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UNIVERSITY OF SOUTHAMPTON

**FACULTY OF NATURAL AND ENVIRONMENTAL
SCIENCES**

Centre for Biological Sciences

Predictive Adaptive Responses in *Drosophila melanogaster*

by

Roger William Ross Shannon

Thesis for the degree of Doctor of Philosophy

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ABSTRACT

FACULTY OF NATURAL AND ENVIRONMENTAL SCIENCES

CENTRE FOR BIOLOGICAL SCIENCES

Doctor of Philosophy

PREDICTIVE ADAPTIVE RESPONSES IN *DROSOPHILA MELANOGASTER*

by Roger William Ross Shannon

Predictive Adaptive Responses are changes in development made in the perinatal period in response to maternally transmitted information, and a mismatch between the diet selected during human evolution and the contemporary Western diet can produce an adult phenotype characterised by weight gain, cardiovascular disease, hypertension and diabetes.

In humans, most evidence is epidemiological. Using *Drosophila melanogaster*, the problem can be approached from an adaptive phenotypic plasticity perspective. Health effects in humans stem from predictive adaptations made to enhance fitness and so it must first be shown that *D. melanogaster* make these responses.

To model the human dietary transition, two equivalent fly diets were designed, one a human Palaeolithic diet and the other a contemporary Western diet. Using isofemale lines, flies were swapped between diets over three generations and fitness indicators measured in the offspring generation. Fitness indicator responses to a range of diets differing in protein: carbohydrate ratio and total macronutrient content were also investigated.

There were adaptive, compensatory effects on survival rate and male thorax size from parental diet, and development time from grandparental diets, but also non-adaptive effects on development time and female thorax size from the parental diets. Higher dietary protein: carbohydrate ratios reduced development time and increased thorax size and survival rate, while increased macronutrient content increased weight, lipid content and survival. Diet had no effect on ommatidia number relative to fly size.

Whether a response to diet is predictive and adaptive depends not only on diet composition, but whether offspring, parents or grandparents consumed the diet, the phenotypic character measured and the genotype of the fly. The variety of responses in relation to parental and grandparental diets show that intergenerational effects are complex, and *D. melanogaster* is a suitable model to help unravel the causes of human diseases.

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DECLARATION OF AUTHORSHIP

I, Roger William Ross Shannon

declare that the thesis entitled

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.....

and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this University;
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Date:.....

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List of abbreviations

BMI	Body Mass Index
Dilp2	Drosophila insulin-like peptide 2
DNA	Deoxyribonucleic acid
dTOR	Drosophila target of rapamycin
H-G	Hunter-gatherer
mRNA	Messenger RNA (ribonucleic acid)
P	“Palaeolithic” diet
PAR	Predictive Adaptive Response
PC	Principal Component
PCA	Principal Component Analysis
P:C ratio	Protein: carbohydrate ratio
PPAR α	Peroxisome proliferator – activated receptor alpha
PPAR γ	Peroxisome proliferator – activated receptor gamma
TOR	Target of rapamycin
TSC	Tuberous sclerosis complex
W	“Western” diet

Chapter One

General Introduction

1.1 The Predictive Adaptive Response hypothesis

1.1.1 Historical background

The origins of the Predictive Adaptive Response (PAR) concept can be found in the search for the reasons behind the rising rates of cardiovascular disease, diabetes, hypertension, obesity and the metabolic syndrome (Gluckman et al. 2005c). These diseases have become the most common cause of death in the West since the beginning of the 20th Century and have traditionally been thought to be “lifestyle” or affluent diseases, with proximate causes including diet and lack of exercise (Gluckman and Hanson 2005; Hanson and Gluckman 2005). Disease precursors include high cholesterol and a build up of truncal fat, although the incidence of these proximate factors in the population was a poor fit with the incidence of heart disease. This was typified by the “French Paradox” as despite high consumption of fatty foods and wine and a more sedentary lifestyle than most, French rates of cardiovascular disease and diabetes were among the lowest in the developed world (Bateson 2001; Gluckman and Hanson 2005). Other clues that the problem might not be purely lifestyle-related came from populations in transition from a traditional diet to a Western one (Diamond 2003). In the Pima tribe of Native Americans, diabetes rates approached 50% of the population (Knowler et al. 1990; Diamond 2003) while in Nauru Island in Polynesia rates were around 25% (Dowse et al. 1991). The incidence of cardiovascular disease was also higher among those who had emigrated to the USA and their descendants, than among the rest of the population of the USA (Gluckman and Hanson 2005).

One explanation for this was the “thrifty genotype” (Neel 1962; Barker 1997; Diamond 2003). This predicted that populations from non-Western and pre-modern Western societies had a genotype which enabled them to cope with a reduced or unpredictable diet but that this made them susceptible to obesity and cardiovascular disease on a modern Western diet. However, the genetic risk factors identified during the 1980s could not explain the incidence of cardiovascular disease either (Gluckman and Hanson 2005).

1.1.2 The “thrifty phenotype”

In the early 1980s, the highest incidence of cardiovascular disease in the UK occurred in the most deprived areas rather than the most affluent, as would be expected if it was a disease of an affluent society (Barker and Osmond 1986; Barker 1997). David Barker led a study into the epidemiology of cardiovascular disease, concluding that the strongest geographical correlation was not only with deprived areas, but with the areas in which infant mortality (a marker of deprivation) was highest 70 years before, that is at the time when the present sufferers were born (Barker and Osmond 1986; Barker 1997; Gluckman and Hanson 2005). A further correlation between infancy and adult heart disease was found in a study of 16,000 births in Hertfordshire between 1911 and 1930. Increased mortality from cardiovascular disease (and also the incidence of altered vascular endothelium function, hypertension, truncal fat and raised cholesterol) was correlated with lower birth weight (Osmond et al. 1993; Barker 1997; Gluckman and Hanson 2005).

In the Hertfordshire Cohort studies, birth size or some other measure of sub-optimal fetal development were linked to adult disease, in particular to cardiovascular disease, obesity and Type 2 diabetes (Barker 1997; Gluckman et al. 2005a). These observations formed the basis of the “thrifty phenotype” hypothesis (Hales and Barker 2001). This is the basis for the PAR concept and states that an adverse fetal environment invokes a developmental response in the fetus. This response reduces the growth trajectory of the fetus, enabling it to conserve energy, but with the result that the adult would be suited to a nutritionally deprived environment (Gluckman et al. 2005c).

The thrifty phenotype hypothesis describes a disruption of the normal developmental programme, where the individual has to cope in later life with the short-term adaptations to a sub-optimal environment in development. The consequences in post-reproductive life do not have any selective advantage and are “capricious” (Gluckman et al. 2005a). There is a range of time-scales over which these responses operate: immediate or acute challenges (e.g. low oxygen) can be countered with a homeostatic response (e.g. channelling blood to vital organs and away from peripheral areas), but if they continue over a longer time-scale they become chronic and can alter the developmental trajectory.

The adaptations to the deprived fetal environment include increased insulin resistance, and so were linked to increased risk of Type 2 diabetes in later life. The thrifty

phenotype model considers fetal adaptations to be a response to a short-term challenge, with long-term consequences that may not manifest until after the individual has passed reproductive age. In this view, the fetal adaptation (and the inherited capacity to make the adaptation) may have a selective advantage in that it allows the individual to develop and subsequently to reproduce. The trade-off comes in the form of increased risk of cardiovascular disease, obesity and diabetes, but does not occur until after the reproductively active phase and so could be beyond the reach of selection pressures, which can only operate on heritable characteristics affecting fitness (Williams 1957; Gluckman et al. 2005a; Kuzawa 2005b). This model cannot explain the continuous variation in later-life disease risk found across birth weights from the Hertfordshire study as it would predict a step or threshold effect rather than a linear correlation between birth weight and disease (Gluckman et al. 2005c).

1.1.3 The PAR hypothesis

A study from Finland similar in scope to the Hertfordshire Cohort showed the importance of the interaction of the current environment with the intra-uterine (maternal) environment (Eriksson et al. 2001). Those who had been small at birth and were now overweight had a much higher risk of cardiovascular disease than those who were merely overweight as adults (Eriksson et al. 2001). Along with the evidence from the Hertfordshire Cohort, this continuous variation and interaction between the maternal and adult environments gave rise to the Predictive Adaptive Response hypothesis (Gluckman and Hanson 2004; Gluckman and Hanson 2005).

If the changes in developmental trajectory made in the fetal environment are not short term but have the effect of fitting the organism for adult life, they are predictive and adaptive responses. The use of 'predictive' is a shorthand for saying that the adaptations made by the fetus have the effect of matching the adult phenotype to the information available during development (Gluckman and Hanson 2005). Physiological markers can vary greatly between populations, including those regarding reproduction. Progesterone levels measured in women in Boston and Chicago were almost twice that found in Tamang and Bolivia, yet each population was equally fertile (Vitzthum et al. 2004). The low levels found in the normally-fertile women in the developing countries would be regarded typical of infertile women in the U.S. (Vitzthum et al. 2004). The marker used to assess the integrated nutritional state experienced prenatally is often birthweight, but this is regarded as an effect of the adaptations rather than a cause of

subsequent disease (Gluckman et al. 2005c). The level of nutrients delivered via the placenta influence the production of insulin and IGF-2 (insulin-like growth factor 2), which in turn set the fetal growth rate (Kuzawa 2005b). Although not perfect as a marker, birth weight has a small genetic component compared to its environmental component and so is a reasonable measure of the quality of the intrauterine environment (Godfrey et al. 2006).

Adaptations made in response to the summed nutritional information from the maternal environment are thought to be in anticipation of the future environment faced by the mature individual (Gluckman et al. 2005a). For instance, one of these adaptations relates to insulin sensitivity – if an individual (with a particular polymorphism in the regulatory gene *PPAR-γ2*) experiences a sub-optimal maternal environment, then as an adult in a modern Western dietary environment it is more likely to suffer from Type 2 diabetes (Eriksson et al. 2002; Gluckman et al. 2005a). The adaptations observed have a similar health outcome if the prediction is wrong: a changed metabolic homeostasis regarding insulin resistance, changed endothelial function, hypertension and obesity (Gluckman et al. 2005c). They are not disruptions to normal development without any adaptive value, as occurs for instance in thyroid aplasia associated with iodine-deficient mothers (Gluckman et al. 2005c).

Currently, the main candidate for the transmission of PAR effects from mother to offspring is epigenesis, by DNA methylation or histone acetylation (Gluckman et al. 2005c; Lillycrop et al. 2005; Gluckman and Hanson 2006a; Godfrey et al. 2006; Burdge et al. 2007a; Daxinger and Whitelaw 2010). Methylated genes are silenced by the methylation of cytosine regions in the promoter regions of the genes, in the same way that genes not required in a particular tissue are silenced during development (Gluckman et al. 2005c).

Particular polymorphisms of genes can interact with the environment to induce PARs, including genes coding for angiotensin-converting enzyme (connected with high blood pressure), plasma cell glycoprotein 1 (pre-eclampsia) and vitamin D receptors (involved in mineralization) (Godfrey et al. 2006). The association between the *PPAR-γ2* gene, low birth weight and insulin resistance was only found in those with the Pro12Pro allele, and not in those with the Pro12Ala. In subjects with the Pro12Pro polymorphism of *PPAR-γ2*, lower birth weight was correlated with the insulin resistance characteristic of Type 2 diabetes (Eriksson et al. 2002).

PARs may stretch across more than one generation and may be caused by epigenetic effects persisting through meiosis. Alternatively there may be changes in the reproductive tract, or the PAR effect in the female offspring may alter the nutritional environment of the fetus in the next generation (Gluckman et al. 2005c; Daxinger and Whitelaw 2010). This last possibility means that the PAR may be a response to several generations worth of information, integrated in the maternal phenotype (Kuzawa 2005b). Mathematical modelling also shows that a strategy of “carry-over” of phenotypic adaptation over several generations is a more successful strategy in a stochastic environment than either pure genetic change (limited by the slow rate of mutation) or complete phenotypic plasticity (Jablonka et al. 1995).

These effects are the modern manifestation of PARs, but the costs and benefits of a PAR mechanism need to be looked at in terms of human evolution. If an individual who had experienced nutritional deprivation in the womb made appropriate adaptations to that environment and subsequently spent its mature life in an unpredictable or nutritionally poor environment, it would enjoy an advantage as it would tend to expend less energy, be able to thrive on less food and have a metabolism which encourages the deposition of fat to tide it through times of food shortage or famine (Gluckman et al. 2005a; Gluckman et al. 2005c). This “survival phenotype” would use less resources for growth, have a lowered basal metabolism and increased energy store (in the form of truncal fat), smaller adult size and may well have been able to complete its development to adult reproductive competence in a low-nutrient or unpredictable environment (Gluckman and Hanson 2007; Gluckman et al. 2011). Periods of negative energy balance would be fewer and so surplus energy could still be directed towards growth in childhood and reproduction in adulthood (Kuzawa 2005b). The environmental deficit would then be spread across the organism as a whole rather than falling disproportionately on reproduction and growth (Kuzawa 2005b). This can be seen in an earlier study of the subsistence farmers of Heliconia, Columbia, where males grow slowly to the age of 26, whereas females reach their full stature at 18, as the demands of reproduction are different in the two sexes (Stini 1971).

The most important aspect of the PAR is that the prediction is correct. It is the mismatch between the fetal environment and the adult environment which is pathological, not the original prediction or even necessarily the post-natal environment (Gluckman and Hanson 2005). However, there is always a constraint on fetal development imposed by the capacity of the mother to deliver nutrients to the fetus via

the placenta and to limit the size of the fetus relative to the mother, and so excessively nutritionally rich environments will not necessarily always be signalled accurately to the fetus (Gluckman et al. 2005a; Gluckman et al. 2005c; Gluckman and Hanson 2006a). This maternal constraint may operate as a failsafe mechanism to ensure that the fetus is able to withstand a slightly worse environment than the external one during pregnancy (Gluckman and Hanson 2006a).

One criticism levelled at the PAR hypothesis is that maternal malnutrition during pregnancy does not produce disease in later life in subsistence farming societies, such as in the Gambia (Wells 2007). In this case, the PAR is appropriate and accurate (Gluckman et al. 2008). There are many instances of societies similar to the Gambian farmers changing their diet to a more Western diet, and subsequently developing higher rates of cardiovascular disease and diabetes than found in Western populations (Lev-Ran 2001; Ulijaszek 2003; Willcox et al. 2007). The main two reasons why the PAR is often inappropriate today, and leads to disease, are firstly that food is often now present in abundance in the west (and less energy is expended obtaining it), and secondly that people are living well past their reproductively competent years (Hanson and Gluckman 2005). The effects of an inappropriate prediction are usually not seen until middle age or later, and by this time selection no longer operates (Williams 1957; Hanson and Gluckman 2005).

1.1.4 Epidemiological evidence

Evidence for the interaction of prenatal and postnatal environments has come from a study using the very comprehensive medical records of Finland, where the hazard of dying from heart disease was greatly increased in those born small but subsequently catching up to the average Body Mass Index (BMI) (Eriksson et al. 2001) and studies in India which showed a similar relationship despite overall lower birth weight (Yajnik et al. 2003; Gluckman and Hanson 2005).

More evidence came from the “Dutch Hunger Winter” of 1944-45, when severe rationing was imposed on the Western Netherlands in reprisal for Resistance activity. The rationing lasted for seven months until the Netherlands were liberated, but in that time the daily ration fell from around 1800 cal person⁻¹ day⁻¹ to less than 800 cal person⁻¹ day⁻¹ between December 1944 to April 1945, and recovered quickly after liberation to its former level. Women whose pregnancies ended during this time gave birth to babies with a low birth weight, while those who became pregnant during the Hunger Winter gave

birth to babies of a normal or slightly above average birth weight, who as adults suffered disproportionately from cardiovascular disease and obesity (Roseboom et al. 2000; Gluckman and Hanson 2005). In the case of the babies conceived during the famine, the maternal signals would have been independent of actual nutrition received from the mother, as there is no nutrient transfer to the fetus during the embryonic (eight week) period (Roseboom et al. 2000). So, if in the Hunger Winter the fetal environment was very poor, this gave a signal that the future environment would be poor and allowed the babies to adjust their physiology, especially as relates to insulin (Roseboom et al. 2000). This showed that the early period of pregnancy had a greater effect on development compared to later in gestation. Two examples are that almost all of the cells of the heart and the nephrons of the kidney are formed before birth (Hanson and Gluckman 2005; Godfrey et al. 2006)

A similar result has been obtained in studies of the Chinese famine (1959-1961) and the Biafra civil war (1967-1970), where those affected by the famine during gestation or infancy were more likely to be overweight or show signs of metabolic syndrome, especially if they ate a western diets as adults (Hult et al. 2010; Li et al. 2011). However, the Siege of Leningrad did not affect the subsequent health risks of babies or infants who had suffered malnutrition (Stanner et al. 1997). This may be a result of the continued malnutrition after the end of the war in Russia, but not in the Netherlands (Bateson 2001).

1.1.5 Experimental evidence in other animals

Apart from the “natural experiments” of famine, evidence in humans is as yet largely epidemiological (Bateson and Gluckman 2011). Studies in other animals allow the manipulation of the maternal and offspring environments and so can lead to firmer conclusions on the influence of each.

In rats, increased grooming of the pups by the mother is associated with being less fearful and having a lower response to stress when the pups become adults (Weaver et al. 2004). These effects are irreversible, and are associated with the methylation of a glucocorticoid receptor promoter in the hippocampus of the pups. In rats who had less grooming by their mothers as pups, methylation was lower and stress responses much higher, but pups from non-grooming mothers could have their responses changed by fostering with a grooming mother (Weaver et al. 2004). In sheep, under-nutrition in the periconceptual period can result in premature birth (Bloomfield et al. 2003). Watkins et

al. (2008) fed mice two diets, low- and normal-protein, and this induced a heavier phenotype in the offspring of the low-protein group, who also had higher blood pressure and displayed more anxiety as adults. Pregnant rats on a protein-restricted diet had offspring with reduced methylation of the PPAR α gene, involved in lipid and carbohydrate homeostasis (Lillycrop et al. 2005).

Animals responses' to the maternal environment can be in the form of a polymorphism or a more graduated response (Monaghan 2008). In some species, two or more distinct morphs may be produced from the same genotype in response to maternal or juvenile conditions (West-Eberhard 2003; Jablonka 2009). These include the seasonal coat length of the meadow vole *Microtus pennsylvanicus* (Lee and Zucker 1988; Lee et al. 1989), shell polymorphism in response to predators in the freshwater snail *Physa heterostrophus* (DeWitt 1998), the wet- or dry-season eyespots in the butterfly *Bicyclus anynana* (Brakefield et al. 2007) or the carnivore morph in spadefoot toad *Spea multiplicata* tadpoles (Martin and Pfennig 2010). For polymorphisms, a “mismatch” between the predicted and actual environment could result in greatly reduced fitness or a failure to reach adulthood at all (Bateson and Gluckman 2011).

Where the maternal or juvenile environmental stimulus is graduated, for instance temperature or food availability rather than the presence or absence of a predator or an abrupt seasonal change, the response of the animal may also be graduated (Monaghan 2008; Bateson and Gluckman 2011), in the form of more subtle changes in size, development time or mobility (Huey et al. 1995; Gibert et al. 2001; Gilchrist and Huey 2001; Steigenga and Fischer 2007; Monaghan 2008; Bateson and Gluckman 2011). This may be a result of a constraint or “carry-over” effect from the previous generation (or juvenile stage), also known as a “hot parents better” or “silver-spoon” effect (Gilchrist and Huey 2001; Jasienska et al. 2006; Ellison and Jasienska 2007; Monaghan 2008), where animals from a good parental or developmental environment are fitter in any environment than those from a poor environment. Alternatively, there may be a compensatory or predictive adaptive response induced by the poor environment, which may again give an advantage in all environments (Gilchrist and Huey 2001; Monaghan 2008).

Two experiments have explicitly tested the PAR hypothesis in *Drosophila melanogaster* in relation to diet. Prasad et al. (2003) used a standard and a poor diet reduced in protein and found that flies whose mothers were raised on poor diet had a

higher survival rate than those raised on rich diet, but only when they developed on the rich diet. Vijendravarma et al. (2010) performed a similar experiment and found that the eggs from poor-diet parents were larger and the offspring developed faster on both rich and poor diets than those from rich diet parents, suggesting a compensatory predictive adaptive response to the poor diet.

1.1.6 Recognising a PAR

It is important to distinguish the potential effects of a PAR on fitness from effects on health. The PAR is directed at maintaining or enhancing fitness in a predicted environment, particularly by altering growth and metabolism to suit that environment, but is not directed at enhancing health, except where it would impinge on fitness (Bateson et al. 2004; Bateson and Gluckman 2011). The effects of a mismatched parental and offspring environment on fitness might therefore be different to those on health outcomes.

To show that a Predictive Adaptive Response has occurred, firstly the manipulated environmental conditions of the experiment must produce a response. If the response is a qualitative one, for instance colour or shape of a body part, such as the development of a protective carapace in response to predation threat in *Daphnia* (Agrawal et al. 1999), then given the correct environmental conditions this is relatively straightforward. When the response is quantitative, for instance the time to eclosion or size of thorax in a fly, there must be some method of distinguishing a PAR from other types of parental effects (Nunney and Cheung 1997; Huey et al. 1999; Ellison and Jasienska 2007; Monaghan 2008).

A predictive response would be one in which the diet of a previous generation exerts an influence on the fitness indicators of the offspring generation. Gluckman and Hanson (2005) define a predictive adaptive response as one in which a signal from the parental environment influences the phenotype of the offspring to maximise its fitness in the predicted environment, which is normally expected to be the same as the parental environment. Information may be integrated over several generations, or in some cases, phenotypic optimisation may be to a different environment to that experienced by the parent, due to the predicted season in which the offspring will be born (Lee and Zucker 1988; Gluckman and Hanson 2004; Gluckman et al. 2005d).

An inter-generational effect of diet need not be an adaptive response (Ellison and Jasienska 2007; Monaghan 2008). Ellison and Jasienska (2007) suggest that responses can be constrained by the previous generation's environment, or may be pathological, or adaptive. In the hypothetical examples given below in Figures 1.1 – 1.3, it is assumed that the α environment is a “good” dietary environment and β is “bad” (Fig. 1.1a). This environment could however relate to temperature or any other factor that has an effect on the fitness of the organism (Monaghan 2008).

Constraint would be evident as an influence of parental environment, where the direction of influence is the same as that in the offspring environment (Ellison and Jasienska 2007), also known as the “silver spoon” effect (Monaghan 2008) (Fig 1.1b and c). This may operate in conjunction with the offspring environment (Monaghan 2008) (Fig. 1.1c). If the response is pathological this could be a disruption to development or the expected response. This may be difficult to detect except by very high variability on one diet, or a low survival rate.

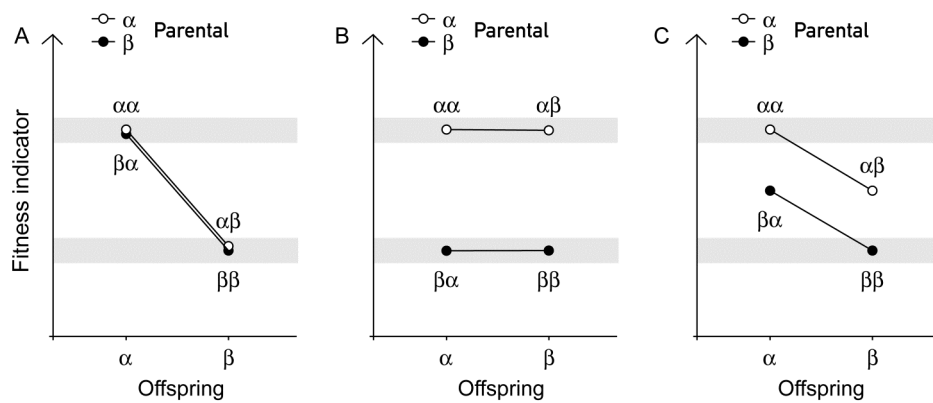


Figure 1.1: Three possible outcomes of a hypothetical two-generation, two-diet experiment. The y-axis represents a fitness indicator (F.I.), where a higher figure indicates higher fitness. The x-axis represents the offspring diet. Open circles represent parental diet α , closed circles represent parental diet β . Diet β is assumed to induce a lower figure for the fitness indicator than diet α when parental and offspring diets are the same. Grey bars indicate the region in which the F.I. is not different to $\alpha\alpha$ (top) or $\beta\beta$ (bottom). Error bars are omitted, but it is assumed that all factors discussed are significant. **A:** F.I. is controlled by the offspring diet. **B:** F.I. is controlled by the parental diet. **C:** F.I. is controlled by both parental and offspring diets, operating in the same direction in each generation.

If a prediction is correct and adaptive, the expected outcome is a very similar response to a diet in the offspring as in the parent. The condition in which the hypothesis can be tested is one in which the parental and offspring diets are different (hereafter called a “mismatch”), with the opposite condition (matched diets) acting as the

controls (Ellison and Jasienska 2007). In the case of the two theoretical diets α and β in Figures 1.1–1.3, a parental/offspring diet history of $\alpha\beta$ or $\beta\alpha$ are the mismatched diets, and $\alpha\alpha$ and $\beta\beta$ the matched. If a mismatch lowers a fitness parameter, this is measured against a matched diet history, and this should be the match in which the offspring diets are the same (e.g. $\alpha\beta$ against $\beta\beta$). If both mismatched conditions have lower fitness than the matched conditions, the situation would look like that in Figures 1.2a and 1.2b. If a mismatch diet combination produces a response between those of the matched diet conditions (Fig. 1.2c), it is difficult to say whether the $\beta\alpha$ condition is caused by a combination of offspring diet and PAR or offspring diet and constraint, although in the case of $\alpha\beta$ in Figure 1.2c, the $\alpha\beta/\beta\beta$ comparison would indicate that $\beta\beta$ might produce an adaptive response (Nunney and Cheung 1997).

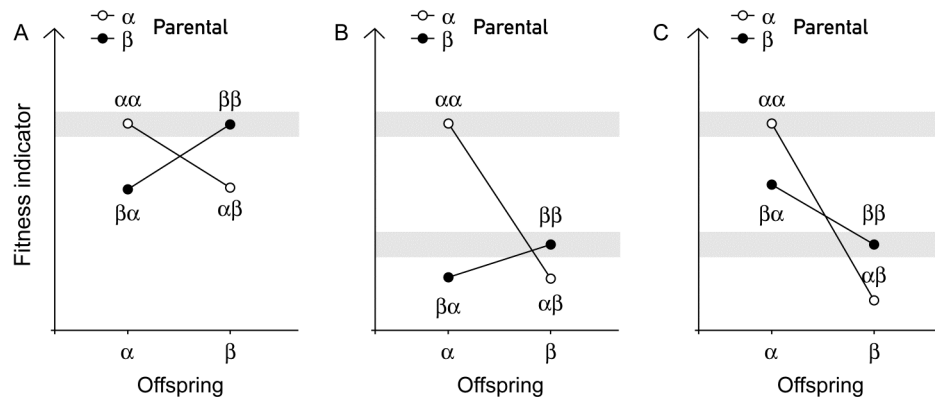


Figure 1.2: Three possible outcomes of a two-generation, two-diet experiment where a significant interaction between different generations' diets is present. All formats are the same as Figure 1.1 above, except that in A, the two diets produce equal F.I. values when parental and offspring diets are matched **A:** There is a significant adaptive effect, and when diets are mismatched the F.I. is lower than when they are matched. **B:** Diet β induces a lower F.I. than diet α , and mismatched parental and offspring diets have lower F.I. than matched diets **C:** A combination of offspring diet and parental diet, but where each group has higher F.I. where parental and offspring diets match.

A mismatch resulting in an incorrect PAR could occur in one direction (e.g. $\alpha\beta$), but not in the other ($\beta\alpha$) (Fig 1.2c). This would indicate that some form of predictive adaptive response is operating to optimise an organism to diet β but not diet α . The difference between $\alpha\alpha$ and $\beta\alpha$ could be a direct effect of the parental diet β , with an improvement in P.I. due to the better quality offspring diet. It may also be the result of a wrongly-predicted PAR, counteracted by a higher quality offspring diet, but if the value of the fitness indicator is between that of the matched groups, this might only be

detected if the PAR induces a qualitative change as well as a quantitative one (e.g. a different wing shape).

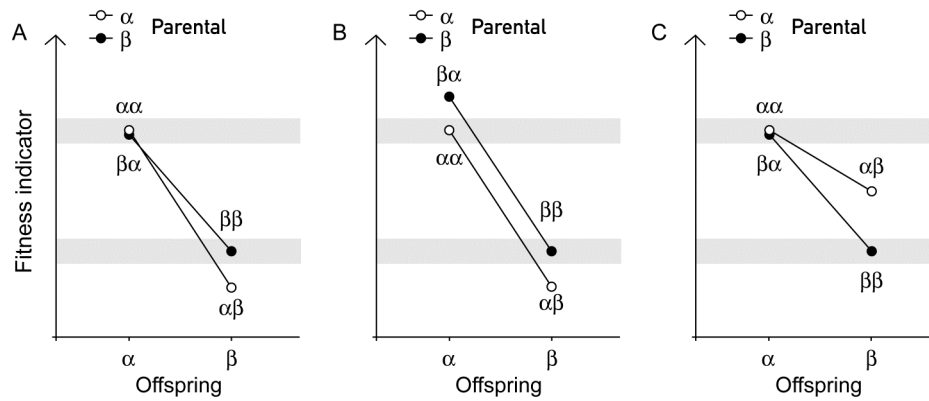


Figure 1.3: Three possible outcomes of a two-generation two-diet experiment. All formats are the same as Figure 1.1 above. **A:** A PAR may be operating when parents are on diet β , as the $\alpha\beta$ group have lower F.I. than $\beta\beta$, but not on diet α . **B:** A compensatory PAR. The mismatched conditions operate in different directions: $\beta\alpha$ has a higher P.I. than $\alpha\alpha$ and $\alpha\beta$ a lower P.I. than $\beta\beta$. **C:** A significant interaction between parental and offspring diets caused by the influence of the parental diet α , but not β .

In Figure 1.3a, the situation is similar to Figure 1.2c, with a PAR operating on diet β , but no parental diet effect in the $\beta\alpha$ condition. In Figure 1.3b, the mismatched diets have an opposite effect, which would be detected as a significant effect of the parental diet and of the offspring diet, where one diet raises fitness if it occurs as a parental diet but lowers fitness if it occurs as an offspring diet, in the same way observed for intergenerational effects of temperature on pupal mass by Steigenga et al. (2007). As an example, if there is some inhibitory factor in diet β which delays development, this might induce a PAR with the effect of accelerating development if an organism's parent is on diet β . In this case, $\alpha\beta$ would not have the benefit of this optimisation, while it may still be acting in the $\beta\alpha$ condition to accelerate or enhance growth, but now in the absence of the inhibitory factor and with the benefit of the higher quality α diet, resulting in a higher P.I. than the $\alpha\alpha$ condition. There may also be significant interactions between the diets of different generations, which are not PARs (Fig. 1.3c). Here, there is no effect of a parental diet β , but diet α does still have a positive effect in the next generation. This could be the result of a rich diet enhancing the provisioning of eggs, for example.

Where a significant effect of parental environment is found, the direction of its effect and the nature of its interaction with the offspring environment must therefore be

examined carefully (Gilchrist and Huey 2001; Monaghan 2008), as not all significant effects or interactions between the two can be considered as showing any evidence of adaptation. Any evidence for adaptation will come from the responses to the mismatched condition (Ellison and Jasienska 2007) and these must show significant effects not reasonably attributable to either the parental or offspring diet, by comparison with the matched condition.

Illustrated below are graphical representations of the criteria for deciding between constraint, adaptation or pathology (maladaptation), adapted from Ellison & Jasienska (2007) and Monaghan (2008), to be considered if significant effects or interactions between parental and offspring environments have been found (Fig. 1.4).

In the first set of diagrams in Figure 1.4 (1-4b), the mean value of the fitness indicator is no different between the good and bad parental diets, although when there is a significant interaction between parental and offspring diet, with (Fig 1.4 4a) or without (Fig 1.4 2a) a significant effect of offspring diet, fitness indicators are higher when parental and offspring diets are matched, and lower when they are mismatched, as described by Gluckman et al. (2005) and Godfrey et al. (2010). It is also a logical possibility that fitness indicators could be higher when diets are mismatched, although the adaptive significance of what this would mean is unclear (Fig. 1.4 2b and 4b).

