 2010; Sisodia and Singh, 2012; Kristensen *et al.*, 2016; Henry, Overgaard and Colinet, 2020). As expected (e.g. Chown and Terblanche, 2006; Bubliy *et al.*, 2012; Bauerfeind *et al.*, 2014; van Heerwaarden, Kellermann and Sgrò, 2016; Overgaard and MacMillan, 2017), development at the warmer rearing temperature (28°C) resulted in lower adult cold resistance compared to 25°C.

3.4.4 | Impact of temperature/diet interactions during development on adult starvation and desiccation resistance

Larval developmental temperature and diet interacted to affect adult starvation, desiccation and heat resistance, while they acted independently to affect cold resistance. We found that higher protein to carbohydrate ratios in the larval diet increased adult starvation and desiccation resistance when larvae were reared at 25°C, consistent with previous work in *D. melanogaster* (Andersen *et al.*, 2010; Kristensen *et al.*, 2016) and *D. ananassae* (Sisodia and Singh, 2012), although Henry, Overgaard and Colinet, (2020) found that a protein rich diet actually reduced adult desiccation resistance. In contrast at 28°C we show that desiccation resistance was not affected by protein levels in the diet, but was reduced when flies developed on diets high in carbohydrates. No other studies have examined how temperature modifies the effect of diet on desiccation resistance, so it remains unclear if this is a generalizable response. Nonetheless, our results suggest that flies may become increasingly susceptible to desiccation stress if the carbohydrate content of primary producers increases with climate change, as is often predicted (Myers *et al.*, 2014; Rosenblatt and Schmitz, 2016; Zhu *et al.*, 2018; Asseng *et al.*, 2019).

Our results for starvation resistance at 25°C, where highest starvation resistance was observed when flies were reared on high P:C diets, are inconsistent with previous work (Sisodia and Singh, 2012; Kristensen *et al.*, 2016; Henry, Overgaard and Colinet, 2020) that report increased adult starvation resistance when larvae were reared on low P:C diets. However, two of these studies (Kristensen *et al.*, 2016; Henry, Overgaard and Colinet, 2020), also maintained the adults on the experimental diets (not standard diet as in the present study) on which they had been reared prior to the resistance assays. Similarly, Weldon *et al.* (2019) found that raising larvae on high P:C diets increased adult starvation resistance when resistance was measured immediately after emergence, but feeding adults for 10 days (both on a sugar-yeast diet or on a carbohydrate sugar-only diet) before testing their starvation resistance eliminated any effect of larval diet in the tephritid fruit fly *Ceratitis cosyra*. Interestingly, Jang and Lee (2018) found that adult starvation resistance increased

when adults were fed high carbohydrate diets, when larvae were fed standard balanced diets. Combined, these results suggest that nutrients acquired during adult feeding are most important for adult starvation resistance.

We also found that diet had little effect on starvation resistance when larvae were reared at 28°C, suggesting that developmental temperature may become a more significant driver of variation in starvation resistance than developmental diet in a warming world.

Finally, the effect of temperature on both starvation and desiccation resistance depended on the rearing diet but our response surfaces indicate that, on most diets, resistance to both stressors was lower when larvae were reared at 28°C than 25°C. This is consistent with our prediction and the results of Bauerfeind *et al.* (2014) who found that rearing *D. melanogaster* larvae at a higher temperature (27°C) decreased desiccation and starvation resistance compared to larvae reared at 22°C. These results suggest that any beneficial effects of nutrition on desiccation and starvation resistance may be negated in an increasingly warmer world.

3.4.5 | Linking our results to thermal and nutritional stress in nature.

Our results show that nutritional and thermal stress experienced during development has only a minimal effect on adult stress traits when adults are not exposed to either stress themselves. However, in nature, *Drosophila* larvae *and* adults are exposed to a broad range of macronutrients derived from both rotting fruits and the yeast communities that they contain (Burke and Waddell 2011; Chng et al 2017; Starmer and Fogleman 1986). Shifts in temperature and water availability will result in changes in both carbohydrate (increase, *Moretti et al 2010*) and amino acid (decrease) content of decaying fruit (Wu et al 2019; Gutierrez-Gambo et al 2021) and the yeast communities which facilitate fruit decay, and in turn the macronutrient composition of the fruit (Matavelli et al., 2015). Changes in macronutrient composition and availability combined with elevated temperature may be met by shifts in larval and adult feeding behaviour to achieve their nutritional targets (Rodrigues et al., 2015; Silva-Soares et al., 2017). While adults are more able to choose oviposition

and feeding sites (Silva-Soares et al., 2017), it is nonetheless likely that adults as well as larvae will face a more restricted nutritional space, and thus more nutritional stress, under increased temperature (Kutz et al., 2019). Whether adult feeding will be able to compensate for larval nutritional stress when adults are also exposed to nutritional stress remains unknown.

Finally, the current study used constant temperatures, while in nature larvae and adults are exposed to thermal fluctuations. It is possible that the small effect of larval thermal and nutritional conditions on adult stress resistance reported here might differ, and potentially become more pronounced, under fluctuating temperature regimes that incorporate periodically stressful temperatures. In addition, we only examined females, however *D. melanogaster* exhibits sexual dimorphism in the stress resistance traits examined in the current study (Lasne et al., 2018). It would be interesting to see if adult males and females varied in their response to the effect of larval nutritional and thermal stress, especially when the adults were also exposed to stress.

3.5 | Conclusion

We assessed, for the first time, how developmental temperature and nutrition across a broad nutrient space combine to affect ecologically important adult stress resistance traits. We found that diet modulates the effects of temperature on key stress resistance traits in complex ways. We show that a combination of elevated developmental temperatures and carbohydrate-rich diets which are projected to become more common under climate change may increase the susceptibility of adult flies to starvation, desiccation, and heat stress, although the effects were small. We also found that adult feeding can compensate for deficiencies in the larval diet with respect to energy stores. Overall, these findings suggest that adults may have the capacity to minimise any carry-over effects of combined nutritional and thermal stress during development, provided they are not themselves also stressed. Further consideration of combinations of stress within and across juvenile and adult life stages is required in order to better assess animal responses to global change.

3.6 | References

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TABLES

Table 1

Effects of dietary carbohydrate (C) and protein (P) and their squares (P^2 and C^2), developmental temperature (T), and their interactions, and model R^2 values on stress resistance traits, energy stores at the time of eclosion and after 7 days on standard conditions.

Trait		T	P	C	P^2	C^2	P x C	T x P	T x C	T x P^2	T x C^2	T x P x C	R^2
Starvation Resistance	**β**	1.9060	**0.2360**	-0.038	**-2.65E-04**	**-2.65E-04**	**-0.001**	**-0.184**	-	**4.22E-04**	-	-	4.95%
	F value	0.499	**30.482*** **	3.484	**5.599***	**9.580****	**28.638*** **	**12.504*** **	-	**8.084****	-	-	
Desiccation Resistance	**β**	-0.550	**0.026**	**-0.012**	-	**2.61E-05**	**-6.90E-05**	**-0.027**	**0.018**	-	**-3.75E-05**	**5.83E-05**	3.30%
	Chisq	2.020	**31.187*** **	**12.636*** **	-	**18.541*** **	**17.513*** **	**16.552*** **	**15.230*** **	-	**19.221*** **	**6.272***	
Heat-Shock Knockdown	**β**	**6.922**	-	0.009	-	-2.20E-05	-	-	**-0.035**	-	**6.27E-05**	-	5.60%
	Chisq	**43.086*** **	-	1.068	-	2.097	-	-	**8.877****	-	**8.601****	-	
Chill-Coma Recovery Rate	**β**	**-0.001**	**-7.59E-05**	**3.20E-05**	**1.61E-07**	**-3.20E-08**	-	-	-	-	-	-	4.60%
	Chisq	**21.969*** **	**35.143*** **	**16.655*** **	**20.920*** **	**6.021***	-	-	-	-	-	-	

Linear mixed models or linear models were fit to the data and reduced based on AIC values to the minimum adequate model. Significant coefficients are in bold. Significance level codes: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Dashes indicate terms that were eliminated from the model during reduction.

Table 2

Effects of dietary carbohydrate (C) and protein (P) and their squares (P^2 and C^2), developmental temperature (T), and their interactions, and model R^2 values on energy stores at the time of eclosion and after 7 days on standard conditions.

Trait		T	P	C	P^2	C^2	P x C	T x P	T x C	T x P^2	T x C^2	T x P x C	R^2
Lipid content eclosion	β	-	**-0.001**	**2.76E-04**	**1.62E-06**	-	-	-	-	-	-	-	50.80%
	Chisq	-	**40.224*** **	**148.047*** **	**22.391*** **	-	-	-	-	-	-	-	
Lipid content 7-day-old	β	**0.009**	**-2.19E-04**	-	**6.54E-07**	-	-	-	-	-	-	-	7.00%
	Chisq	**4.161***	**6.903****	-	**6.380***	-	-	-	-	-	-	-	
Glycogen content eclosion	β	-0.001	**-2.15E-04**	2.58E-05	3.38E-07	-	2.24E-07	1.35E-04	4.26E-05	-	-	-5.69E-07	4.90%
	F value	0.024	**7.448****	1.011	3.246	-	0.755	2.183	1.644	-	-	2.635	
Glycogen content 7-day-old	β	-	**-2.30E-04**	**5.19E-05**	5.27E-07	-	-	-	-	-	-	-	2.00%
	F value	-	**4.991***	**7.294****	3.268	-	-	-	-	-	-	-	

Linear mixed models or linear models were fit to the data and reduced based on AIC values to the minimum adequate model. Significant coefficients are in bold. Significance level codes: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Dashes indicate terms that were eliminated from the model during reduction.

Table 3

Comparisons between the response surfaces of flies reared at 25°C and flies reared at 28°C for stress resistance traits.

Trait	df	Chisq/F-value	p-value
Starvation Resistance	2	7.519	**5.64E-04*** **
Desiccation Resistance	8 12	29.7563	**5.49E-06*** **
Heat Shock Knockdown	6 8	8.918	**0.012***
Chill-Coma Recovery Rate	8 12	0.895	0.925

All response surfaces were compared using partial F tests or chi-square tests. Significant p-values are in bold. Significance level codes: * $p < 0.05$, *** $p < 0.001$

Table 4

Comparisons between the response surfaces of flies reared at 25°C and flies reared at 28°C for energy stores at the time of eclosion and after 7 days on standard conditions

Trait	df	Chisq/F-value	p-value
Lipid content eclosion	7 10	5.822	0.121
Lipid content 7-day-old	6 8	1.344	0.511
Glycogen content eclosion	3	0.878	0.453
Glycogen content 7-day-old	3	1.119	0.342

All response surfaces were compared using partial F tests or chi-square tests.

Table 5

Effects of dietary carbohydrate (C) and protein (P), their squares (P^2 and C^2) and interaction, and model R^2 values, subsetted by rearing temperature (25°C or 28°C) for traits where response to larval diet depended on the developmental temperature.

	Rearing Temp.		P	C	P^2	C^2	P x C	R^2
Starvation Resistance	25	β	**0.253**	**-0.063**	**-3.29E-04**	**1.26E-04**	**-4.46E-04**	3.97%
		F-value	**26.496*** **	**4.526***	**7.268****	**5.834***	**9.918****	
	28	β	0.034	-0.012	2.18E-04	8.69E-05	**-0.001**	3.71%
		F-value	0.545	0.191	3.550	3.332	**19.287*** **	
Desiccation Resistance	25	β	**0.026**	**-0.012**	-	**2.67E-05**	**-7.03E-05**	4.62%
		Chisq	**31.186*** **	**12.647*** **	-	**18.888*** **	**17.747*** **	
	28	β	-	-	-	**-2.78E-06**	-	0.56%
		Chisq	-	-	-	**4.242***	-	
Heat Shock Knockdown	25	β	-	-	-	-	-	0.00%
		Chisq	-	-	-	-	-	
	28	β	-	**-0.026**	-	**4.03E-05**	-	1.24%
		Chisq	-	**8.334****	-	**6.010***	-	

Linear mixed models or linear models were fit to the data and reduced based on AIC values to the minimum adequate model. Significant coefficients are in bold. Significance level codes: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Dashes indicate terms that were eliminated from the model during reduction.

Table 6

Effects of dietary carbohydrate (C) and protein (P) and their squares (P^2 and C^2), developmental temperature (T), fly age (A) -either at eclosion or after 7 days on standard conditions-, and their interactions, and model R^2 values, on adult lipid and glycogen content.

		A	T	P	C	P^2	P x C	T x P	T x C	T x P^2	T x P x C	T x A	A x P	A x C	A x P x C	R^2
Lipid content	β	**0.101**	**-0.016**	**-0.001**	**2.16E-04**	**1.27E-06**	3.60E-07	-	**4.47E-05**	-	-	**0.017**	**4.53E-04**	**-1.52E-04**	**-1.03E-06**	76.7%
	Chisq	**158.686*** **	**6.261***	**51.622*** **	**36.777*** **	**27.556*** **	1.147707	-	**4.107***	-	-	**6.123***	**14.587*** **	**12.697*** **	**5.171***	
Glycogen content	β	**0.064**	-0.001	-1.31E-04	7.55E-06	1.14E-07	2.42E-07	-9.88E-05	**1.06E-04**	**7.41E-07**	**-7.71E-07**	-	-	-	-	54.8%
	t-value	**560.284*** **	0.035	2.103	0.074	0.189	0.850	0.595	**7.090** **	**3.995***	**4.307***	-	-	-	-	

Linear mixed models or linear models were fit to the data and reduced based on AIC values to the minimum adequate model. Significant coefficients are in bold. Significance level codes: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Dashes indicate terms that were eliminated from the model during reduction.