In the next set of diagrams, the effect of the “good” parental diet is to raise the fitness of the offspring on all diets, an effect known as “silver spoon” (Monaghan 2008) or as “constraint” (Ellison and Jasienska 2007) due to the effect of lowering the fitness of offspring from the “bad” parental diet. In the “mismatch” conditions (Fig. 1.4 6a and 8a), when the offspring of the “bad” diet parents develop on their own diet this reveals an adaptive effect compared to when they do not, although the additional silver spoon effect makes it difficult to declare that other diet combinations are adaptive.

In the last set (Fig 1.4 9-12), the “bad” parental diet induces an adaptive response in the offspring generation resulting in a higher value of the fitness indicator on average than that of offspring of the “good” diet parents. This may be apparent across both diets (Fig. 1.4 9 and 11) or in only the matched (bad-bad) condition (Fig. 1.4 10a and 12a). To be able to distinguish between models 5 and 9, further information is needed to establish whether the diets are “good” or “bad” in their effects on fitness.

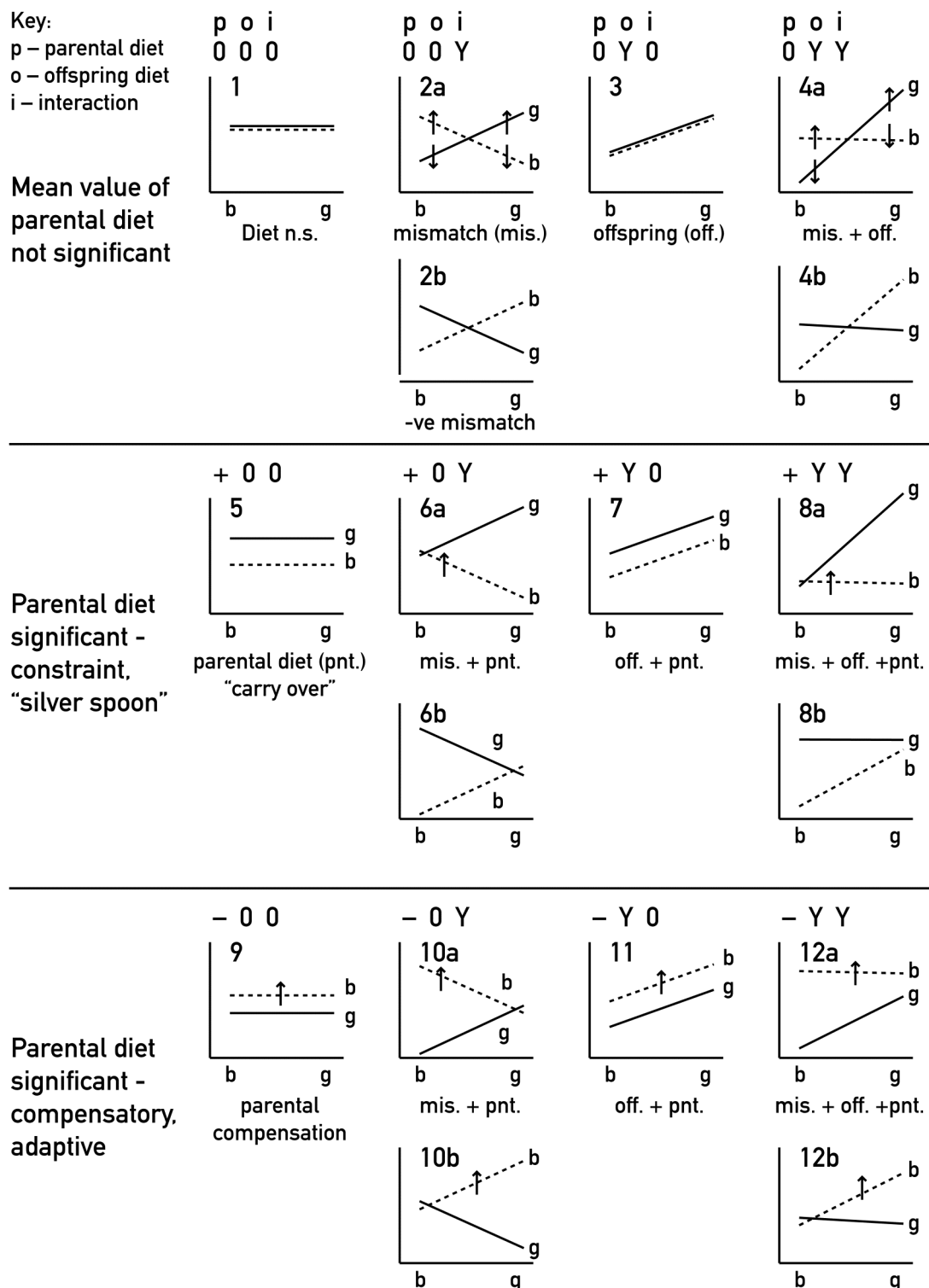


Figure 1.4: Possible outcomes of a factorial two-generational diet experiment, using two diets, b (bad and g (good), which have differential effects on fitness. Key: "0 0 0" represents the significance of parental diet, offspring diet and interaction between diets, where 0 = not significant, Y = significant, + = significant parental in the same direction as offspring and - = significant parental in the opposite direction, up arrows represent adaptive effects, down arrows maladaptive. Y-axes of the reaction norm diagrams represent values of a fitness indicator where more positive values indicate higher fitness. X-axes represent the offspring diet.

1.1.7 Summary and conclusions

Predictive Adaptive Responses are adaptations made in response to the grandparental, parental or perinatal environment, directed at enhancing fitness, and normally tailored to a predicted continuation of the parental environment, or to the next phase of a seasonal environment (Gluckman and Hanson 2004; Monaghan 2008; Bateson and Gluckman 2011). They may have adverse effects on health if this prediction is incorrect, but the effects on fitness might not be the same. Detecting a PAR requires careful consideration of the relative fitness of organisms in situations where their own environment matches their parents', and when it does not (Jasienska et al. 2006; Rickard and Lummaa 2007; Monaghan 2008).

The PAR concept in humans relates to a transition between a traditional, or ancestral, environment and a modern Western one (Bateson et al. 2004; Gluckman and Hanson 2004; Godfrey et al. 2010). In the next section this transition is examined, mainly from a dietary perspective, as before any attempt is made to model the PAR hypothesis for humans, using *Drosophila melanogaster*, it is first necessary to know what the environmental causes might be.

1.2 Human evolution and diet

1.2.1 Early Human evolution and diet

The genus *Homo* has existed for around the last 2 million years and evolved from one of the earlier *Australopithecus* species (Eaton and Konner 1985; Bromage et al. 1995; White et al. 2003; Prat 2007). Anatomically modern *Homo sapiens* evolved in East Africa between 200,000 and 100,000 years ago (Stini 1971; Bromage et al. 1995; McHenry and Coffing 2000; White et al. 2003; McDougall et al. 2005). Humans spread around the world as hunter-gatherers and had a significant proportion of meat in their diets (Stini 1971), probably initially obtained by scavenging carrion (Cordain et al. 2000c; Teaford and Ungar 2000).

The diet of these early humans may have influenced the evolution of modern *Homo sapiens* (Aiello and Wheeler 1995; Leonard et al. 2007). The tripling of brain size during hominin evolution (McHenry and Coffing 2000) is thought to have required high-quality nutrition, which may have been offset by a smaller, less energetically expensive colon relative to other primates, and a comparatively high-protein, high energy-density diet to compensate for this (Aiello and Wheeler 1995; Leonard et al. 2007). The skeletal remains of the Early Upper Palaeolithic people (older than 20,000 BP) show few signs of pathology or of nutritional stress and indicate that a rich environment was enjoyed by these hunter-gathering communities (Formicotal and Holt 2007). Skeletal samples from after this period show more disruption to the process of growth, increased dental caries and degeneration of the joints and a decline in stature (Formicotal and Holt 2007). This is interpreted to mean that the shift from hunter-gatherer lifestyle to subsistence farming was detrimental to the health of humans and the increased consumption of cereals is closely linked to this (Formicotal and Holt 2007). The increased frequency of Harris lines (pauses during growth) in fossilised bones also indicates more frequent disruption of the food supply, slowing normal growth (Formicotal and Holt 2007).

The invention of agriculture around 10,000 BP (Diamond 1997) did not necessarily improve human health, but may have actually caused a decline by changing the composition of the diet, and by tying a population to a fixed spot it was left open to climatic variation causing periodic famine (Diamond 1997; Cordain et al. 2002; Trevathan 2007). Modern subsistence farmers show a similar pattern of malnutrition and ill-health (Stini 1971). For instance, the children of the village of Heliconia in Columbia showed a reduction in growth, with females less affected than males, on a low-protein

high-carbohydrate diet (Stini 1971). Undernourished males appeared to continue growing until the age of approximately 26, rather than reaching full stature at around 18, and this may be an adaptation to reduce metabolic demand in their nutritional environment, in contrast to the females and to the well-nourished male controls (Stini 1971). The females underwent a normal adolescent growth spurt and this is thought to make reproduction possible, whereas in males increased stature is not necessary for reproduction (Stini 1971). Stini thought that given the short span of time since the beginning of agriculture, and the response of the controls, that this reduced stature and growth was likely to be a result of human phenotypic plasticity (Stini 1971).

1.2.2 Palaeolithic and modern nutrition, protein and energy density

The nutrition and energy balance environment of the Palaeolithic period has been extensively studied over the past few decades as it offers a way of examining the diet on which modern *Homo sapiens* evolved, and so gives a baseline for what is likely to be the ideal diet for humans (Eaton and Konner 1985; Eaton et al. 1997; Kuipers et al. 2010). Modern humans evolved in the environment of the Palaeolithic, and the historic availability of food would have influenced their food preferences and macronutrient intake target (Cordain et al. 2000c; Eaton and Eaton 2000; Simpson et al. 2003; Eaton 2006). Evidence is gathered by studying modern hunter-gatherer (H-G) societies, archaeological data and a by taking a modelling approach (Eaton and Konner 1985; Eaton et al. 1997; Cordain et al. 2000b; Cordain et al. 2000c; Kuipers et al. 2010). Using H-G diets as a proxy gives a wide range of macronutrient ratios in the diet, reflecting the food available in different regions (Cordain et al. 2000c; Richards et al. 2003; Ulijaszek 2003; Eaton 2006; Strohle et al. 2007).

Eaton's model (based largely on H-G diets) has been refined over many years (see Table 1.2), with typical values for macronutrients of over 30% of energy being derived from protein, around 45% from carbohydrate and the remainder from fats (Eaton and Konner 1985; Eaton et al. 1988; Eaton 1992; Eaton et al. 1997), with the majority of the protein being derived from animal sources (Hedges and Reynard 2007). More recent studies have tended to show a lesser role for protein, at around 27% of energy (Cordain et al. 2000a; Kuipers et al. 2010). This is in contrast to typical values in modern Western diets, of protein between 12 and 17%, carbohydrate around 50% and fat contributing the remainder (Henderson et al. 2003; Wright et al. 2003; Bates et al. 2010) (see Table 1.1 for details).

According to Simpson and Raubenheimer (2005), the need to maintain protein intake can explain much current obesity, if the contribution of protein relative to carbohydrate and fat has dropped in freely available food. They tested this by first allowing two groups of volunteers free access to various foodstuffs while in a controlled environment. Over the first two days of free choice, they chose foods with the overall macronutrient ratio of one part protein to four parts combined carbohydrate and fat (Simpson et al. 2003). On the second two days, one group was offered foods high in protein relative to carbohydrate and fat, and the second group offered foods low in protein. The high-protein group consumed less overall, while the low-protein group almost maintained their protein intake but as a result consumed more calories (Simpson et al. 2003). On the final two days subjects were offered a free choice again and the high-protein group appeared to compensate by reducing their protein intake, although the low-protein group did not increase theirs. The scarcity of readily available carbohydrate and fat in the Palaeolithic diet may have resulted in the mechanisms controlling their intake being less important than the ones controlling protein intake (Simpson et al. 2003; Simpson and Raubenheimer 2005).

The effect of lack of exercise would be to lower energy expenditure and so also the combined carbohydrate and fat target, but as protein requirements are unchanged, an individual whose energy requirements were low might still overeat carbohydrate and fat unless their diet changed to one higher in protein (Simpson and Raubenheimer 2005). A further complication of less exercise is that in itself it can lead to insulin resistance and so to gluconeogenesis. Over the long term, the effect would be to increase protein requirements while decreasing carbohydrate and fat requirements. The lack of regulation of carbohydrate and fat intake together with the evolved human capacity to store energy against times of famine (Lev-Ran 2001) will combine to increase the individual's weight (Simpson and Raubenheimer 2005).

Another approach to modelling ancestral and modern diets is to look at the difference in the energy density between Palaeolithic/H-G diets and diets in the West. Energy density is the total amount of energy available from a set weight of a foodstuff, expressed as $\text{kJ } 100\text{g}^{-1}$ (Prentice and Jebb 2003) and from analysis of diet survey data appears to have a direct relationship with obesity (Kant and Graubard 2005). In the Palaeolithic/H-G model, daily intake and expenditure of energy is much higher than the present, though on a diet with less energy density than the Western diet, due to the

unrefined nature of the sources of carbohydrate (Cordain et al. 2000c; Simpson and Raubenheimer 2005; Eaton 2006).

Prentice and Jebb (2003) compared the overall energy density of three diets: fast foods (from data published on company websites); a typical British diet (Henderson et al. 2003); and that of the diet of subsistence farmers in Gambia, as an example of a “traditional” diet, using data from McCrae and Paul (1996). The energy density of a typical modern hunter-gatherer diet was calculated as around 450 kJ 100g⁻¹ (McCrae and Paul 1996), the British diet in the early 2000s had an energy density of 670 kJ 100g⁻¹, UK supermarket ready meals at around 800 kJ 100g⁻¹ and that of “fast-food” (calculated from menus and data published by three fast food chains in the UK) is typically around 1100 kJ 100g⁻¹ (Prentice and Jebb 2003).

Prentice (1998) covertly manipulated foods so that the energy density varied from 480 kJ 100g⁻¹ to 700 kJ 100g⁻¹, while palatability was the same, and allowed subjects to eat freely for seven days. Another group were given foods whose fat content differed, but not their energy density. After seven days, a similar weight of food had been consumed by all groups, but those subjects who had been provided with high energy-density foods had gained an average of 65g d⁻¹ of body fat, while those on the low energy-density diet had not (Prentice 1998). In the other group, there was no difference in either amount eaten or weight gain in the groups where fat content was different but energy density was not. This suggests that humans may follow a rule of “eat until full”, that is that satiation occurs when a set amount (for each individual) has been consumed (Prentice 1998; Rolls 2000; Prentice and Jebb 2003). This also suggests that fat content is not important in itself in encouraging overeating, but that its higher energy density of 37.6 kJ 100g⁻¹, compared to 16.7 kJ 100g⁻¹ (Rolls 2000) for protein and carbohydrate, tends to increase the overall energy density of a food (Prentice and Jebb 2003) without a control mechanism to discourage over-consumption (Prentice 1998; Prentice and Jebb 2003). Food volume is a limiting factor, but only when volume is high. A study of the effect of a “Simian” diet on human health found that while eating a diet consisting mainly of high-fibre vegetables and nuts had a significant cholesterol-lowering effect, the subjects had to be pressured to eat the full calorie-maintaining 5.5 kg each day (Jenkins and Kendall 2006).

There is therefore evidence that human diet choices are influenced by both the need to maintain protein intake (Simpson et al. 2003) and by overall volume of food

consumed (Prentice 1998), with carbohydrate and especially fat content playing a relatively minor role (Prentice 1998; Rolls 2000; Simpson et al. 2003). This suggests that the two most important changes affecting the Western diet compared to a Palaeolithic one may be the halving of the protein to combined carbohydrate and fat ratio (P:C+F) (Eaton et al. 1997; Cordain et al. 2000b; Simpson et al. 2003; Simpson and Raubenheimer 2005; Kuipers et al. 2010) while simultaneously increasing and perhaps doubling energy density (Eaton and Konner 1985; Eaton et al. 1997; Prentice 1998; Rolls 2000; Prentice and Jebb 2003).

1.2.3 Summary and conclusion

Humans in the Palaeolithic enjoyed a wide range of food, in which protein played a larger role than in contemporary Western diets, and there was a much smaller role for carbohydrate, which would also have consisted of more complex carbohydrates than make up the Western diet (Eaton and Konner 1986; Cordain et al. 2000c; Cordain et al. 2005; Konner and Eaton 2010; Kuipers et al. 2010). It is unlikely that early humans could have subsisted on a diet consisting solely of fruits, nuts and vegetables (Jenkins and Kendall 2006). Archaeological evidence suggests that malnutrition was not as prevalent as in later periods (Formicola and Giannecchini 1999; Formicola and Holt 2007). Evidence on the control of human appetite suggests that it may be controlled both by the need to maintain protein intake (Simpson and Raubenheimer 2005, 2007) and by the amount of food eaten (Rolls 2000; Prentice and Jebb 2003; Prentice 2005).

Palaeolithic / H-G diets

Reference	Group	Macronutrient intake (% of energy intake)				P:(C+F) ratio 1:	Protein g d ⁻¹	Carbohydrate g d ⁻¹	Fat g d ⁻¹	Total energy kJ d ⁻¹	Energy density kJ 100g ⁻¹
1	Palaeo / H-G	34	45	21	66	1.9	251	334	71	12439	558
2	Palaeo / H-G	33	46	21	67	2.0					
3	Palaeo / H-G	32	43	25	68	2.1					
4	Palaeo / H-G	37	41	22	63	1.7					
5	H-G	27	31	43	74	2.7					
	midpoint range	19 – 35	22 – 40	28 – 58	65 – 81	1.9 – 4.3					
6	Palaeo E. Africa	27	39.5	34.5	74	2.7					
	midpoint range	25 – 29	39 – 40	30 – 39	71 – 75	2.4 – 3.0					
7	Gambian traditional										490

Contemporary Western diets

Reference	Group	Macronutrient intake (% of energy intake)				P:(C+F) ratio 1:	Protein g d ⁻¹	Carbohydrate g d ⁻¹	Fat g d ⁻¹	Total energy kJ d ⁻¹	Energy density kJ 100g ⁻¹
8	US 1999–2000	14.7	51.9	32.7	84.6	5.8					
9	UK Adults 2001	16.6	48.1	35.4	83.5	5.0	76	239	74	8043	
10	UK Adults 2007–08	17.6	47.3	35.1	82.4	4.7	77	227	71	8190	
7	UK Fast Food										1100
7	UK Supermarket ready meal										800
7	UK female										670
11	US average 1988–94										803

Table 1.1: Comparison of estimates of the macronutrient percentages, energy content and energy density of Palaeolithic human, Hunter-gatherer and contemporary Western diets. References: **1** Eaton and Konner, (1985), **2** Eaton et al., (1988), **3** Eaton, (1992), **4** Eaton et al., (1997), **5** Cordain et al., (2000b), **6** Kuipers et al., (2010), **7** Prentice and Jebb, (2003), **8** Wright et al., (2003), **9** Henderson et al., (2003), **10** Bates et al., (2010), **11** Kant and Graubard, (2005).

1.3 Using *Drosophila melanogaster* to investigate PARs

1.3.1 Suitability of *Drosophila melanogaster* for investigating PARs

Drosophila melanogaster was chosen as an animal to investigate PARs as it offers many advantages for the study of the effects of diet and phenotypic plasticity. It has complex organ systems which although do not exactly parallel those of humans perform similar functions (Ashburner et al. 2005). Its response to diet is similar to that of humans (Baker and Thummel 2007; Lee et al. 2008; Musselman et al. 2011) and it displays phenotypic plasticity in relation to diet (Prasad et al. 2003; Baker and Thummel 2007; Lee et al. 2008; Kolss et al. 2009; Vijendravarma et al. 2010) and other environmental factors (David et al. 1994; David et al. 1997). Plasticity is also variable between populations of *Drosophila*, suggesting that it may have been selected for in response to different environments (Noach et al. 1996). Finally, it is easy to culture in large numbers, with a short generation time and its genetics have been extensively studied (Ashburner et al. 2005; Markow and O'Grady 2005).

1.3.2 Development and metabolism

Drosophila melanogaster Meigen (Insecta; Diptera; Brachycera; Drosophilidae) is an insect that undergoes a complete metamorphosis. Eggs are held within the ovary in arrested meiosis until fertilisation, after which they begin development into an embryo (Ashburner et al. 2005). Among the first cells to differentiate are the pole cells at the posterior end of the embryo, which are the precursors of the gonads (Ashburner et al. 2005). Eggs hatch at around 18 to 22 hours after oviposition, and the three larval instars (L1 – 24 hours, L2 – 24 hours and L3 – 48 hours at a temperature of 25°C) develop over four days, during which the major amount of feeding takes place, before pupation. The larval stages have the greatest influence on adult size, with adult size being related to the final weight of the L3 instar (Prasad and Joshi 2003; Ashburner et al. 2005). The larva reaches a critical size at the beginning of the L3 stage and a pulse of 20-hydroecdysone then commits the larva to pupariation six hours later and a smaller pulse 10 hours after that, two hours before the transition to pupation (White et al. 1997; White et al. 1999; Prasad and Joshi 2003). The first of these pulses causes the development of the adult head and the exoskeleton of the abdomen and the second causes puparium formation and the formation of rudimentary appendages (White et al. 1999). Pupation itself takes around four days. Temperature affects the rate of development of all stages (Prasad and Joshi 2003). After eclosion, females can lay eggs after about 24 hours, with peak

fecundity beginning at about three days (Ashburner et al. 2005). In the wild, life expectancy may be between 1.5 to 6 d, although in the laboratory it can be much longer (Rosewell and Shorrocks 1987).

There are three main areas where lipids are found in *D. melanogaster*: the fat body, the midgut epithelial cells and the oenocytes (Gutierrez et al. 2007). *Drosophila* diets are generally low in lipids, but any present can be taken up by the midgut epithelial cells and transported in the haemolymph to the fat body, where they are stored as intracellular lipid droplets (Canavoso et al. 2001; Gutierrez et al. 2007). Male and female *Drosophila* have similar lipid content when scaled by body weight (Wang et al. 2005).

Although the specific organs involved are different, *Drosophila* lipid metabolism has many parallels with mammal lipid metabolism (Gutierrez et al. 2007). In both, lipids are critical energy stores (Gutierrez et al. 2007), and when needed in response to starvation are released from storage locations (adipocytes in mammals and the fat body in *Drosophila*) and accumulate in specialised cells (hepatocytes in mammals and in oenocytes via the haemolymph in *Drosophila*) where they are broken down (Gutierrez et al. 2007). When energy in food is in excess of requirements, the opposite process occurs; lipids are synthesised into triglycerides in hepatocytes and delivered to adipocytes as lipoproteins (Gutierrez et al. 2007). In *Drosophila*, transport of lipids to the fat body occurs via lipophorins in the haemolymph (Dean et al. 1985; Keeley 1985; Canavoso et al. 2001).

The storage and mobilisation of lipids in *Drosophila* is regulated by TSC/TOR (target of rapamycin) signalling (Zhang et al. 2000; Colombani et al. 2003). This also links with another major function of the fat body, where amino acid levels are monitored and translation of mRNA is regulated by dTOR (Colombani et al. 2003), mutations in which inhibit the growth of larvae, mimicking the effects of starvation and protein deprivation (Zhang et al. 2000). This links nutrient availability to cell growth, cell proliferation (via cyclin E) and development (Zhang et al. 2000). The fat body appears to function as a nutrient sensor, monitoring amino acid levels and directing the overall growth of the organism (Colombani et al. 2003). The growth of vital tissues such as the imaginal disks is partly under the control of this system. When amino acid levels are low, dTOR in the fat body is downregulated, reducing growth in peripheral tissues by a reduction in PI3K (phosphoinositide 3-kinase), a signalling protein which has cellular effects including increased protein synthesis, glucose import and lipid storage (Britton et al. 2002). However in the case of the imaginal disks this signal can be over-ridden by

autocrine production of Dilp2 (*Drosophila* insulin-like peptide) (Colombani et al. 2003). In this way the fat body can direct scarce resources to where they are most needed (Colombani et al. 2003).

Carbohydrate levels in the developing larvae appear to be controlled by a group of seven insulin-producing neurosecretory cells in the protocerebrum (Rulifson et al. 2002). When these cells were genetically ablated the resulting adults were smaller than the wild-type and larval phenotypic effects included developmental delay, retarded growth and raised haemolymph carbohydrate, all of which could be rescued by systemic induction of Dilp2 (Rulifson et al. 2002). Feeding excess carbohydrates to *D. melanogaster* results in a diabetes-like phenotype, an up-regulation of lipogenesis and associated high levels of triglycerides (Musselman et al. 2011).

1.3.3 *Drosophila* fitness parameters

Morphological traits known to be associated with high or low fitness can be used to assess fitness without measuring fecundity. Survival from egg to eclosion is very important, as is the time to eclosion, as completing development quickly allows an individual to mate and produce offspring sooner than its rivals. The time to eclosion is determined during the larval stages by the growth rate of the L3 instar (Bakker 1959; Prasad and Joshi 2003).

In the adult fly, a larger thorax or higher dry weight is associated with increased fitness (Partridge et al. 1987; Bublly et al. 2001; Prasad and Joshi 2003), and there are several morphological traits relating to this that can be measured. Among these are thorax length, wing length and dry weight, all of which show both genetic and phenotypic variability in response to stressful environments (Bublly and Loeschcke 2000; Bublly et al. 2001). Wing size and thorax size are expected to be broadly correlated (David et al. 1994).

Males and females are different sizes in *D. melanogaster*, with the females being consistently larger than the males, although there is no consensus on why this is so (David et al. 2003). The genes controlling the traits should be differentially expressed in each sex (David et al. 2003) and environmental conditions can have different effects on males and females. Larger size in both males and females is associated with higher fecundity (David et al. 2003). In females this is due to more ovarioles in the ovary of larger flies (Boulétreau-Merle et al. 1982), and in males, larger flies have greater mating

success. This may be due to their louder mating songs and greater ability to follow decamping females (Partridge et al. 1987).

1.3.4 Diet and *Drosophila melanogaster*

Surprisingly little is known about the diet composition of wild *Drosophila melanogaster* (Lachaise et al. 1988) although its nutritional needs in the laboratory are well-characterised (Sang 1959; Markow and O'Grady 2005). *Drosophila melanogaster* is a human commensal and will breed on a wide variety of rotting fruit and vegetation, eating both the fruit itself and moulds, especially yeast, growing on it. In its natural East African range it is known to favour bananas, but also mangoes, papaya and guava. These sources vary in their carbohydrate and protein content and decay also alters the macronutrient content of the fruit (Lachaise et al. 1988; Keller 2007; Stephan and Li 2007).

A recent study established a self-selected intake target for *D. melanogaster* adults (Lee et al. 2008). The flies were given a free choice of liquid diets, measured by metering the amount of liquid food dispensed through a capillary tube. The self-selected target was found to be in the ratio of 1P:4C (approximately 30 μ g of protein d⁻¹ to 120 μ g carbohydrate d⁻¹) when carbohydrate was high, although at lower carbohydrate concentrations they overcompensated by eating more of the yeast solution, showing that there is a constraint on the volume of diet consumed regardless of its content (Lee et al. 2008).

By offering a series of no-choice diets of varying P:C ratios (termed a “nutritional rail” in the geometric framework model), it was also established that the flies on the 1P:4C diets had the greatest lifetime egg production (LEP), taken to be a good measure of fitness. The greatest longevity was found in flies on 1P:16C diets and highest egg-laying rate on 1P:2C diets. This may indicate a trade-off of egg-laying against lifespan, with a point in-between being the ratio of P:C at which this balance is most successful, or it may show that a high protein diet has life-shortening effects, for instance caused by organ damage by its breakdown products (Lee et al. 2008).

1.3.5 Epigenetic mechanisms in *Drosophila melanogaster*

The mechanism by which these intergenerational effects are caused is currently not fully understood (Youngson and Whitelaw 2008; Daxinger and Whitelaw 2010). Aside from changes in gene methylation persisting through meiosis (Lillycrop et al. 2005), a

possibility in mammals but not in *Drosophila* is behavioural transmission, for instance the transmission of the “stress phenotype” in rats (Weaver et al. 2004).

In *Drosophila*, epigenetic inheritance (rather than effects) has been confirmed for some phenomena, but these are generally failures of gene regulation which are stably passed on to the next generation causing, for instance, enhanced tumorigenesis (Xing et al. 2007). A possibility in insects is that parental mRNAs are transferred to the developing embryo (Fox and Mousseau 1998) via the nurse cells, which may affect not only that embryo but also the following generation, as the pole cells which will form the gonads are present from an early stage of embryogenesis (Ashburner et al. 2005). Another possibility is by RNAi transmission, which has been shown to occur in *Drosophila* (Daxinger and Whitelaw 2010). In *C. elegans*, injection of RNAi can cause phenotypic change which can be passed down from the offspring to grand-offspring, for up to three generations (Grishok et al. 2000). Protein complexes acting to silence developmental regulation genes have also been shown to persist through meiosis in *Drosophila* (Cavalli and Paro 1998). Another possible mechanism could include the effect of egg provisioning or size (Vijendravarma et al. 2010), in the first or the second offspring generation.

1.3.6 Isofemale lines in *Drosophila melanogaster*

Phenotypic plasticity represents the capacity for one genotype to produce a range of phenotypes in response to environmental variation (David et al. 2005). To investigate the effect of environmental conditions on phenotypes, the same individual animal cannot be used more than once, and clones are not available in the same way that they can be used in plant studies, but isofemale lines can be used (David et al. 2005). These are strains of flies, founded by one female and one male selected at random from a natural population (Hoffmann and Parsons 1988; David et al. 2005). Once selected, their progeny are collected and form a closed population. This isofemale line can be split and subjected to different environments and the effect of different environments on the phenotype measured (Hoffmann and Parsons 1988; David et al. 2005).

The difference in phenotypic trait values between isofemale lines is assumed to be genetic rather than a result of uncontrolled variation between culturing conditions, resulting for instance from variation between culturing vials (Moreteau et al. 1995; David et al. 2005a). Comparison between isofemale lines and isogroups (founded from 10 wild-collected pairs) reveals that when kept under similar conditions there is far more

variation between the mean trait values (wing length) of different isofemale lines than different isogroups, suggesting that the differences are genetic (Moreteau et al. 1995). The relative lack of genetic variation within one line means that observed phenotypic variation within a line, across treatments or generations, can be ascribed to environmental factors (Hoffmann and Parsons 1988).

Although the genetic variation between lines can potentially increase over the number of generations due to genetic drift (David et al. 2005) or conversely to decrease due to selection (Gibert et al. 1998), it has been shown that there was no significant effect on a quantitative trait (wing length) over nine generations of culturing isofemale lines in the laboratory (Gibert et al. 1998). The isofemale line technique represents a way of sampling a population and this method of sampling dictates that each isofemale line (rather than each individual within a line) should be regarded as the replicate in any experimental treatments (Hoffmann and Parsons 1988; David et al. 2005).

Each isofemale line has a high heritability of phenotypic characteristics (Hoffmann and Parsons 1988; Gibert et al. 1998), which is important in the context of these experiments as they are designed to test phenotypic plasticity. Using isofemale lines allows phenotypic variation in response to a treatment to be partitioned into genetic (between lines) and environmental (between treatments) factors (Hoffmann and Parsons 1988). The ability to distinguish between genetic and non-genetic influences on a morphometric trait make the isofemale line technique well-suited to analysing the range of phenotypic morphometric variation in a population (Hoffmann and Parsons 1988; David et al. 1994; David et al. 2005).

1.4 Experimental Aims

1.4.1 Establishing *Drosophila melanogaster* as a model animal to investigate PARs

The main purpose of this thesis is to establish whether *Drosophila melanogaster* shows PARs in response to diet. To model the human transition between a “Palaeolithic” diet, which is assumed to be closer to the diet humans evolved to eat (the intake target) and the contemporary Western diet, two fly diets were designed (Chapter 2). These were not intended to model a starvation versus “ideal” diet, as has been investigated previously over two generations of flies (Prasad et al. 2003; Vijendravarma et al. 2010) as the human diet in the Palaeolithic is not thought to have been inadequate (Formicola and Holt 2007; Konner and Eaton 2010). Instead, the comparison was between a diet, which is thought to be at or near the intake target for macronutrients and one which is significantly richer.