FIGURES

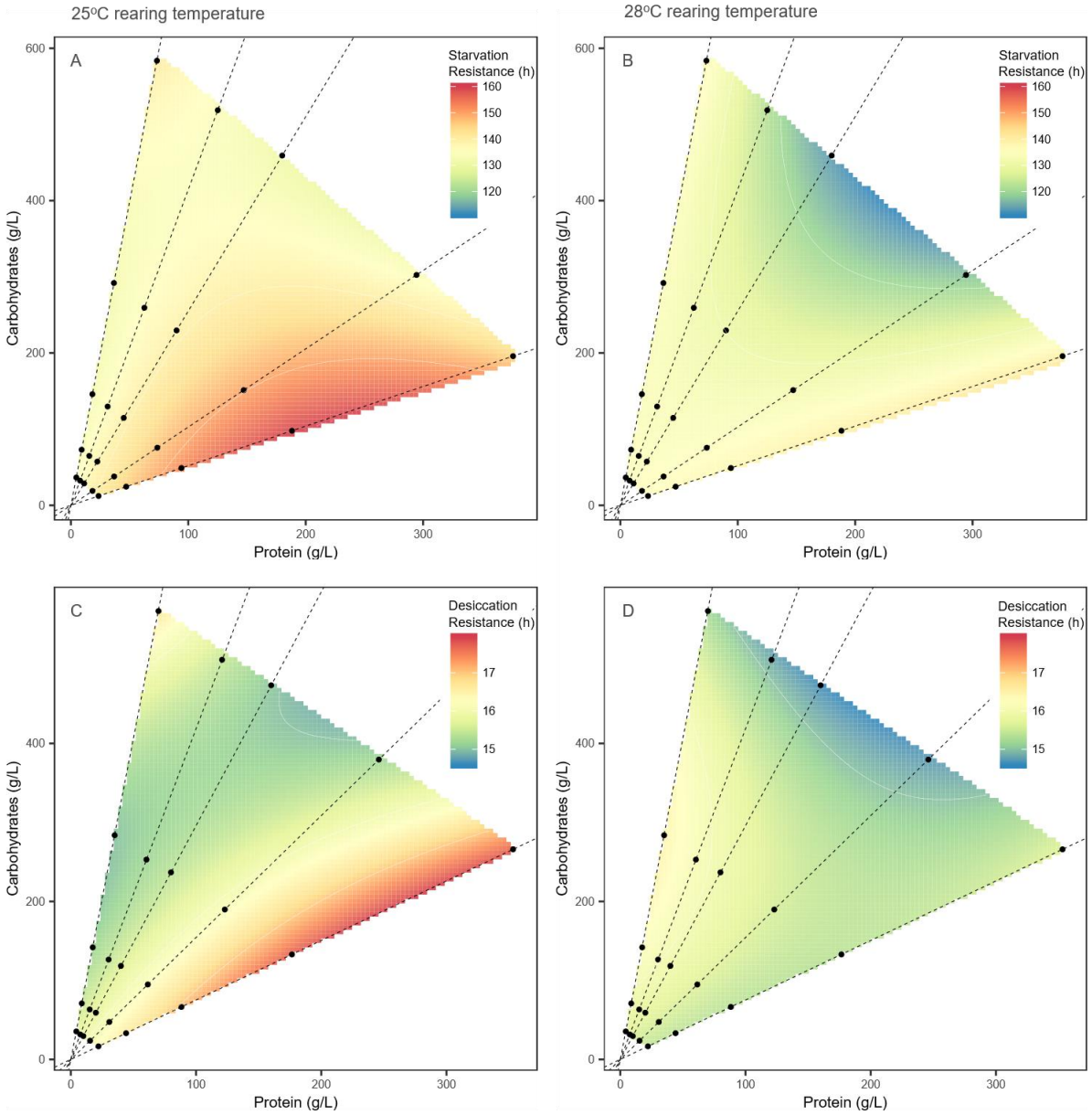

Fig. 1. The effects of protein and carbohydrate content of the larval diet, represented as the fitted response surfaces of the effects of 25 different diets varying in protein and carbohydrate content. *Dashed black lines* represent P:C ratios 1:8, 1:4, 1:3, 2:3 and 3:2 (from left to right). *Filled black circles* represent the respective nutritional coordinates of each of the 25 diets used. A-B: hours surviving starvation; C-D: hours surviving desiccation. *Left column*: 25°C rearing temperature; *right column*: 28°C rearing temperature.

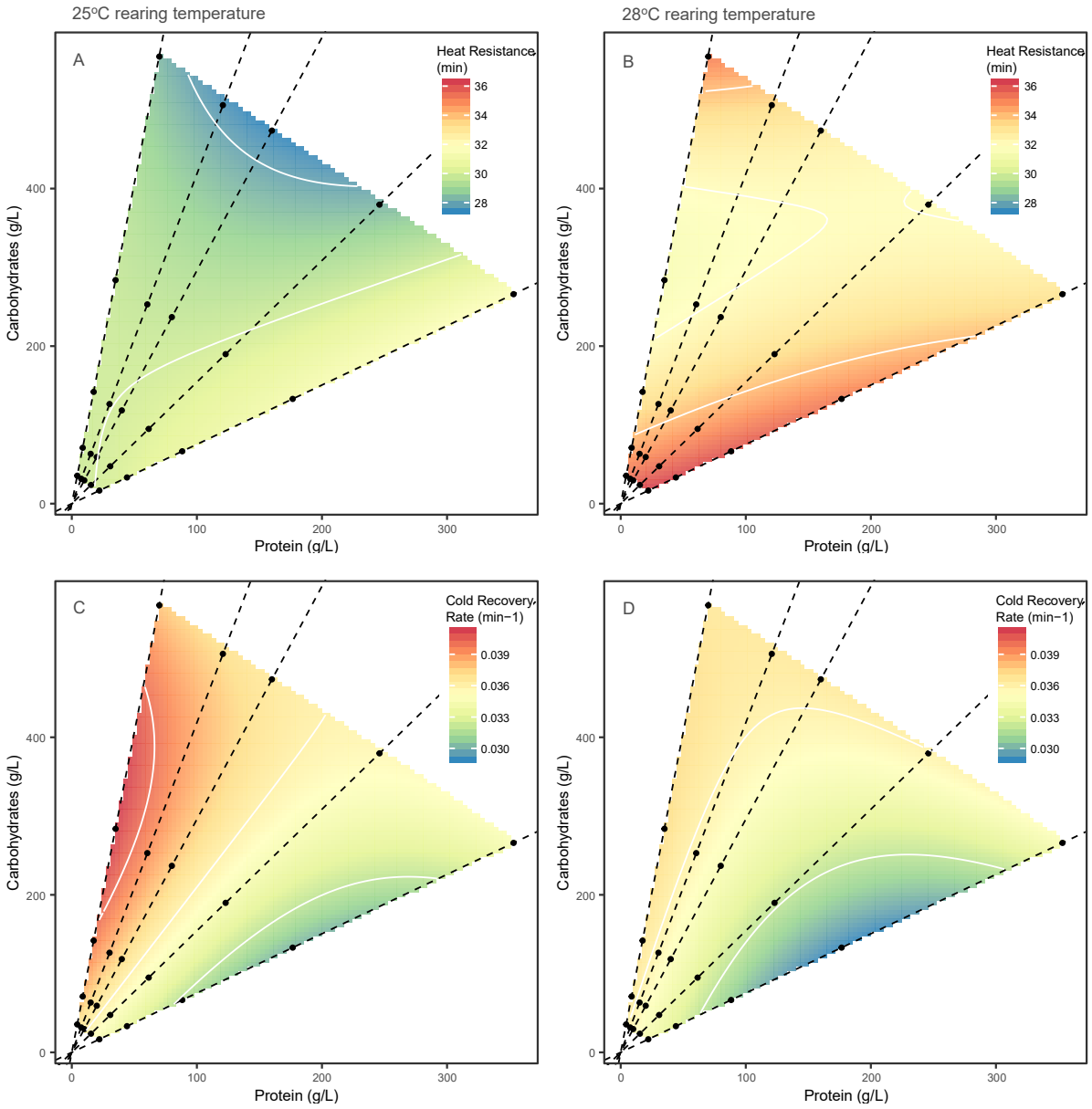

Fig. 2. The effects of protein and carbohydrate content of the larval diet, represented as the fitted response surfaces of the effects of 25 different diets varying in protein and carbohydrate content. *Dashed black lines* represent P:C ratios 1:8, 1:4, 1:3, 2:3 and 3:2 (from left to right). *Filled black circles* represent the respective nutritional coordinates of each of the 25 diets used. A-B: Heat-shock knockdown time; C-D: Chill-coma recovery rate. *Left column*: 25°C rearing temperature; *right column*: 28°C rearing temperature.

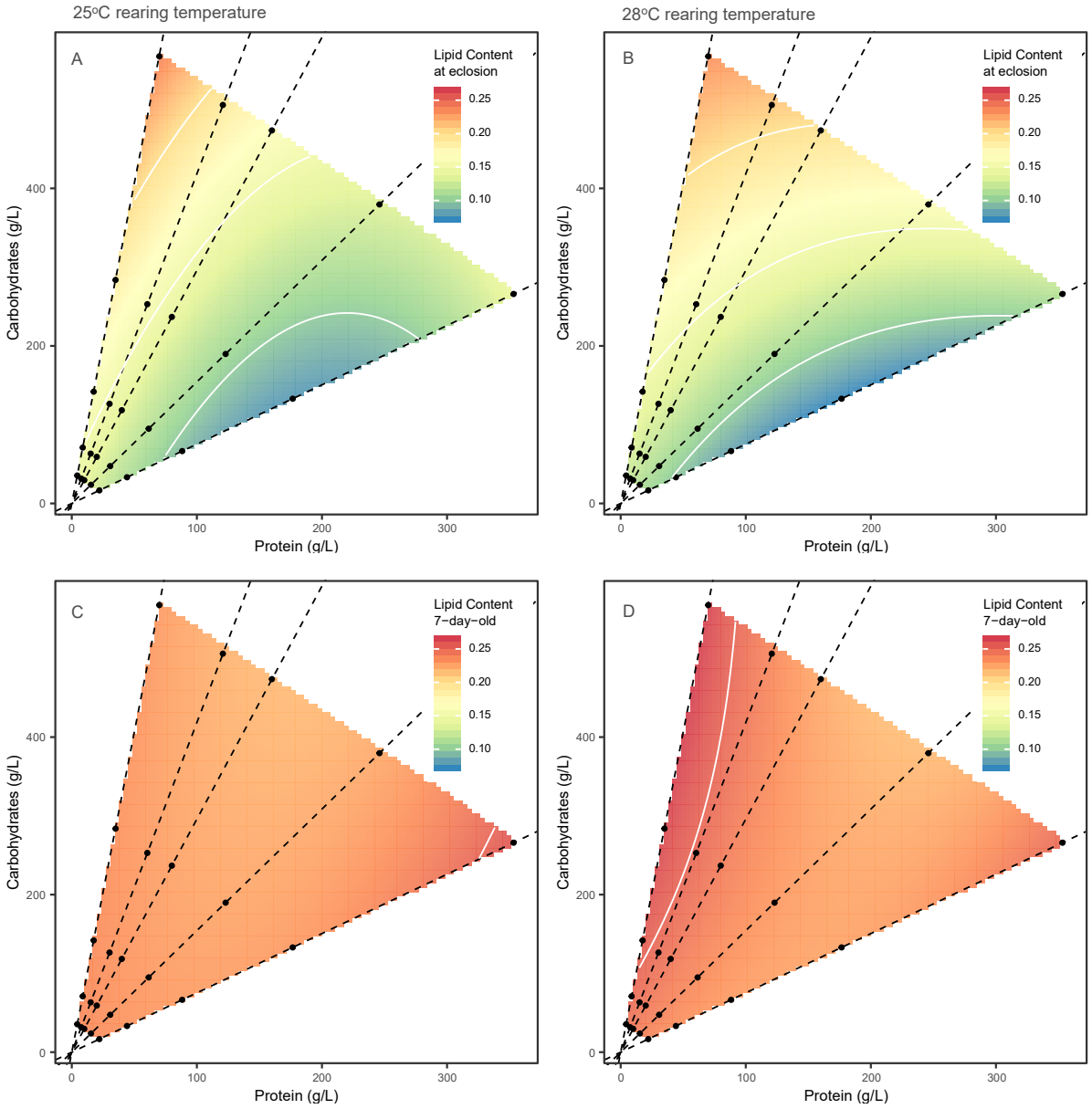

Fig. 3. The effects of protein and carbohydrate content of the larval diet, represented as the fitted response surfaces of the effects of 25 different diets varying in protein and carbohydrate content. *Dashed black lines* represent P:C ratios 1:8, 1:4, 1:3, 2:3 and 3:2 (from left to right). *Filled black circles* represent the respective nutritional coordinates of each of the 25 diets used. A-B: Mass-specific lipid content at the time of eclosion; C-D: Mass-specific lipid content after 7 days on standard conditions. *Left column*: 25ºC rearing temperature; *right column*: 28ºC rearing temperature. Units are in µg of lipids per µg of dry weight.