The extent to which *D. melanogaster* shows phenotypic plasticity in relation to the maternal effects was measured, and to investigate the possibility of PARs stretching across multiple generations, three generations’ diets were included (Chapters 3 and 6). This looked for longer term effects from the grandparental generation on the grand-offspring, as is also thought to occur in humans (Kaati et al. 2002). The nature of any phenotypic plasticity was assessed according to the criteria set out in Section 1.1.6 (Fig. 1.4) as not all maternal effects may be adaptive. Although PARs in humans are usually discussed in terms of their effects on health (Gluckman et al. 2009a; Godfrey et al. 2010), the measures used to detect a PAR must relate to fitness. Therefore a range of fitness indicators were used to assess whether PARs had occurred in response to the diet of the parental and grandparental generations.

The responses of the flies to parental and grandparental diets were considered at the population level in the above experiments, using the isofemale line as the replicate, but the responses of individual isofemale lines were also analysed to test for differences in response and plasticity. If individual isofemale lines have different characteristic responses, this could be the basis for selection on phenotypic plasticity (Chapter 7). If isofemale lines vary in their plasticity towards parental and grandparental diets this raises the question of whether there is a cost to plasticity. Thorax length, a morphological characteristic related to fitness and fixed in the larval stages, was tested to discover if increased plasticity for thorax length reduced fitness measured by survival and time to eclosion (Chapter 7). Also of interest was whether, if some lines are more plastic than others, this applies to all characters or whether plasticity is different for each phenotypic

characteristic (Chapter 7). At a different scale, although each phenotypic character was measured separately, they may vary together in ways too subtle to be detected when analysed separately. A Principal Components Analysis was carried out to determine if together the fitness indicators constituted distinct phenotypes in response to parental and grandparental diets (Chapter 7).

Behaviour might also be altered by previous diet experience and might act to reinforce the effects of parental or grandparental diets and any PARs caused by them, if adults exposed to one or other of the diets change their feeding or oviposition preferences. This is tested in Chapter 4.

An experiment has already been carried out which measured the fecundity and lifespan of *Drosophila melanogaster* across a range of adult diets differing in P:C ratio and calorific content, and found strong effects of both (Lee et al. 2008). This was extended to ask whether varying the P:C ratio and calorific value of larval diets altered morphological fitness indicators, survival and time to eclosion in the adults. This showed how fecundity may relate to morphological indicators and also looked at whether varying aspects of the larval diet caused trade-offs in morphological characters.

1.5 Summary and conclusion

PARs in humans are thought to be phenotypic adaptations made in relation to the parental or perinatal environment, aimed at maintaining fitness in a continuation of that environment. If this prediction is wrong, the effects on the health of the offspring may include increased risk of cardiovascular disease, hypertension and diabetes. This was tested in *Drosophila melanogaster*, by modelling the transition between a “Palaeolithic” diet and a modern Western diet, over three generations of flies using diets differing in their protein: carbohydrate content and energy density. Other potential effects of parental and grandparental diets are explored including genotypic differences in response to previous generations’ diets, coordination of response between different phenotypic characters, behavioural changes in response to diet and how the quality and quantity of macronutrients in the larval diet affects the phenotype of adult flies.

Chapter Two

Human diet, *Drosophila melanogaster* diets and general methods

2.1 Introduction

One of the bases of the Predictive Adaptive Response (PAR) hypothesis (Gluckman and Hanson 2004; Gluckman and Hanson 2005; Gluckman et al. 2005b; Gluckman et al. 2005d; Gluckman et al. 2009a; Bateson and Gluckman 2011) is that humans evolved to be adapted to the pre-agricultural environment and diet. A major part of the change between then and the present day is in diet and there are major differences between the composition of the diet of the Palaeolithic and today's diet in the West (Rolls 2000; Konner and Eaton 2010; Kuipers et al. 2010). To model this transition between two dietary environments, two *Drosophila melanogaster* diets were designed and are introduced here. The final section of this chapter contains general methods used throughout Chapters 3 to 7.

2.2 Human evolution and diet

2.2.1 The “Palaeolithic” diet

The exact composition of the Palaeolithic human diet may never be known, and it is not likely that it would have been the same for populations in different parts of the world, as different food sources were available in different regions (Cordain et al. 2000c; Richards et al. 2003; Uliaszek 2003; Eaton 2006). In the human pre-agricultural dietary environment, food resources available to humans were variable but adequate, and this environment would have shaped human food preferences and macronutrient intake targets (Southgate 1991; Cordain et al. 2000c; Eaton and Eaton 2000; Simpson and Raubenheimer 2005; Eaton 2006). It is thought though that protein content was almost twice the Western level, and fat content higher than recommended today, with a much smaller role for carbohydrates. Humans are estimated to have had a much higher daily energy intake than currently recommended, although this was balanced by a higher energy expenditure and on a diet with less energy density than the Western diet (Southgate 1991; Cordain et al. 2000c; Simpson and Raubenheimer 2005; Eaton 2006).

2.2.2 The “Western” diet

Worldwide, one billion people are estimated to be overweight (with a Body Mass Index (BMI) of over 25) (Simpson and Raubenheimer 2005). The causes of this are not clear, as there is an interplay between exercise and the quantity and quality of diet (Simpson and Raubenheimer 2005). The contribution of dietary fat to the western obesity epidemic is not certain (Bray and Popkin 1998; Willett 1998), but increased access to carbohydrates, especially refined carbohydrates is thought to play a part (Stubbs et al. 2001; Simpson and Raubenheimer 2005). Protein intake remains similar (in terms of grams per day) compared to estimated pre-agricultural diets and between populations (Simpson and Raubenheimer 2005). For westerners around the turn of the last century, protein intake for men was 88 g d^{-1} , representing 14% of calorie intake (1500 kJ d^{-1}), with the remaining 9200 kJ d^{-1} coming from carbohydrate and fat combined, a ratio of approximately 1P:6C+F (Simpson and Raubenheimer 2005). According to Simpson and Raubenheimer (2005), the need to maintain protein intake can explain much current obesity, as the mean level of protein relative to carbohydrate or fat in the diet is lower in the modern Western diet than historically. The intake target for protein is maintained though and this results in a compensatory increase in carbohydrate and fat intake to maintain protein intake. The scarcity of readily available carbohydrate and fat in the Palaeolithic diet has resulted in their intake being less regulated than protein intake (Simpson and Raubenheimer 2005). Some corroboration for this comes from the different responses of the appetite-suppressing hormone ghrelin to protein, fat and carbohydrate (Foster-Schubert et al. 2008). In an experiment using human volunteers, shakes containing high protein suppressed appetite for longer and more strongly than those containing fat or carbohydrate (Foster-Schubert et al. 2008).

The Western diet varies between, and within, countries where it is prevalent, but there are some common features which make it distinctly different from the Palaeolithic diet (Fig. 2.1). A common feature of estimates of Western diet is the relatively low protein and high combined carbohydrate and fat components of the diet, compared to estimates of Palaeolithic or hunter-gatherer (H-G) diets. (Fig. 2.1 and Table 1.1). Other differences include a much higher carbohydrate content in the Western diet, the refined nature of the carbohydrates in the diet and lower micronutrient levels (Cordain et al. 2000c; Henderson et al. 2003; Eaton 2006).

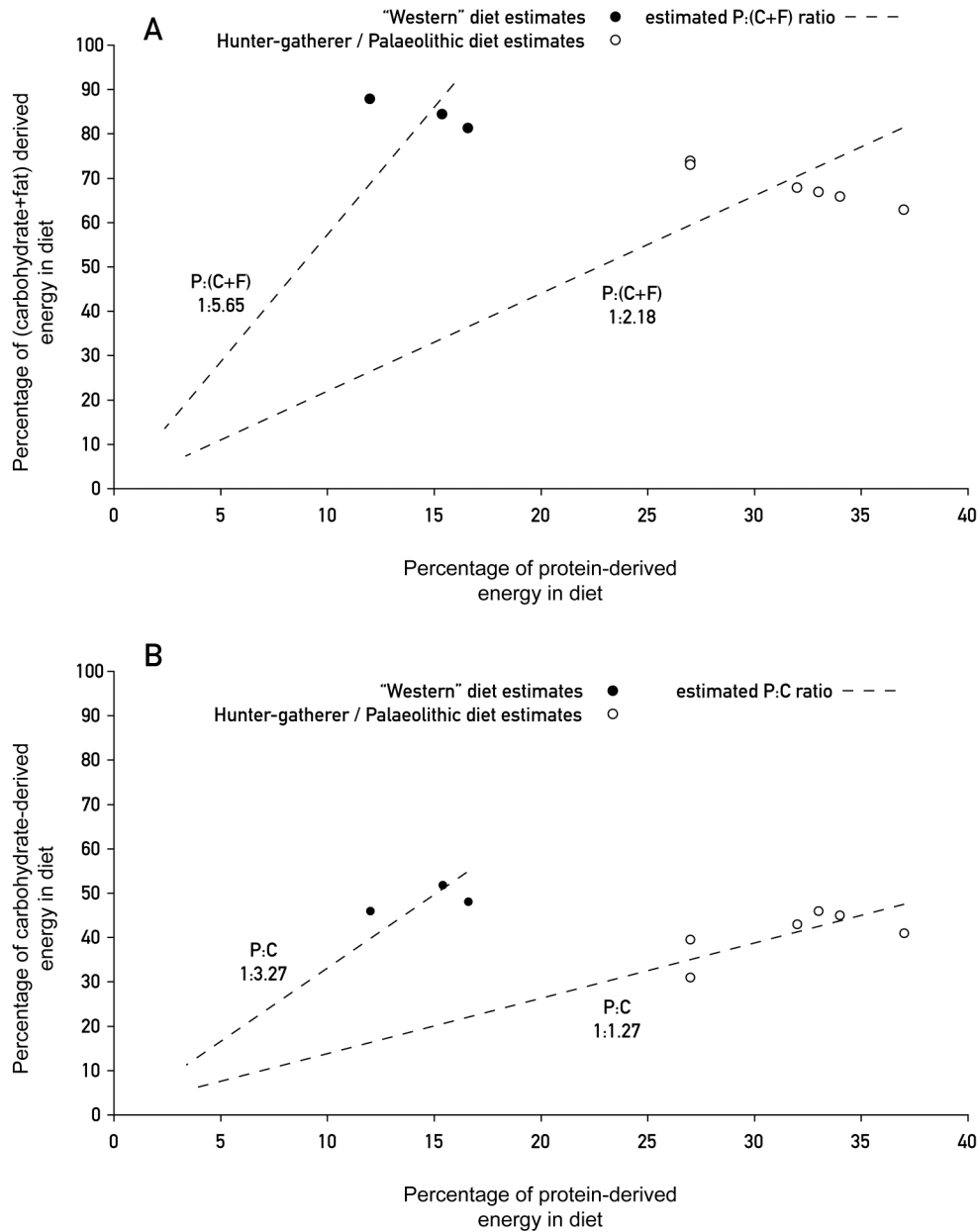


Figure 2.1: Estimated percentage of protein-derived energy in human diets against **A:** combined percentage of carbohydrate- and fat-derived energy; **B:** carbohydrate-derived energy. Closed circles represent modern Western diets, open circles represent Palaeolithic / Hunter-gatherer diets. Dashed lines indicate the estimated ratio of protein-derived energy to energy derived from the other dietary components. P – protein; C – carbohydrate; F – fat. For references, see Table 1.1.

2.2.3 *Drosophila melanogaster* experimental diets

To model the transition between a human pre-agricultural and a modern Western diet, two fly diets were designed which stand in the same relation to each other as the estimated human diets. The diet representing the Palaeolithic / H-G diet (hereafter –

“Palaeolithic”) is based on work done by Lee et al. (2008). Adult *Drosophila melanogaster* were allowed to self-select the macronutrient constituents of their diet from two capillary tubes, one filled with a sucrose solution and the other with a suspension of yeast. The ratio that the flies converged on was 1 protein to 4 carbohydrate (P:C 1:4) (Lee et al. 2008). They also conducted a series of no-choice trials, where the sugar and yeast were combined in the diet at different P:C ratios, and found that flies eating the P:C 1:4 food also had the highest lifetime egg-production, P:C 1:2 flies had the highest egg-laying rate and P:C 1:16 flies were the longest-lived (Lee et al. 2008). The conclusion was that P:C 1:4 was the macronutrient target for *Drosophila melanogaster* as it was self-selected maximised fly fitness (Lee et al. 2008). The “Palaeolithic” diet was based on the diet used to culture the flies, with a slightly increased protein content (2% w vol⁻¹) and with carbohydrate at 8% w vol⁻¹, a ratio of 1:4 P:C (Table 2.1).

This macronutrient ratio was therefore chosen as the P:C ratio to be the *Drosophila* equivalent of the human Palaeolithic diet protein content (Fig. 2.1). To simplify experimental diets, the protein: combined carbohydrate and fat ratio was chosen (1:2.18 P: C+F). The relationship of either of these “Palaeolithic” ratios to their Western equivalents is also almost identical (Fig. 2.1), and using either would give a P:C ratio of 1:10 for the “Western” fly diet, if the “Palaeolithic” ratio was P:C 1:4 (Fig. 2.1).

Using the nutritional geometry approach of Lee et al. (2008) and Simpson and Raubenheimer (1995) implies a decision rule based on protein consumption, but an alternative rule is based on the energy density of diets and implies that appetite is controlled by the amount eaten. As noted in Chapter 1 there is experimental evidence from humans (Prentice 1998; Prentice and Jebb 2003; Bowman and Vinyard 2004) and from flies (Bowman and Vinyard 2004; Mair et al. 2005; Lee et al. 2008) to support both models.

The energy density of the human diet varies greatly between the ‘traditional’ diet and the ‘fast food’ diet (Prentice and Jebb 2003). In the ‘traditional’ diet (from western Gambia), energy density of the typical diet was around 450 kJ 100g⁻¹, while in typical fast foods it was around 1100 kJ 100g⁻¹. In three surveys of the British diet between 1986 to 1997 the average energy density was 670 kJ 100g⁻¹ (Prentice and Jebb 2003). The typical UK diet from 1986-1997 has an energy density of 144% of a contemporary H-G diet from Western Gambia, while typical fast foods have an energy density of 244% of the hunter-gatherer diet (Prentice and Jebb 2003).

Two diets were used in an initial feasibility trial (“100” and “500”, with a P:C ratio of 1:4 and 1:16 respectively). The 500 diet was unsuitable for culturing as it became too wet due to the very high sugar content, but the 100 did not cause culturing problems.

“Western” fly diet

Figure 2.2 below shows the two trial diets (100 and 500), the “Palaeolithic” fly diet (P) and three proposed equivalent Western fly diets. The protein content of the Western diets was kept the same as the P diet as a control. Two of these diets, “UK Energy” and “UK Fast-food” are based on changes in energy density from hunter-gatherer to the UK 1986-1997 diet and the typical energy density of fast food. The UK Fast-food diet, while it would represent one extreme of contemporary diets, is not representative of the average Western diet. The UK Energy diet does model the differences in energy density between traditional and contemporary diet, but not the change in the P:C ratio. Midway between these is the UK Protein diet, which although it is on a slightly lower P:C ratio (1:9) than the P:C 1:10 suggested by comparison with human H-G and Palaeolithic diets, also takes into consideration the differences in energy density (Fig. 2.2, Table 2.1). Therefore the “UK Protein” diet was chosen as the fly equivalent of a human Western (W) diet (Fig 2.2, Table 2.1).

Diet	Yeast	Sucrose	Protein*	Carbohydrate*				Energy Density kJ g ⁻¹
	g L ⁻¹	g L ⁻¹	g L ⁻¹	g L ⁻¹	P:C ratio	Protein % (w vol ⁻¹)	Carb % (w vol ⁻¹)	
P	42.6	70	20	80.2	1:4	2	8	170.42
W	42.6	170	20	180.2	1:9	2	18	340.42

Table 2.1: Macronutrient composition of the two experimental diets, “Palaeolithic” (P) and “Western” (W). * Taking the composition of dried yeast to be 47% protein and 24% carbohydrate (Caballero-Córdoba and Sgarbieri 2000; Yamada and Sgarbieri 2005).

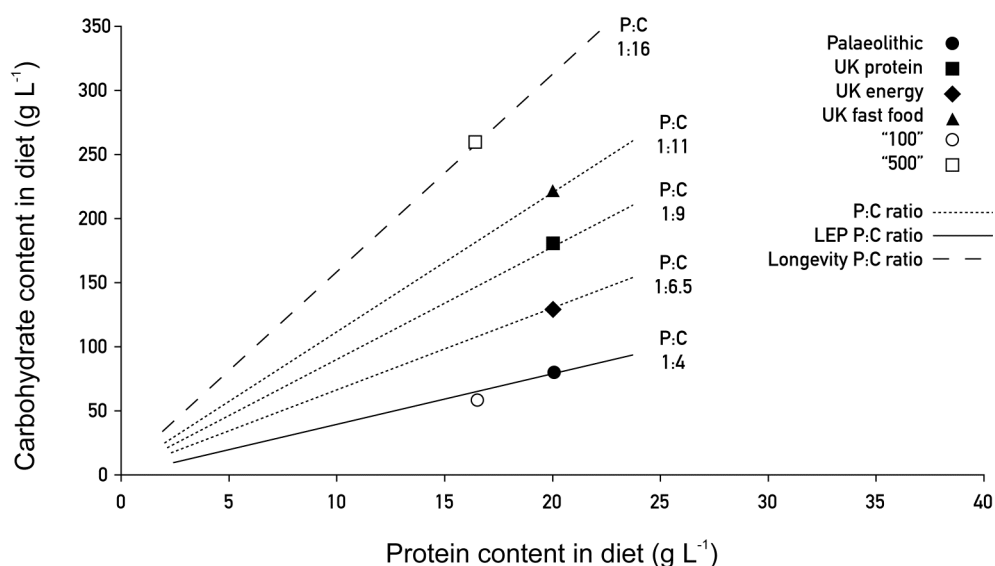


Figure 2.2: Proposed experimental fly diets to model the change between human Palaeolithic / hunter-gatherer diets and modern Western diets. “100” and “500” – initial trial diets; Palaeolithic (P) – diet based on standard culturing diet but with increased protein content to 2% w vol⁻¹; UK Protein – based on the difference between the P:C ratio of human Paleolithic and modern Western diets, and the P diet; UK Energy – based on the difference in energy density between subsistence and modern Western diets; UK Fast-food – based on the difference in energy density between subsistence diets and a typical selection of UK fast-foods. P:C ratio – ratio of protein to carbohydrate; LEP P:C ratio – ratio of P:C associated with highest lifetime egg-production; Longevity P:C ratio – ratio of P:C associated with greatest longevity. For references, see text.

2.3 General methods

2.3.1 Fly stocks

All the experiments carried out throughout this thesis used a culture of 25 isofemale lines derived from a strain of *Drosophila melanogaster* collected in Avigliano, Italy in 2001 (Wertheim et al. 2005) and cultured in the laboratory since then. This “AV” culture has been maintained as a heterogeneous culture in a set of six 300 ml oil bottles (Fisher Scientific Ltd., Loughborough). The flies from the AV culture were mixed between bottles when being collected after eclosion (adult emergence from the pupa) to ensure the culture remained as one population. Both the AV and the isofemale lines were cultured on a three-week cycle at 20°C under a 16:8 hr light: dark regime.

2.3.2 Culturing *Drosophila melanogaster*

The 25 isofemale lines were started from the main AV culture and maintained alongside. Each line was started from one female and one male taken randomly from the AV culture. Eggs laid were allowed to develop and each female's offspring were subsequently treated as a closed culture.

Standard culturing took place on a three-week cycle. Adult flies were placed in a fresh bottle with standard food medium (Table 2.2) and yeast paste in an incubator at 20° C (LMS Ltd., Kent) and allowed to lay eggs. After one day, the adult flies were shaken back into the holding bottles in the 7.5° C incubator (LMS Ltd., Kent). After five days the developing larvae were washed out of each bottle and a pea-sized amount (containing approximately 50 larvae) were placed in a fresh bottle. This ensured that there was little competition between larvae, which if allowed to occur could alter some life-history parameters, for instance size, or time to eclosion (Kraaijeveld and Godfray 1997). The larvae developed, pupated and emerged after approximately 15 days from egg-laying, and were then stored at 7.5° C in fresh bottles with a small piece of Kimwipe (Kimberley-Clark, Wisconsin) to soak up excess moisture. Collection continued until all flies had emerged from all lines, after which bottles containing the adult flies were moved to the 20° C incubator for three days before the cycle started again with egg-laying.

The standard diet (Table 2.2) was based on yeast (*Saccharomyces cerevisiae*) and sugar set in agar with additional nutrients (Kraaijeveld and van der Wel 1994). This was heated in a saucepan and poured into the culture bottles to cool, with the opening secured with a wad of cotton wool. When possible, the diet was prepared a day in advance to allow condensation to clear. The standard diet (Table 2.2) includes live yeast paste on top of the food medium. This could not be included in the experimental diets P and W as it was necessary to control the exact composition of the food ingested by the flies. To control fungus growth in the absence of live yeast, 5 ml l⁻¹ of propionic acid was added to the P and W diets and thoroughly mixed in just before the mixture was poured into the bottles.

Two more sets of the 25 isofemale lines were kept on the experimental diets P and W. The culturing procedure was identical to that of the standard lines except that the time to eclosion was longer necessitating a longer culturing cycle. Typically, an extra week was added to allow all the isofemale lines to emerge.

Ingredient	Amount	Supplier
Water	1 litre	
potassium phosphate	1.5 g	Fisher Scientific
tartaric acid	5 g	Fisher Scientific
ammonium sulphate	2 g	Fisher Scientific
magnesium sulphate	0.5 g	Fisher Scientific
Sugar (sucrose)	50 g	Tate & Lyle
Dried yeast	35 g	Allinsons
Agar	20 g	Acros Organics

Table 2.2 The ingredients used in the standard diet for culturing *Drosophila melanogaster*. Additionally, a thick paste of live yeast was placed in the culturing bottles.

2.3.3 Handling *Drosophila melanogaster*

During the normal culturing cycle, the only technique necessary to collect flies was to shake them down to the bottom of their bottles, remove the cotton wool, invert one bottle over the collecting bottle and shake again. However, for other procedures, such as sexing or transferring to glass specimen vials, it was necessary to anaesthetize and then to move them gently using a soft paintbrush.

Anaesthetising the flies with CO₂ was the preferred method, as detrimental effects have been shown to be no worse than chilling the flies (Barron 2000). Whenever flies were anaesthetised, their exposure to CO₂ was kept as short as possible as this has been shown to decrease fecundity (Perron et al. 1972).

2.3.4 Controlling sucrose and yeast concentration in experimental diets

Adding sugar and yeast to the water for making culture medium tended to expand its volume, but as the agar cooled and set, the volume of the medium reduced to below the starting volume (for 2% w vol⁻¹ of agar). It was not possible to simply add the ingredients, cook the medium and, while still liquid, top it up with water to achieve the final required volume as this changed as the agar set. The quantities and relative proportions of protein and carbohydrate in the diet media needed to be accurately controlled and so the reduction due to 2% w vol⁻¹ agar, and the expansion due to adding yeast and sucrose was measured by making up a series of batches of diet using 100 ml of water for each.

Three samples each were made up at a concentration of 0, 5 and 25 g 100 ml⁻¹ for sucrose and 0, 3.5 and 7 g 100 ml⁻¹ for yeast, spanning the range used for the experimental diets P and W. These were cooked and mixed as described above (Section

2.3.2), poured into a 1000 ml measuring cylinder and allowed to cool. Once the diet had set the volume was recorded.

The final set volume without either yeast or sugar was 93.1 ± 1.7 ml, but as the accuracy of the yeast concentration was more important, the intercept from a regression line ($F_{1,7} = 8.42, p < 0.05$) using the data from the yeast concentration set of measurements, 92.0 ml, was taken to be the set volume of a solution containing 2% w vol⁻¹ of agar (Fig. 2.3.a). The coefficient for the volume increase caused by 1 g of yeast was +0.71 ml g⁻¹ of yeast, and the coefficient for the volume increase caused by adding sucrose was +0.51 ml g⁻¹ of sucrose added (Fig. 2.3 b).

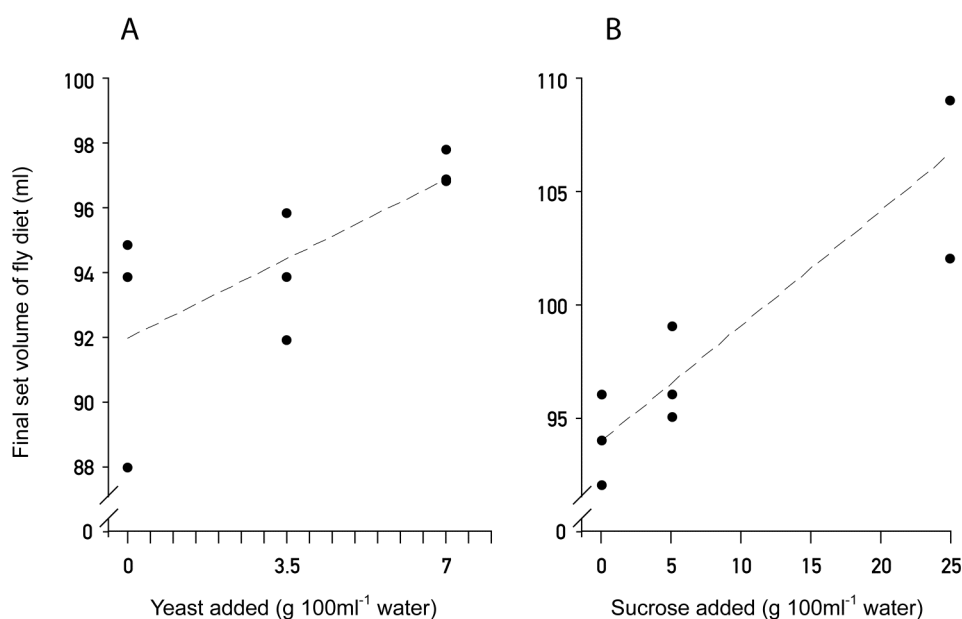


Figure 2.3: The effect on volume of adding **A:** yeast and **B:** sucrose to a solution of 2% w vol⁻¹ of agar, starting with 100 ml of water. Dashed lines indicate regression lines.

The two coefficients for yeast and sucrose and the intercept for the effect of agar were combined into Equation 2.1, which describes the effect of adding yeast and sucrose on the volume of a 2% w vol⁻¹ solution of agar, starting with 1000 ml of water.

$$V = 0.07y + 0.05s + 0.92w$$

Equation 2.1 The effect on final set volume (V, litres) of adding yeast (y, grams), sugar (s, grams) and water (w, ml) to culture media containing 2% w vol⁻¹ of agar.

Once the target yeast and sucrose amounts have been decided on, the amounts (in g) needed for 1000 ml of a fly diet are put into the equation, and an estimated volume calculated. This new volume can then be used to adjust the yeast and sucrose amounts to give the required concentration. The adjusted volumes for the P and W diets are shown below (Table 2.3).

Ingredient	Diet		Supplier
	P	W	
dried yeast	39.5 g	39.7 g	Allinsons
sugar (sucrose)	64.9 g	158.4 g	Tate & Lyle
agar	20 g	20 g	Acros Organics
water	1 litre	1 litre	
potassium phosphate	1.5 g	1.5 g	Fisher Scientific
tartaric acid	5 g	5 g	Fisher Scientific
ammonium sulphate	2 g	2 g	Fisher Scientific
magnesium sulphate	0.5 g	0.5 g	Fisher Scientific
Propionic acid	5 ml	5 ml	Fisher Scientific

Table 2.3 Ingredients used in the P and W experimental diets for *Drosophila melanogaster*. The amounts of yeast and sucrose have been adjusted using Equation 2.1.

2.3.5 Osmolality

The osmolality of the P and W diets were measured with an Advanced Instruments Model 3300 osmometer (AI Inc., Mass.) using freezing-point analysis. Five samples from each diet were measured. The two experimental diets P and W were found to have significantly different osmolality (two sample t-test, $t = 21.76$, 8 d.f. $p < 0.001$). The t-test was carried out using SPSS Statistics 19.0 (SPSS Inc., Chicago).

The Western diet (1754.2 ± 42.1 mOsm kg^{-1} H_2O) had more than twice the mean osmolality of the Palaeolithic (820.6 ± 8.3 mOsm kg^{-1} H_2O) reflecting the greater amount of sucrose present.

2.3.6 Storing fly samples

When samples needed to be stored for later analysis, flies were first anaesthetised with CO_2 and immediately flash-frozen in liquid nitrogen. Samples for morphometric measurements and lipid analysis were stored at -18°C .

Chapter Three

Do parental and grandparental diets influence fitness and health indicators in the offspring?

3.1 Introduction

3.1.1 Predictive adaptive responses and offspring fitness

The Predictive Adaptive Response (PAR) hypothesis states that the phenotype of an organism will be optimised to suit the environmental conditions which it is anticipated that it will encounter in later life (Gluckman et al. 2005c; Gluckman et al. 2005d; Hanson and Gluckman 2005; Gluckman and Hanson 2007; Gluckman et al. 2009b). An example of the conditions in which this might be advantageous is in a variable climate (Saastamoinen et al. 2010). Climate is very variable over geological time scales, but also on the scale of decades and years, due to phenomena including, for instance, sunspot cycles, volcanic eruptions and El Niño events, and the annual cycle of the seasons (Roberts 1998). These fluctuations also change the productivity of ecosystems and the availability of food resources (Roberts 1998). Change may occur too quickly for a population to track genetically by mutation and natural selection, and so an epigenetic mechanism may alter the phenotype to suit the predicted environment (West-Eberhard 2003; Gluckman et al. 2005d). This is thought to operate by sensing the maternal nutritional condition and levels of stress hormones (Gluckman and Hanson 2005) and epigenetically altering the offspring's phenotype in anticipation of the continuation of that environment (Burdge et al. 2007a). Numerical modelling of three phenotypic plasticity strategies (no plasticity; intergenerational; and offspring only) suggests that an induced phenotype persisting across several generations is the most successful strategy in an unpredictable environment (Jablonka et al. 1995; Kuzawa 2005a).

3.1.2 Experimental evidence for PARs

In humans, studies have usually focussed on the adverse health consequences rather than the fitness gained by making a PAR. A long-term epidemiological study from Finland, made possible partly because of the very complete medical records available from that country, showed that babies born small, measured by their ponderal index (a measure similar to the Body Mass Index, but suitable for all heights, calculated using the equation: $P.I. = (\text{mass (kg)})/(\text{height (m)})^3$) remained small in infancy, but then caught up in childhood, and were more likely to suffer from obesity and cardiovascular disease in

middle age (Eriksson et al. 2001). This may be partly due to reduced numbers of muscle cells formed before birth, leading to higher adiposity for a given height and weight (Eriksson et al. 2001; Gluckman et al. 2011).

Maternal energy intake in pregnancy is a very important influence on the likelihood of the offspring developing heart disease in later life. Gale et al. (2006) recorded the energy intake of pregnant women. Nine years later, using ultrasound, they measured the thickness of the carotid artery wall (a marker of developing atherosclerosis) in their children (Gale et al. 2006). They concluded that low maternal energy intake during pregnancy was inversely proportional with the thickness of the artery wall, having taken into account maternal macronutrient proportions in the diet, smoking and BMI before pregnancy (Gale et al. 2006). The weight of the children at nine years old was also a contributory factor, but only when maternal energy intake was low in the last part of pregnancy (Gale et al. 2006). Children whose mothers had a low energy intake during pregnancy and whose current environment allowed them to put on weight had the thickest carotid artery walls. This suggests that poor maternal nutrition may set the child on a different trajectory, advantageous to early survival but not to long-term health in a food-rich environment, compared to children whose mothers had a higher energy intake (Gale et al. 2006; Gluckman et al. 2011).

However, despite these serious health effects when the perinatal and adult environments are mismatched, any disease which does not affect health until after the end of reproductive years cannot be selected against as it does not affect fitness (Williams 1957; Gluckman et al. 2005a). This may change if the current demographic trend of later reproduction continues, with the birth rate for women in their thirties and forties increasing by between 30 and 50% in the United States between 1990 and 2004, while the birth rate for women in their early twenties fell by 11% (Sutton and Mathews 2004). It is not however the disease outcome after middle age which is the target of the PAR, but altered metabolism in earlier life, in anticipation of a lower energetic environment, eventually leading to disease if this prediction is incorrect (Gluckman and Hanson 2004; Gluckman et al. 2005a).