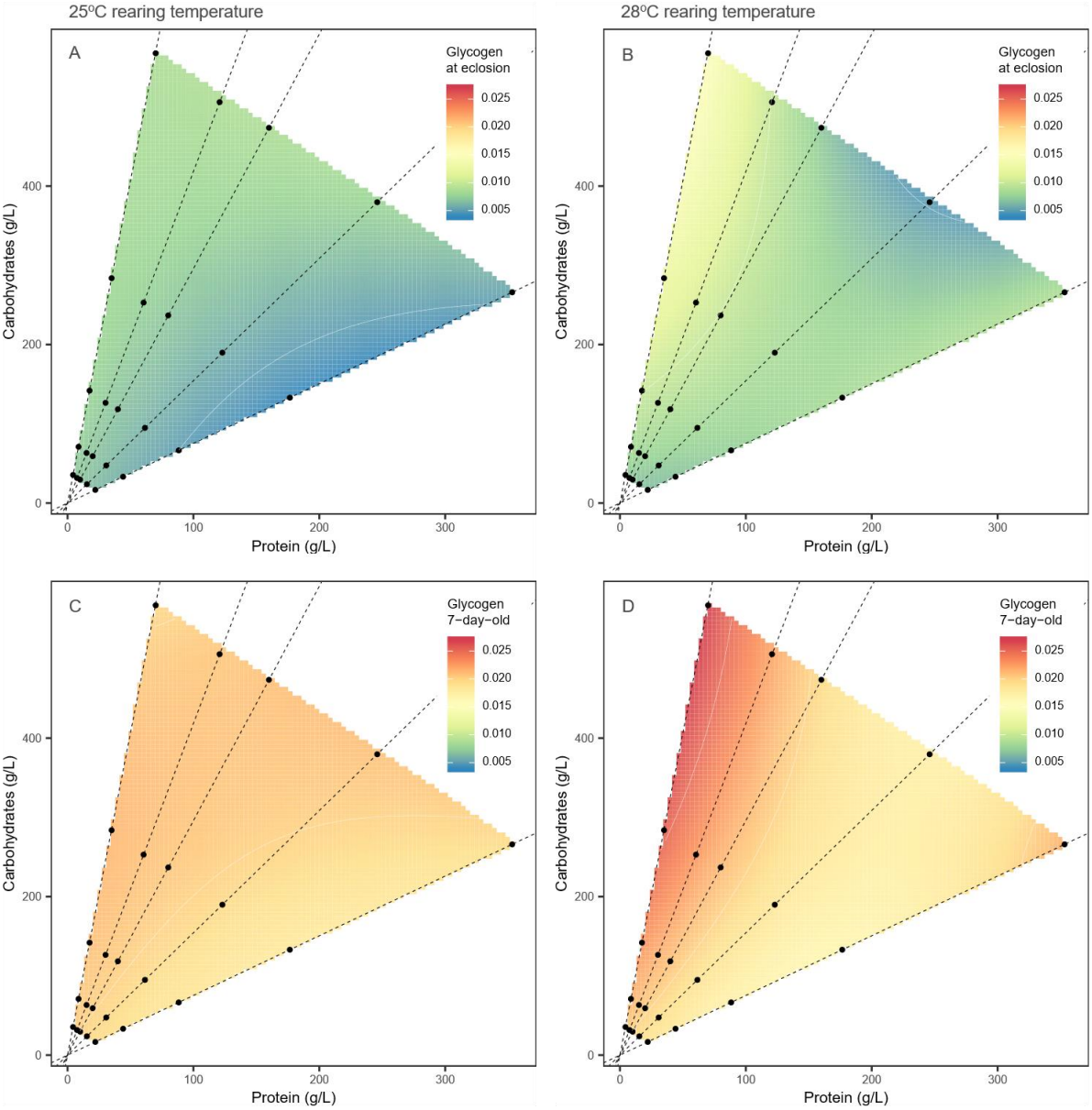

Fig. 4. The effects of protein and carbohydrate content of the larval diet, represented as the fitted response surfaces of the effects of 25 different diets varying in protein and carbohydrate content. *Dashed black lines* represent P:C ratios 1:8, 1:4, 1:3, 2:3 and 3:2 (from left to right). *Filled black circles* represent the respective nutritional coordinates of each of the 25 diets used. A-B: Mass-specific glycogen content at the time of eclosion; C-D: Mass-specific glycogen content after 7 days on standard conditions. *Left column*: 25°C rearing temperature; *right column*: 28°C rearing temperature. Units are in µg of glycogen per µg of dry weight.

Chapter 4 | Impacts of adaptation to combined thermal and nutritional stress on trait plasticity

ABSTRACT

Temperature and nutrition are amongst the most common stressors for small ectotherms and will become increasingly so under current climate change. One key outstanding question is whether animals will be able to adapt quickly enough to survive. Most studies investigating adaptation focus on stressors singly, however, insects will experience changes in their thermal and nutritional environment simultaneously. Further, phenotypic plasticity can play a key role under climate change, especially because environmental variability (including temperature fluctuations and food availability) is expected to increase in the future. Therefore, it is important to understand how evolutionary adaptation affects plasticity. However, changes in plasticity are often overlooked in studies investigating adaptation. In this study, we used experimental evolution in *Drosophila melanogaster* to investigate adaptation of key life history traits – development time, viability, and adult size – to the combined effects of increased temperature and suboptimal diet: either a low-calorie diet, which simulates reduced food abundance, or a low-protein diet, which simulates reduced food quality. Because larval and pupal stages are less mobile than adults, and thus less likely to escape stressful conditions, selection was imposed only on the pre-adult stages. We showed that co-adaptation to elevated temperature and poor food can result in non-additive effects for development time and viability, indicating these effects cannot be predicted from adaptation to each variable singly. We also found that thermal adaptation can change plasticity to diet and vice versa, suggesting an overlap between the mechanisms leading to thermal and nutritional adaptation and the mechanisms controlling nutritional and thermal plasticity, respectively. Our findings suggest that both plastic and evolved responses, the primary mechanisms through which animals can persist in their environments, are altered under combined

stress. This highlights the need for more studies of adaptation to environmental variables in combination when assessing organisms' responses to climate change.

Key words: experimental evolution, wing size, development time, egg-to-adult viability, thermal adaptation, developmental diet, nutritional adaptation, climate change

4.1 | Introduction

Under current climate change, animals will face challenging thermal environments including higher average temperatures (IPCC, 2014; IPBES, 2019). It is therefore likely that organisms will be under increasing selective pressure to withstand elevated temperatures. Current predictions suggest that the rate of environmental change will exceed the capacity of many species to adapt (Etterson and Shaw, 2001; Quintero and Wiens, 2013); however, rapid adaptation has already been observed in many taxa (Franks, Sim and Weis, 2007; Skelly *et al.*, 2007; Sinclair, Williams and Terblanche, 2012; Krehenwinkel, Rödder and Tautz, 2015). In addition to evolutionary adaptation, phenotypic plasticity may also play an important role in animal responses to climate change (Seebacher, White and Franklin, 2015; Sgrò, Terblanche and Hoffmann, 2016). Indeed, because extreme weather events are becoming increasingly more frequent (IPCC, 2014; Stott, 2016; Chevin and Hoffmann, 2017) having high levels of plasticity could be crucial for animals in the future. However, while evolutionary adaptation may improve chances of survival in an altered environment in the medium to longer-term, it is not clear how adaptation will affect animals' plasticity (Falconer, 1952; Via and Lande, 1985; Via, 1993), which allows them to adjust their phenotype in response to their environment in the short-term (Chevin and Hoffmann, 2017). Therefore, more research is needed to understand how animals will adapt to climate change stressors, and how this will affect their ability to adjust their phenotypes with plastic responses, to better reconcile predictions of vulnerability with reality and improve risk assessments.

Thermal stress and extreme weather events are not the only stressors animals will face under climate change. Organisms will be exposed to multiple, interacting stressors simultaneously. In particular, nutritional stress (the most common stressor for animals in nature) will become increasingly relevant as climate change modifies plant distribution and abundance (Raubenheimer, Simpson and Tait, 2012; Cross *et al.*, 2015) and nutritional value (Rosenblatt and Schmitz, 2016). Changes in the thermal and nutritional environment of animals are likely to interact in shaping evolutionary responses to environmental change (Kaunisto, Ferguson and Sinclair, 2016). However, very few studies in terrestrial systems have attempted to uncover the outcomes of adapting to combinations of environmental stressors simultaneously, and how that differs from adapting to each stressor independently (but see Bochdanovits and Jong, 2003).

While adaptation to thermal and nutritional stress in combination has not received much attention, much more is known about adaptation to each of these stressors individually. Adaptation to new thermal environments and its life history consequences has often been studied using laboratory experimental evolution (e.g. Cavicchi *et al.*, 1985, 1989, 1991; Kolss *et al.*, 2009; Vijendravarma, Narasimha and Kawecki, 2012; Mallard *et al.*, 2018). Experimental evolution studies have shown that, in *Drosophila* species, adaptation to cooler temperatures usually results in faster development and larger size under common garden conditions (Anderson, 1966; Huey, Patridge and Fowler, 1991; James and Partridge, 1995; Neat *et al.*, 1995). Hence, adaptive responses to new thermal environments include changes in developmental traits in *Drosophila* species.

Experimental evolution has also been used to study adaptation to challenging nutritional environments in *Drosophila* species and caterpillars (Warbrick-Smith *et al.*, 2006; Kolss *et al.*, 2009; Vijendravarma, Narasimha and Kawecki, 2011; Vijendravarma *et al.*, 2015; Erkosar *et al.*, 2017; May *et al.*, 2019; Zajitschek *et al.*, 2019; Cavigliasso *et al.*, 2020; Kawecki *et al.*, 2020). Most of these studies focused on adaptation to calorie-diluted diets (containing ¼ of the nutrients of the standard diet; Kolss *et al.*, 2009; Vijendravarma, Narasimha and Kawecki, 2011; Vijendravarma *et al.*, 2015; Erkosar *et al.*, 2017; Asseng *et al.*, 2019; May *et al.*, 2019; Cavigliasso *et al.*, 2020;

Kawecki *et al.*, 2020). They found that adaptation to a low-calorie diet led to faster development (Kolss *et al.*, 2009; May *et al.*, 2019) and higher viability when reared on poor food (Kolss *et al.*, 2009), lower adult weight (Kolss *et al.*, 2009; May *et al.*, 2019), lower early life fecundity (Kolss *et al.*, 2009), and smaller wing size when reared on standard food (Vijendravarma, Narasimha and Kawecki, 2011), lower dependence on gut microbiota for nutrient digestion (Erkosar *et al.*, 2017), and increased nutrient assimilation (Cavigliasso *et al.*, 2020). Therefore, adaptation to low-calorie diets leads to changes in life-history traits and digestive mechanisms.

While calorie-diluted food approximates an environment where food resources are scarce, climate change is also altering the macronutrient composition of plants (Rosenblatt and Schmitz, 2016; Medek, Schwartz and Myers, 2017; Beach *et al.*, 2019) often forcing animals to adapt to low protein diets (Rosenblatt and Schmitz, 2016; Medek, Schwartz and Myers, 2017; Beach *et al.*, 2019). Because climate change may decrease the abundance of some plants as well as change their macronutrient composition, it is also important to understand how animals will adapt to changes in the macronutrient composition of their food.

Zajitschek *et al.* (2019) investigated adaptation to macronutrient imbalance and found that adaptation to high-protein foods led to increased fecundity, but low lifespan when animals were reared on standard and low-protein diets. In contrast, adaptation to low-protein foods reduced lifespan without increasing fecundity, when animals were reared on standard and low-protein diets. Further, Warbrick-Smith *et al.* (2006) found that caterpillars adapted to carbohydrate-rich food could eat excess carbohydrate without laying it down as fat, while caterpillars adapted to low-carbohydrate food stored more fat. These studies suggest that dietary adaptation affects a wide range of life-history and fitness traits, as well as post-ingestive mechanisms.

The few studies that have explored how animals adapt to combined thermal and nutritional stress have shown that their responses differ from those expected from adaptation to either stressor individually. Flies adapted to a low-calorie diet in combination with either 17.5°C (cold-adapted) or 27.5°C (warm-adapted) conditions showed unexpected synergistic responses in body weight and

viability (Bochdanovits and Jong, 2003). For example, under common garden conditions, cold-adapted flies were always heavier than warm-adapted flies. In addition, flies adapted to warm temperature and low-calorie food were lighter than those adapted to warm temperatures and high-calorie food. However, flies adapted to both low-calorie food and cold were even heavier than flies adapted to high-calorie food and cold under common garden conditions (Bochdanovits and Jong, 2003). That is, adaptation to a low-calorie diet resulted in lighter flies when flies were also adapted to warmer temperatures, but heavier flies when also adapted to lower temperatures; the effect of nutritional adaptation on body size depended on the thermal environment to which the populations had also adapted.

These synergistic effects of diet and temperature are likely to result from differences in adapative capacity across environmental conditions. Sgrò and Hoffmann (1998) found increased additive genetic variance and adaptive capacity for fecundity when larvae were exposed to combinations of cold shock, ethanol, and low-protein diet during development when compared to unstressed controls. Taken together, the above studies suggest that exposure to combined thermal and nutritional stress during development could lead to unexpected phenotypic outcomes and increased expression of adaptive genetic variation, which might in turn facilitate adaptation to combinations of stressors.

Importantly, experimental evolution studies can shed light on how plasticity might also change during adaptation to individual or combined stressors. For instance, thermal adaptation can result in shifts in thermal plasticity. Cavicchi *et al.* (1995) found that lines adapted to 18°C showed increased heat tolerance when reared at 25 or 28°C, but lines adapted to 28°C did not; they showed equal heat tolerance across rearing temperatures. These results suggest that adaptation to warmer temperatures can result in a loss of thermal plasticity. In contrast, Stazione *et al.* (2020) found that *D. buzzatii* lines selected for mating success at high temperature improved heat resistance through hardening (i.e., the plastic response of increasing heat resistance after a short

exposure to sublethal heat stress), but control lines did not, indicating that selection at elevated temperatures actually increased thermal plasticity of their flies.