Two studies looked directly at the adaptive nature of the PAR hypothesis, one on male fertility and one on female. The female study related P.I. at birth to body characteristics as an adult, and to the level of the oestrogen estradiol in Polish women. No relationship was found between P.I. at birth and height, weight, B.M.I., fat

percentage, length of menstrual cycle or physical activity as an adult (Jasienska et al. 2006). However, P.I. at birth was correlated with the increased sensitivity of estradiol levels to energetic stress as an adult, as moderate levels of physical activity lowered estradiol in the low P.I. birth group, while low activity left them at the same level as the high P.I. group (Jasienska et al. 2006). This was considered adaptive rather than pathological as it altered the sensitivity to stress rather than induce an overall reduction in response (Jasienska et al. 2006). In males, patients with unexplained low sperm-counts were more likely to be underweight at birth (Francois et al. 1997).

3.1.3 PARs in other mammals

Using mice, Watkins et al. (2008) tested the impact on their offspring of two diets given to pregnant mice. There were three groups in the study, one on 18% protein diet (NPD) from conception to birth, one on 9% protein (LPD) and one group on LPD for 3.5 d, but then the embryos were implanted into mothers on NPD for the remainder of the pregnancy (Watkins et al. 2008). Both LPD groups showed higher blood pressure as adults, and the females had smaller hearts compared to the NPD group. The transferred embryo group were also significantly heavier and displayed more anxiety-related behaviour as adults (Watkins et al. 2008). Watkins et al. (2008) attribute this to the periconceptual induction of a phenotype, which protects against a “poor” nutritional environment, and then subsequently develops in an inappropriately rich nutritional environment. In another experiment, pregnant rats on a protein-restricted diet had offspring with reduced methylation of the PPAR α gene, involved in lipid and carbohydrate homeostasis (Lillicrop et al. 2005). Increased expression of PPAR α could explain hypertension and altered carbohydrate metabolism in animals born to restricted-diet mothers (Lillicrop et al. 2005).

When mouse diets were altered to induce weight gain, obese pregnant mice were less likely to have a successful pregnancy than controls (Igosheva et al. 2010). Their offspring also had increased mitochondrial activity and a higher reactive oxygen species concentration, suggesting that very rich maternal diets also have a detrimental affect on the offspring (Igosheva et al. 2010).

3.1.4 PARs in *Drosophila melanogaster* and other insects

Gilchrist and Huey (2001) tested four different hypotheses regarding thermal maternal adaptive effects using *Drosophila melanogaster*. The first of these was that for each of the three temperatures tested (18, 25 and 29°C), fitness would be maximised when parents and offspring were reared at the same temperature (the “beneficial acclimation hypothesis”, similar to the match-mismatch hypothesis). Secondly, that “cold parents” would have fitter offspring in all thermal environments, due to a compensatory adaptive response to the cold parental environment, which would also increase fitness in other environments. The third hypothesis was “hot parents best”, that is that a good parental environment would give the offspring an advantage in all environments and finally that there was an optimum medium temperature (Gilchrist and Huey 2001). The results indicated that maternal effects were important as well as offspring environment, and that the “hot parents” hypothesis best fitted the data (Gilchrist and Huey 2001). This does not preclude the possibility that an even hotter temperature might lower fitness, and this might mean that the 29°C group were at the optimal temperature. The mismatch hypothesis was not supported (Gilchrist and Huey 2001).

Steigenga and Fischer (2007) reared two groups of the tropical butterfly *Bicyclus anynana* at two different temperatures, 20°C (corresponding to the dry season in the butterflies’ native environment) and 27°C (corresponding to the wet season), for two generations. The eggs laid by the second generation were then divided between the two temperatures, and development time and pupal mass (normally greater at low rearing temperatures) measured as fitness indicators (Steigenga and Fischer 2007).

The results indicated that parental environment was significant, and although not producing as large an effect as rearing temperature in time to pupation, time to eclosion and growth rate, was the most important factor in determining pupal mass (Steigenga and Fischer 2007). Offspring raised at the same temperature as their parents had a similar mass in the two matched groups (20-20 and 27-27), while those raised at different temperatures showed opposite effects, with the 20-27 group being larger than the 27-27 group, and the 27-20 group being substantially smaller than the 20-20 group (Steigenga and Fischer 2007). This produced the surprising result that lower parental temperature resulted in a greater pupal mass across all temperatures. This suggests that the parental 20°C group (poor environment) may have been phenotypically adapted to be more efficient at gaining weight, while the parental 27°C group did not have this “cold parents

better” advantage (Gilchrist and Huey 2001; Fischer et al. 2003; Steigenga and Fischer 2007). Males responded slightly less strongly than females. Egg laying in the offspring generation did show some influence of parental temperature, but this difference declined over time indicating that acclimatisation to the new temperature was taking place (Steigenga and Fischer 2007).

A similar hypothesis to the mismatch, but excluding maternal influence, is that organisms will be best suited as adults to the environment in which they developed, and this was tested for temperature and walking speed in *D. melanogaster* by Gibert et al. (2001). Here the optimal developmental temperature hypothesis was supported as flies that had developed in medium temperatures performed better in all environments (Gibert et al. 2001). This may reflect a difference between developmental and parental effects. Gibert et al. (2001) also tested the effect of temperature on two different populations of *D. melanogaster*, and found that the population from a cooler climate had a peak walking speed at a lower temperature than the African population, though this is likely to be result of genetic adaptation rather than a maternal effect. Similar results were obtained from the seed beetle *Stator limbatus* from two populations from different thermal environments (Stillwell and Fox 2005). In another study using crossed larval and adult stress conditions in the butterfly *Bicyclus anynana*, larval and adult food stress significantly increased the relative thorax size in the adults, while having a negative effect on early-life fecundity and egg size, although only adult food stress had an effect on lifespan (Saastamoinen et al. 2010). The difference in fecundity was not significant however if the butterflies were subsequently flight-stressed. These larval food- and flight-stressed individuals also had a longer lifespan than the flight-stressed individuals that had not been food-stressed as larvae (Saastamoinen et al. 2010). This was interpreted as being a possible adaptation for dispersal ability, using larval food-stress as the cue (Saastamoinen et al. 2010).

Two experiments with *Drosophila melanogaster* used high and low temperature as a factor to examine adaptive phenotypic plasticity (equivalent to a Predictive Adaptive Response), one where the effect of the previous generation’s environment was included in the larval treatment (Nunney and Cheung 1997) and another in which it was tested explicitly (Huey et al. 1995). Nunney and Cheung (1997) raised the parental generation and the larvae at one of two temperatures, 18 and 25°C, and then split these groups as adults into one low and one high temperature group. Early fecundity followed a mismatch pattern, where the females kept at the same temperature as they had been

raised on had higher early fecundity than the equivalent mismatched group. Lifespan followed this same model, but displayed a trade-off where it appeared that the groups, but not individual females, with high early fecundity had a shorter lifespan (Nunney and Cheung 1997). Neither temperature raised early fecundity on its own, but only in interaction with the larval environment, and so they concluded that the observed phenotypic plasticity was adaptive (Nunney and Cheung 1997).

Huey et al. (1995) conducted a similar experiment, except that they used male and female development temperatures as factors as well as offspring development temperature and laying temperature. They found that by far the largest effect came from the laying temperature, but that the male development temperature also had a significant effect on offspring fecundity, as females whose fathers were raised at the lower 18°C temperature laid 8% more eggs than those whose fathers were raised at 25°C (Huey et al. 1995). Although the size of the fly (thorax or dry-weight) is regarded as being a marker of fecundity, both of these studies note that there appeared to be little effect of the larger size when flies developed at low temperatures (Huey et al. 1995; Nunney and Cheung 1997).

Two studies have examined the PAR hypothesis in *Drosophila* in relation to diet, although both of these used a standard *Drosophila* diet as the “rich” diet and a reduced energy (Vijendravarma et al. 2010) or protein (Prasad et al. 2003) diet as the “poor” diet, which may mimic a borderline famine situation rather than two different but adequate diets. Prasad et al. (2003) investigated the effect of mismatched parental and offspring nutrition on egg weight, survival and offspring dry weight, with “rich” and “poor” food differing in the proportion of yeast used, although this was possibly compensated for by an increase in flour in the poor diet, making it difficult to estimate the relative protein and carbohydrate content. Parental diet was significant in that larval survivorship was higher in the offspring of poor-diet mothers raised on rich food, suggesting that the “cold parents best” hypothesis might extend to diet, although there was no corresponding drop in survivorship in the offspring of the rich diet mothers raised on poor food (Prasad et al. 2003).

Vijendravarma et al. (2010) used a standard fly diet, and as a “Poor” diet, a diluted version with the same proportion of macronutrient ingredients. As the majority of feeding is in the larval stage, adults of the parental generation were taken off the test diets and placed on a standard diet plus live yeast prior to laying. However, Lee et al.

(2008) have reported significant changes in fecundity and longevity patterns solely as a result of adult diet, so this may have masked the parental effects on egg size and eclosion time in the offspring. One hypothesis was that a poor larval diet may cause an increase in egg size compared to a standard diet as a trade off between quality and quantity of eggs, and this was supported as eggs laid by flies on the larval poor diet were significantly heavier than those from the standard diet flies (Vijendravarma et al. 2010). There were no differences in time spent as a pupa in the offspring generation, but the larval stage was significantly affected by the parental diet. Offspring of poor diet parents reached pupation faster on both poor and standard diet, and offspring of rich diet parents were slower on both offspring diets, despite the standard offspring diet having a shorter mean time to pupation across all groups (Vijendravarma et al. 2010).

As with the significant adaptive parental effects caused by temperature, when one of two test diets is less suitable for development, the effects, as a parental diet or as an offspring diet, are reversed. This appears to be an adaptive compensatory effect caused by poor parental diet or other environmental condition (Vijendravarma et al. 2010). When the effect occurs, it is the more unsuitable of the two diets or environments, which induces an adaptive response in the next generation. Whether this is a general effect of any type of adverse condition is not certain, but the above examples show that in *Drosophila*, temperature and diet do seem to be capable of eliciting a response to parental conditions, which is both predictive and adaptive.

3.1.5 Experimental aims

Effects of diet

Previous studies on PARs have concentrated on using a low-calorie or low-protein diet as one of the diets. These experiments are designed to test the PAR hypothesis by modelling the dietary transition that would be experienced by humans moving between a hunter-gatherer diet and a modern Western diet and so in these experiments the contrast is between one diet which is thought to be close to ideal, and one with excessive energy in the form of carbohydrate (see Chapter 2 for details of the diets).

The two diets were not designed to model a “normal” against “starvation” situation, but rather an approximation of an ideal diet and an energy dense diet. The context of this is that of the transition between a traditional (not an inadequate) human diet and a diet typical of developed countries (“Western” diet), which is suspected to be a

cause of the lifestyle diseases prevalent in developed countries and especially in those who have migrated, either to those countries or to their diets (Iseki et al. 2002; Simpson et al. 2003; Gluckman and Hanson 2005; Willcox et al. 2007; Gluckman et al. 2009b).

The main features of the diets are that the P (Palaeolithic) diet is close to the protein: carbohydrate ratio self-selected by *Drosophila* (P:C 1:4), which produced the maximum lifetime egg-production in an experiment using a nutritional geometry approach to test the effects of macronutrients on *Drosophila* adults (Lee et al. 2008). The P diet in this experiment represents a human baseline or “traditional” diet, and so a Western (W) diet was developed, with double the energy density of the P diet and a lower P:C ratio (1:9), reflecting a compromise between estimates of the difference between traditional or ancestral human diets and the Western diet (Eaton 1990; Bunn and Ezzo 1993; Popkin 1994; Eaton et al. 1997; Cordain et al. 2000b; Rolls 2000; Prentice and Jebb 2003; Wright et al. 2003; Cordain et al. 2005; Bates et al. 2010; Kuipers et al. 2010). To control for protein content in the P and W diets, the amount of protein was identical and only carbohydrate (sucrose) was altered.

Grandparental and parental generations

The effects of PARs are hypothesised to possibly extend over several generations (Gluckman and Hanson 2005; Godfrey et al. 2010) and so in contrast to the two-generation studies outlined above, the grandparental generation will also be included in these experiments and the diets arranged to give all eight combinations of P or W diets across the three generations. The point at which the diets were swapped was when the eggs were laid, so that the maternal environment influence is confined to effects during embryogenesis and egg-provisioning.

Detecting a PAR and other forms of parental diet effects

The flies’ response to the diets was quantified by measures of development and fitness indicators. These included time from laying to eclosion, survival to eclosion, thorax size, dry weight and total lipid content of the flies. If the flies are influenced by parental or grandparental diet this should be apparent either as a simple additive effect (e.g. being more similar to a grandparental treatment than an offspring treatment than could be expected by chance), or as an interaction, which may result in the offspring phenotype having a higher or lower value than that of either of the parental fitness indicators.

PARs are a subset of maternal effects, directed at maintaining fitness, but may have the effect of producing adverse health consequences as a result (Gluckman et al. 2005d; Ellison and Jasienska 2007; Godfrey et al. 2010). They may be induced by an unfavourable environment, whether in diet, temperature or predation pressure, at either extreme of too poor or too rich (Gluckman et al. 2005d; Ellison and Jasienska 2007; Monaghan 2008). An adaptive effect might be impossible to detect if the prediction is accurate, as the compensatory adaptive effect may mask the unfavourable fitness effects of the predicted environment (Gluckman et al. 2005d; Ellison and Jasienska 2007; Monaghan 2008). Therefore it is only possible to detect a PAR when the maternal environment does not match that of the adult offspring, either poor maternal followed by rich offspring or vice versa (Ellison and Jasienska 2007).

The effects of a PAR need not be in the form of a mismatch (see model 2a, Fig. 1.4), as the “cold parents best” response (e.g. model 11, Fig. 1.4), or compensatory effect from a poor environment, is also predictive and adaptive (Gilchrist and Huey 2001). There are other types of maternal effects, which may not be adaptive but are a consequence of a failure to overcome poor circumstances, where offspring from poor parental backgrounds are worse off than those from rich backgrounds in any environment – the “silver spoon” effect (Ellison and Jasienska 2007; Monaghan 2008). Alternatively, the adaptive effects of the parental diet might specifically be targeted at the same offspring diet and so any other offspring diet will result in lowered fitness – the mismatch or beneficial acclimation effect (Ellison and Jasienska 2007; Monaghan 2008; Godfrey et al. 2010). In practice more than one of these may occur in response to the same circumstances, and the effect sizes of parental and offspring environment may not be symmetrical (Monaghan 2008).

The fitness effects of a PAR might be different to the health effects. The related “mismatch” concept (Gluckman and Hanson 2004; Gluckman and Hanson 2005; Godfrey et al. 2010) describes the effects on health of an inaccurate prediction. The effect of the same conditions on fitness need not be the same, as the adverse health effects are hypothesised to be produced as a consequence of the attempt to maintain fitness (Gluckman and Hanson 2004; Monaghan 2008; Godfrey et al. 2010), especially if the predicted environment is not the same as the actual environment. When measuring fitness as a response variable, the consequences for health may not always be apparent.

Hypotheses

The aim of these experiments is to test whether *Drosophila melanogaster* shows significant Predictive Adaptive Responses (PARs) to parental or grandparental diets, in a situation set up to model the human dietary transition between an “ancestral” diet and the modern diet in the West, in the same way as is hypothesised to lead to human metabolic and cardiovascular disease (Gluckman and Hanson 2004; Gluckman et al. 2005d). The responses to the diets will be measured using the fitness indicators (survival, time to eclosion, thorax size, wing size and dry weight, lipid content and number of ommatidia) discussed in Chapter 1 and the two diets P and W discussed in Chapter 2. Although measured at the same time, ommatidia number will be analysed in Chapter 6.

The null hypothesis for all of the response variables in all statistical tests was that neither sex, offspring diet, parental diet, grandparental diet, nor any interaction of diets would influence fitness indicators. Where a significant effect of previous diet is found, the direction of its effect and the nature of its interaction with the offspring environment must therefore be examined carefully (Gilchrist and Huey 2001; Ellison and Jasienska 2007; Monaghan 2008), as not all significant main effects or interactions between two (or three) generations’ diets can be considered as showing any evidence of adaptation. Any evidence for adaptation will come from the responses to the mismatched conditions (Ellison and Jasienska 2007) and these must show significant effects not reasonably attributable to the offspring diet, and adaptive, by comparison with the matched condition. The criteria set out in Chapter 1 (section 1.1.6) will be used to distinguish adaptive effects from non-adaptive.

3.2 Methods

The purpose of these experiments was to test for Predictive Adaptive Responses by examining the effect of previous generations' diets on the offspring generation. Two sets of 25 isofemale lines of *D. melanogaster* were cultured on one of the two experimental diets as described in Chapter 2 (differing in protein:carbohydrate ratio and energy density) for six generations to control for any possible parental effects of switching diet. This method has been used before when investigating thorax and wing area changes caused by switching between high and low temperatures (Partridge et al. 1994).

In the sixth generation, one set of adults were allowed to lay eggs on their own diet while the other set laid eggs on the other diet. These were allowed to develop into adult flies, and then these groups laid eggs on either their own or a different diet, giving eight diet combinations over three generations (Fig. 3.1). All measurements and data on survival and time to eclosion were obtained from the offspring generation in the experiment.

3.2.1 Pre-experiment culturing

The isofemale lines of *D. melanogaster* were derived from the AV strain, collected in Avigliano, Italy in 2001 (Wertheim et al. 2005). This has been maintained as a large heterogeneous culture over several years, from which single unmated females (with one male each) were selected and bred into 25 isofemale lines.

The 25 lines were maintained on two separate diets: "Palaeolithic" (P) with a protein:carbohydrate (P:C) ratio of 1:4 and an energy density of 170.42 kJ l^{-1} , and "Western" (W) with a P:C ratio of 1:9 and an energy density of 340.42 kJ l^{-1} , twice that of P (see Chapter 2 for further details). The P and W isofemale lines had been cultured on their diets for six generations at the start of the experiment, to ensure that any effect of a mismatch between the standard culturing diet and the experimental diets was minimised. Although the standard diet isofemale lines had been cultured on a three-week cycle, when placed on the two experimental diets, the isofemale lines maintained on the W diet took longer to develop than those maintained on the P diet, taking four weeks for all isofemale lines to go through a reproductive cycle. Therefore the length of the breeding cycle was set by the slowest of the isofemale lines, ensuring that all lines were ready to lay eggs in synchrony at the start of the mismatch experiment. The 25 starting isofemale lines were numbered 1, 2, 3, 4, 5, 7, 8, 9, 10, 11, 12, 16, 17, 18, 19, 20, 22, 25, 28, 29, 31,

32, 33, 35. One line from each of the P and W cultures was lost; W16 by a low rate of egg-laying resulting in no offspring after generation four, and P22 due to an unidentified infection. However, lines W22 and P16 were included in the experiment although they had no counterparts in the other group.

3.2.2 Experimental set-up

The 23 pairs of isofemale lines on diets P and W and the two unmatched lines (P16 and W22) were placed in an incubator (LMS Ltd., Kent) at 20°C, with a light: dark cycle of 16:8, with lights on at 8 am (hereafter referred to as the 20°C incubator). After 3 d, 2 groups of approximately 40 flies, male and female, from each line / diet combination were removed under light CO₂ anaesthetic and placed in an upturned tricorner beaker (Fisher Scientific, Loughborough). An inverted 55 mm Petri dish (Fisher Scientific, Loughborough) containing one of the diets P or W was secured on top with masking tape. Once the flies had recovered from the CO₂, the laying chamber was placed in the 20°C incubator.

After 24 h, the flies were removed from the laying chambers and using a scalpel, 25 eggs were cut from the diets under an Olympus SZX7 dissecting microscope (Olympus Imaging and Audio Ltd., Southend-on-Sea), and placed in one of eight 75 mm x 25 mm soda glass specimen tubes (Fisher Scientific, Loughborough) containing 7 ml of the same diet as the isofemale lines had previously experienced. The tube was secured using a polyfoam bung (Fisher Scientific, Loughborough) and placed in one of two metal racks in the 20° C incubator. Each rack was rotated through 90° daily, and alternated between two shelves in the incubator.

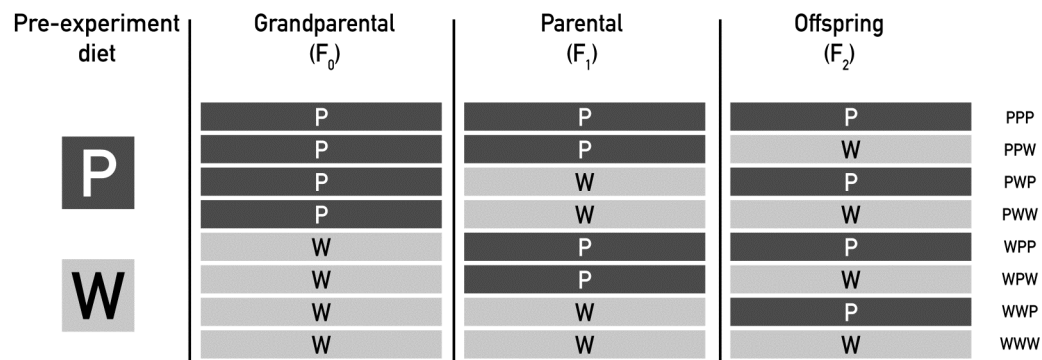


Figure 3.1: Design of multigenerational diet experiment. ‘P’ is the low carbohydrate “Palaeolithic” diet, ‘W’ is the high-carbohydrate “Western” diet. Each solid block represents 25 flies from each of the isofemale lines, in two vials each.

The vials were checked beginning at 10 am daily after Day 10 for emerging flies. When flies had emerged they were sexed and counted under CO₂ using a dissecting microscope, before transfer to a “holding” specimen tube in an incubator at 8°C (LMS Ltd.) under 16:8 hours light: dark (hereafter the 8°C incubator). Three days after the last recorded emergence from a set of A and B tubes (of one isofemale line and diet combination), the two tubes containing adult flies of the grandparental generation were transferred to the 20°C incubator for 3 d, in readiness for egg laying on the fourth day, on the next diet in the sequence (Fig. 3.1).

After 24 hours of egg laying, 25 eggs were transferred to a tube (containing the same diet as the eggs were laid on) for development. Occasionally flies laid less than 25 eggs, and so all available eggs were transferred. The number transferred was noted. After these eggs had developed and adult flies eclosed, the procedure was repeated for the emerged F₁ flies, with a further switch in diet to give the final eight combinations of PPP, PPW, PWP, PWW, WWW, WWP, WPW and WPP (grandparental; parental; offspring), kept in the 20°C incubator. The third generation were counted and transferred on eclosion to the 8°C incubator until all had eclosed, then placed in the 20°C incubator. After 3 d, they were flash frozen in liquid nitrogen, and flies from one set of tubes were kept at -18°C for size and lipid content measurements, while the other set were kept at -80°C for future gene-expression analysis.

3.2.3 Measuring response variables

All responses were measured on the adults of the offspring generation.

Survival

As the number of eggs placed in each vial was known, the proportion surviving was calculated from the number of flies emerging from eclosion, relative to the number of eggs in the vial.

Time to eclosion

Vials were checked daily at 10 am from day 10 after laying for any eclosed flies. Any eclosed flies were sexed under CO₂, counted and transferred to the holding vials at 8°C.

Thorax length, wing length, eye size and ommatidia number

Five males and five females, if available, were selected randomly from each frozen (-18°C) sample and measured using a wide-field microscope (Nikon Eclipse E400) with camera lucida (Nikon Y-1DT) and cold light source (Schott KL1500), with measurements being calibrated with a 1mm stage graticule (Pyser-SGI, Kent). Tracings were made using the camera lucida and subsequently converted to measurements in millimetres, calibrated by the tracings taken from the stage graticule at the same time. Thorax, wing length and eye size were measured using a 10x objective and ommatidia with a 20x objective. Eye size and ommatidia measurements are analysed in Chapter 6.

The thorax was measured from the anterior end of the first thoracic segment to the posterior tip of the scutellum, measured from the side. Wings were measured without first removing them from the fly, along the longitudinal vein L3 from the edge of the wing to where L3 meets the L1 vein.

Dry weight and lipid content

After thorax, wing and eye measurements, samples were transferred to 2 ml glass vials (Fisher Scientific) and, in a procedure similar to that used by Warbrick-Smith et al. (2006) dried for 24 h in an oven (N6C, Agar Scientific, Kent) at 60° C. They were then weighed five at a time using a Sartorius balance capable of weighing 10µg, and soaked in diethyl ether (Fisher Scientific) for 24 h to dissolve their lipids. Ether was changed once

during this procedure and the vials were kept on a rotator at 4 r.p.m. The ether was then pipetted off, and any remainder allowed to evaporate for another 24 hours. Once no ether remained, the samples were then re-dried for 1 hour at 60°C before being re-weighed to obtain the lipid-free dry weight (Warbrick-Smith et al. 2006).

3.2.4 Statistical analysis

Response variables were measured on individual flies, but the mean from each treatment, isofemale line and sex combination was used as the replicate in the statistical analysis. All response variables except for lipid proportion and survival were tested for a normal distribution using a Kolmogorov-Smirnov test, and equality of variance between sex and diet groups using Levene's test in IBM SPSS Statistics 19 (SPSS Inc, Chicago). Thorax length and wing length were no different from a normal distribution at. Proportion data do not follow a normal distribution as they are bounded by 0 and 1 (Quinn and Keough 2002; Crawley 2007). Therefore the lipid content proportion data were first transformed using the arcsine of the square root (Crawley 2007). After this transformation the data conformed to a normal distribution, confirmed by a Kolmogorov-Smirnov test ($p > 0.05$) in IBM SPSS 19 (SPSS Inc., Chicago). Survival data were treated as a two-vector response variable in a generalised linear model with binomial errors in R 2.8.1 (R Foundation for Statistical Computing, Vienna, Austria). This approach allows the numbers of dead and surviving flies to be taken into account, rather than just the proportion surviving, and does not require transformation of the original data (Crawley 2007). The data for time to eclosion and dry weight showed variance increasing with the mean, and so were transformed by taking the base 10 log of the mean (Quinn and Keough 2002; Crawley 2007).

The data were then analysed using a Generalized Linear Model in R 2.1.8 (R Development Core Team 2008), using a model building approach where factors or interactions were removed from the full model if their removal did not significantly increase the residual variance. Terms were removed from each full model in the order of highest-order interaction and least significant first, assessed by an ANOVA procedure on each model. The effect of removing a term was assessed by comparing the total deviance explained by the models with the term included and without, using an analysis of variance and a Chi-squared test. If the change in deviance was not significant, the term was left out, or if significant it was retained. Once the minimal adequate model was

arrived at, the significance of each term was then assessed by building the model up, using the day of egg laying and isofemale line in all models (and for lipid and weight measurements, batch process date), and then sequentially adding the other factors in the minimal adequate model in the order: sex, offspring diet, parental diet and grandparental diet. Interactions between factors were added only after the factors themselves had been added. Isofemale line represents the genetic variability in this model, with the 25 isofemale lines being a randomly-selected sample from the original AV population (see Chapter 1, Section 1.3.5).

3.3 Results

3.3.1 Survival

The survival of a fly from egg to eclosion is fundamental to its prospects of leaving offspring in the next generation. The effect of different combinations of grandparental, parental and offspring diets was tested and the null hypothesis tested was that no diet or combination of diets had an effect on survival, and then if any were significant, the data were examined to determine the nature of the influence of previous diets, as described in Chapter 1, Section 1.1.6.

Overall, 7124 eggs out of 9003 laid by all isofemale lines in all diet combinations (78.4%) at the start of the offspring generation survived to eclosion (Fig 3.2). The offspring diet had a significant effect on survival (change in deviance 22.9, 1 d.f., $p < 0.0001$, Table 3.1), with a higher percentage surviving on the W diet ($78.8 \pm 1.7\%$) than the P diet ($75.2 \pm 1.7\%$), and a significant effect of parental diet (change in deviance 8.0, 1 d.f., $p < 0.01$, Fig 3.3), but no effect of grandparental diet as a main effect (change in deviance 0.01, 1 d.f., $p = 0.91$).

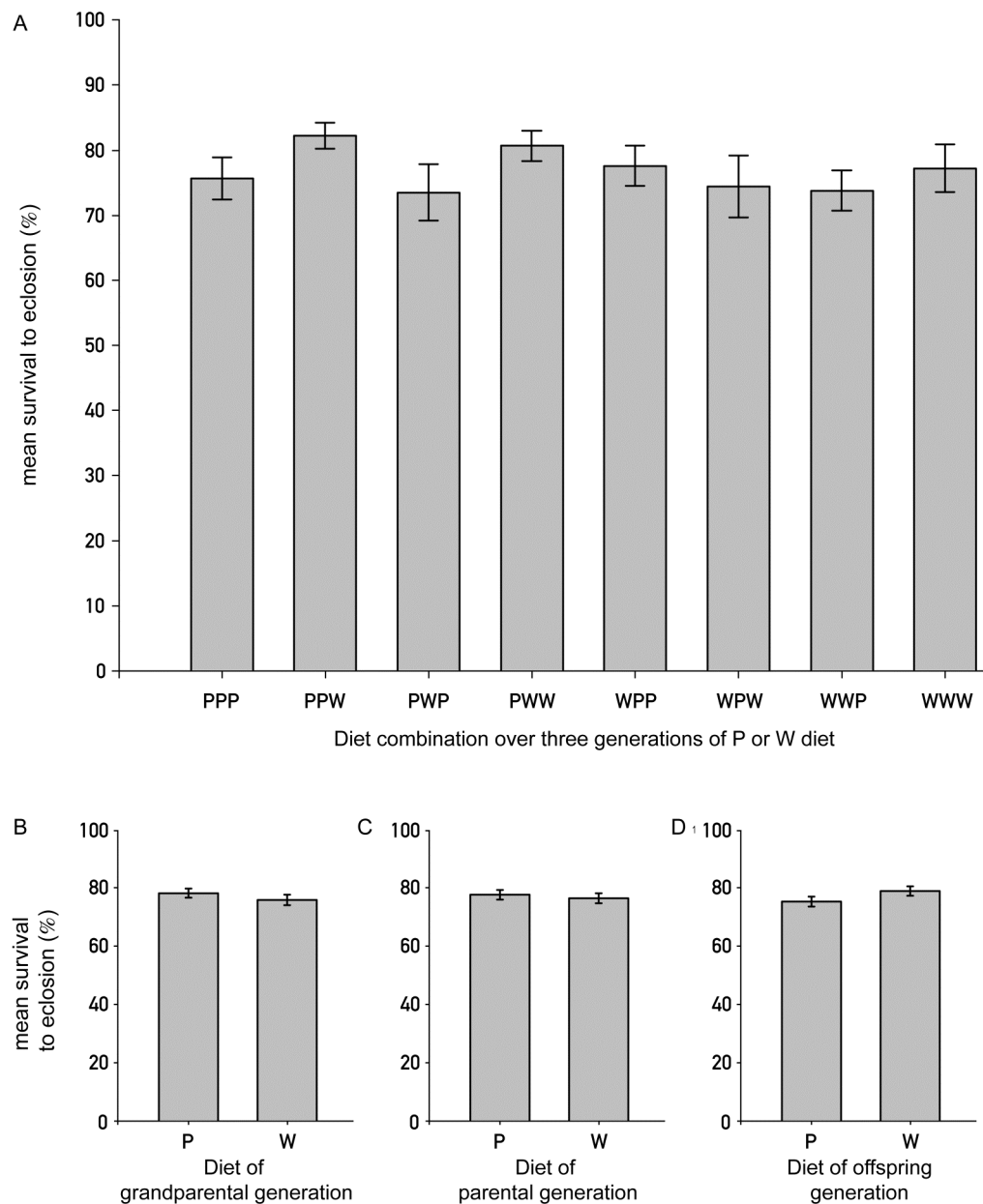


Figure 3.2 Mean percentage of eggs surviving to eclosion, in the third generation of a combination of P or W diets. **A:** each histogram bar represents the mean percentage of eggs surviving to eclosion from 25 isofemale lines with differing diet combinations over three generations. The categories on the x-axis show the combination of diets (P or W) over three generations, with grandparental diet first, then parental diet and offspring diet. For example, PPW indicates P grandparental diet, P parental diet and W offspring diet. **B:** Mean percentage of eggs surviving to eclosion, grouped by the grandparental diet. **C:** Mean percentage of eggs surviving to eclosion, grouped by the parental diet. **D:** Mean percentage of eggs surviving to eclosion, grouped by the offspring diet. All error bars represent ± 1 standard error of the mean.