There are other examples where adaptation to temperature and nutrition individually or in combination has resulted in shifts in plasticity. For instance, Bochdanovits and Jong (2003) found that the increase in body weight (regardless of diet) resulting from adaptation to cold conditions was apparent only when flies were reared under warm, but not cold, conditions, meaning that warm-adapted flies displayed higher plasticity for size than cold-adapted flies. James and Partridge, (1995) found that flies selected at 16.5°C developed faster than flies selected at 25 or 28°C, but this difference was larger when flies were reared at 16.5°C than at 25 or 28°C; this was because the 16.5°C selected lines were less plastic than 25 or 28°C selected lines. Tobler, Hermisson and Schlötterer (2015) found that *D. melanogaster* populations adapted to either cyclic cold or hot temperatures showed increased fitness in both environments, but that both types of selected lines were less plastic than controls in their fitness. Finally, Kolss *et al.* (2009) found that flies adapted to low-calorie food were smaller and developed faster than controls, but this effect was larger when flies were reared on low-calorie food than on standard food. The interaction was due to control lines being more plastic than the low-calorie adapted lines. Thus, adapting to thermal or nutritional conditions can reduce plasticity.

Theoretically, in a selection experiment where animals are not exposed to variable environments but rather are maintained in a uniform environment, plasticity would increase or decrease depending on whether genes controlling the mean phenotypic response also control the plastic response (Falconer, 1952; Via and Lande, 1985; Via, 1993). If the genes involved in adapting to a stressful environment are also genes that play a role in increasing plasticity to that environmental factor, we would expect an increase in plasticity as a correlated response to selection (Via and Lande, 1985; Via, 1993). For instance, Stazione *et al.* (2020) suggest that selection for mating success at high temperature also increased the hardening capacity of selected flies because

selection for heat resistance reduced the expression of Hsp70, and low rates of heat-shock protein synthesis correlates with increased heat hardening capacity.

It is clear that both temperature and nutrition affect growth and development in insects (Clissold and Simpson, 2015). On a basic level, temperature and nutrition are linked through the metabolic rate because higher temperatures require relatively more energy to fuel growth and this energy is obtained through the diet (Kingsolver and Huey, 2008). However, temperature and nutrition are also known to affect insulin signalling and ecdysone synthesis, both of which are key in the regulation of growth and development in insects (reviewed in Mirth, Saunders and Amourda, 2021). Therefore, through their effects on insulin and ecdysone production, diet and temperature can interact to shape plastic developmental responses to environmental conditions. Whether adaptation to combined thermal and nutritional stress will result in correlated increases or decreases in plasticity across a range of life history traits remains largely unknown.

The aim of this project was to investigate changes in mean phenotype and in plasticity of key life history traits as a consequence of adaptation to nutritional and thermal stress singly or in combination in *D. melanogaster*. We subdivided the nutritional stress into low-calorie or low-protein food, to simulate the effects of changes in abundance and macronutrient balance (quality) of food predicted to occur with climate change (Raubenheimer, Simpson and Tait, 2012; Cross *et al.*, 2015; Rosenblatt and Schmitz, 2016; Medek, Schwartz and Myers, 2017; Beach *et al.*, 2019). We also investigated adaptation to a 3^{o}C increase in temperature, from 25 to 28^{o}C, to mimic projected average temperature increase under climate change (IPCC, 2013; Rogelj *et al.*, 2018). We examined the effects of thermal and nutritional stress on both mean responses and plastic responses in egg-to-adult viability, egg-to-adult development time, and adult size, as these have been shown to depend on both nutrition and temperature during development (Clissold and Simpson, 2015; Kutz, Sgrò and Mirth, 2019). We hypothesized that if genes involved in adapting to thermal or nutritional stress also decreases trait plasticity, and if combined stress exerts stronger selective pressure on phenotypes, we would see the lowest plasticity across thermal and

nutritional conditions in lines adapted to combined stress. Furthermore, we predicted that if the genes involved in adaptation to thermal stress, low calorie diet, and low protein diet overlapped, we would see that lines adapted to combined stress would also show improved trait values across these conditions. Finally, because macronutrient composition of the diet can elicit stronger phenotypic responses than calorie content on development time and size (Kutz, Sgrò and Mirth, 2019), we expected that the selective pressure of a low-protein diet would be stronger than that of a low-calorie diet, resulting in a stronger evolved response. The results of this study will indicate how phenotypes change in response to combined stress as well as provide insight into whether adapting to particular thermal/dietary conditions can provide cross-protection to other types of thermal/dietary stress.

4.2 | Methods

4.2.1 | Fly stocks and experimental evolution protocol

The population for this study was obtained from 200 wild-caught isofemale lines of *D. melanogaster* collected from Duranbah (latitude 28.3021° S) in January 2018. All isofemale lines were treated with tetracycline to remove Wolbachia and, two generations later, 5 virgin females and 5 males from each isofemale line were collected and pooled together to form the base population.

This base population was kept at constant 25°C, on standard medium (potato flakes 20 g/L; dextrose 30 g/L; Brewer's yeast 40 g/L; agar 7 g/L; nipagen 6 ml/L; and propionic acid 2.5 ml/L) and a 12-hr light (L):12-hr dark (D) photoperiod and was expanded for two generations, resulting in 60 bottles containing 750 to 1000 flies each. Then, eggs were collected and divided into 6 selection treatments with 5 replicate lines per treatment and 400 to 500 eggs per replicate line, following (Sgrò and Blows, 2004). The six selection treatments included one of two selection temperatures, 25°C or 28°C, and one of three selection diets: standard, low-calorie (same macronutrient

proportion as the standard, but with ¼ of the calories), and low-protein (same calorie content as the standard but with ¼ the amount of protein) (Table 1). We treat the 25°C and standard diet treatment as our control.

At the start of each generation, each replicate line was placed under their corresponding selection treatment at a density of 200-250 eggs per 70mL of medium per fly bottle to equalise density across all treatments and avoid competition and inadvertent density selection, using 4 bottles per replicate line / condition (total of 800-1000 individuals per generation for each replicate line in each condition). Adults were collected from all four bottles upon eclosion each morning until no more adults eclosed to avoid selection for fast development. Adults were then placed together to mate under control conditions (25°C and standard medium) for 3 days after the last adults were collected. Then, eggs were collected and allocated to their respective experimental treatments as described above, to initiate the next generation of selection.

Due to the differences in development time caused by the selection treatments (which varied from 7 to 23 days), our lines were under selection for different numbers of generations. Lines selected under 25°C and either low-calorie or low-protein diet had undergone selection for 22 generations at the time of the experiments described here, whereas lines selected under 28°C and either low-calorie or low-protein diet had undergone selection for 25 generations. Controls (25°C and standard diet) were on generation 27, while lines selected under 28°C and standard diet had undergone selection for 30 generations.

4.2.2 | Assessing evolved and plastic shifts in response to selection

Before assessing the effects of adaptation to thermal and nutritional conditions on life history traits, all lines were bred for two generations under common garden conditions at 25°C and on the standard food to reduce effects due to maternal or grand-maternal environment. For the assays of the life history traits described below, eggs were collected and placed into vials containing 7 mL of medium at a density of 20 eggs per vial to avoid density effects. Because of the number of eggs

needed, the egg transfer was done over two consecutive days (blocks), with 3 vials per line / rearing condition prepared during the first day and 2 vials per line and rearing condition prepared during the second day. Because we were interested in assessing the effects of adaptation on changes in both mean trait values and trait plasticity, all replicate selection lines were reared under all 6 temperature-diet treatment combinations in a full-factorial design, with 5 replicate vials per selection line and rearing condition.

4.2.3 | Egg-to-adult viability

Egg-to-adult viability was measured as number of adults eclosed relative to the 20 eggs transferred into each vial.

4.2.4 | Egg-to-adult development time

To assess egg-to-adult development time, vials seeded with eggs (as described above) were placed under constant light to minimize circadian eclosion peaks. Vials were checked for emerged adults every 8 hours, and adults were removed from the vials. For each vial, the assay ended when four consecutive time periods yielded no flies, at which point vials were discarded. Each fly received a development time score counted as the hours to eclosion from the mid-point of the egg laying period.

4.2.5 | Wing centroid size

Body size was estimated using wing centroid size. After they emerged, adults were kept under standard conditions for 2-3 days. Then they were frozen and females were preserved in a glycogen-ethanol solution. Wings of 20 females per selection line were collected, mounted on a microscope slide and photographed (Leica M80 stereo microscope; Leica). Although size is a sexually dimorphic trait, understanding sex-specific responses to the combined effects of nutrition and temperature was not the focus of the present study, and so, only females were used.

Wing centroid size was calculated following Clemson, Sgrò and Telonis-Scott (2016) with the exception that landmarks were automatically placed using wingNet, a neural network created for

this purpose (https://github.com/TimoStoff/wingNet). Landmark placement was visually inspected and corrected where necessary. Centroid size was automatically calculated as the square root of the sum of the square distance from each landmark to the centroid.

4.2.6 | Statistical analysis

The effect selection treatment and rearing environment was explored with a full model that included the fixed effects of selection temperature, selection diet, rearing temperature, rearing diet and all their interactions as explanatory variables. To test for evolved differences in trait means, the subset of flies reared on standard common garden conditions (standard diet, 25^{o}C) were analysed in a separate model including the fixed effects of selection temperature and selection diet, and their interaction as explanatory variables. Data were fit with linear mixed models for development time and size, and generalized linear mixed models using a binomial distribution for viability. The random factors were block (day when eggs were collected) and replicate line. Model fit was validated by visual inspection of the residuals. Models were simplified using stepwise backwards elimination (Kuznetsova, Brockhoff and Christensen, 2017) based on Akaike's Information Criterion to arrive at a minimum adequate model. The significance of fixed effects was tested using Type III Wald $\chi 2$ tests in the *car* package (Fox and Weisberg, 2019). All statistical analyses were done in R (version 3.6.1, R Core Team, 2019, URL https ://www.r-project.org/), and the complete dataset and scripts are available on Figshare (DOI: 10.26180/15032697).

4.3 | Results

Under climate change, animals are predicted to experience elevated temperatures and both changes in abundance and quality of food. This study aimed to test the phenotypic and plastic effects of adapting to these combined stressors. To simulate changes in abundance of food, we let *D. melanogaster* larvae evolve on a low-calorie diet. To mimic changes in food quality, we let flies

evolve on a low-protein diet. To impose thermal stress, lines for each diet type were reared at one of two temperatures (25°C or 28°C), resulting in a total of six diet by temperature combinations.

We hypothesized that adaptation to combined stress could change the plastic response of traits across multiple conditions. We expected that if genes targeted by selection were involved in reducing thermal and nutritional plasticity, the plasticity of the selected lines would be reduced, leading to lowest plasticity in lines adapted to combined stress. Additionally, we expected that the low-protein diet would exert stronger selective pressure than low-calorie diet resulting in a stronger evolved response.

The full model containing all interaction terms between selection and rearing conditions revealed complex interactions for all three traits (Table 2). All three traits showed significant 2 and 3-way interactions between selection temperature, selection diet, rearing temperature, and rearing diet. For development time, we found a significant four-way interaction. This indicates that adaptation to diet and temperature significantly affects both trait means and trait plasticity.

4.3.1 | Egg-to-adult viability

If we first focus on the results from the common garden conditions (25°C rearing temperature on standard diet), we see a significant effect of selection diet, but not selection temperature, on viability; viability was highest when flies evolved on the low protein diet irrespective of their selection temperature (Table 3; Fig 1A & B).

However, a more complex response to thermal and dietary selection is revealed when we consider the full data set where all experimentally evolved lines were reared and tested across all diet and temperature combinations (Table 2). First, we found that rearing temperature had a direct effect on viability, independent of all other factors; rearing flies at 28°C increased their viability (~2% increase on average) independently of selection treatment, selection diet, and rearing diet (Table 2, Fig. 1 C&D cf. A&B).

Second, we saw a significant effect of selection temperature, not evident from the common garden assessment, that depended on rearing and selection diet (Table 2). For the lines adapted to 25°C, the viability of standard and low-calorie diet lines was lower than that of the low-protein diet selection lines when they were reared on standard and low-protein rearing diets (Fig. 1A&C). However, in lines adapted to 28°C (Fig. 1B&D), the effect of selection diet disappeared on the standard and low-protein rearing diets, such that the viability was equal across all diet selection lines. Thus, adaptation to 28°C led to an increase in viability for the standard and low-calorie lines when reared on standard and low-protein diet (Fig. 1B&D cf. 1A&C).

Finally, low-calorie lines showed higher viability than controls when reared on the low-calorie diet but not on the other diets or when co-adapted to 28°C. This means that, for the low-calorie selection lines, co-adaptation to 28°C increased viability when they were reared on standard and low-protein diets, but decreased viability when they were reared on low-calorie diet. As a result, low-calorie selection lines displayed reduced plasticity to diet when selected at 25°C.

4.3.2 | Egg-to-adult development time

When we consider only the results from the common garden conditions (25°C rearing temperature on standard diet), we see a significant effect of selection diet, but not selection temperature, on development time; development time was fastest when flies evolved on the low-calorie diet irrespective of their selection temperature (Table 3; Fig 2A & B).

However, when we considered all rearing conditions, we found a four-way interaction between selection temperature, selection diet, rearing temperature, and rearing diet (Table 2). This interaction implies that adaptation to both thermal and nutritional stress changed the plastic response to the combined effects of temperature and nutrition. We elaborate on these results below.

First, we explored nutritional plasticity in development time after flies had evolved on the three diets at a non-stressful (25°C) temperature. When 25°C selection lines were reared at 25°C,

development time was slowed on low-calorie and low-proteins rearing diets as expected (Fig. 2A). Rearing diet interacted with selection diet such that flies adapted to the low-calorie diet developed faster than controls, but only when reared on low-calorie or low-protein diets (Fig. 2A). This means that the plastic response of development time to nutrition shifted during adaptation to different diets when flies were selected at 25°C.

When the 25°C selection lines were reared at 28°C, we found that development was slower when animals were reared on low-calorie and low-protein diets (Fig. 2C). We also found a clear plastic response to rearing temperature, whereby rearing at 28°C led to faster development across all treatments (Fig. 2C cf. 2A). However, lines from all three dietary selection treatments did not differ in their development time on each rearing diet (Fig. 2C). This means that differences in the nutritional plasticity of development time after adaptation to different diets disappeared when the rearing temperature was higher than the selection temperature. Additionally, this implies that adaptation to diet changed the thermal plasticity of our lines.