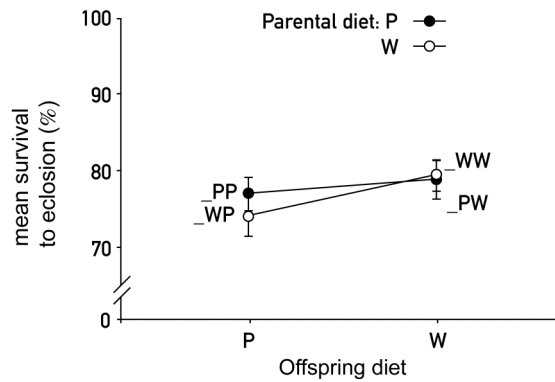


Figure 3.3: Mean survival to eclosion by parental diet and offspring diet (x-axis). Labels indicate the parental and offspring diets, and underscore indicates combined figures for grandparental P and W diets. All error bars are ± 1 standard error of the mean.

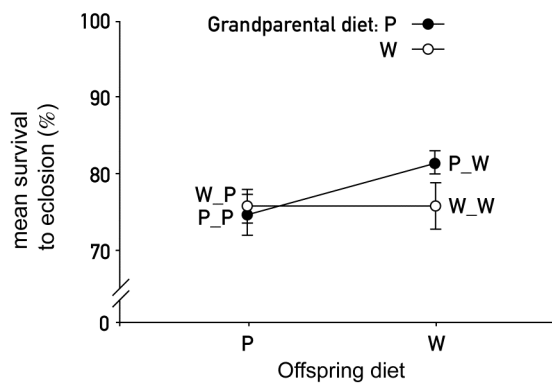


Figure 3.4: Mean survival to eclosion by grandparental diet and offspring diet (x-axis). Labels indicate the grandparental and offspring diets, and underscore indicates combined figures for parental P and W diets. All error bars are ± 1 standard error of the mean.

The only significant interaction between the diets of different generations was between the grandparental diet and the offspring diet (change in deviance 8.4, 1 d.f., $p < 0.01$). Flies whose grandparental diet was P had higher survival when their offspring diet was W (Fig 3.4) than when raised on the P diet. All other combinations of grandparental and offspring diets had very similar percentages of eggs surviving, including the opposite mismatch group W_P, which did not show higher or lower survival than either P_P or W_W. Different isofemale lines had different survival rates across all diet combinations (change in deviance 245.04, 24 d.f., $p < 0.0001$). This will be analysed more fully, along with all other isofemale line interactions for all the response variables in this chapter, in Chapter 7.

Minimal adequate model:
survival is controlled by:

Factor	Res. dev.	– dev	Res. d.f.	– d.f.	<i>p</i>	
Null	1007.6		180			
Day	902.1	105.5	170	10	< 0.0001	***
Iso	657.1	245.0	146	24	< 0.0001	***
Offspring	634.2	22.9	145	1	< 0.0001	***
Parental	626.2	8.0	144	1	< 0.01	**
Grandparental	626.2	0.0	143	1	0.91	
Offspring: Grandparental	617.8	8.4	142	1	< 0.01	**

Table 3.1 The minimal adequate model and analysis of factors influencing survival to eclosion. The minimal adequate model was arrived at by removing non-significant factors and interactions from the full model. The significance of each factor was then assessed by adding factors in turn to the model (Day + Iso) and assessing the reduction (– dev) in residual deviance (Res. dev.). Key to factors: Day – date of egg laying; Iso – isofemale line; Sex – sex of fly; Offspring - offspring diet; Parental – parental diet; Grandparental - grandparental diet.

3.3.2 Time to eclosion

The time taken between egg laying and eclosion is an important fitness indicator in flies, reflecting the speed of development and allowing faster-eclosing flies to produce offspring before slower-eclosing flies. To test the relative influence of the offspring diet, parental diet and grandparental diet, 25 isofemale lines were swapped each generation between two diets (P and W), differing in carbohydrate content (and so also in energy density and Protein: Carbohydrate ratio). After three generations this produced eight different combinations of grandparental, parental and offspring diet, PPP, PPW, PWP, PWW, WPP, WPW, WWP and WWW. Flies from this offspring generation were counted daily as they eclosed.

The null hypothesis was that no diet from any generation would influence eclosion time, nor would any of the interactions between generations' diets, and then if any were significant, the data were examined to determine the nature of the influence of previous diets, as described in Chapter 1. A second null hypothesis was that sex, or its interaction with any generation's diet, would not influence time to eclosion.

The largest influence of diet on time to eclosion was the offspring diet. Flies on the P diet emerged significantly faster (change in deviance 2.8295, 1 d.f., $p < 0.0001$) than those developing on the W diet for both sexes, a difference of 1.63 d for females and 1.46 d for males (Fig 3.5 and Fig. 3.6). Sex was a significant influence on eclosion time,

with females being faster to eclose overall by a mean of 0.32 d (change in deviance 0.1103, 1 d.f., $p < 0.01$).

The parental diet also influenced eclosion time (change in deviance 0.0104, 1 d.f., $p < 0.05$) in the same direction as offspring diet (Fig. 3.7a), but the grandparental diet significantly altered eclosion time in the opposite direction to the offspring diet (change in deviance 0.1196, 1 d.f., $p < 0.0001$). Flies whose grandparental diet was P took 0.24 d longer for females and 0.33 d for males to eclose, than grandparental diet W flies (Fig 3.7b and Table 3.2).

The effect of grandparental diet was slightly but significantly stronger when the offspring diet was W than when it was P (change in deviance 0.0042, 1 d.f. $p < 0.05$, Fig. 3.7c), and there was also a significant interaction of the effects of all three generations' diets (change in deviance 0.0122, 1 d.f., $p < 0.05$, Fig. 3.7d).

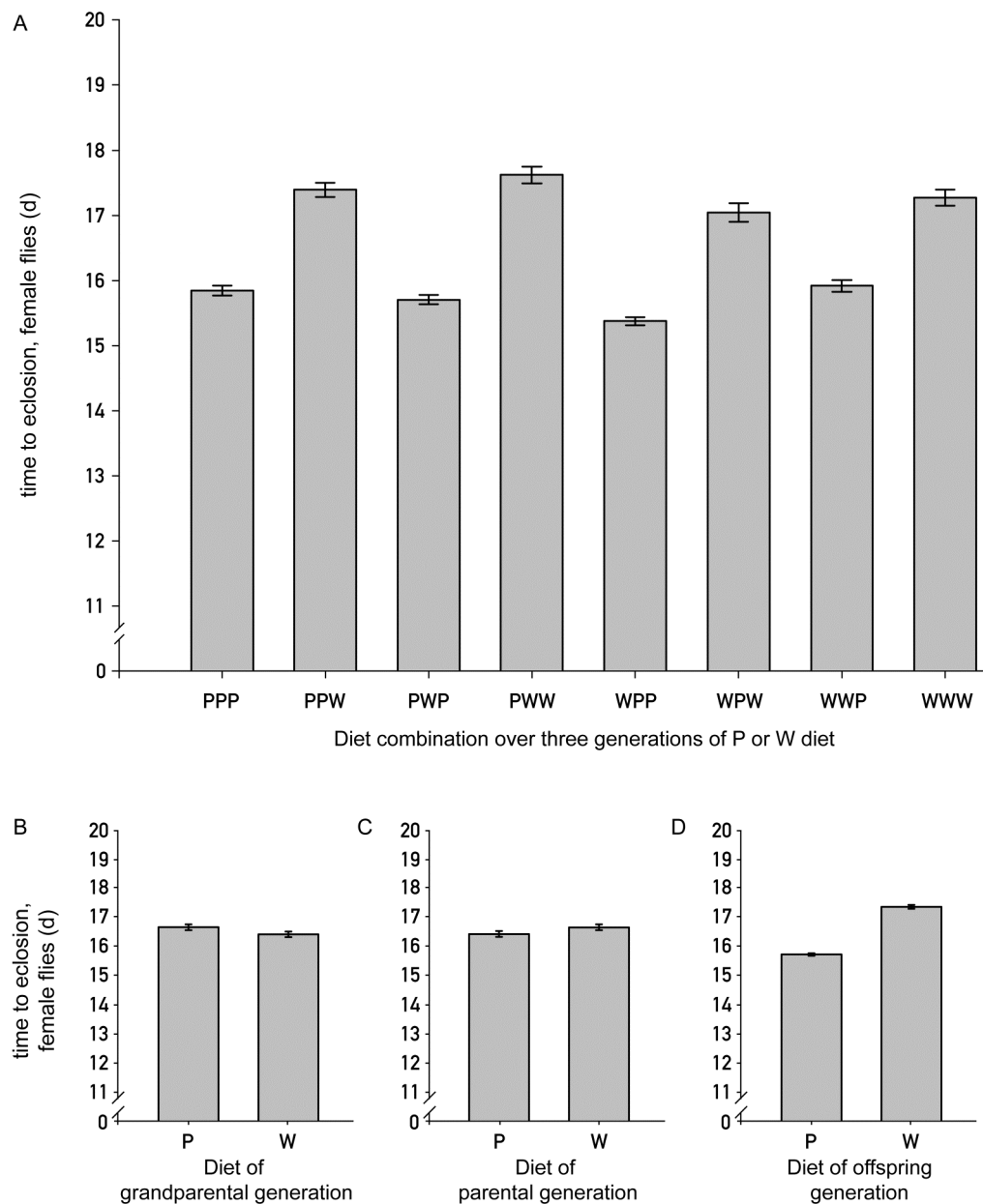


Figure 3.5 Mean time to eclosion (d) for female flies, in the third generation of a combination of P or W diets. **A:** each histogram bar represents the mean time to eclosion from 25 isofemale lines with differing diet combinations over three generations. The categories on the x-axis show the combination of diets (P or W) over three generations, with grandparental diet first, then parental diet and offspring diet. **B:** Mean time to eclosion, grouped by the grandparental diet. **C:** Mean time to eclosion, grouped by the parental diet. **D:** Mean time to eclosion, grouped by the offspring diet. All error bars represent ± 1 standard error of the mean.

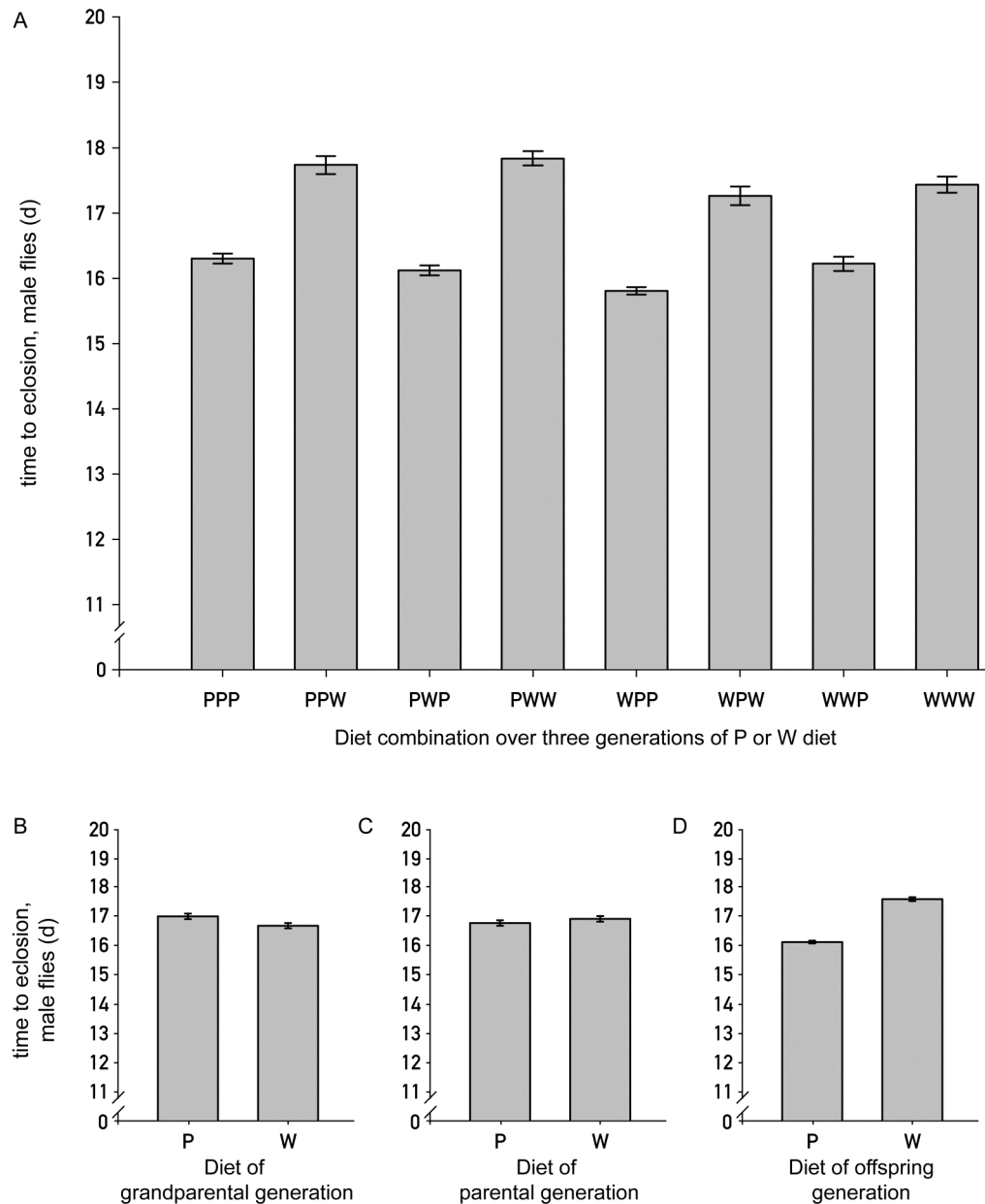


Figure 3.6 Mean time to eclosion (d) for male flies, in the third generation of a combination of P or W diets. **A:** each histogram bar represents the mean time to eclosion from 25 isofemale lines with differing diet combinations over three generations. The categories on the x-axis show the combination of diets (P or W) over three generations, with grandparental diet first, then parental diet and offspring diet. **B:** Mean time to eclosion, grouped by the grandparental diet. **C:** Mean time to eclosion, grouped by the parental diet. **D:** Mean time to eclosion, grouped by the offspring diet. All error bars represent ± 1 standard error of the mean.

Minimal adequate model:

Log 10 (Time to eclosion) is controlled by:

factor	res dev	– dev	Res d.f.	– d.f.	<i>p</i>	
Null	4.4995		378			
Day	4.1894	0.3101	368	10	< 0.01	**
Iso	3.7699	0.4195	344	24	< 0.05	*
Sex	3.6596	0.1103	343	1	< 0.01	**
Offspring	0.8301	2.8295	342	1	< 0.0001	***
Sex:Offspring	0.8222	0.0080	341	1	0.069	
Parental	0.8118	0.0104	340	1	< 0.05	*
Offspring: Parental	0.8097	0.0021	339	1	0.353	
Grandparental	0.6901	0.1196	338	1	< 0.0001	***
Offspring: Grandparental	0.6874	0.0028	337	1	0.243	
Parental: Grandparental	0.6740	0.0133	336	1	< 0.01	**
Offspring: Parental: Grandparental	0.6618	0.0122	335	1	< 0.05	*

Table 3.2 The minimal adequate model for time to eclosion, arrived at by removing non-significant factors and interactions from the full model. The significance of each factor was then assessed by adding factors in turn to the model (day+iso) and assessing the reduction (– dev) in residual deviance (Res. dev.). Key to factors: Day – date of egg laying; Iso – isofemale line; Sex – sex of fly; Offspring - offspring diet; Parental – parental diet; Grandparental - grandparental diet.

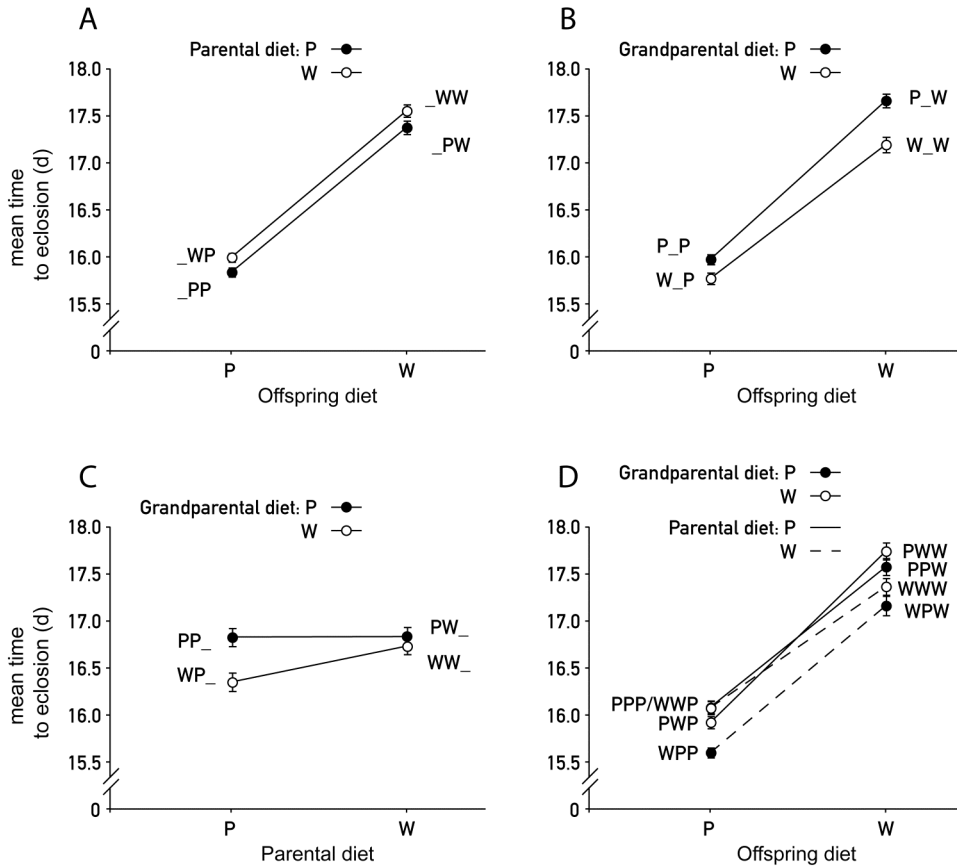


Figure 3.7: Mean time to eclosion (d) in the offspring generation by **A:** grandparental diet and offspring diet **B:** grandparental diet and offspring diet **C:** Grandparental: parental diet interaction **D:** The interaction of all generations' diets. Labels indicate the grandparental, parental and offspring diets and underscore indicates combined figures for P and W for that generation. All error bars are ± 1 standard error of the mean.

3.3.3 Thorax length

Thorax length in *Drosophila melanogaster* is an indicator of potential breeding or mating success, and for each sex a larger thorax is associated with greater success (Partridge et al. 1987). Females are on average larger than males (David et al. 2003). If a fly can optimise the thorax length of its offspring in anticipation of a future nutritional environment, it is expected that thorax length should be different if the offspring develop on a different diet to the parental or grandparental diet.

To test this, some groups of flies were swapped from their parental P or W diets over three generations, while other groups remained on their parental diets. The null hypothesis was that no diet from any generation would influence eclosion time, nor would any of the interactions between generations' diets, and then if any were significant,

the data were examined to determine the nature of the influence of previous diets, as described in Chapter 1. A second null hypothesis was that sex, or its interaction with any generation's diet, would not influence time to eclosion.

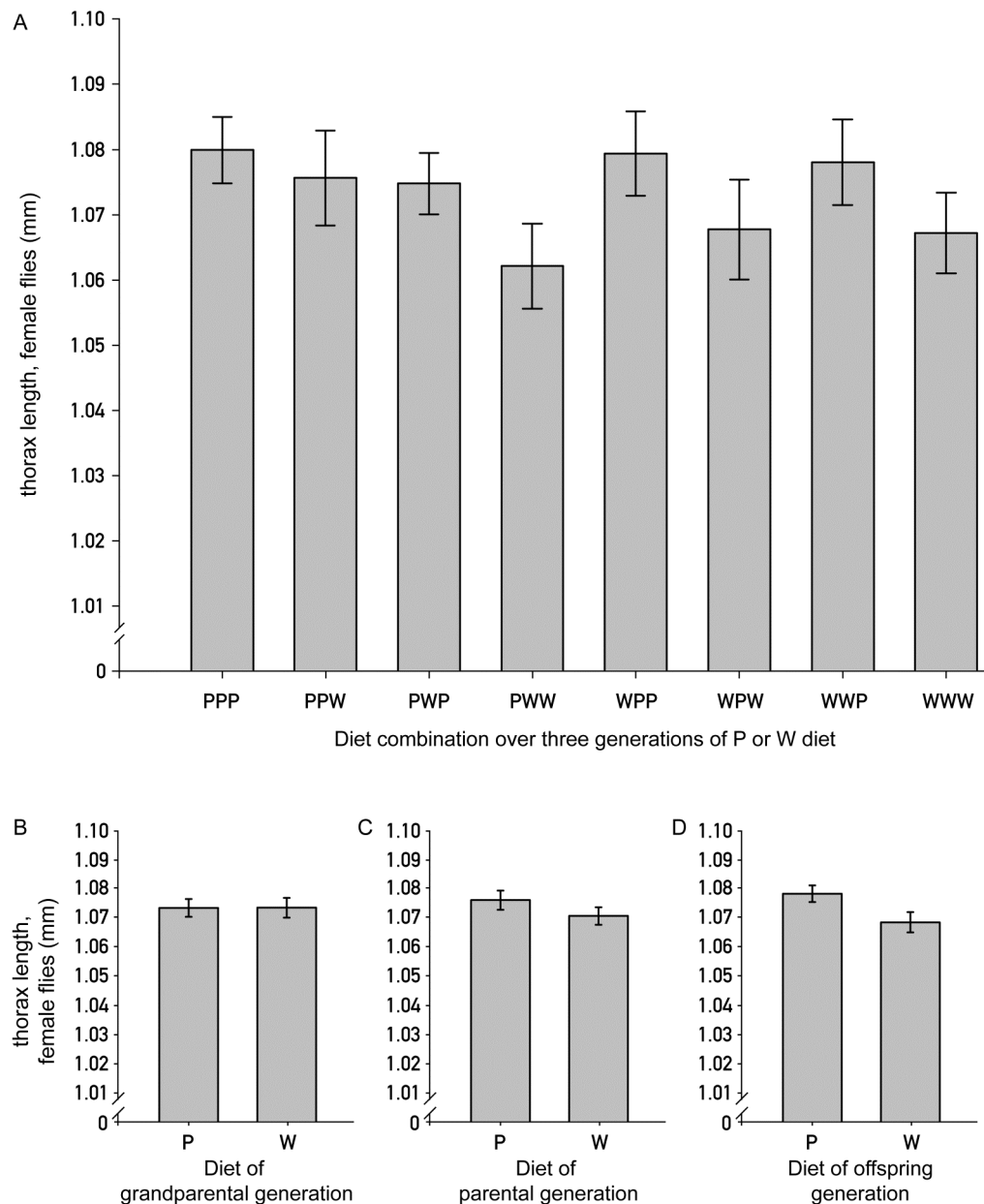


Figure 3.8 Mean thorax length (mm) for female flies, in the third generation of a combination of P or W diets. **A:** each histogram bar represents the mean thorax length from 25 isofemale lines with differing diet combinations over three generations. The categories on the x-axis show the combination of diets (P or W) over three generations, with grandparental diet first, then parental diet and offspring diet. **B:** Mean thorax length, grouped by the grandparental diet. **C:** Mean thorax length, grouped by the parental diet. **D:** Mean thorax length, grouped by the offspring diet. All error bars represent ± 1 standard error of the mean.

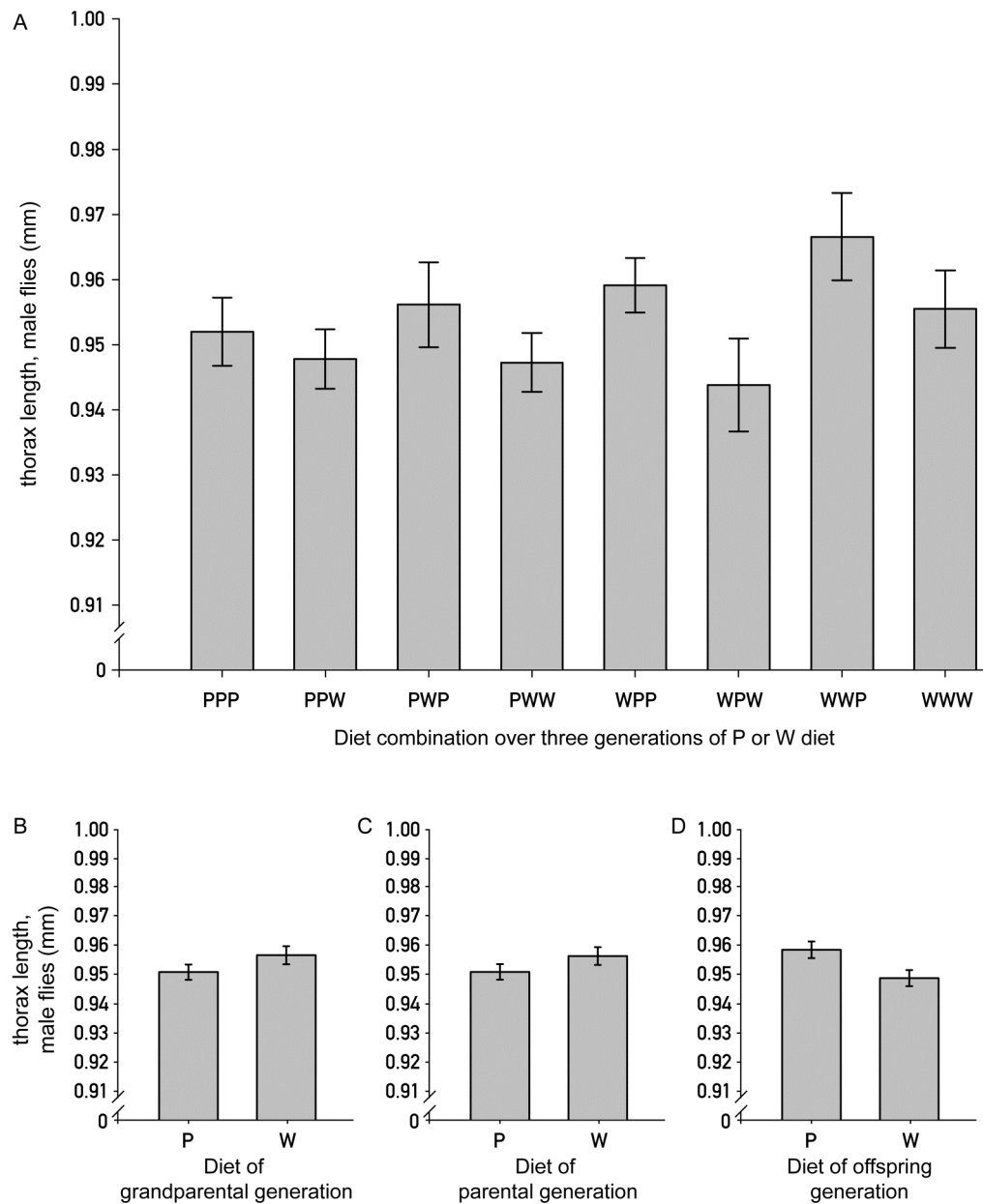


Figure 3.9 Mean thorax length (mm) for male flies, in the third generation of a combination of P or W diets. **A:** each histogram bar represents the mean thorax length from 25 isofemale lines with differing diet combinations over three generations. The categories on the x-axis show the combination of diets (P or W) over three generations, with grandparental diet first, then parental diet and offspring diet. **B:** Mean thorax length, grouped by the grandparental diet. **C:** Mean thorax length, grouped by the parental diet. **D:** Mean thorax length, grouped by the offspring diet. All error bars represent ± 1 standard error of the mean.

Sex was the main factor in determining the size of the thorax (change in deviance 6.1708, 1 d.f., $p < 0.0001$), with females (Fig 3.8) having a mean thorax length of $1.069 \pm$

0.002 mm, and males (Fig 3.9) having a mean length of 0.954 ± 0.002 mm. The current diet of the fly (Fig 3.8d, Fig. 3.9d) was also a significant factor (change in deviance 0.0393, 1 d.f., $p < 0.001$). Both males and females had larger thoraxes when their current diet was P than when it was W regardless of previous generation's diets. No previous generation's diet had a significant effect on its own, and there were no significant interactions between the diets of different generations (Table 3.3).

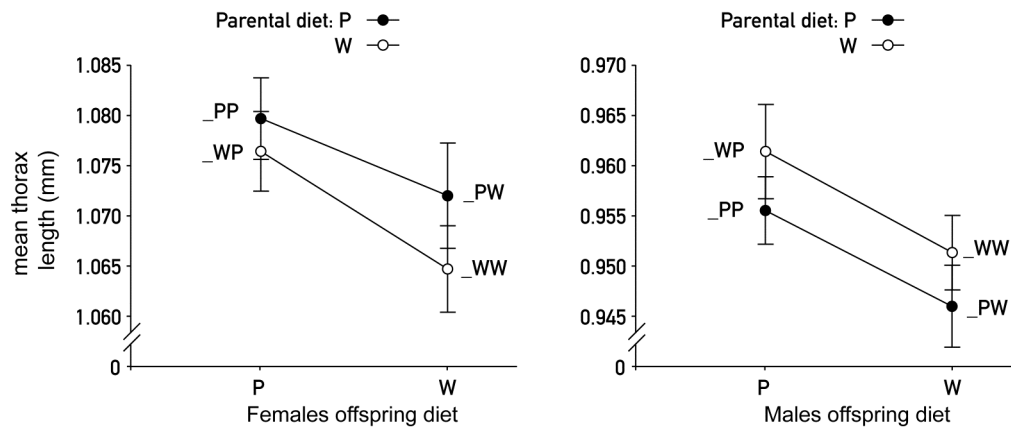


Figure 3.10: Mean thorax length of female flies (left) and male flies (right), by offspring diet (x-axis) and parental diet (closed circles – P, open circles – W). All error bars are ± 1 standard error of the mean.

Males and females of the offspring generation responded differently to a parental diet of P or W (change in deviance 0.01023, 1 d.f., $p < 0.05$). Females whose parental diet was P were larger than those whose parents developed on a W diet, while males were smaller if their parental diet was P than if it was W (Fig. 3.10).

Minimal adequate model:

Thorax length (mm) is controlled by:

Factor	Res. dev.	– dev.	Res d.f.	– d.f.	p	sig
Null	7.6073		367			
Day	7.5345	0.0728	357	10	0.9688	
Iso	7.0702	0.4643	333	24	0.5871	
Sex	0.8994	6.1708	332	1	< 0.0001	***
Offspring	0.86013	0.03926	331	1	< 0.0001	***
Parental	0.85606	0.00407	330	1	0.2105	
Sex: Parental	0.84583	0.01023	329	1	< 0.05	*

Table 3.3 The minimal adequate model for thorax length (mm), arrived at by removing non-significant factors and interactions from the full model. The significance of each factor was then assessed by adding factors in turn to the model (Day + Iso) and assessing the reduction (– dev) in residual deviance (Res. dev.). Key to factors: Day – date of egg laying; Iso – isofemale line; Sex – sex of fly; Offspring – offspring diet; Parental – parental diet.

3.3.4 Wing length

Wing length, along with thorax size and dry weight, is an indicator of body size and is correlated with mating speed in the closely related *D. subobscura* and with fitness in *D. melanogaster* (Gilchrist and Partridge 2001). Wing measurements along the third wing vein were taken from the same flies as for thorax length. The null hypothesis was that diet combinations over the three generations would not affect wing length, and also that neither sex nor any one generation's diet would affect wing length. As with the other fitness indicators, the direction of any significant effects and interactions was then assessed to determine the nature of the effect of previous generations' diets.

Sex was the major contributory factor determining wing length (change in deviance 33.12, 1 d.f., $p < 0.001$), as females' wings were a mean length of 2.31 ± 0.01 mm as opposed to the males' mean of 2.02 ± 0.01 mm (Fig. 3.11 and Fig. 3.12). The offspring diet was significant (change in deviance 0.1371, 1 d.f., $p < 0.01$). Both males and female had longer wings overall when they had developed on the P diet than on the W (Fig. 3.11d, Fig. 3.12d). Considered on their own, none of the other generations' diet had any significant influence over wing length in the offspring generation (Table 3.4).

There was a significant influence on wing length of the combination of all three generations' diets (change in deviance 0.0893, 1 d.f., $p < 0.05$, Fig. 3.13), although none of the two-way interactions between diets were significant (Table 3.4). When the grandparental and parental diets were the same, the effect of the P or W offspring diet was smaller (Fig. 3.13, and groups PPP, PPW, and WWP, WWW in Fig. 3.11 and Fig. 3.12). In the groups where the grandparental and parental diets were different, the offspring diet had a larger effect on wing size, with flies on a W diet having smaller wings than those on the P diet (Fig 3.13, and groups PWP, PWW, and WPP, WPW in Fig. 3.11 and Fig. 3.12).

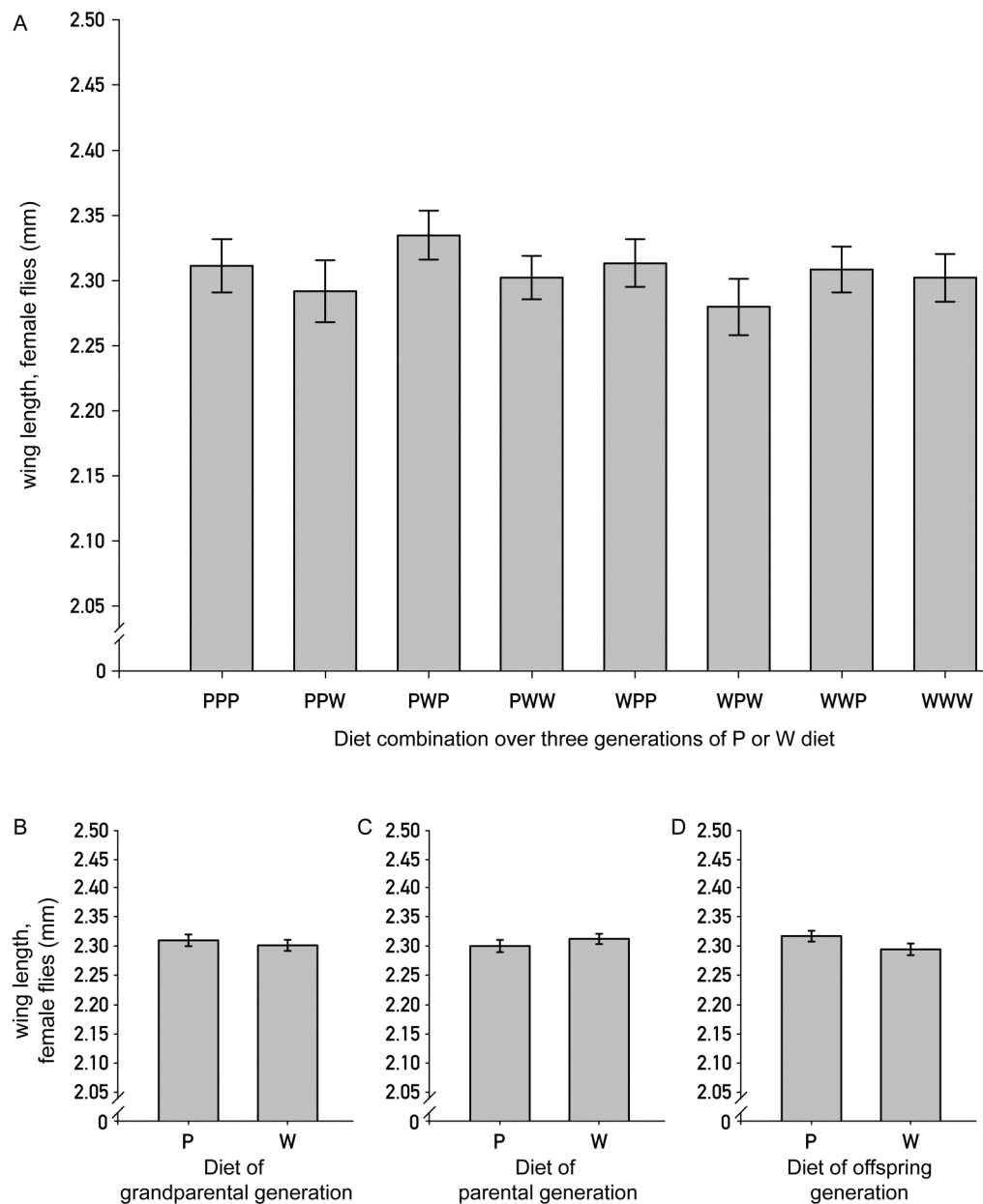


Figure 3.11 Mean wing length (mm) for female flies, in the third generation of a combination of P or W diets. **A:** each histogram bar represents the mean thorax length from 25 isofemale lines with differing diet combinations over three generations. The categories on the x-axis show the combination of diets (P or W) over three generations, with grandparental diet first, then parental diet and offspring diet. **B:** Mean wing length, grouped by the grandparental diet. **C:** Mean wing length, grouped by the parental diet. **D:** Mean wing length, grouped by the offspring diet. All error bars represent ± 1 standard error of the mean.

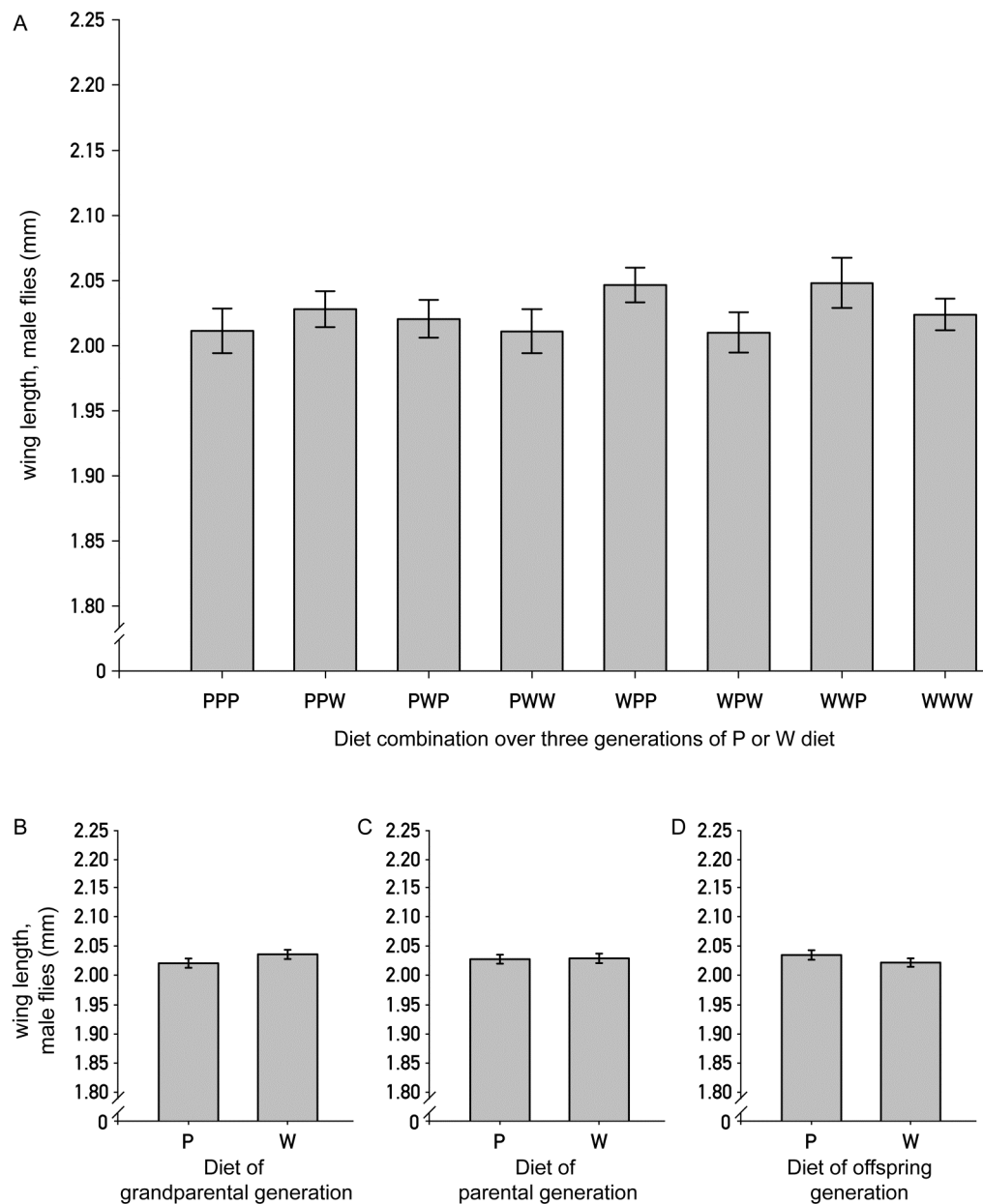


Figure 3.12 Mean wing length (mm) for male flies, in the third generation of a combination of P or W diets. **A:** each histogram bar represents the mean thorax length from 25 isofemale lines with differing diet combinations over three generations. The categories on the x-axis show the combination of diets (P or W) over three generations, with grandparental diet first, then parental diet and offspring diet. **B:** Mean wing length, grouped by the grandparental diet. **C:** Mean wing length, grouped by the parental diet. **D:** Mean wing length, grouped by the offspring diet. All error bars represent ± 1 standard error of the mean.

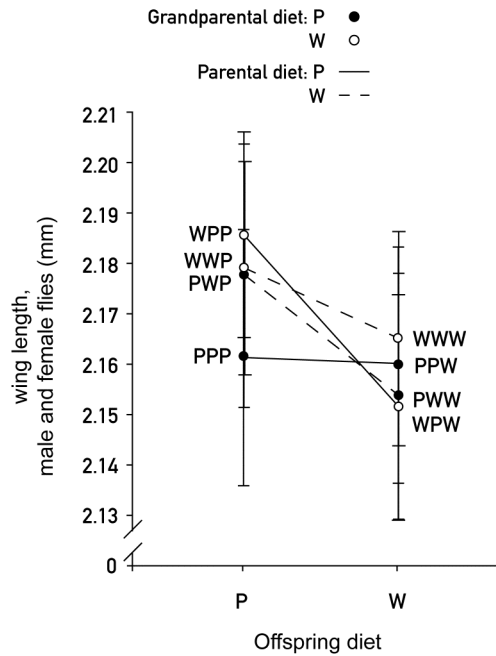


Figure 3.13: Interaction of the three generations' diets and its effect on mean wing length of male and female flies. All error bars represent ± 1 standard error of the mean.

Minimal adequate model:

Wing length (mm) is controlled by:

Factor	Res. dev.	– dev.	Res d.f.	– d.f.	<i>p</i>	Sig
Null	44.884		367			
Day	44.254	0.63	357	10	0.8850	
Iso	38.867	5.387	333	24	< 0.01	**
Sex	5.747	33.12	332	1	< 0.001	***
Offspring	5.6097	0.1371	331	1	< 0.01	**
Parental	5.6096	0.000116	330	1	0.9752	
Offspring:Parental	5.6029	0.0068	329	1	0.5279	
Grandparental	5.6021	0.0007	328	1	0.8381	
Offspring: Grandparental	5.5995	0.0026	327	1	0.6969	
Parental: Grandparental	5.5966	0.003	326	1	0.6766	
Offspring: Parental: Grandparental	5.5072	0.0893	325	1	< 0.05	*

Table 3.4 The minimal adequate model for wing length (mm), arrived at by removing non-significant factors and interactions from the full model. The significance of each factor was then assessed by adding factors in turn to the model (Day + Iso) and assessing the reduction (– dev) in residual deviance (Res. dev.). Key to factors: Day – date of egg laying; Iso – isofemale line; Sex – sex of fly; Offspring – offspring diet; Parental – parental diet; Grandparental – grandparental diet.

3.3.5 Dry weight

Dry weight is related to the size of an adult fly, hence to fitness, and is thought to be largely determined during the larval stages of the fly lifecycle (Bakker 1959). Larger flies have greater mating success and so if flies display PARs in relation to the two diets P and W, it would be expected that flies with a consistent generational diet history would have a different dry weight than those on the same current diet but with changing diets over previous generations. Therefore the null hypothesis was that no combination of diets or single diets would affect dry weight, and if any were significant the nature of the effect was assessed as set out in Chapter 1. Sex was included as a factor, with the null hypothesis that sex did not affect dry weight.

The sex of the fly was the main influence on dry weight (change in deviance 8.298, 1 d.f., $p < 0.001$, Table 3.5). Females had a mean dry weight of $345 \pm 2 \mu\text{g}$, higher than the males' $248 \pm 2 \mu\text{g}$ (Fig. 3.14 and Fig. 3.15). Although the offspring diet had no significant influence (change in deviance 0.002, 1 d.f., $p = 0.503$), the grandparental diet did, as flies whose grandparents developed on the W diet had a higher mean dry weight (change in deviance 0.049, 1 d.f., $p < 0.01$, Fig. 3.16a). There were also significant interactions between grandparental and parental diets (change in deviance 0.003, 1 d.f., $p < 0.01$, Fig 3.16b) and between all three generations' diets (change in deviance 0.058, 1 d.f., $p < 0.001$, Fig 3.16c).

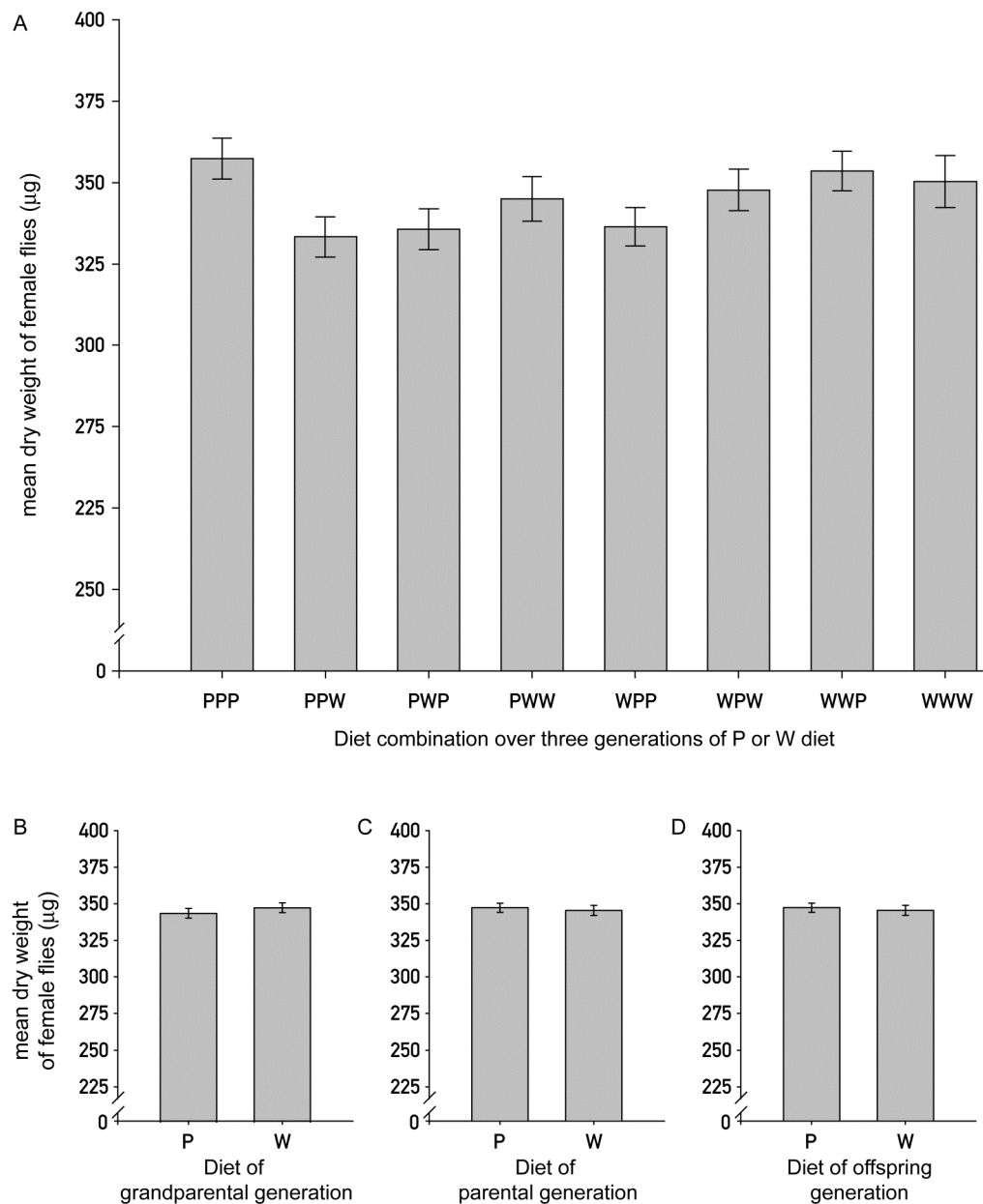


Figure 3.14 Mean dry weight (μg) for female flies, in the third generation of a combination of P or W diets. **A:** each histogram bar represents the mean dry weight from 25 isofemale lines with differing diet combinations over three generations. The categories on the x-axis show the combination of diets (P or W) over three generations, with grandparental diet first, then parental diet and offspring diet. **B:** Mean dry weight, grouped by the grandparental diet. **C:** Mean dry weight, grouped by the parental diet. **D:** Mean dry weight, grouped by the offspring diet. All error bars represent ± 1 standard error of the mean.

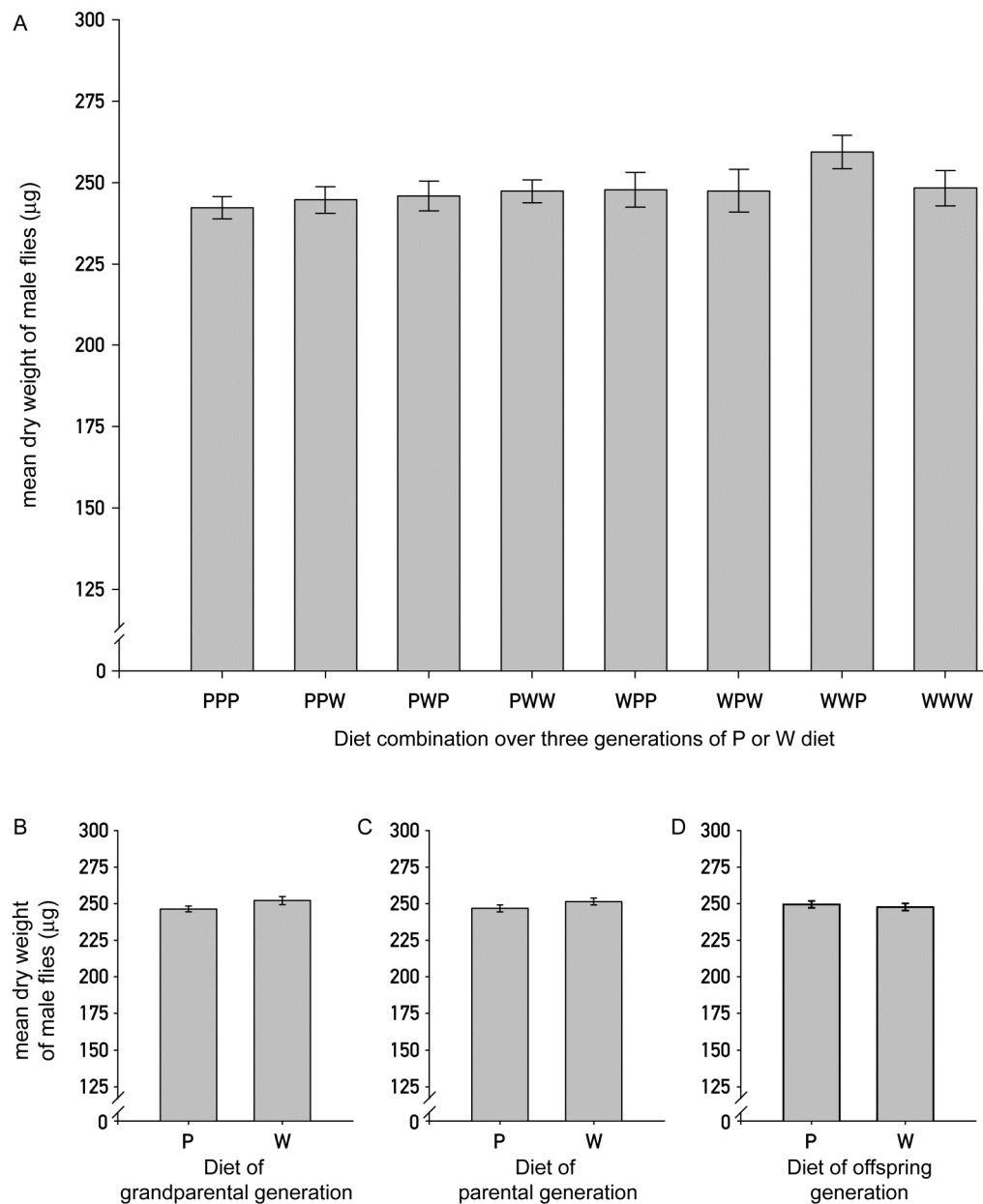


Figure 3.15 Mean dry weight (μg) for male flies, in the third generation of a combination of P or W diets.

A: each histogram bar represents the mean dry weight from 25 isofemale lines with differing diet combinations over three generations. The categories on the x-axis show the combination of diets (P or W) over three generations, with grandparental diet first, then parental diet and offspring diet. **B:** Mean dry weight, grouped by the grandparental diet. **C:** Mean dry weight, grouped by the parental diet. **D:** Mean dry weight, grouped by the offspring diet. All error bars represent ± 1 standard error of the mean.

Minimal adequate model
Log 10 (dry weight (μg)) is controlled by:

factor	res dev	– dev	Res df	– d.f.	<i>p</i>	sig.
Null	11.144		360			
Day	10.923	0.221	350	10	0.718	
Iso	10.314	0.610	326	24	0.738	
Process	9.834	0.480	313	13	0.291	
Sex	1.536	8.298	312	1	< 0.001	***
Offspring	1.533	0.002	311	1	0.503	
Parental	1.532	0.001	310	1	0.673	
Offspring: Parental	1.532	0.000	309	1	0.944	
Grandparental	1.484	0.049	308	1	< 0.01	**
Offspring: Grandparental	1.483	0.001	307	1	0.692	
Parental: Grandparental	1.481	0.003	306	1	< 0.01	**
Offspring: Parental: Grandparental	1.423	0.058	305	1	< 0.001	***

Table 3.5 The minimal adequate model for dry weight (μg), arrived at by removing non-significant factors and interactions from the full model. The significance of each factor was then assessed by adding factors in turn to the model (Day + Iso + Process) and assessing the reduction (– dev) in residual deviance (Res. dev.). Key to factors: Day – date of egg laying; Iso – isofemale line; Process – batch for lipid assay; Sex – sex of fly; Offspring - offspring diet; Parental – parental diet; Grandparental - grandparental diet.

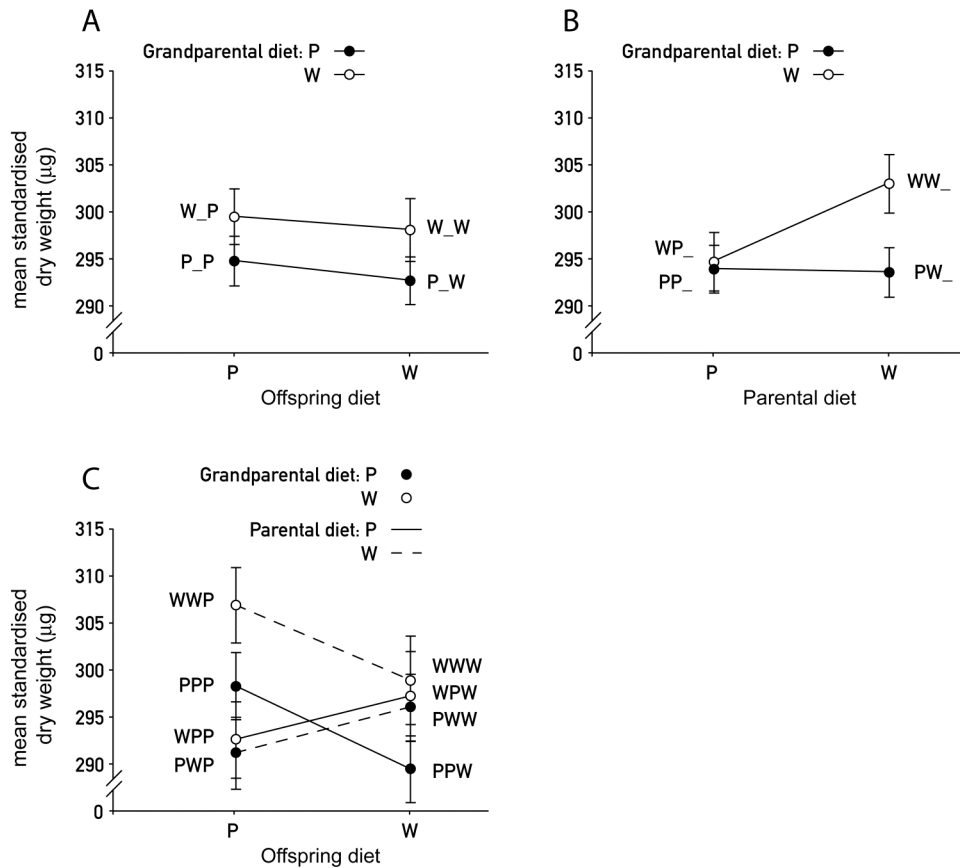


Figure 3.16: Mean dry weight (μg) for males and females in the offspring generation. Male and female dry weights have been adjusted for the difference accounted for by gender. **A:** grandparental diet and offspring diet **B:** Grandparental: parental diet interaction **C:** The interaction between all generations' diets. Labels indicate the grandparental, parental and offspring diets and underscore indicates combined figures for P and W for that generation. All error bars are ± 1 standard error of the mean.

3.3.6 Lipid content by weight

The lipid content of a fly can relate to fitness as it may indicate a greater resistance to starvation (Chippindale et al. 1996; Baldal et al. 2006) but it is also included here as a “health” indicator as flies, like humans, store excess energy from the diet as lipids (Musselman et al. 2011) and may be significantly affected as a result of adaptive changes directed at fitness indicators, for example, time to eclosion. The null hypotheses were that neither sex, any generation's diet nor combination of diets would affect the lipid content.

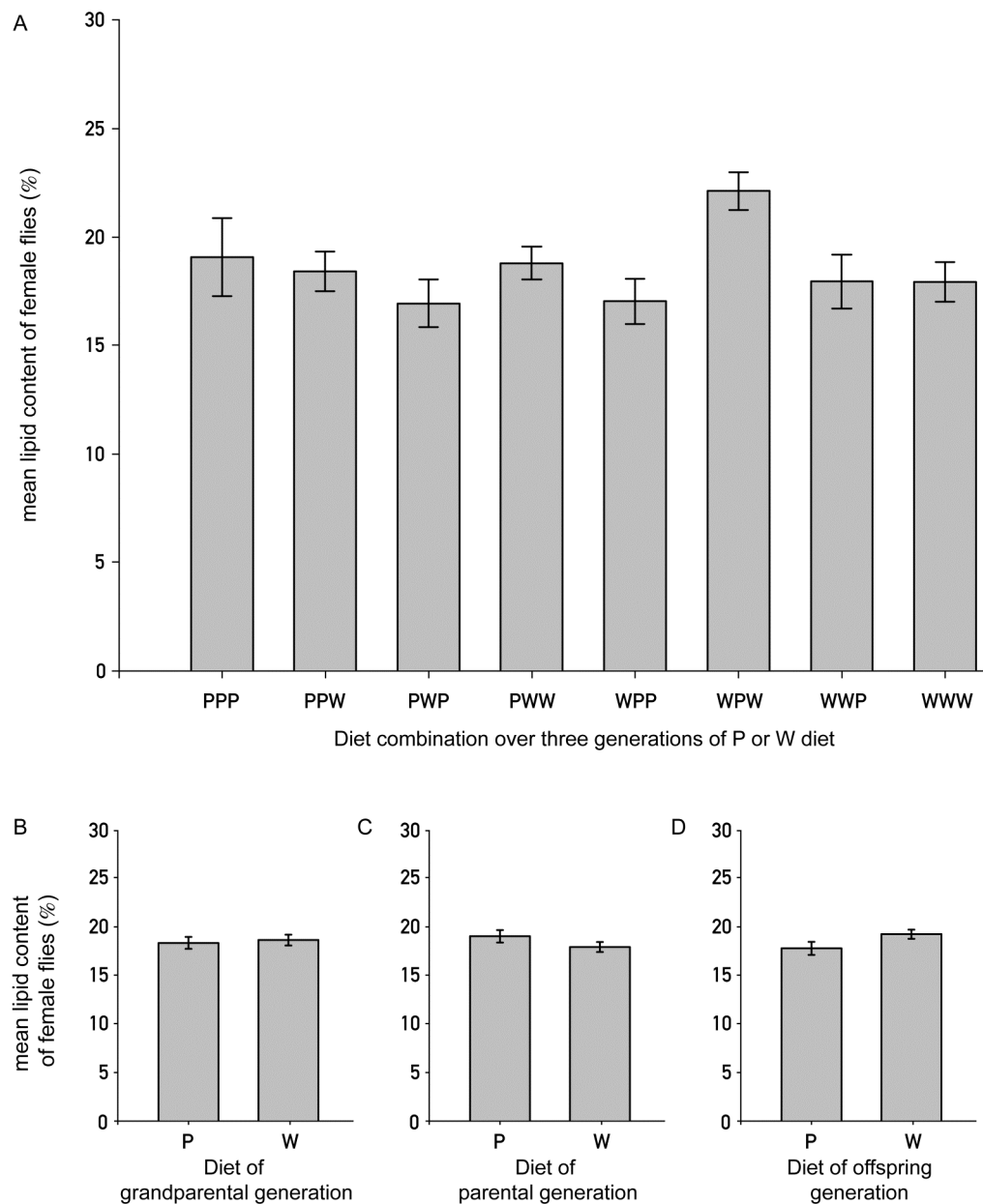


Figure 3.17 Mean lipid percentage by weight for female flies, in the third generation of a combination of P or W diets. **A:** each histogram bar represents the mean lipid percentage from 25 isofemale lines with differing diet combinations over three generations. The categories on the x-axis show the combination of diets (P or W) over three generations, with grandparental diet first, then parental diet and offspring diet. **B:** Mean lipid percentage, grouped by the grandparental diet. **C:** Mean lipid percentage, grouped by the parental diet. **D:** Mean lipid percentage, grouped by the offspring diet. All error bars represent ± 1 standard error of the mean.