We then asked whether combined adaptation to both diet and elevated temperature would reveal differences in nutritional plasticity across diets and evolved lines. When the 28°C selection lines were reared at 25°C, we saw the same plastic response to diet described above – development on poor diets slowed development (Fig 2B). Selection at 28°C led to slower development compared to 25°C selection, but only when flies were also adapted to the standard diet (Fig. 2B cf. 2A). Flies co-adapted to low-calorie or low-protein diets and 28°C developed faster on most diets than those adapted to standard diet (Fig. 2B cf. 2A). The exception was that flies adapted to low-protein diet and 28°C developed at the same rate as those adapted to standard diet and 28°C. This suggests that adaptation to combined stress reduced total developmental time and plasticity in developmental time when compared to adaptation to thermal stress alone. Furthermore, adaptation to either diet reduced plasticity in development time when reared on low-calorie diets.

Finally, when the 28°C selection lines were reared at 28°C we again found a strong plastic response both to rearing diet and rearing temperature: flies on all rearing diets developed faster than their counterparts reared at 25°C and flies reared on poor diets had longer development times than flies reared on standard diet (Fig. 2D). Developing at 28°C for flies selected at 28°C (Fig. 2D) further increased the differences caused by adaptation to diet when compared to the same lines reared at 25°C. Also, low-calorie and low-protein lines co-adapted to 28°C developed faster than their counterparts selected at 25°C (Fig. 2C cf. 2D). Thus, overall, we saw that adaptation to combined stress reduced the plasticity in development time to temperature and to diet.

4.3.3 | Wing centroid size

The common garden assessment of size (25°C rearing temperature on standard diet) revealed a significant effect of selection temperature, although not in the direction expected (Cavicchi *et al.*, 1985; Huey, Patridge and Fowler, 1991; James and Partridge, 1995; James, Azevedo and Partridge, 1997); adaptation to 28°C increased, not decreased, overall body size (Fig 3A&B; Table 3). Adaptation to either diet did not alter body size under common garden conditions (Fig 3A&B; Table 3).

The full model revealed a significant three-way interaction between selection temperature, rearing diet, and rearing temperature (Table 2). The differences in plasticity with thermal adaptation were driven by the fact that 28°C adapted lines were larger than the 25°C adapted lines only when reared on standard diet and at 25°C (Fig 3A&B), not when reared on other diets (Fig. 3A&B) or at 28°C (Fig 3C&D). Additionally, 28°C selection lines showed greater plasticity to diet than 25°C selection lines (Fig. 3A cf. 3B). This indicates that selection temperature not only changed the plastic response to temperature but also to diet.

4.4 | Discussion

There is growing evidence that both plasticity and evolutionary adaptation will be important for determining how populations respond to climate change (Franks, Sim and Weis, 2007; Skelly *et al.*, 2007; Sinclair, Williams and Terblanche, 2012; Krehenwinkel, Rödder and Tautz, 2015). Laboratory experimental evolution has often been used to ask questions about adaptability to changing environments. However, current global change is multifaceted and adaptation to a single environmental factor is likely to misrepresent the outcome of adapting simultaneously to various environmental variables (Kaunisto, Ferguson and Sinclair, 2016). Here, we aimed to determine the extent to which adaptation to environmental change is facilitated by exposure to combinations of stressors, and whether plastic shifts in response to combined thermal and nutritional stress change as a consequence of adaptation.

4.4.1 | Co-adaptation can increase the response to selection

Previous research suggested that simultaneous exposure to two stressors might result in greater response to selection (Sgrò and Hoffmann, 1998; Bochdanovits and Jong, 2003). We found some instances for development time and viability where lines co-adapted to warmer temperature and a stressful diet outperformed their counterparts adapted only to one stressor. For example, low-protein selection lines developed faster than standard diet lines when co-adapted to 28°C and reared on low-calorie food, and low-calorie selection lines developed faster than standard diet lines only if co-adapted to 28°C when reared at 28°C on low-calorie or low-protein food. In addition, low-calorie lines co-adapted to 28°C had higher viability than if they evolved on low-calorie diet and 25°C when reared on standard or low-protein diet. Previous studies (Kolss *et al.*, 2009; Vijendravarma, Narasimha and Kawecki, 2011; May *et al.*, 2019) similarly found that flies adapted to nutrient poor larval food developed faster than controls. However, in our study, faster development of lines evolved on poor foods depended on co-adaptation to high temperature and on the rearing environment.

The instances in which co-adapting to poor diet and elevated temperature improved viability or reduced development time could be due to an increase in the expression of additive genetic variance induced by the combination of stressful conditions leading to greater response to selection, as suggested by Sgrò and Hoffmann (1998). Increased genetic variance and heritabilities under stressful conditions have been found for some traits (reviewed in Hoffmann and Merilä, 1999). For example, Bubliy, Loeschcke and Imasheva (2001) found increased genetic variance of thorax length under poor nutrition in isofemale lines. In addition, Clare and Luckinbill (1985) reported a significant response to selection for longevity in *D. melanogaster* only when flies were maintained under high density, a stressful condition, and not when density was controlled (low) during selection. Similar results were found by Service, Hutchinson and Rose (1988). Further, Husby, Visser and Kruuk (2011) found that the expression of genetic variance in timing of breeding of a natural bird population was positively associated with warmer spring temperatures. The authors point out that high spring temperatures, in their study system, are generally associated with adverse environmental conditions because they lead to a mismatch between the time of reproduction and the peak in food abundance. Their results indicated a stronger response to selection under adverse environmental conditions (Husby, Visser and Kruuk, 2011). Additionally, we previously showed how poor diets exacerbated the negative impact of elevated temperature during development while optimal diets had a buffering effect against a high developmental temperature (Kutz, Sgrò and Mirth, 2019). This suggests that the selective pressure of elevated temperature may be higher when high temperature is combined with poor nutrition. Therefore, the results of the present study add to the list of studies supporting the hypothesis that stressful conditions may increase the response to selection.

However, our results also provide examples where co-adaptation did not provide greater benefits than adaptation to temperature only, and show that any increased response to selection through co-adaptation depends on the type of nutritional stress (low-calorie or low-protein), the rearing environment, and the trait studied. For instance, viability of low-protein selection lines did not

change when they were also selected at 28°C. Additionally, when reared on low-protein food at 28°C, flies co-adapted to either of the poor diets and 28°C had development times equal to flies adapted only to 28°C or only to poor diet. Finally, diet selection had no effect on size.

4.4.2 | Non-additive effects of adapting to thermal and nutritional stress in combination

The increased responses to selection through co-adaptation described above could have been the result of additively combining the effect of adapting to each stressor individually. However, the interactions between selection diet and selection temperature found for viability and development time indicate that the effects of adapting to each stressor individually are not additive. Similar non-additive effects were reported by Bochdanovits and Jong (2003) for body weight and viability. For instance, they found that flies adapted to 17.5°C were always heavier than flies adapted to 27.5°C, and flies adapted to 27.5°C and low-calorie food were lighter flies adapted to 27.5°C and high-calorie food. However, flies adapted to 17.5°C and low-calorie food were even heavier than flies adapted to high-calorie food and cold under common garden conditions. This indicates that the effects of adapting to a cooler environment and nutrient-poor food were not additive.

Similarly to Bochdanovits and Jong (2003), we found many instances of non-additive effects in our data. For example, while adaptation to low-calorie diet reduced development time (in agreement with Kolss *et al.*, 2009; Vijendravarma, Narasimha and Kawecki, 2011; May *et al.*, 2019) and adaptation to 28°C extended development time, flies co-adapted to 28°C and low-calorie developed equally fast as flies adapted only to low-calorie diet. Therefore, our results indicate that the outcome of combined thermal and nutritional selection cannot be predicted from the results of adaptation to temperature and diet independently, as their effects are interactive resulting in non-additive responses.

Interestingly, while Bochdanovits and Jong (2003) concluded that in their experiment adaptive changes in development time were negligible, our results showed that development time was the trait that was most clearly affected by our selection treatments. In addition, we found little

response of size to thermal selection and no response at all to nutritional selection, in contrast with Bochdanovits and Jong (2003) who found clear evolved responses of body weight to both temperature and diet. Based on previous work, we expected that adaptation to low-calorie food would result in smaller flies (Kolss *et al.*, 2009; Vijendravarma, Narasimha and Kawecki, 2011; May *et al.*, 2019). However, our findings contrast with previous results: we found no effect of selection diet on size under any rearing condition.

It is unlikely that the lack of evolutionary response to diet for size was due to the selective pressure being too weak, as we used the same calorie dilution (1/4 of the calories of the standard diet) as previous studies (Bochdanovits and Jong, 2003; Kolss *et al.*, 2009; Vijendravarma, Narasimha and Kawecki, 2011; May *et al.*, 2019). It is also unlikely that the lack of evolutionary response of size to diet was due to a lack of standing genetic variation in our founding population to allow for the evolution of differences in size (Blows and Hoffmann, 2005) since we maintained the selection lines at population sizes consistent with previous studies that have found significant levels of genetic variation in quantitative traits in *Drosophila* (e.g. Sgrò and Blows, 2003), and *D. melanogaster* populations originating from an area close to our collection size have been shown to harbour abundant genetic variation for wing size (Hangartner *et al.*, 2020). In addition, it is unlikely that the lack of response of size to selection was due to differences in the number of generations exposed to selection; while varying generations of exposure to selection can result in different evolved responses (Langmüller and Schlötterer, 2020), the number of generations used in the present study were similar to previous studies (Kolss *et al.*, 2009: 29 generations; May *et al.,* 2019: 19-38 generations) who found decreases in body size in response to selection to low-calorie food. Finally, some studies (Bochdanovits and Jong, 2003; Kolss *et al.*, 2009; Vijendravarma, Narasimha and Kawecki, 2011; May *et al.*, 2019) measured body weight, rather than wing size as in the present study; however, (Vijendravarma, Narasimha and Kawecki, 2011) measured both body weight and wing size and found similar evolved responses to diet selection; albeit body weight showing greater

effect sizes. Hence, it is possible that the response to selection of wing size is not perfectly correlated with that of body weight.

We also expected that adaptation to higher temperature would result in smaller adults because previous studies showed that cold-adapted flies (either to 18°C, Cavicchi *et al.*, 1985; or to 16.5°C, Huey, Patridge and Fowler, 1991; James and Partridge, 1995) were larger than flies adapted to 25°C, and flies adapted to 28°C were smaller than flies adapted to 25°C (Cavicchi *et al.*, 1985). However, we observed an increase in wing size in lines adapted to 28°C when reared on standard conditions. The discrepancy between our study and previous findings could be due to the fact that in the current study selection was imposed on the larval stage only, whereas previous studies had kept both adults and larvae under constant selection temperatures (Cavicchi *et al.*, 1985; Huey, Patridge and Fowler, 1991; James and Partridge, 1995), and that we controlled larval density while previous studies did not (Cavicchi *et al.*, 1985; Huey, Patridge and Fowler, 1991; James and Partridge, 1995). Additionally , the contrast in our results could also be due to genetic differences in the founding population (Blows and Hoffmann, 2005). This highlights that each of these factors might alter the adaptive response to thermal stress, and warrants future study.

4.4.3 | Changes in plasticity in response to thermal and nutritional selection

In previous experimental evolution studies, the response to selection changed in intensity, or even in sign, depending on the rearing conditions (Huey, Patridge and Fowler, 1991; James and Partridge, 1995; Bochdanovits and Jong, 2003; Santos, Brites and Laayouni, 2006; Kolss *et al.*, 2009). This was a consequence of selection affecting the plasticity of flies. We hypothesised that if adaptation to nutritional or thermal stress targeted genes involved in plasticity, plasticity would be affected (increased or reduced) by selection (Via, 1993).

We found significant interactions between selection treatment and rearing conditions for all three traits studied, indicating that selection affected the plasticity of our lines. For instance, flies co-adapted to 28°C and low-calorie diet were not significantly different than flies adapted to just 28°C

or just low-calorie diet if they were reared on low-protein diet at 28°C. Hence, our results show that the expression of evolved phenotypic change is highly dependent on the rearing environment. This finding is also in agreement with Bochdanovits and Jong (2003), who also found that the rearing environment influenced the interaction between selection treatments. For instance, the increase in body size through simultaneous adaptation to a cold environment and poor food quality was more visible at the lowest rearing temperature.

In addition, for development time we found that adaptation to 28°C changed plasticity to temperature but also to diet, and that adaptation to diet modulated both thermal and nutritional plasticity. Similarly, for size, adaptation to 28°C changed both the thermal and nutritional plasticity of our flies. Therefore, our results suggest that there must be an overlap between genes underpinning developmental sensitivity to diet and temperature, at least for development time and size. Locally-adapted populations of *D. melanogaster* along a latitudinal cline are known to be differentially adapted to temperature (Lasne *et al.*, 2018) and show differences in their plastic response to combined thermal and nutritional stress (Chakraborty, Sgrò and Mirth, 2020). This supports our finding that adaptation to temperature can change plasticity to diet.