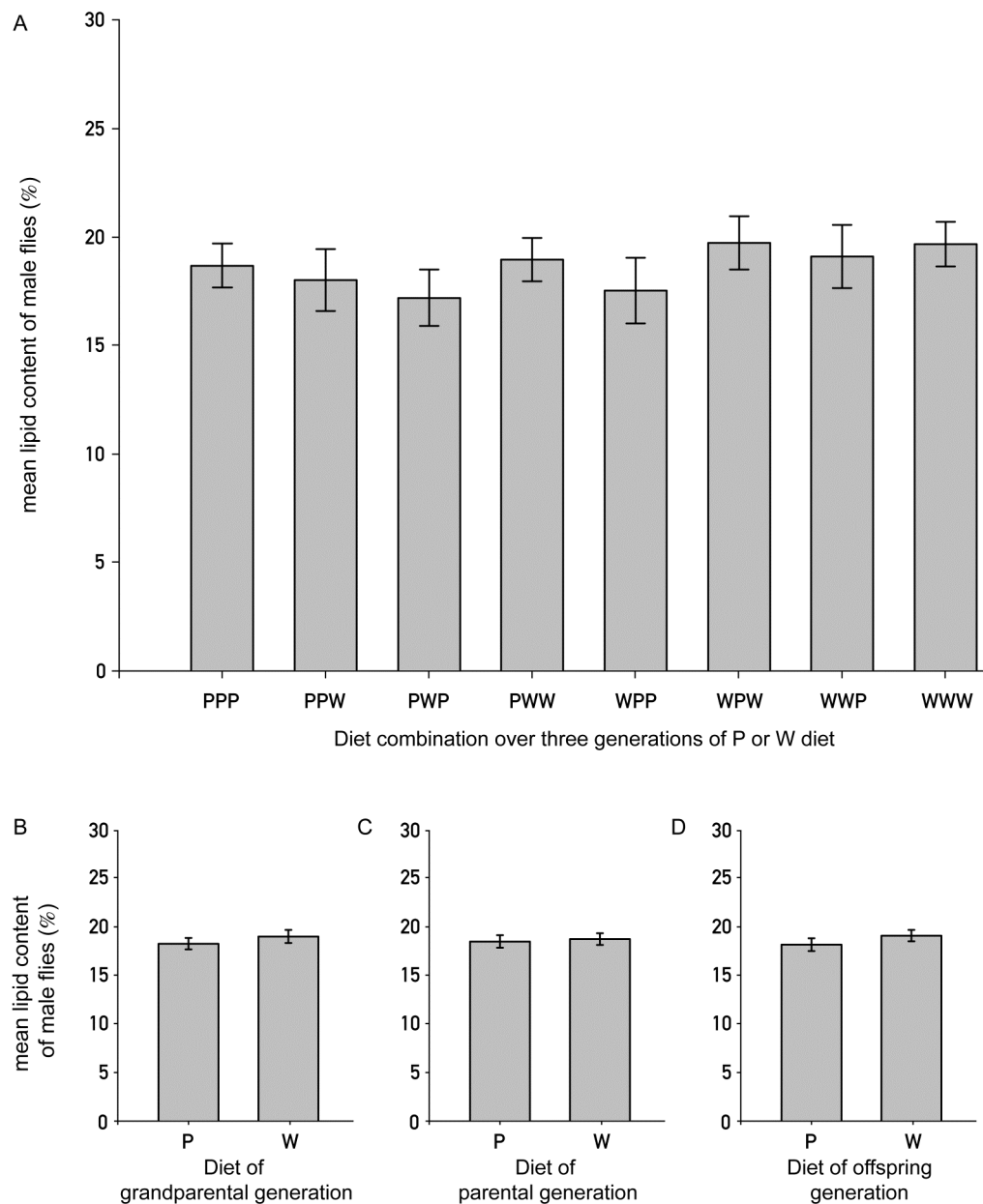


Figure 3.18 Mean lipid percentage by weight for male flies, in the third generation of a combination of P or W diets. **A:** each histogram bar represents the mean lipid percentage from 25 isofemale lines with differing diet combinations over three generations. The categories on the x-axis show the combination of diets (P or W) over three generations, with grandparental diet first, then parental diet and offspring diet. **B:** Mean lipid percentage, grouped by the grandparental diet. **C:** Mean lipid percentage, grouped by the parental diet. **D:** Mean lipid percentage, grouped by the offspring diet. All error bars represent ± 1 standard error of the mean.

Males and females did not have significantly different lipid content, and so the term for sex was removed from the model (Table 3.6). Flies on the W offspring diet had significantly higher lipid content (Fig. 3.17 and 3.18) at 19.2 ± 0.5 % for females and 19.1

$\pm 0.6\%$ for males, than those developing on the P diet, at $17.8 \pm 0.7\%$ for females and $18.1 \pm 0.7\%$ for males (change in deviance 0.091, 1 d.f., $p < 0.01$). The grandparental diet was also a significant factor and acted in the same direction as the offspring diet (change in deviance 0.061, 1 d.f., $p < 0.05$, Fig 3.19a), and there was also a significant interaction between all three diets (change in deviance 0.112, 1 d.f., $p < 0.01$, Fig 3.19b).

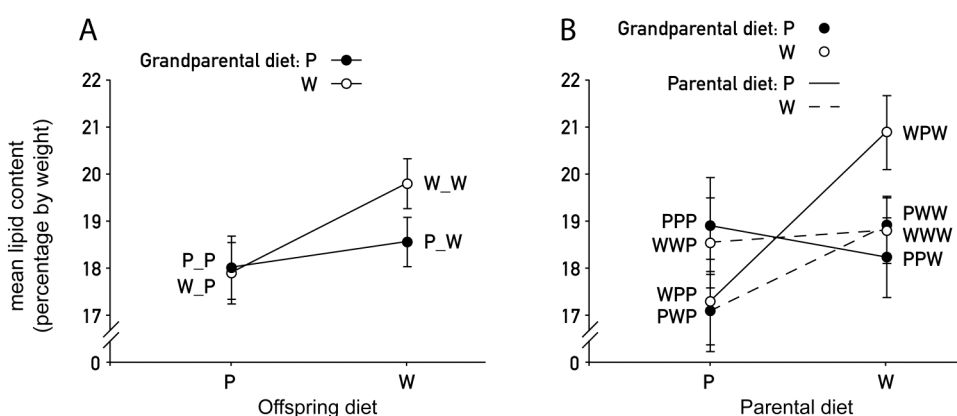


Figure 3.19: Mean lipid percentage by weight for males and females in the offspring generation.

A: grandparental diet and offspring diet **B:** Grandparental: parental: offspring diet interaction. Labels indicate the grandparental, parental and offspring diets and underscore indicates combined figures for P and W for that generation. All error bars are ± 1 standard error of the mean.

Minimal adequate model

Lipid percentage (arcsine square-root transformed) is controlled by:

Factor	Res. dev.	– dev.	Res d.f.	– d.f.	p	sig.
Null	7.894		360			
Day	7.570	0.324	350	10	0.133	
Iso	4.200	3.370	326	24	< 0.0001	***
Process	3.475	3.475	313	13	< 0.0001	***
Offspring	3.384	0.091	312	1	< 0.01	**
Parental	3.384	0.001	311	1	0.809	
Offspring: Parental	3.384	0.000	310	1	0.941	
Grandparental	3.323	0.061	309	1	< 0.05	*
Offspring: Grandparental	3.306	0.017	308	1	0.210	
Parental: Grandparental	3.295	0.012	307	1	0.300	
Offspring: Parental: Grandparental	3.182	0.112	306	1	< 0.01	**

Table 3.6 The minimal adequate model for lipid percentage by weight, arrived at by removing non-significant factors and interactions from the full model. The significance of each factor was then assessed by adding factors in turn to the model (Day + Iso + Process) and assessing the reduction (– dev) in residual deviance (Res. dev.). Key to factors: Day – date of egg laying; Iso – isofemale line; Process – batch for lipid assay; Sex – sex of fly; Offspring – offspring diet; Parental – parental diet; Grandparental – grandparental diet.

3.3.7 Reaction norm models

Having determined whether the effect of parental and grandparental diets were statistically significant, the direction of the significant main effects and two-way interactions for each response variable were compared with the models set out in Chapter 1, Section 1.1.6 (Fig. 1.4). For parental diets, there were two effects that agreed with the adaptive model 11 (a P parental diet increased survival in the offspring, and increased thorax length in male offspring), while the parental effect on time to eclosion and female thorax was in the same direction as the offspring diet (constraint, model 7). For grandparental diets, the effect on time to eclosion agreed with the adaptive model 11 (grand-offspring of flies developing on W diet eclosed faster than those on P diets), and for lipid content, agreement with model 7 (constraint), as the effect of grandparental diet was the same as offspring. Two significant grandparental effects had unclear adaptive significance. The grandparental effect on survival was not significant in itself, but the interaction with offspring diet was. The direction of this interaction was however a negative mismatch, that is that when grandparental and offspring diets were dissimilar, survival was higher (model 4b). Grand-offspring of flies developing on W diet had higher dry weight, but as there was no significant difference between the offspring P and W flies, this agreed either with model 5 or with model 9. Table 3.7 below sets out the significant main effects and two-way interactions of parental and grandparental diets with the offspring diet, and the fit with the models set out in Chapter 1.

Parental diet:**Main effect and 2-way interaction with offspring diet**

Response variable	Parental sig.	Offspring sig.	Interaction sig.	Model	Significance
Survival (%)	Y, P higher	Y, W higher	n.s.	–Y0 11	adaptive effect of parental P diet
Eclosion (d) (lower is fitter)	Y, P lower	Y, P lower	n.s.	+Y0 7	constraint effect
Thorax length male (mm)	Y, W higher	Y, P higher	n.s.	–Y0 11	adaptive effect of parental W diet
Thorax length female (mm)	Y, P higher	Y, P higher	n.s.	+Y0 7	constraint effect

Grandparental diet:**Main effect and 2-way interaction with offspring diet**

Response variable	Grandparental sig.	Offspring sig.	Interaction sig.	Model	Significance
Survival (%)	0	Y, W higher	Y (-ve).	0YY 4b	unclear
Eclosion (d) (lower is fitter)	Y, W lower	Y, P lower	n.s.	–Y0 7	adaptive effect of gparental W diet
Dry weight (µg)	Y, W higher	n.s.	n.s.	Y00 5 or 9	higher if gparental diet was W
Lipid content (%)	Y, W higher	Y, W higher	n.s.	+Y0 7	constraint effect

Table 3.7: Significant main effects and interactions of parental and grandparental diets with the offspring diet, and reaction norm models. Key: sig. – significance; Model – reaction norm model (see Chapter 1, section 1.1.6, Figure 1.4); Y – significant effect; 0 – effect not significant; gparental – grandparental.

3.4 Discussion

The purpose of these experiments was firstly to identify whether *Drosophila melanogaster* shows any evidence of the influence of parental or grandparental diet in the offspring and secondly to examine whether this influence was adaptive, whether as a compensatory effect or mismatch, or a constraint (“silver spoon”) effect. To show that any effects were adaptive, it is necessary to distinguish between a simple effect of positive or negative constraint by the previous generations’ diets, and an adaptive effect (Ellison and Jasienska 2007).

Across the whole experiment, offspring on P diets eclosed faster, and had a longer thorax and wings than those on W diets, but also had a lower survival rate and lipid content. The main differences between the two diets were the increased carbohydrate content of the W diet, giving it twice the energy density of the P diet for the same amount of protein.

3.4.1 Intergenerational effects

There were significant effects on the survival rate of offspring, from both the parental and grandparental diets. Survival was higher when the offspring diet was W (Fig. 3.2d), but was significantly increased by having a parent on the P diet (Fig 3.2c and Fig. 3.3). This indicates that there may be an adaptive effect acting to compensate for the lower survival and agrees with the reaction norm model 11. Although the diets were not designed as a famine or shortage model versus an adequate diet, as they were in Prasad & Joshi (2003) and Vijendravarma et al. (2010), but rather to model an “adequate” (P) and an “excess” (W) human diet, the pattern of parental diet influence on survival was similar. The effect may be linked to efficiency of energy use in the offspring being enhanced when parents developed on the less energy-dense diet. Alternatively, Prasad and Joshi (2003) suggested this effect may be due to enhanced provisioning of eggs in preparation for an expected poorer environment, as found by Vijendravarma et al. (2010). The size of eggs laid was not measured in the multigenerational diet experiment, but if this is the case then the enhanced survival of the offspring of P parents indicates that the effect may be caused by the contrast by any two diets differing in energy levels, and not solely as a response to a very poor diet. There was also an effect of grandparental diet which is harder to interpret as the grandparental diet was only significant as an interaction with the offspring diet (reaction norm model 4b, as survival

is enhanced when grandparental and offspring diets are dissimilar). A grandparent on the P diet may enhance survival, but only if the offspring diet was W. Offspring diet did not influence survival if the grandparental diet was W.

Time to eclosion reflects the speed of development of the larvae, as pupariation begins during the L3 stage and is triggered by reaching a threshold weight (Bakker 1959). High-carbohydrate, low protein diets slow time to eclosion (Matzkin et al. 2011) and the main effect on time to eclosion in the multigeneration diet experiment was from offspring diet, with all groups on the W diet taking longer to eclose than those developing on the P diet. Matzkin et al. (2011) attribute the longer developmental period to the lack of protein in their high-carbohydrate diet, but the amount of protein contained in the P and W diets here was the same, so it may either be an effect of carbohydrate or of the protein: carbohydrate ratio. This has been shown to have significant effects on fly lifespan and fecundity (Lee et al. 2008).

Having a parent on the P diet in the multigenerational diet experiment shortened time to eclosion, and offspring of W parents took significantly longer, a constraint effect similar to reaction norm model 7 (Fig. 1.4, Table 3.7). While this effect appears adaptive if the parent developed on the P diet, it does not if the parent developed on the W diet. However, the grandparental diet induced the opposite effect, decreasing the offspring time to eclosion when it was W. This effect was more pronounced when the parental diet was P (Fig. 3.7c and d), suggesting that for some measures, adaptive effects might reinforce constraining effects if they are in the same direction. The acceleration of development in reaction to a less suitable environment in a previous generation is same effect observed in relation to development under different temperature regimes by Nunney and Cheung (1997), and in relation to diet by Vijendravarma et al. (2010), except that in the multigenerational diet experiment, the effect came from the grandparental generation's diet.

It was expected that the thorax and wing lengths should show a similar response to diet. This was true when just the offspring diet was considered, as both wings and thorax were larger when the offspring diet was P. However, their responses to previous generations' diet were different. In females, parental diet influenced the size of the thorax in the same direction as in the offspring diet (reaction norm model 7), but in males this was reversed, in the same pattern as the other adaptive effects described above (reaction norm model 11). This may reflect the differing requirements of males and females

regarding mating and reproduction, where males with larger thoraxes enjoy greater mating success for behavioural reasons. Size is an advantage because it allows them to be more competitive for females and increases their ability to follow females (Partridge et al. 1987). When developing in the same environment, female thorax size is correlated with more ovarioles in their abdomen (Drummond-Barbosa and Spradling 2001) and ovary size also responds rapidly to diet quality (Drummond-Barbosa and Spradling 2001) so this may mean that females do not gain the same benefit from having a large thorax as males do. For females, the cost of implementing adaptive plasticity for thorax size may outweigh the advantage.

Wing length was increased in the offspring by the P diet, and there were no main effects or two-way interactions of previous generations' diets. Wing size is often controlled more by cell size than cell number (Partridge et al. 1994) and it is perhaps more under control of the developmental (offspring) environment than other traits. However there was a significant interaction between the diets of all three generations. There are no clear adaptive effects, although it is noticeable that when grandparental and parental diets were P, there was very little effect of offspring diet.

Dry weight presents a different pattern, as the grand-offspring of flies on the W diet had higher dry weight, although the offspring diet itself was not a significant factor. The matched diet histories, PPP and WWW, were also the same, making it impossible to know whether the grandparental diet effect was adaptation or constraint by the criteria set out in Chapter 1 (section 1.1.6). The interaction of all generations' diets means that the pattern of the reaction norms is different depending on parental diet. The grandparental: parental diet interaction is also significant, with a noticeably higher dry weight for the matched W grandparental and parental conditions over any other. This suggests that for WWP and WWW, the matched diets act to increase dry weight over the PPP and PPW conditions, in an adaptive way. However, the other mismatched conditions mean it is not possible to generalise this to the grandparental effect on all diet conditions.

Lipids

The response of the lipid content needs to be considered in a different way to the fitness indicators above, as well as being adaptive, promoting resistance to starvation, a higher lipid value might indicate a potentially deleterious effect as the consequence of too much energy in the diet (Matzkin et al. 2011; Musselman et al. 2011). Lipid content was

higher in offspring developing on the W diet, along with a slower eclosion time and a smaller thorax, which in themselves indicate that the high energy W diet was not as suitable for development as the self-selected P diet. Grandparental diet is also significant as a main effect and increases lipid content in the same way as in the offspring diet, although on inspection of the significant interaction between the three generations' diets, this is mainly a result of the much higher figure for the WPW condition (Fig. 3.19b). Lipid levels are less variable when grandparental and parental diets are matched, but when they are mismatched they appear to be more sensitive to offspring diet, as the WPP and PWP conditions have the lowest lipid contents of any diet groups, and WPW the highest. The pattern of the response of lipid content to diet did not match any of the fitness indicators, and was not opposite to any either, except that where development was fastest (WPW and WPP), lipid content was most sensitive to offspring diet.

3.4.2 Significance of intergenerational effects of diet in *Drosophila melanogaster*

Some fitness indicators which responded to a parental or grandparental P or W diet did so in the same manner as is proposed by the Predictive Adaptive Response hypothesis (Gluckman and Hanson 2004) increasing fitness on the W diet when parents or grandparents had also developed on the W diet. Their response also supports the “cold parents better” hypothesis (Gilchrist and Huey 2001), where parents raised on a poor (here, the W diet in terms of its effect on development) environment or food produce offspring whose fitness is maximised in both their own and other environments. Similar effects of a poor parental environment on some morphological traits or on aspects of fecundity have been observed in *Drosophila* in relation to diet (Prasad et al. 2003; Vijendravarma et al. 2010) and temperature (Huey et al. 1995; Crill et al. 1996; Nunney and Cheung 1997) and in other insects (Stillwell and Fox 2005; Steigenga and Fischer 2007). The two fitness indicators that showed a constraint effect both did so in relation to the parental diet (eclosion time and female thorax length).

However, in the other experiments on parental nutrition in *Drosophila melanogaster* (Prasad et al. 2003; Vijendravarma et al. 2010), the “poor” condition is a diet deficient in some or all macronutrients. Here, carbohydrate is present in greater amounts in the diet producing the lowest fitness indicators, the Western diet, and *Drosophila* responded in a similar way to this rich maternal diet as it had to a very low-nutrient maternal diet, by a compensatory adaptive response. This suggests that the effect may not be directional with diet composition, but rather that any maternal diet which is sub-optimal in terms of

development might produce a compensatory adaptive response from the offspring or grand-offspring.

There may be differences in the mechanisms causing the effects of parental and grandparental generations on development. Vijendravarma et al. (2010) recorded a decrease in development time in offspring of flies from the slower-developing, less energy-dense, but in this case poor, parental diet that could be attributed partly to increased egg size (an adaptive effect). In the multigenerational diet experiment, the less energy-dense diet produced the fastest development, and this effect was increased if this was also the parental diet (a constraint), although egg size was not measured in this experiment. It was the grandparental diet that produced the adaptive response, by accelerating development if grandparents had developed on the slower-eclosing W diet. There may therefore be two different effects, one caused by greater egg provisioning when the energy content of the parental diet is lower (Vijendravarma et al. 2010) and a different adaptive effect which acts to speed development when the parental or grandparental diet is sub-optimal in either direction from an “ideal diet”. This could be sub-optimal for instance either by containing a large amount of carbohydrate, or at the other extreme, by having a very low energy content. This effect might only be detected when grandparental diets are considered, as this rules out the direct effect of egg-provisioning. This could account for the particularly decreased time to eclosion in the multigenerational diet experiment when the grandparental diet was W and the parental diet was P, and also the decreased time to eclosion in the offspring of the poor parental diets in Vijendravarma et al. (2010).

3.4.3 Predictive adaptive responses and mismatches

There were several instances of responses to previous generations’ diets that were in support of the PAR hypothesis. In the important fitness indicators survival rate, time to eclosion and thorax size, there were responses which were adaptive and predictive in the sense that in the offspring, the response corrected or compensated for a fitness-lowering effect of the parental or grandparental diet, alongside some other effects which reinforced the effect of parental diets and might be accounted for by egg-provisioning or another direct maternal effect. The grandparental and parental effects were not related to each other and the case where both were significant (eclosion time) their influence was adaptive from the grandparents and constraining from the parents. The “mismatch” or “beneficial acclimation” pattern of reaction norm was not detected in any fitness

indicator, nor in a measure of offspring health, lipid content. However this does not rule out that health effects could occur as a result of adaptive responses, as it is expected that there should be a cost involved in raising fitness.

If there were no cost associated with the acceleration of development observed on the carbohydrate rich W diet here, or on the “Poor” diet of Vijendravarma et al. (2010), then it would be expected that flies would display an accelerated development no matter what their parental or grandparental diet was and there would be no measurable intergenerational effect of a poor or sub-optimal diet. This cost may be paid in the form of a decreased lifespan, decreased overall fecundity or reduced mating success. Nunney and Cheung (1997) found that although early fecundity was increased by a low (poor) rearing/parental temperature, longevity was reduced. In view of the fitness-increasing effects described above, there may be a similar trade-off in relation to the diet of previous generations.

Drosophila melanogaster in the wild have a life expectancy of around 6 d, (Rosewell and Shorrocks 1987), and as they can be kept alive in the laboratory for much longer than this, it is possible that deleterious effects of an early accelerated development would become more apparent as they age. *Drosophila* is established as ageing (Mair et al. 2005; Partridge et al. 2011) heart function (Ocorr et al. 2007; Enell et al. 2010) and diet (Warbrick-Smith et al. 2006; Lee et al. 2008; Musselman et al. 2011) models. The effects of parental and grandparental diets, which accelerate development on poor diets, could affect these and *Drosophila* can offer a way of exploring the developmental origins of disease.

3.4.4 Conclusion, improvements and future directions

The evidence from the multigenerational diet experiment shows that *Drosophila melanogaster* exhibits adaptive effects in relation to the diet of the parental or grandparental generation, in thorax, survival and time to eclosion. This experiment was designed to mimic the transition between “Palaeolithic” and “Western” human diets, but it could be refined with an understanding of the limits of fly diets rather than human diets, as although they appear to respond in similar ways to diet, the parameters of fly diets may be different.

A range of diets that produce greater phenotypic differences in the flies should also produce a greater main effect of diet, and so the comparatively small intergenerational effects might also be larger. These could be tested against a common diet, to eliminate the effect of differing offspring diets. Having established an adaptive effect, this experiment could be extended to include both fecundity and offspring viability, which would directly tie parental diet to offspring fitness instead of through fitness indicators. It could also be extended to look at more outcomes relevant to human health, and explore further the effects of parental diets on obesity and lipid content, longevity, heart function, insulin resistance, activity levels and gene expression relating to these traits, which would provide a useful experimental model for exploring the Predictive Adaptive Response hypothesis.

Chapter Four

Does diet experience influence adult oviposition and feeding preferences?

4.1 Introduction

4.1.1 Predictive Adaptive Responses, oviposition and feeding preferences

The Predictive Adaptive Response hypothesis states that an organism's physiology may be partly shaped by the maternal environment, to adapt it to that environment (Gluckman and Hanson 2004; Gluckman and Hanson 2005; Gluckman et al. 2005a; Gluckman et al. 2005c; Gluckman and Hanson 2007). In Chapter 3, it was demonstrated that flies could respond to the diet of parental and grandparental generations with adaptive phenotypic plasticity in the proportion of eggs surviving, time to eclosion and male thorax length.

If the phenotype of the female fly is optimised to its own dietary environment by an adaptive response induced by the diet of its parents (or grandparents), it might be adaptive for it to feed on or lay its own eggs on that diet and may seek out this diet as an adult, in a similar way to that proposed by the Natal Habitat Preference Induction hypothesis (Davis and Stamps 2004). This developed from studies of Hopkins' host selection principle (Hewitt 1917). Hopkins observed that the mountain pine beetle *Dendroctonus monticolae* (Coleoptera: Scolytidae Hopk.) has two alternative hosts, the lodgepole pine and the yellow pine (Hewitt 1917; Barron 2001). He made the observation that beetles which had emerged on one species of pine tended to then breed on that species of pine, even if the alternative species was close by (Hewitt 1917; Barron 2001). A preference for the parental diet could have fitness consequences for the offspring, either by encouraging rapid suitable habitat selection or by the offspring ignoring habitats or food which might be more appropriate than the parental habitat (Davis and Stamps 2004; Dukas 2007). If this effect was strong it could ultimately lead to the formation of a new host race (Hewitt 1917; Thorpe and Jones 1937; Smith and Cornell 1979; Feder and Filchak 1999; Barron 2001).

Whether or not the parental diet would be the optimum environment for the fly, if it chooses the same environment as it already has experience of, for feeding or oviposition, it can be assumed that it at least recognises that environment as an

appropriate one. This would be the first requirement for claiming that the developmental environment of the fly has induced a preference for feeding or oviposition on that diet.

4.1.2 The influence of pre-imaginal and adult conditioning on insect oviposition and feeding preferences

Following on from Hopkins' ideas on host selection, several studies demonstrated that insects' pre-imaginal environment appeared to influence adult feeding choices (Thorpe 1939; Manning 1967) or oviposition choices (Thorpe and Jones 1937; Cushing 1941). Thorpe and Jones (1937) found that the preference for the odour of the normal host species (the moth *Ephestia kühniella*) of the ichneumoid wasp *Nemeritis canescens* could be altered by larval or adult exposure to an alternative host *Meliphora grisella* (Thorpe and Jones 1937). Later, Thorpe (1939) also found that exposure of *D. melanogaster* larvae or adults to peppermint oil lessened their adult aversion to the substance (Thorpe 1939) and Cushing (1941) found that larval experience altered oviposition preferences regarding novel substrates in *Drosophila guttifera* (Cushing 1941). Manning (1967) used geraniol in *D. melanogaster* larval diet and subsequently the adult flies were tested using a Y-tube olfactometer. They chose an arm at random, rather than showing aversion to geraniol as did normally-reared flies. This showed that the flies may have become habituated to the smell of geraniol (Manning 1967). An odour (peppermint oil) was also successfully associated with the presence of food in larval *D. melanogaster* by Herschberger and Smith (1967) and this preference persisted into the adult flies (Herschberger and Smith 1967). These experiments have been subsequently criticised as not having controlled sufficiently for the possibility of contamination of the pupal case by a chemical legacy from the larval environment (Jaenike 1982, 1983; Barron and Corbet 1999; Barron 2001).

To eliminate this possibility, when Barron and Corbet (1999) tested for an induced preference for menthol, *D. melanogaster* raised on a menthol-containing medium had their pupal cases washed before eclosion. They subsequently showed no greater preference for menthol than flies raised on menthol-free medium (Barron and Corbet 1999), suggesting that any pre-imaginal conditioning effect was caused by a chemical legacy on the pupal case. In an earlier study, prior experience as adults, but not as larvae, increased the proportion of eggs laid on tomato, tested versus apple, and banana versus squash (Jaenike 1983). Adult exposure to peppermint oil also increased subsequent attraction to substrates containing peppermint (Jaenike 1982).

Drosophila larvae have been shown to be capable of associative learning (Aceves-Pina et al. 1979; Dukas 1999) so any failure to retain information from the larval environment need not be due to a failure to learn it initially. Aceves-Piña et al. (1979) repeatedly paired an electric shock with either octanol or ethyl acetate odours as stimuli to *D. melanogaster* larvae, and wild-type larvae learned to avoid the odour associated with the electric shock, although they only retained this aversion for 30 min. Larvae which were mutants for the *dunce*, *cabbage* and *turnip* genes associated with learning did not subsequently avoid those odours (Aceves-Pina et al. 1979). Larvae can also learn to associate odours with predation risk and with food quality (Dukas 1999). Tully et al. (1994) trained *D. melanogaster* larvae in a similar way to Aceves-Pina et al. (1979), but then tested their odour preferences after eclosion. They found that even eight days after training as third-instar larvae, the flies retained an aversion to the odour of (Tully et al. 1994) ethyl acetate after it had been paired with electric shocks (Tully et al. 1994). Taken together, these experiments show that *D. melanogaster* larvae are capable of associative learning and of retaining memory through metamorphosis (Aceves-Pina et al. 1979; Tully et al. 1994; Dukas 1999). A similar result has been recorded in the moth *Manduca sexta* by Blackiston (2008), where the pairing of ethyl acetate with the stimulus of an electric shock produced an aversion to ethyl acetate in caterpillars which was still present in adult moths (Blackiston et al. 2008).

The mushroom bodies of the *Drosophila* brain are important in processing sensory inputs, olfactory learning and associative learning (Armstrong et al. 1998; Ito et al. 1998) and made up of neurons called Kenyon cells (Zars et al. 2000). One possible mechanism for this retention of memory through metamorphosis may be the persistence of Kenyon cell bodies through metamorphosis in *D. melanogaster* (Armstrong et al. 1998). During metamorphosis, the mushroom bodies are remodelled (Technau and Heisenberg 1982). However, although the number of Kenyon cell fibres within the mushroom bodies drops briefly at the onset of metamorphosis, a large proportion of the Kenyon cell bodies are retained through metamorphosis, which could provide a site for the retention of larval memory into adulthood (Technau and Heisenberg 1982; Armstrong et al. 1998). The gustatory pharyngeal sensillae are also retained from the larval to the adult animal in a modified state (Gendre et al. 2004), although gustatory information is thought to be processed in the sub-oesophageal ganglion rather than the mushroom bodies (Cobb et al. 2009). Many other larval sensory neurones play a role in the guiding the development of the adult nervous system (Williams and Shepherd 1999, 2002). Ray (1999) used larval

houseflies (*Musca domestica*) exposed to either peppermint oil or geraniol and washed before pupariation. The adult flies showed a attraction to the odours they had been exposed to as larvae, and this response could be transferred from one fly to another by injecting nervous tissue before pupariation had been reached, suggesting that this preference was remembered through metamorphosis (Ray 1999).

Drosophila melanogaster has also been shown to be influenced by the early adult environment. Stamps and Blozis cultured two groups of *D. melanogaster* on either banana or kiwi fruit for six months, then exposed females to their own larval environments for one day before allowing them to choose between two environments containing either banana or kiwi. Although whether the flies chose these environments as suitable for feeding or oviposition was not measured, significantly more flies chose their own environment over the alternative (Stamps and Blozis 2006).

In addition to habituation to a chemical which they previously showed aversion towards (Jaenike 1982, 1983), adult *D. melanogaster* are also capable of associative learning, regarding both food and adverse stimuli. Adults can be trained to associate odours with a reward of sugar-water and subsequently preferred the odour associated with the sugar-water. This effect lasted for 24 hr after 100 min exposure (Tempel et al. 1983). Aversive situations can also be associated with stimuli, for instance particular colours of lights were used at the same time as shaking and these colours were subsequently avoided (Folkers 1982). Similarly, when certain visual patterns were presented at the same time as heat from an infra-red beam, flies learned to fly away from these patterns and towards alternatives (Liu et al. 2006).

Diet and other environmental factors may influence adult insect oviposition or feeding choices as a result of exposure at different life stages. It is conceivable that conditioning may occur as a result of the maternal environment, at the larval stage, from residues on the pupal case or early adulthood. Although many of the experiments detailed above were conducted on olfaction rather than gustation, it is not unreasonable to assume that gustation may produce the same effects (Cobb et al. 2009).

The experiments detailed above that showed an induction of preference, by any means, were conducted using hosts or substrates that differed qualitatively from each other (Thorpe and Jones 1937; Thorpe 1939; Cushing 1941; Hershberger and Smith 1967; Manning 1967; Jaenike 1982, 1983; Stamps and Blozis 2006). If, as suggested by the Predictive Adaptive Response hypothesis (Gluckman and Hanson 2004; Gluckman

et al. 2005d), an organism's physiology may be optimised in anticipation of the continuation of the maternal environment, it could be adaptive behaviour on the part of a mother to bring her offspring up in that environment, and so maximise their survival and fitness. In the case of *D. melanogaster*, this parental care is limited to the choice of oviposition site, which also becomes the food source for the larvae.

In the context of the transition between a “traditional” and “modern” human diet (Popkin 2002; Eaton 2006), the difference between the two diets is more quantitative, relating to macronutrient ratios, rather than the choice between a “normal” and a “novel” diet. If flies are phenotypically adapted by parental or grandparental diet, and either this or their own experience also alters their macronutrient intake target, this could have implications for human dietary preferences.

4.1.3 Experimental aims

As *D. melanogaster* are capable of regulating their macronutrient intake target, to a ratio of 1:4 protein: carbohydrate (Lee et al. 2008), this experiment will test whether previous dietary experience can alter that intake target, and whether previous experience can alter oviposition preferences as the choice between the P and W diets will have fitness consequences for the next generation (Yang et al. 2008).

If feeding and oviposition choices are determined by previous diet, they may be induced in the egg (maternal), larval or early adult stages. Before attempting to distinguish between these stages, it is first necessary to show that any previous experience of the “P” low-carbohydrate or “W” high-carbohydrate diets actually influences adult *D. melanogaster* oviposition and feeding choices. If previous experience does change preferences, further work can be carried out to determine what part of a fly's experience causes the change. Adult *Drosophila* feed intermittently, although females, having the greater nutritional demands of egg production, feed more than the males (Carvalho et al. 2006; Melcher et al. 2007). It is possible that males and females may have different feeding preferences, and so only female flies were chosen for the feeding experiments to make a comparison between feeding and oviposition preferences.

Experimental hypotheses

The first hypothesis to be tested is that if *D. melanogaster* females are physiologically optimised to their current diet and their offspring will also be optimised to that diet, the current diet should be recognised as a more suitable oviposition site over a different diet.

Flies taken from cultures raised on a P or W diet over several generations (see Chapter 2) are expected to choose their own diet for oviposition rather than the alternative. If both are equally suitable then equal numbers should choose each diet, or if one diet is intrinsically more suitable for oviposition this should be chosen by both groups of flies. The null hypothesis is that there will be no difference in preference between the two substrates.