The reciprocal effect of adaptation to diet and temperature on development and plasticity, although complex, is not unexpected. It is well known that both temperature and nutrition affect growth and development in insects (Clissold and Simpson, 2015) and, on a basic level, temperature and nutrition are linked through metabolic rate because higher temperatures require relatively more energy to fuel growth and this energy is obtained through the diet (Kingsolver and Huey, 2008). However, the relationship between food, temperature and variation in life history traits is mediated by more than their link to metabolism. How nutrition regulates growth has been the focus of a great deal of research (e.g. Wigglesworth, 1934; Nijhout, 1979; Brogiolo *et al.*, 2001; Ikeya *et al.*, 2002; Géminard, Rulifson and Léopold, 2009; Hietakangas and Cohen, 2009; Koyama and Mirth, 2018). It is well established that, in insects, nutrition changes the concentration of insulin-like peptides which directly affect tissue growth (Brogiolo *et al.*, 2001; Ikeya *et al.*, 2002;

Géminard, Rulifson and Léopold, 2009; Hietakangas and Cohen, 2009). Insulin also regulates the synthesis of the hormone ecdysone (Caldwell, Walkiewicz and Stern, 2005; Colombani *et al.*, 2005; Mirth, Truman and Riddiford, 2005; Koyama *et al.*, 2014), which is necessary for the onset of moulting and can extend development time and result in overlarge adults if its production is delayed (reviewed in Mirth, Saunders and Amourda, 2021). Interestingly, temperature also modulates both ecdysone synthesis and insulin signalling (Ghosh, Testa and Shingleton, 2013; Li and Gong, 2015) meaning that temperature does not just affect growth by generally accelerating metabolic processes (through an increased metabolic rate) but directly targets hormones that regulate growth. Through their effects on insulin and ecdysone, diet and temperature can interact to shape plastic developmental responses to environmental conditions. Our data provides evidence that the genes regulating the sensitivity of growth to the thermal and nutritional environment were affected by adaptation to thermal and nutritional stress. Therefore, differences in genes involved in insulin signalling and ecdysone synthesis make excellent targets for future work, as they could explain the differences in thermal and nutritional plasticity found after selection.

4.4.4 | Evidence for cross-protection across selection diet types

Under climate change, abundance and macronutrient composition of primary producers are expected to change affecting nutrient flow in the food chains (Cross *et al.*, 2015; Rosenblatt and Schmitz, 2016; Beach *et al.*, 2019). We used two dietary selection treatments to simulate reduced nutrient availability or reduced protein content to investigate which aspect of nutritional stress would have greater impact on adaptation. We know from nutritional studies that macronutrient balance can have more marked effects than calories on the plastic response of life history traits in insects (Lee *et al.*, 2008; Simpson and Raubenheimer, 2012; Kutz, Sgrò and Mirth, 2019), therefore we expected selection on low-protein food to result in a stronger evolved response than selection on low-calorie food.

We found that development time and viability (but not size) were affected by nutritional adaptation. Improvements in viability were seen for flies adapted to low-protein diet when reared on all diets. This would agree with our prediction that adaptation to macronutrient imbalance would result in stronger evolved responses. However, for development time, low-calorie diet selection resulted in accelerated development under more rearing conditions that adaptation to low-protein diet, including development on the low-protein diet, contrasting with our prediction. Interestingly, adaptation to either diet type accelerated development when flies were reared low-calorie diets. The reduced development time of low-calorie lines on low-protein food and of low-protein lines on low-calorie food indicates that adaptation to both diet types must share some of the same underlying mechanisms.

One mechanism that may underpin adaptation to both low-calorie and low-protein diets is increased feeding rate (Bochdanovits and Jong, 2003; Simpson and Raubenheimer, 2012). In the case of the low-calorie diet, increased feeding rate simply allows animals to ingest more calories, whereas on the low-protein diet it allows the animal to reach its protein requirements necessary for growth and development by ingesting more food. Another shared mechanism could be the increased ability to extract and assimilate nutrients from food (Cavigliasso *et al.*, 2020). Trehalose metabolism also seems to play a key role for survival both on low-sugar and on low-protein diets (Yasugi, Yamada and Nishimura, 2017).

On the other hand, flies adapted to low nutrient intake (comparable to low-calorie diets) have been shown to increase lipid deposition (Zhang *et al.*, 2019) whereas flies adapted to low protein content in diet seemed to have reduced lipid storage, as they needed to eliminate carbohydrate excess (Cavigliasso *et al.*, 2020). This would suggest that adaptation to low-calorie or low-protein diet is not interchangeable. Further, a shared adaptive mechanism would not explain why adaptation to low-protein diet accelerated development on low-calorie food but not on low-protein food. It would be interesting to investigate feeding rate, nutrient assimilation, trehalose metabolism, and

lipid storage in our evolved lines to better understand the mechanisms contributing to the observed differences in development time in response to diet.

4.4.5 | Relating our results to thermal and nutritional stress in nature

Our results showed that selection imposed by thermal and nutritional stress only during the larval stages of flies can lead to differential evolution of life-history traits. While direct exposure to elevated temperatures and decreased P:C ratios (as expected under climate change) had the direct plastic effect of reducing viability, we showed that some function was regained through adaptation (i.e., animals adapted to low-protein diet and 28^{o}C raised under those had highest viability). However, this improvement was not large enough to fully abrogate the effect of climate change in the course of our experiment. Because, in this study, experimental evolution was only conducted for about a year, it would be interesting to continue with selection for a period more similar to the timeframe of climate change to investigate wether longer adaptation time could lead to better outcomes for the adapted lines.

In nature, *D. melanogaster* larvae feed on rotting fruit and other decomposing plant material (Markow and O'Grady, 2008). The microbial community growing on rotting fruit (bacteria, moulds, and yeasts) is the primary source of protein for both the larvae and the adults of *D. melanogaster* (Starmer and Fogleman, 1986; Markow and O'Grady, 2008) and they often obtain carbohydrates from the decomposing fruit and plant material directly (Burke and Waddell, 2011; Chng, Hietakangas and Lemaitre, 2017). Under elevated temperatures and reduced water, amino acid content of fruits and other above-ground plant organs is predicted to decrease (Wu *et al.*, 2019; Gutiérrez-Gamboa *et al.*, 2020) while carbohydrate content is predicted to increase (Moretti *et al.*, 2010; Rosenblatt and Schmitz, 2016). Changes in the P:C ratio of fruit and other plant material would translate in changes to the abundance and composition of the yeast communities growing on them, because they are the primary source nitrogen for yeast (Wu *et al.*, 2019; Gutiérrez-Gamboa *et al.*, 2020). As a result, *D. melanogaster* will likely be exposed to higher carbohydrate concentrations in their diet, particularly in the larval stages because larval development will be

limited to the area of the oviposition site for food (Medina-Muñoz and Godoy-Herrera, 2005), while adults are more mobile, and, therefore, more able to choose feeding sites (Silva-Soares *et al.*, 2017). Whether adult adaptation to combinations of nutritional stress would lead to similar or different evolved responses than larval adaptation remains unknown.

Additionally, the effects of larval adaptation to diet and temperature could be accompanied by other selective pressures in nature and lead to different adaptive outcomes. For example, in nature, insects can experience intra and interspecific competition and predation risks that add their own selective pressures on growth and development (e.g., Santos *et al.*, 1997; Gotthard, 2000; Schneider, Takken and McCall, 2000). Further, it is unclear how larval or adult adaptation to thermal and nutritional stress in combination could impact other life-history traits important for fitness, such as fecundity and lifespan.

Finally, here we used constant temperatures for selection, however, ectotherms are exposed to daily and seasonal thermal fluctuations. It is possible that the evolved responses found in the present study would be different if flies adapted to fluctuating temperature regimes that incorporated periodically sublethal temperatures (Schou *et al.*, 2014; Colinet *et al.*, 2015).

4.5 | Conclusion

While the questions about specific mechanisms driving evolved changes in performance in our study remain unresolved, our results showed that co-adaptation to nutrient-deficient diets and high temperature can lead to synergistic effects in the evolved response of key life history traits, which cannot be predicted from the adaptation to each variable singly. We also show that adaptation can, in some cases, decrease the plastic response to the thermal and nutritional environment. However, this depends on the trait and on the condition to which they are adapting, and might only be observed under specific rearing conditions. Additionally, we found that thermal adaptation can change plasticity to diet and vice versa, indicating that thermal and nutritional

sensitivity share at least some underlying mechanisms. Finally, we found evidence that in some conditions, adaptation to poor quality diets improved trait performance across diet types. Our findings emphasise the complex nature of adaptation to combined stressors and the important role that plasticity may play. Further, they highlight the importance of studying environmental variables in combination to make better predictions of organisms' responses to climate change.

4.6 | References

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TABLES

Table 1. Nutrient content of diets used for selection and as rearing medium for experiments.

Diet	Protein (g/L)	Carbohydrate (g/L)	Calories (Kcal/L)	P:C ratio
Standard	20	59	320	1:3
Low-calorie	5	15	80	1:3
Low-protein	6	74	321	1:12

Table 2. Effects of selection treatment and rearing environment on egg-to-adult viability, development time, and adult wing centroid size.

Trait	ST	SD	RT	RD	ST x SD	ST x RT	SD x RT	ST x RD	SD x RD	RT x RD	ST x SD x RT	ST x SD x RD	ST x RT x RD	SD x RT x RD	ST x SD x RT x RD
Viability	**9.9****	26.3	**10.9****	**123.6*****	5.9	-	-	1.1	**19.0*****	-	-	**13.737****	-	-	-
Dev. Time	1.5	**15.4*****	**6817.5*****	**23478.8*****	**6.0***	1.6	**21.5*****	**22.3*****	**44.9*****	**296.2*****	1.7	**36.853*****	2.354	**13.599****	**10.971***
Size	**5.3***	**-**	**233.3*****	**514.1*****	-	**4.5***	-	**8.3***	-	**25.5*****	-	-	**7.225***	-	-

Chi square values and significance levels from the reduced model for all traits. Mixed models were fitted to the data. Viability was analysed with a generalized mixed model with binomial family and a logit link. *ST*: Selection temperature; *SD*: selection diet; *RT*: rearing temperature; *RD*: rearing diet. Significant coefficients are in bold. Significance level codes: $^{*}p<.05$, $^{**}p<.01$, $^{***}p<.001$. Dashes represent terms that were removed from the model during model reduction.

Table 3. Effects of selection treatment on egg-to-adult viability, development time, and adult wing centroid size for flies reared under a common garden of standard conditions (25ºC and standard diet).

Trait	ST	SD	ST x SD
Viability	1.555	**6.665 ***	3.109
Dev. Time	-	**7.092***	-
Size	**5.447***	-	-

Chi square values and significance levels from the models fit to the subset of lines reared at 25sºC on standard diet. Mixed models were fitted to the data. Viability was analysed with a generalized mixed model with binomial family and a logit link. *ST*: Selection temperature; *SD*: selection diet. Significant coefficients are in bold. Significance level codes: $^{*}p < .05$. Dashes represent terms that were removed from the models during model reduction.

FIGURES

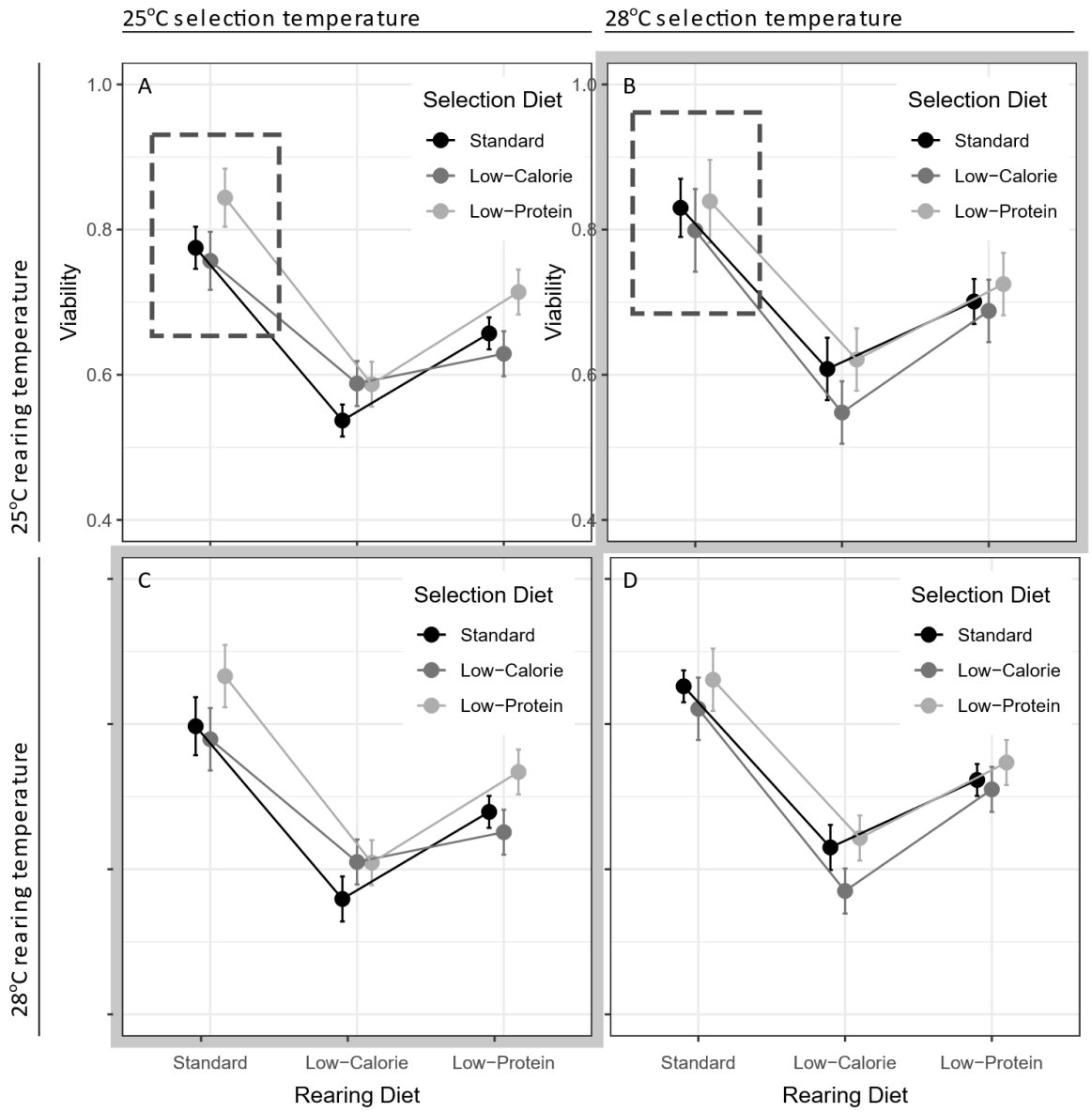

Fig. 1. Effects of selection treatment and rearing environment on egg-to-adult viability. *A* Lines evolved at 25°C, reared at 25°C. *B:* Lines evolved at 28°C, reared at 25°C. *C:* Lines evolved at 25°C, reared at 28°C. *D:* Lines evolved at 28°C, reared at 28°C. *Grey margin* represents selection lines reared at a temperature different to their home temperature. *Dashed boxes* represent lines reared under standard common garden conditions. Means and standard errors were taken from the minimum adequate model.

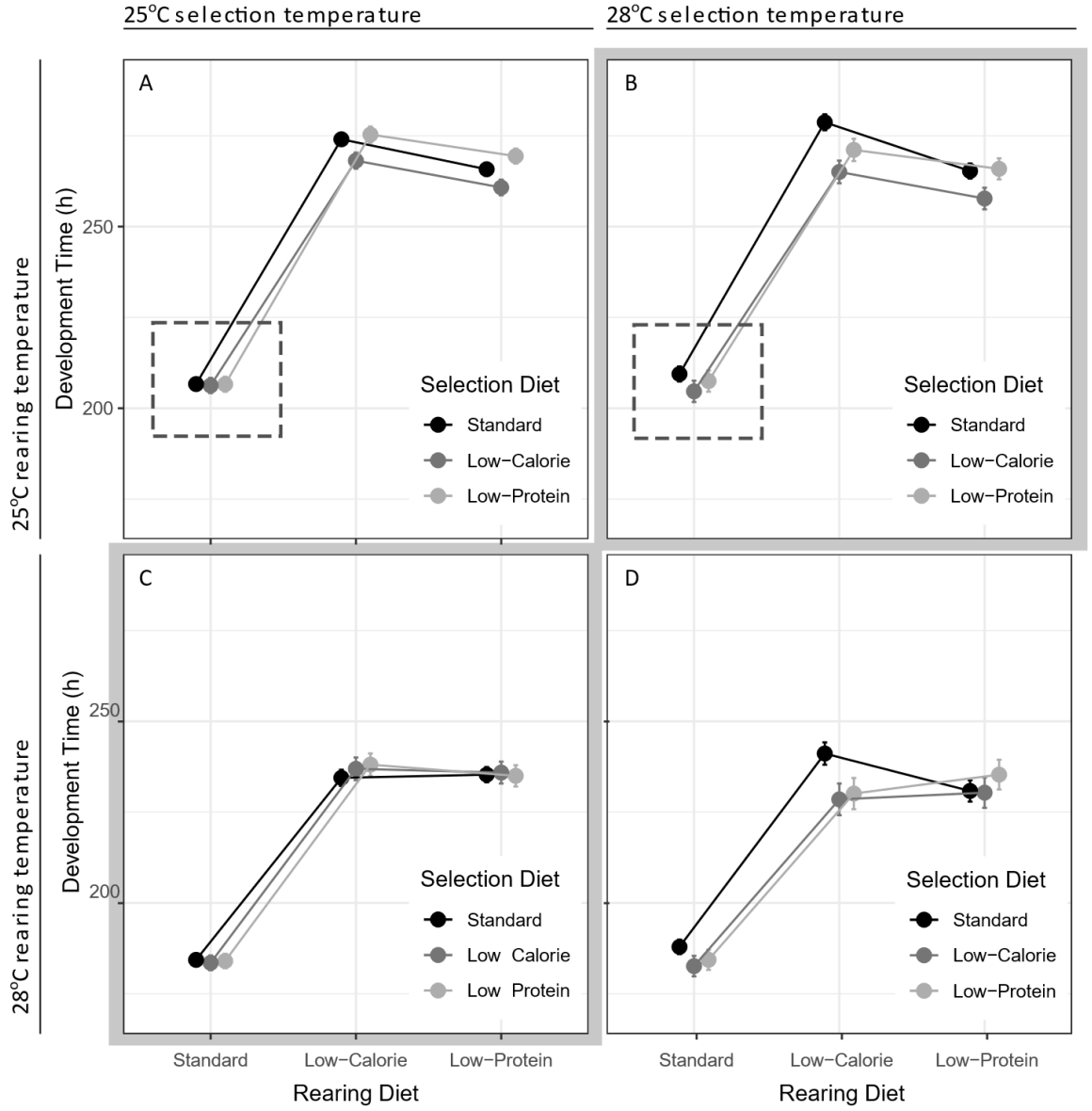

Fig. 2. Effects of selection treatment and rearing environment on egg-to-adult development time. *A:* Lines evolved at 25°C, reared at 25°C. *B:* Lines evolved at 28°C, reared at 25°C. *C:* Lines evolved at 25°C, reared at 28°C. *D:* Lines evolved at 28°C, reared at 28°C. *Grey margin* represents selection lines reared at a temperature different to their home temperature. *Dashed boxes* represent lines reared under standard common garden conditions. Means and standard errors were taken from the minimum adequate model.

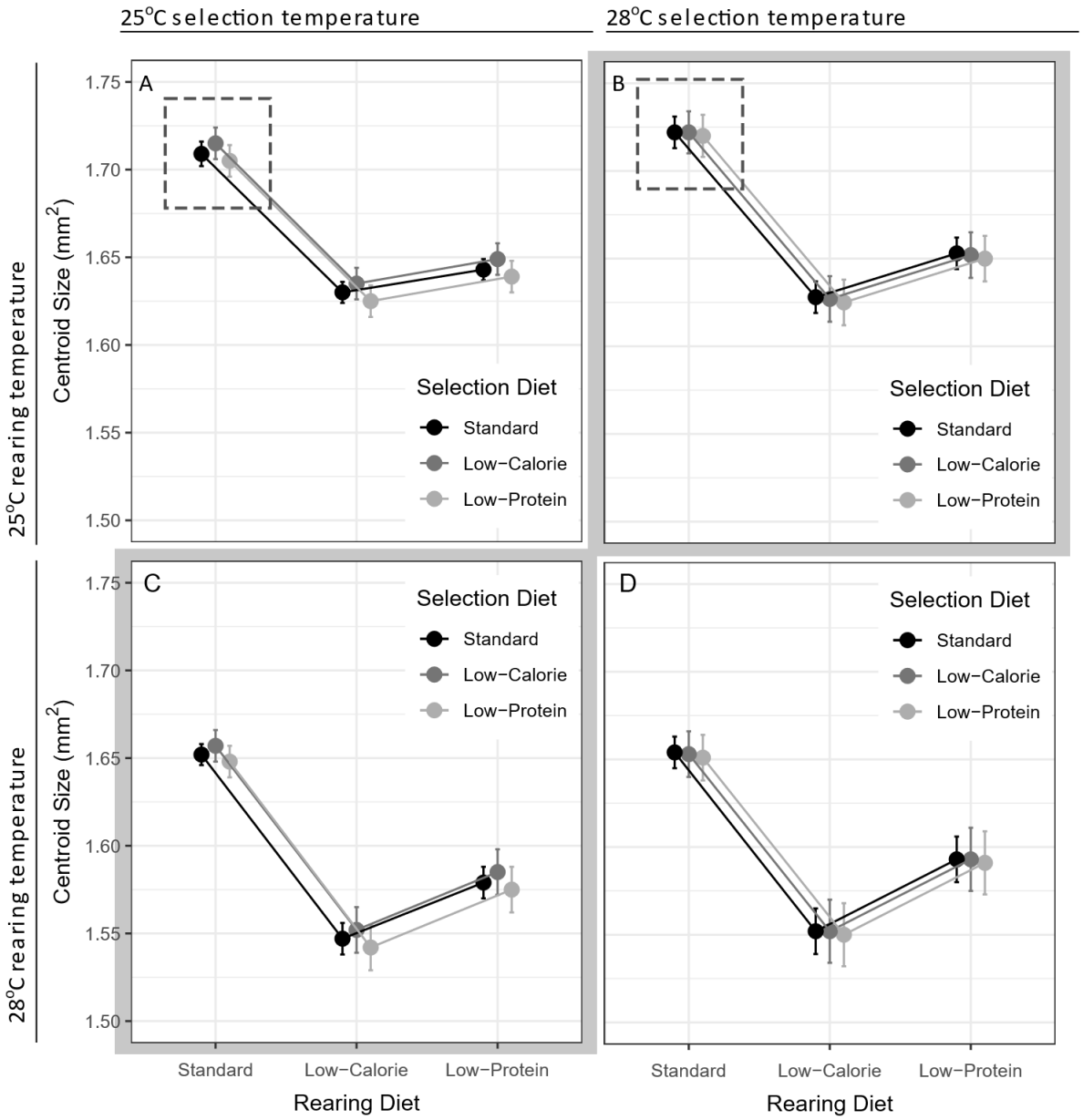

Fig. 3. Effects of selection treatment and rearing environment on wing centroid size. *A:* Lines evolved at 25°C, reared at 25°C. *B:* Lines evolved at 28°C, reared at 25°C. *C:* Lines evolved at 25°C, reared at 28°C. *D:* Lines evolved at 28°C, reared at 28°C. *Grey margin* represents selection lines reared at a temperature different to their home temperature. *Dashed boxes* represent lines reared under standard common garden conditions. Means and standard errors were taken from the minimum adequate model.

Chapter 5 | General discussion

The goal of this thesis was to investigate the role diet could have in mediating insect responses to high temperature. The types of responses I explored were twofold: first, plastic changes of key life history and adult stress resistance traits in response to the combined effects of larval diet and developmental temperature (Chapter 2 and Chapter 3 respectively); second, the evolutionary response of key life history traits after selection to suboptimal larval diet and high developmental temperature (Chapter 4).

5.1 | Temperature and nutrition interact to shape the plastic response of life-history traits

In *D. melanogaster*, developmental life history traits are known to be affected by the thermal environment (e.g., Atkinson, 1994; Atkinson and Sibly, 1997; Angilletta, Jr., and Dunham, 2003; Angilletta, Jr. *et al.*, 2004; Kozłowski, Czarnołęski and Dańko, 2004; Miller *et al.*, 2009) and by both the calorie content and the macronutrient balance of the larval diet (Rodrigues *et al.*, 2015; Shingleton *et al.*, 2017; Jang and Lee, 2018a; Deas, Blondel and Extavour, 2019; Chakraborty, Sgrò and Mirth, 2020). More recently, research has focused on the interaction between diet and temperature in insect development (Kingsolver *et al.*, 2006; Lee and Roh, 2010; Lee *et al.*, 2015; Lemoine and Shantz, 2016; Kim, Jang and Lee, 2020; Min, Jang and Lee, 2021). However, these studies have typically covered a limited range of dietary conditions, which in turn limits our understanding of the nature of the effects of combined thermal and nutritional stress.

In Chapter 2, I investigated plastic responses of egg-to-adult viability, egg-to-adult development, adult wing size, and adult femur length to combinations of diet and temperature during larval development. I used nutritional geometry (Simpson and Raubenheimer, 2012) to study the responses of these four life-history traits to 36 diets containing varying amounts of protein and

carbohydrate and combined those diets with either a standard rearing temperature of 25°C or a hotter rearing environment of 28°C, representing the average temperature increase projected under climate change (IPCC, 2013; Rogelj *et al.*, 2018).

In general, the responses to diet of viability, development time, and size when *D. melanogaster* larvae where reared at 25°C were in agreement with findings from previous studies (Rodrigues *et al.*, 2015; Gray, Simpson and Polak, 2018). Increased temperature overall led to faster development that resulted in smaller individuals. However, when reared at 28°C I found traits had narrower nutritional optima resulting in harsher trade-offs between viability, development time, and body size traits. This highlights the importance of considering temperature and diet together when assessing species' vulnerability to climate change.

Interestingly, at 28°C, despite there being fewer diets that optimized performance, those that did also minimized the impact of temperature. This means that on these diets there was little difference between larvae raised at 25°C or at 28°C. This is result is in agreement with (Lee *et al.*, 2015) but contrasts with (Lee and Roh, 2010; Lemoine and Shantz, 2016). Therefore, more research is necessary to see if this finding can be generalized across traits and species.

While I studied life history responses to 36 diets varying in their caloric content and P:C ratio at a benign temperature (25°C) and at a temperature relevant to climate change projections (28°C), a recent study (Kim, Jang and Lee, 2020) looked at responses to eight diets that varied in P:C ratio from 1:18 - 8:1 over six temperatures ranging from 13 to 33°C, spanning the natural range of temperatures encountered by *D. melanogaster* in the wild (Petavy *et al.*, 2001; Hoffmann, 2010). They also found that traits differed not just in their nutritional optima, in accordance with numerous studies including ours (Rodrigues *et al.*, 2015; Shingleton *et al.*, 2017; Jang and Lee, 2018a; Deas, Blondel and Extavour, 2019; Kutz, Sgrò and Mirth, 2019; Chakraborty, Sgrò and Mirth, 2020), but also in their thermal optima. Additionally, they found that the effects of larval thermal environment carried over to affect the responses of adult traits to temperature. The results from Kim, Jang and Lee (2020) reinforce our finding that it is crucial to consider

temperature and nutrition in combination in order to better understand of how insects and other ectotherms will respond to climate warming.

Additionally, Chakraborty, Sgrò and Mirth (2020) recently used a similar design to explore differences in the response to developmental diet and temperature among three populations along a latitudinal cline. Their experimental design allowed them to investigate differences in the plasticity of locally-adapted populations along a latitudinal gradient in response to combined environmental stress. In agreement with my results, Chakraborty, Sgrò and Mirth (2020) found that nutritional optima depended on the rearing temperature and the trait, but also that populations of *D. melanogaster* responded differently to temperature and nutrition, and that these differences did not follow a clinal pattern. Their results indicate that the adaptive history within a species can affect their response to the larval environment in ways that are difficult to predict, and more research is needed to find generalizable rules in the way populations and species react to combined nutritional and thermal stress.

Recently, Min, Jang and Lee, (2021) investigated how the match and mismatch of larval and adult nutritional and thermal environments affected adult reproductive success and survivorship. Their work explores the effects of dietary macronutrient composition and environmental temperature independently on adult performance. They found that larval diet had a strong influence on egg production during the first 5 days, after which its effects became weaker and adult diet gained more relevance. Future work should investigate the effects of matching and mismatching temperature and nutrition in combination across life stages, adopting the framework used by Min, Jang and Lee (2021); that is, it should include changes in the larval environment, changes in the adult environment, and changes in both. Additionally, it would be beneficial if dietary calorie content was studied alongside macronutrient composition when studying the effects of diet, as both P:C ratio and calories in the diet affect life history traits of insects (Lee *et al.*, 2008; Fanson and Taylor, 2012; Jensen *et al.*, 2012; Le Gall and Behmer, 2014; Matavelli *et al.*, 2015; Kutz, Sgrò and Mirth, 2019; Wilson, Ruiz and Davidowitz, 2019).

5.2 | Adult feeding could compensate for nutritional stress during development

While in holometabolous insects the adult niche is not tied to the larval niche due to their complex lifecycle (Kingsolver *et al.*, 2011), many studies have shown that the larval environment can have carry-over effects on a range of adult traits such as size, stress resistance, physiology, fecundity, and lifespan (e.g. Crill, Huey and Gilchrist, 1996; Kingsolver *et al.*, 2011; Jang and Lee, 2018; Galarza *et al.*, 2019). In *Drosophila*, the thermal (e.g. Crill, Huey and Gilchrist, 1996; Bauerfeind *et al.*, 2014; Kellermann, van Heerwaardenf and Sgrò, 2017; MacLean *et al.*, 2017; Zheng *et al.*, 2017) and nutritional (Andersen *et al.*, 2010; Sisodia and Singh, 2012; Kristensen *et al.*, 2016; Henry, Overgaard and Colinet, 2020) larval environments are known to affect adult stress resistance traits. However, the effects of the larval thermal and nutritional environment on adult stress resistance traits had never been tested in combination.

In Chapter 3, I tested the effect of larval thermal and nutritional environment on four adult stress resistance traits: starvation, desiccation, heat, and cold resistance. To measure this, I reared larvae on 25 diets of varying carbohydrate and protein content, using a nutritional geometry approach (Simpson and Raubenheimer, 2012), at either 25 or 28°C. Because I was interested in the effects of the larval environment, newly eclosed adults were maintained on standard conditions (i.e., 25°C and standard diet) for 7 days. To identify physiological changes underpinning differences in stress resistance, I measured relative lipid and glycogen content of the flies at the time of eclosion and also after 7 days on standard food at 25°C.

I found that temperature and nutrition interacted to affect heat, starvation, and desiccation resistance, but that the effects of temperature and nutrition on cold resistance were additive. However, despite finding a significant carry-over effect from the larval environment on adult stress resistance, the size of the effect for all adult stress resistance traits was small, with larval nutrition explaining only a very small portion of the variation found in the resistance to stress in adults.

The minimal effect of larval diet could be explained by the changes found in the energy stores between eclosion and the time of the stress resistance assays. Both lipids and glycogen were optimized in adult flies after 7 days on standard conditions, resulting in similar lipid and glycogen content across all larval dietary and thermal treatments at the time of the stress resistance assays. Therefore, I surmised that I only found a minimal effect of larval diet on stress resistance because adult feeding had reduced differences in lipid and glycogen content, which play an important role in stress resistance (Djawdan *et al.*, 1998; Folk and Bradley, 2004; Ballard, Melvin and Simpson, 2008; Lee and Jang, 2014; Slocumb *et al.*, 2015). Consequently, I expect that the effects of the larval diet on adult stress resistance would be exacerbated if adults had also been held on experimental diets (e.g., Kristensen *et al.*, 2016; Henry, Overgaard and Colinet, 2020) and at elevated temperatures prior to trait assessments. This assumption is supported by the fact that, for fecundity, larval diet has a greater impact during the first 5 days of adult life, but adult diet becomes more important after that (Min, Jang and Lee, 2021). A similar switch is likely to have happened for adult stress resistance in our study.

While my results shed light on the relevance of the larval environment on adult stress resistance and highlight that developmental temperature can change how larval diet affects adult stress resistance, I also show that adult feeding can alleviate the negative carry-over effects from the larval environment for these traits. Given this result, future work focusing on how adult nutritional stress, in combination with larval nutritional stress or on its own, affects adult stress resistance could help determine the extent of the carry-over effects from the larval diet and to what degree adult feeding is able to compensate for nutritional stress during the larval stages.

Additionally, in our study I used fixed thermal conditions which do not realistically represent the thermal fluctuations experienced by flies in nature. Because daily temperature fluctuations on their own have important consequences for ectotherms (Colinet *et al.*, 2015) it is possible that the carry-over effects from the larval environment would affect adult stress resistance differently.

Future studies of carry-over effects on stress resistance should employ fluctuating temperatures to obtain a more realistic representation of stress resistance in nature.

Finally, due to logistic constraints, in Chapter 3 I measured stress resistance of female individuals only; however, it is known that in *D. melanogaster* the stress resistance traits studied are sexually dimorphic (Lasne *et al.*, 2018). Therefore, in future work it would be interesting to investigate how males and females differ in their response to their thermal and nutritional environment with regards to stress resistance.

5.3 | Thermal and nutritional stress interact to shape evolved plastic responses

In response to rapid environmental change, species must either adjust to the new conditions via phenotypic plasticity and/or adapt through evolution in order to persist in their environment (Hoffman and Parsons, 1991). After investigating plastic responses to the larval thermal and nutritional environment in Chapters 2 and 3, I focused on the evolutionary responses to the larval environment (Chapter 4). There is some evidence suggesting that combinations of stressful conditions can increase the expression of additive genetic variance leading to greater response to selection (Sgrò and Hoffmann, 1998). This means that adapting to stressors in combination could lead to synergistic adaptive responses, such as those found by (Bochdanovits and Jong, 2003). In addition, under selection, plasticity is predicted to increase, decrease, or remain unchanged depending on whether or not genes controlling the phenotypic response under selection also control the plastic response, (Falconer, 1952; Via and Lande, 1985; Via, 1993).

To test the outcome of adapting to thermal and nutritional stress in combination, I reared flies for 22 to 30 generations under temperature selection, diet selection, or both and tested them across all conditions used for selection. I found interactive effects of diet and temperature on development time and viability in my selected lines, similar to the results for viability and size from

Bochdanovits and Jong (2003). In some cases, co-adaptation accelerated development and improved viability, supporting the theory that combinations of stressful conditions can lead to greater response to selection, as suggested by Sgrò and Hoffmann (1998). However, our results also show that an increased response to selection through co-adaptation did not always occur. It depended on the trait studied, the type of selection pressure (low-calorie or low-protein diet), and the plastic responses to rearing conditions.

Next, I explored how selection affected the plasticity of our lines. Previous experimental evolution studies have often reported differences in the adaptive responses depending on the rearing environment at the time of testing (e.g. Huey, Patridge and Fowler, 1991; Partridge *et al.*, 1994; Santos, Brites and Laayouni, 2006), which implies changes in the plasticity of the selected lines. I found that some selective pressures affected plasticity (for example, low-calorie diet adaptation led to a decrease in the plastic response of viability to diet) and others did not (for example, adaptation to a low-protein diet or 28^{o}C did not change plasticity of viability to diet) and that this was trait-dependent. This could indicate that adaptation to some types of stressors target genes involved in plasticity for some traits but not others.

Additionally, I found that thermal adaptation led to changes in nutritional plasticity and vice versa for all three traits studied suggesting that there must be an overlap between mechanisms underpinning developmental sensitivity to diet and to temperature. In particular, because temperature and nutrition are linked through the metabolic rate (i.e., growth at higher temperatures requires more energy and this energy is obtained through the diet; Kingsolver and Huey, 2008), testing the metabolic rate of our selection lines across all selection environments in the future could potentially help explain the cross-over between thermal and nutritional adaptation and changes in thermal and nutritional plasticity found in this study. Further, because both temperature and nutrition regulate growth in *D. melanogaster* via insulin and ecdysone production (Mirth, Saunders and Amourda, 2021), future work should investigate the plasticity in the production of these hormones in our evolved lines to see if changes in the sensitivity of these

hormones are driving the interaction between temperature, diet, and the plastic response to the two.

In this study, I used two different selection diets: low-calorie or low-protein because under climate change organisms will face changes in the abundance and composition of plants (Cross *et al.*, 2015; Rosenblatt and Schmitz, 2016). I wanted to test if nutrient limitation (in the form of low-calories in the diet) and macronutrient imbalance would result in different adaptive responses. I found that adaptation to the low-calorie diet accelerated development when reared on the low-protein diet and that adaptation to the low-protein diet accelerated development when reared on the low-calorie diet. This cross-over suggests that adaptation to low-calorie and low-protein diets could have some shared underlying mechanism.

Increased feeding rate (Bochdanovits and Jong, 2003; Simpson and Raubenheimer, 2012) and the increased ability to extract and assimilate nutrients from food (Cavigliasso *et al.*, 2020) are known mechanisms used to improve development both on low-calorie and low- protein foods. Further, trehalose metabolism also seems to play a key role for survival on both these diets (Yasugi, Yamada and Nishimura, 2017). In future work, it would be interesting to investigate whether any of these mechanisms are responsible for the cross-over I found.

The results of Chapter 4, highlight the complexity of the adaptive response to combinations of stressors, both in terms of mean trait value and of plasticity, and stress the importance of studying responses to environmental variables in combination. The selection lines produced for this chapter are a valuable tool to explore adaptive responses to the thermal and nutritional larval environment. In addition to investigating evolved differences in feeding rate, nutrient assimilation, glycogen stores, metabolic rate, and insulin and ecdysone synthesis, as mentioned above, future work could make use of these lines to investigate the effects of larval thermal and nutritional adaptation on adult stress resistance or adult life history traits such as lifespan and fecundity.

Additionally, here I focused on the effects of selection on the larval stages only. Future work focusing on the effects of thermal and nutritional selection on the adult stage could shed light on which part of the lifecycle is more responsive to selection and how the evolved response of life history traits differs depending on the stage at which flies experience the selective pressure. Finally, because in nature organisms experience fluctuating temperatures, future studies investigating adaptation to fluctuating thermal regimes in combination with diet selection could provide a closer representation of how insects might evolve in the wild.

5.4 | Final conclusions

In conclusion, my results show that larval diet and developmental temperature interact to shape plastic and evolved responses. For instance, I showed that development in a warmer environment led to narrower nutritional optima for development time, viability, and size, resulting in harsher trade-offs between the three traits (Chapter 2; Kutz et al 2019). Additionally, I demonstrated that developmental diet and temperature interacted to shape adult stress resistance traits. However, the carry-over effects found were small because adults had access to nutrient rich, balanced food, suggesting that adult feeding could alleviate or exacerbate the effects of a stressful larval environment depending on their access to optimal diets (Chapter 4). Finally, in Chapter 4 I showed that adaptation to increased temperature in combination with poor diet led to non-additive effects that cannot be predicted from the response to each stressor individually and that adaptation changed the plasticity of the life history traits studied. In fact, thermal adaptation changed plasticity to diet, and nutritional adaptation changed plasticity to temperature, indicating an overlap between genes controlling sensitivity to diet and genes controlling thermal sensitivity.

The findings of this thesis add to an emerging body of multi-stressor studies (e.g., Crain, Kroeker and Halpern, 2008; Hecky *et al.*, 2010; Holmstrup *et al.*, 2010; Gunderson, Armstrong and Stillman, 2016; Alton and Franklin, 2017) that illustrate the importance of interactions in shaping responses to environmental conditions. Because animals in nature experience many types of environmental

stress simultaneously, understanding the effects of each type individually is not enough to infer organisms' responses in nature (Kaunisto, Ferguson and Sinclair, 2016). While this thesis provides insight on the interactive effect of developmental temperature and diet on plastic and evolutionary responses, more work is needed to include other environmental factors that will become increasingly stressful for insects with global change, such as water deficiency or higher pathogen load (Kaunisto, Ferguson and Sinclair, 2016).

Further, in this work I have considered both plastic and evolutionary responses to altered thermal and nutritional environments. While plasticity allows an individual to respond quickly to new conditions, evolutionary adaptation is considered slower because it requires changes in genetic variation of a population across generations (Sgrò, Terblanche and Hoffmann, 2016). The debate about the relative contribution of plastic and evolved response for surviving climate change is ongoing (e.g., Sgrò, Terblanche and Hoffmann, 2016; Sørensen, Kristensen and Overgaard, 2016; Rodrigues and Beldade, 2020; Terblanche and Hoffmann, 2020). However, the results from this thesis show that, in *D. melanogaster,* both plasticity and evolution have the potential to mitigate the negative impacts of thermal and nutritional stress; for instance, balanced food can reduce the negative impacts of elevated temperature through plasticity, and adaptation to high temperature and poor diet in combination can, at times, increase the response to selection.

Habitat changes caused by climate change are complex and encompass not just modifications to abiotic factors but also new species interactions and altered ecosystem productivity (Scheffers *et al.*, 2016). In turn, species responses to those changes are also complex and, as a consequence, hard to predict. This thesis, while adding to the effort to understand those responses, highlights the intricacy of the effects of stressful developmental conditions in insects, and emphasises that much more research is needed if we are to understand how species will cope with ongoing global change.

5.5 | References

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