The second hypothesis is that if an organism is optimised by the maternal diet, it should choose this diet for feeding as an adult over an alternative diet if this maximises its fitness. Flies taken from cultures raised on a P or W diet over several generations (see Chapter 2) are expected to feed on their own rather than the alternative. Again, if both are equally suitable then each diet should be eaten in equal amounts, or one of the two diets may be chosen by both groups of flies. The null hypothesis is that there will be no difference in feeding preference between the two diets.

If individual isofemale lines' oviposition and feeding preferences are correlated with each other this may indicate that a similar mechanism lies behind their choices. A negative correlation might still indicate that the environment influenced choice, even though this would be in the opposite direction – so that if one isofemale line greatly preferred P for oviposition it might avoid it for feeding and vice-versa. No correlation between the two choices would suggest that oviposition and feeding choices are not linked. The null hypothesis is that there is no correlation between an isofemale line's oviposition and feeding preferences.

4.2 Methods

4.2.1 Culturing

Flies for the oviposition and feeding experiments were cultured as isofemale lines as described in Chapter 2. The flies had been on the experimental P (low-carbohydrate) and W (high-carbohydrate) diets for nine generations at the start of the experiments (see Chapter 2 for an explanation of the diets and Table 2.3 for their composition). All isofemale lines were used except line 16 and line 22, which had failed to lay enough eggs. This left 23 pairs of isofemale lines, one of each pair cultured on the P diet and the other on the W diet. The lines were numbered 1, 2, 3, 4, 5, 7, 8, 9, 10, 11, 12, 17, 18, 19, 20, 24, 25, 28, 29, 31, 32, 33 and 35. Whenever possible all lines were used in each repeat of each experiment. Each repeat was taken from a new generation of flies.

Prior to the start of each experiment flies were moved from the 7.5°C incubator (LMS Ltd.) to the 20° C incubator (LMS Ltd.) for three days before being introduced to the experimental set-ups. This ensured that the females were at the peak of their egg-laying potential (Prasad and Joshi 2003).

4.2.2 Oviposition choice experiment

The oviposition experiment was repeated five times (with each repeat acting as a blocking factor). Ten randomly selected female flies from each of the P and W isofemale lines were anaesthetised under CO₂ and introduced into the egg-laying chambers. These consisted of a 55 mm diameter petri dish (Fisher Scientific, Loughborough) with a ventilated tricorn beaker (Fisher Scientific, Loughborough) secured over the plate with tape. Within each beaker were two disks of fly food, one of the P diet and one of W diet, measuring 15 mm in diameter and 2 mm deep (Fig. 4.1). The disks had been prepared by allowing the food to set inside 100 mm long by 15 mm diameter plastic specimen tubes (Fisher Scientific) with the end removed. These were cooled quickly in a refrigerator at 4°C to ensure the diets did not start to separate before setting. Once set, the food tube was pushed out and sliced to size. Each end of the food tube was often damaged or filled with bubbles and so discarded, as it had been found during trials that rough areas of food tended to have more eggs laid on them than smooth areas. This had also been noted by previous studies (Rockwell and Grossfield 1978; Chess and Ringo 1985). The two food disks were placed equidistant from each other and the walls of the Petri dish.

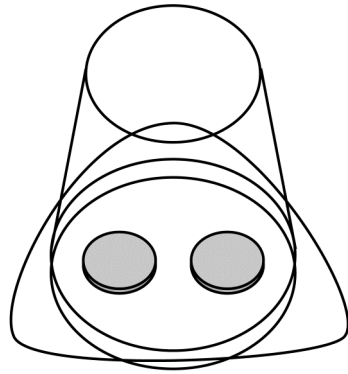


Figure 4.1: The laying chamber used in the oviposition choice experiment. One disk of P and one of W fly food were placed equidistant from each other and from the walls of the Petri dish, with a tricorn beaker secured on top.

Flies were sexed under CO_2 and ten females selected randomly before being placed into the upturned tricorn beaker. The petri dish was then secured on top. Once the flies had recovered from the CO_2 , the beaker was gently turned over so that the food was at the bottom. This ensured that flies did not start the experiment closer to one food or another, but would have to move towards the disks. The laying chambers were then placed in an incubator (LMS Ltd., Kent) set at 20°C on a 16:8 light: dark cycle. After 24 hours, the chambers were removed from the incubator and the flies discarded. The Petri dishes with the plates were frozen at -18°C to kill the eggs before the larvae could hatch. Previous trials had established that flies would choose to oviposit eggs on food instead of plain agar disks (Young 2007), showing that a decision was being made on oviposition sites. To ensure there was no effect of freezing on the number of eggs counted, flies were allowed to oviposit on 15 plates of fly food, the eggs counted and then frozen at -18°C for two days and recounted. There was no difference between counting freshly laid egg plates and frozen egg plates (paired t-test, $t=0.355$, 14 d.f., $p=0.728$). The eggs were counted under a dissection microscope (Olympus SZX7) at 4x magnification, using a grid placed above the food disk to help ensure no egg was missed or double-counted.

4.2.3 Feeding preferences experimental set-up

Female flies' feeding preferences were assessed by allowing flies to choose between two different diets, coloured either blue or red. Previous research had established that flies had no preference for blue or red food, although they preferred red food over green (Young 2007). The experiment was repeated three times and in each repeat two complete sets of 23 paired isofemale lines were tested, one whose P diet was dyed red and W diet was dyed blue, and another where colours were reversed. Female flies only were used in this experiment.

Initial no-choice trials had also established that, although flies were observed to eat blue food, it was very difficult to detect blue food through their abdomens. Therefore the concentration of blue dye was doubled to 30 ml l⁻¹, ensuring that the dye could be observed reliably. A set of mixed diets was made in the proportions 4Red(R):0Blue(B), 3R:1B, 2R:2B, 1R:3B, 0R:4B and five females introduced to feeding chambers containing only these diets. It was found that not enough food had been consumed to be visible after one day, and that after three days the diets began to dry out, so a feeding period of 48 hours was selected. Five females per diet colour combination were kept frozen at -18°C as reference to judge the subject flies' abdominal colours.

The choice chambers were 55 mm Petri dishes as in the oviposition experiment, but rather than disks of food, these had a 2mm layer of agar solution at a concentration of 20 g l⁻¹ set in the bottom (Fig. 4.2). Out of this, two wells were cut of 10 mm diameter each. The wells were filled with the P or W experimental diets but with two alterations. The amount of agar was reduced from 20 to 10 g l⁻¹, to facilitate the adult flies' feeding, and 15 ml l⁻¹ of red food dye (Supercook) was added to one diet and 30 ml l⁻¹ of blue food dye (Supercook) added to the other. While still liquid, these were pipetted into the wells in the agar until they were slightly over-full. On cooling these shrank so they were level with the flat surface. Ten females from each diet and isofemale line were introduced into the choice chamber under CO₂ as for the oviposition experiment, and the chambers placed in an incubator (LMS Ltd., Kent) at 20°C for 48 hours under a 16:8 hour light:dark cycle. Flies were not starved before the experiment as this may influence them to select the high-carbohydrate diet over the low-carbohydrate (Hoffmann 1985), but were kept on their culturing diet.

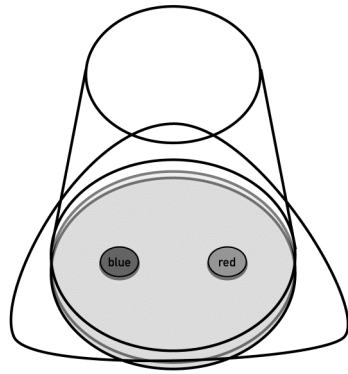


Figure 4.2: The choice chamber used in the feeding choice experiment. The Petri dish had a layer of agar with two wells cut in it. These wells were filled with dyed fly food, one dyed red and the other blue. One was the P diet and the other W diet. A tricorn beaker was secured on top.

After 48 hours the chambers were removed from the incubator and the ten flies from each (excepting the few which had died) were flash frozen in liquid nitrogen and stored at -18°C , so that gut contents were preserved *in situ*. The frozen flies were then randomly assigned codes so that colour scoring would be without prior knowledge of the diet treatment of the flies, measured against the reference flies previously obtained. The flies were scored 1 to 5, equivalent to the scale 4R:0B, 3R:1B, 2R:2B, 1R:3B, 0R:4B.

4.2.4 Statistical analysis

Oviposition

The oviposition trials were scored by the number of eggs laid on the surface of each disk of food (including the vertical side of the disk). The percentage of eggs laid on the P diet was arcsine square-root transformed, as percentage scores, being bound by 0 and 100%, do not follow a normal distribution (Crawley 2007), and tested for normality (Kolmogorov-Smirnov test) and homogeneity of variance (Levene's test) in SPSS 19.0 (SPSS Inc., Chicago). The arcsine square-root transformed scores for oviposition preferences followed a normal distribution (Kolmogorov-Smirnov test, $Z = 0.585$, $p = 0.883$) and the variance between P- and W-cultured isofemale lines was not significantly different (Levene's test. $F = 3.225$, $p = 0.074$). The transformed scores were then analysed using a Generalized Linear Model in R 2.1.8 (R Development Core Team 2008), using a model building approach where factors or interactions were removed from the full model if their removal did not significantly increase the residual variance. Interactions were tested in the sequence of highest order first, and then least significant

interaction first, assessed by an ANOVA on each model. The factor “Isofemale line” was kept in the model regardless of significance and none of its interactions were included in the model, as “Isofemale line” was the replicate in this model, and the 23 isofemale lines are a randomly selected sample from the original AV population (see Chapter 1). The number of eggs laid in each chamber was not used as a weighting factor in the analysis, as the number of eggs laid may not correspond to the number of choices of oviposition sites made by the flies (Stamps and Blozis 2006). In the preliminary experiments, eggs were often observed to be laid in clumps and flies are known to use already-laid eggs as a cue for their own egg laying (del Solar and Palomino 1966; del Solar 1998). Therefore each egg-laying chamber was treated as an unweighted replicate and contributed one data point to the set. Overall oviposition preference was assessed by a one-sample t-test against a 50% model, using the arcsine square-root transformed percentage preferences of each isofemale line, in SPSS Statistics 19.0 (SPSS Inc., Chicago).

Feeding preferences

Flies that had not apparently eaten or had died were excluded from the analysis. Flies were examined under a dissecting microscope (Olympus SZX7) at an objective setting of 4x, and the colour of the abdomen compared to the reference flies collected earlier. Each fly was scored between 0 and 4, with 0 representing all-red diet and 4 all-blue. The scores were converted to a proportion (where 0 represents consuming only W diet and 1 represents consuming only P diet) and transformed using the arcsine square-root transformation (Crawley 2005). The transformed scores were then tested for normality (Kolmogorov-Smirnov test) and homogeneity of variance (Levene’s test) in SPSS 17.0 (SPSS Inc.) and analysed using a generalized linear model in R 2.8.1 (R Development Core Team 2008). The proportion of P food consumed, when transformed using arcsine square-root, followed a normal distribution (Kolmogorov-Smirnov test, $Z = 0.818$, $p = 0.516$) and there was equality of variance between groups (Levene’s test, $t = -1.297$, 226 d.f., $p = 0.196$). The figures below in Section 4.3 use the untransformed percentage data, although the transformed data were used in all statistical analyses.

The colour of the P diet in each repeat was also included in the analysis to check that colour did not influence choice. As for the oviposition analysis, a model building approach was used where factors and interactions were removed from the full model to construct the minimal adequate model and then significant factors added back in.

Isofemale line was once again kept in the model regardless of significance and none of its interactions tested. The food preference scores were not weighted by the number of flies counted as it was not possible to be certain that each fly had made an independent feeding decision. Overall feeding preference was assessed by a one-sample t-test against a 50% model, using the arcsine- square-root transformed percentage preferences of each isofemale line, in SPSS Statistics 19.0 (SPSS Inc., Chicago).

The preferences of each isofemale line for P or W diet were also tested, as isofemale line means for both diets and then separately for the flies cultured on P and W diets, for any correlation between oviposition and feeding preference, using a Pearson's correlation test in SPSS Statistics 19.0 (SPSS Inc., Chicago).

4.3 Results

4.3.1 Oviposition preferences

If flies are physiologically optimised to their diets by a PAR mechanism, it is expected that they will choose to oviposit on the same substrate as their current diet, as this would maximise the fitness of their offspring. Females from 23 isofemale line pairs, one half of each pair raised on P diet and the other on W, were offered the choice of P or W diet oviposition sites. The null hypothesis was that previous diet would not influence the choice of oviposition sites. The response variable was the proportion of eggs laid on the P substrate, and for the null hypothesis to be accepted there should be no difference in the proportion of eggs laid on the P diet oviposition substrate by flies cultured on P or W diets. Each data point corresponds to the proportion of eggs laid on the P diet substrate in one trial of 10 flies from one isofemale line. Figures are given below as percentages, although the data used in the statistical tests were arcsine square-root transformed proportions.

Overall, flies preferred to lay eggs on the P diet, with a mean of $66.86\% \pm 2.06\%$ being laid on P. This was significantly higher than the 50% that could be expected by chance (one sample t-test, $t = 7.899$, 202 d.f. $p < 0.0001$). Previous diet was not a significant factor in determining the flies' egg-laying preferences (change in deviance = 0.0067, 1 d.f., $p = 0.784$, Table 4.1). The mean percentage preferences for the two groups were almost identical, with flies raised on a P diet laying $66.5 \pm 3.1\%$ on P, while flies raised on a W diet laid $67.2 \pm 2.7\%$ on P (Fig. 4.3).

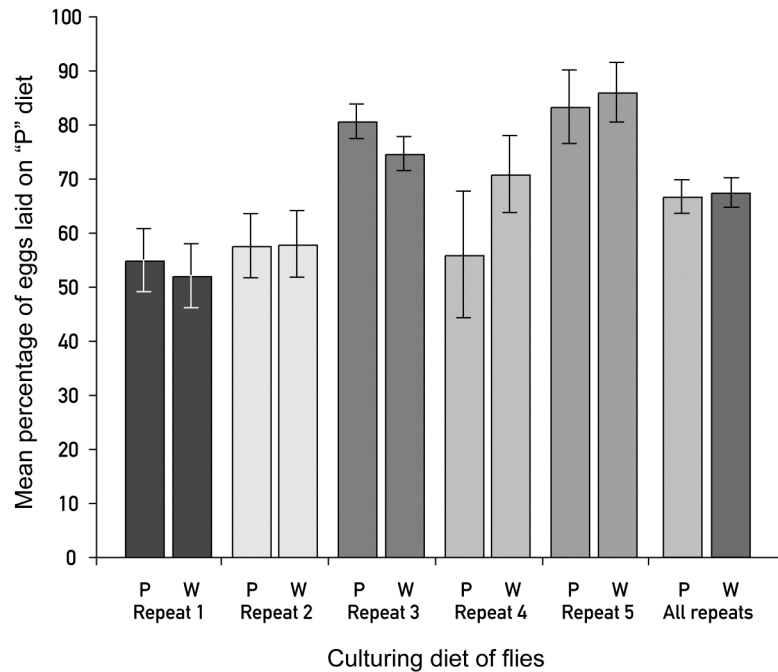


Figure 4.3: The mean of the percentage of eggs laid on “P” (low carbohydrate) substrate in each repeat, depending on the flies’ culturing diet experience (P or W), and the overall mean across all repeats. Left-hand bars: mean percentage of eggs laid on P diet by female flies raised on P diet. There were 101 replicates over the 5 repeats. Right-hand bars: mean percentage of eggs laid on P diet by flies raised on W diet, 102 replicates over the 5 repeats. Error bars represent ± 1 standard error of the mean.

The proportion of eggs laid on the P diet was significantly different between repeats (change in deviance = 4.315, 4 d.f., $p < 0.001$). Different isofemale lines were significantly different in their degree of preference for oviposition substrate (change in deviance = 6.7343, 22 d.f., $p < 0.001$, Fig. 4.4).

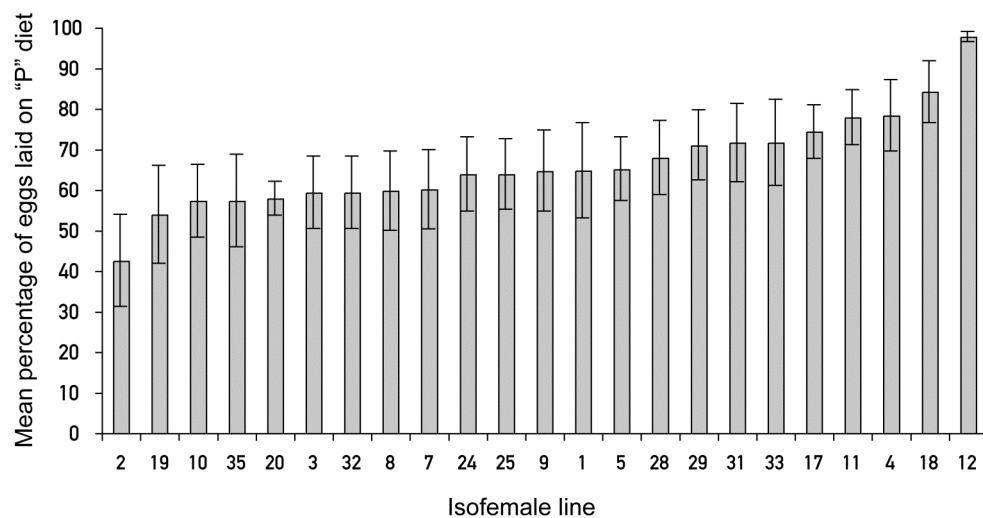


Figure 4.4: The mean of the percentage of eggs laid on “P” (low carbohydrate) substrate by individual isofemale lines. Isofemale lines are ordered by the mean percentage of eggs laid on P. Error bars represent ± 1 standard error of the mean.

Minimal adequate model:

arcsine square root (proportion of eggs on diet P)

Factor	res. dev.	– dev.	res. d.f.	– d.f.	P	sig.
Null	32.8470		202			
Block	28.5320	4.3150	198	4	< 0.001	***
Iso	21.7978	6.7343	176	22	< 0.001	***
block:iso	8.8048	12.9929	99	77	< 0.001	***
Diet	8.7981	0.0067	98	1	0.7840	

Table 4.1: The minimal adequate model for factors controlling oviposition preference on the two diets P or W. Key to model: res. dev. – residual deviance; d.f. – degrees of freedom; block – repeat of experiment; iso – isofemale line; diet – culturing diet of flies.

4.3.2 Feeding Preferences

If flies are physiologically adapted to their diets by an adaptive response to their developmental or maternal diets, as suggested by the mismatch hypothesis (Gluckman and Hanson 2004), their fitness would be maximised by choosing to eat that diet as adults. This was tested by offering a choice of diets (P or W) to two groups of female flies from 23 isofemale lines, one group having been cultured on a lower carbohydrate diet (P) and one on a high-carbohydrate diet (W). The diets were dyed blue or red and in each repeat there were two groups of the 23 isofemale lines, one with the P diet dyed red

and the other with the W diet dyed red. The null hypothesis was that previous diet experience would not influence feeding preferences.

Flies ate significantly less of the low-carbohydrate P diet ($31.26 \pm 1.22\%$ of the total diet consumed) than could be expected by chance, and correspondingly they preferred the high-carbohydrate W diet (one-sample t-test, $t = -13.984$, 227 d.f., $p < 0.001$).

The flies' previous diet was not a significant factor in determining their current diet choices (change in deviance = 0.0594, 1 d.f., $p = 0.262$, Table 4.2). The P diet made up $29.50 \pm 1.74\%$ of the total diet for flies that had been cultured on the P diet, and $32.92 \pm 1.70\%$ for those cultured on the W diet (Fig. 4.5). The effect of culturing diet did not change according to the colour of the experimental diet and so this term was removed from the statistical model. The colour of the dye added to the experimental diets did not influence the flies feeding choices as a main effect (change in deviance = 0.1255, 1 d.f., $p = 0.103$).

Minimal adequate model:
arcsine square root (proportion of
P diet in total diet)

Factor	res. dev.	\pm dev.	res. d.f.	d.f.	p
Null	13.1848		227	-	
Block	10.7855	2.3994	225	2	< 0.001 ***
Iso	9.6211	1.1643	203	22	0.318
Block:iso	7.7763	1.8448	163	40	0.530
Colour	7.6509	0.1255	162	1	0.103
Diet	7.5915	0.0594	161	1	0.262

Table 4.2: The minimal adequate model for factors controlling feeding preference on the two diets P or W. Key to model: res. dev. – residual deviance; d.f. – degrees of freedom; block – repeat of experiment; iso – isofemale line; colour – dye colour in experimental diet; diet – culturing diet of flies.

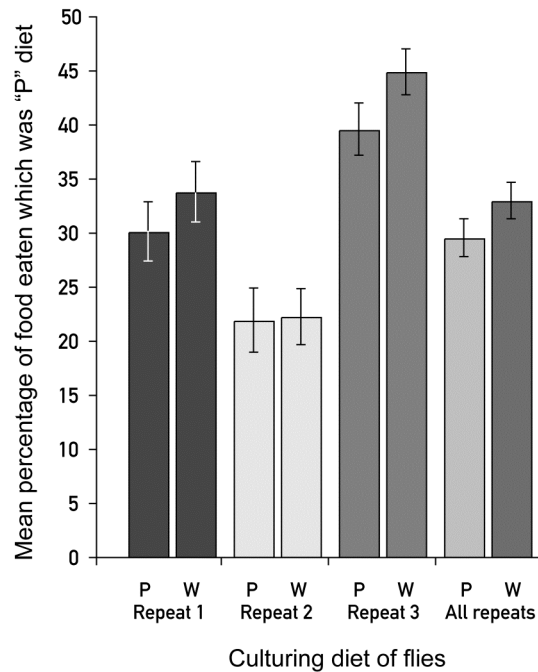


Figure 4.5: The mean proportion of food eaten that was “P” (low carbohydrate) substrate in each repeat and the overall mean across all repeats, as a percentage of total food eaten depending on the flies’ culturing diet experience (P or W). **Left-hand bars:** mean percentage of food eaten by female flies raised on P diet which was “P”. **Right-hand bars:** mean percentage of food eaten by female flies raised on W diet which was “P”. There were 228 trials, each with 10 female flies from an isofemale line, in three blocks. In each repeat ten different flies from each isofemale line and diet group were tested in two groups with P dyed either blue or red in each. Error bars represent ± 1 standard error of the mean.

The feeding experiment was repeated three times, using each repeat as a blocking factor, and this accounted for most of the variation in dietary preferences (change in deviance = 2.3994, 2 d.f., $p < 0.001$). However, there was no progressive change across the repeats (Fig. 4.5) as flies in the second repeat consumed less of the P diet ($22.0 \pm 2.0\%$) than either those in the first repeat ($31.9 \pm 1.96\%$), or the third ($42.4 \pm 1.62\%$). Individual isofemale lines did not vary in their preferences for the P diet (change in deviance = 1.1643, 22 d.f., $p = 0.318$, Fig. 4.6).

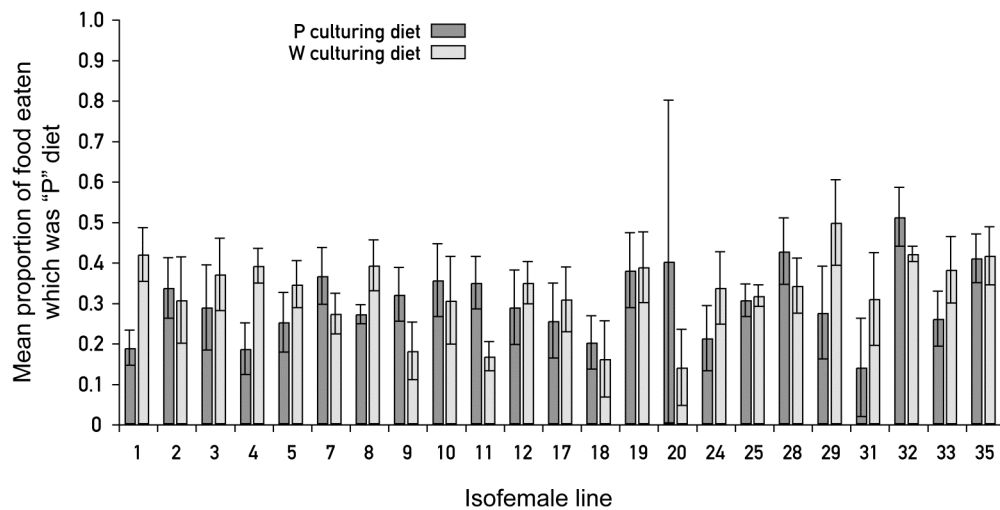


Figure 4.6: The mean proportion of food eaten that was “P” (low carbohydrate) diet by each isofemale line as a percentage of total food eaten depending on the flies’ culturing diet experience (P or W). **Left-hand bars:** mean percentage of food eaten by female flies raised on P diet which was “P”. **Right-hand bars:** mean percentage of food eaten by female flies raised on W diet which was “P”. There were 228 trials, each with 10 female flies from an isofemale line, in three blocks. In each repeat ten different flies from each isofemale line and diet group were tested in two groups with P dyed either blue or red in each. Error bars represent ± 1 standard error of the mean.

4.3.3 Correlation of oviposition and feeding preferences

As noted above, flies preferred to oviposit on the low-carbohydrate P diet while preferring to feed on the high-carbohydrate W diet, and individual isofemale lines varied in their degree of preference for oviposition site. To determine whether for each isofemale line, there was a relationship between the degree of preference for feeding on and for oviposition on the P diet, the correlation between percentage of eggs laid on P diet and the percentage of P diet making up the total diet was measured. The null hypothesis was that would be no correlation and for each isofemale line the degree of preference for P as an egg-laying site could not predict the degree of preference for P as food, or vice-versa.

Most of the isofemale line means were clustered around 50 – 80% for oviposition preference and 20 – 40% for feeding preference on P (Fig. 4.7), but there was no clear pattern to indicate any relationship between the two preferences. There was neither a

negative nor a positive correlation between preference for feeding on P and oviposition on P when the mean preferences of each isofemale line were compared (Pearson correlation test, Pearson correlation = -0.360 , $N = 23$, $p = 0.91$, Fig. 4.7).

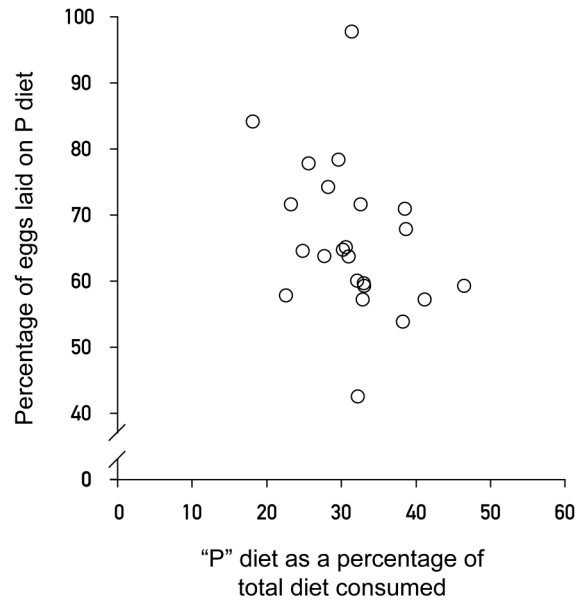
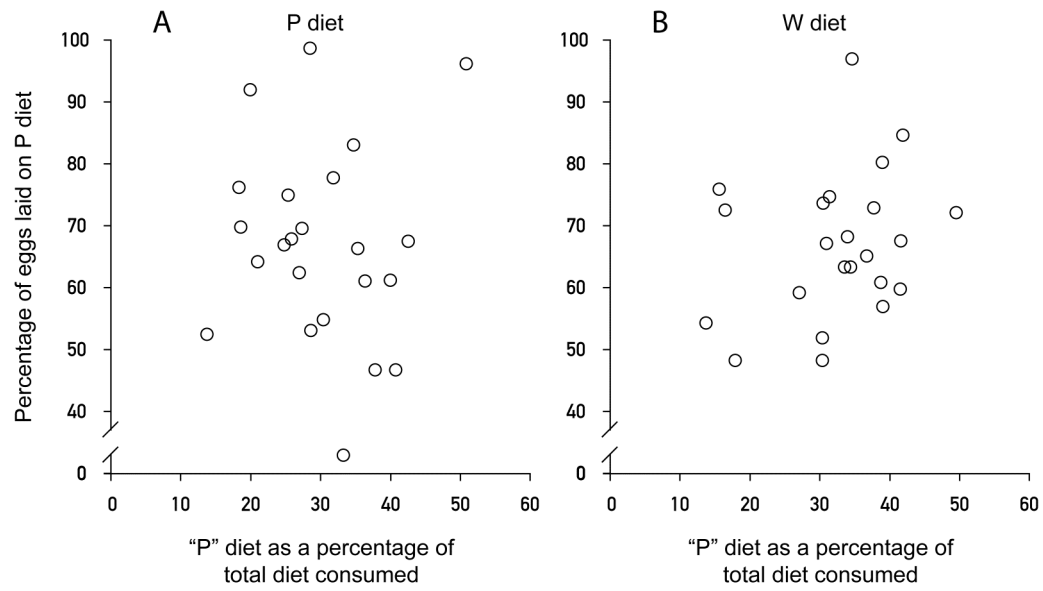


Figure 4.7 Percentage of P diet in the total diet consumed and the percentage of eggs laid on P diet by each isofemale line. Each circle represents the mean preference for an isofemale line over all repeats and diets.

When the isofemale line preferences for the P diet were split by culturing diet, there was no correlation between preference for feeding on P diet and oviposition on P diet whether the flies had been cultured on P (Pearson correlation 0.014 , $N = 23$, $p = 0.950$, Fig. 4.8a) or on W (Pearson correlation 0.238 , $N = 23$, $p = 0.274$, Fig. 4.8b).



4.4 Discussion

4.4.1 Summary of results

The culturing diet of the flies did not influence the feeding or oviposition preferences of the flies. The two groups P and W both strongly preferred to feed on the high-carbohydrate W diet and this made up 68.8% of their total diet with little difference between the groups. They preferred to oviposit on the P diet, with a mean proportion of 66.9% of eggs being laid on this diet. Individual isofemale lines significantly varied in their preference for oviposition substrate, but not in feeding preferences. There was no positive or negative correlation between isofemale lines' preferences for oviposition and feeding.

4.4.2 Preferences and PARs

The mismatch hypothesis, where offspring of parents on a particular diet have higher fitness (or are more healthy) if they develop on the same diet as their parents (Gluckman and Hanson 2004; Godfrey et al. 2010), corresponds to one of the patterns of adaptive response that might be seen in response to parental (or grandparental) diets (Gilchrist and Huey 2001; Ellison and Jasienska 2007; Monaghan 2008). Although the health effects of being an adult in a different dietary environment to the parental or developmental one might be severe, the consequences for fitness might be different (Williams 1957). Adult flies would be expected to make feeding and oviposition choices which maximise their own immediate fitness (and their offspring's by extension) above all else, especially as the lifespan of a wild, as opposed to laboratory, *Drosophila* might be only around six days (Rosewell and Shorrocks 1987).

Previous diet experience did not affect subsequent choice of diet or oviposition site at all, either as a main effect or as an interaction with any other factor. This was unexpected, as early adult experience has been shown to strongly affect subsequent preference (Jaenike 1982, 1983; Stamps and Blozis 2006). However, most previous studies which have claimed to show pre-imaginal or early adult conditioning have used a novel chemical such as peppermint oil (Thorpe 1939) or geraniol (Manning 1967), or a non-chemical stimulus such as an electric shock (Tully et al. 1994). Studies using natural foods have used different kinds of food in their training and choice assays, for example different fruits (Jaenike 1983). In that study, a range of natural foods did provoke a response, but the interpretation of the results was that there were particular novel

chemicals present in some of the foods which were the actual stimulus that the flies responded to. In the oviposition and feeding preference experiments presented here, the difference between the two foods was of degree rather than kind, and so the extra sucrose in the W diet may have represented an irresistible stimulus to flies already used to a diet containing sucrose.

4.4.3 Feeding preferences

The clear preference for feeding on the W diet rather than the P diet may be a consequence simply of the extra energy that may be gained from this diet. Young (2007) found that only a larval diet containing 0% sucrose changed adult preferences, but as the flies in this experiment had adult experience of the diets, there was a possibility that this might have changed their preferences (Young 2007). When Lee et al. (2008) allowed flies to self-select the proportions of macronutrients in the diet, they allowed a free choice of sugar or yeast, separately (Lee et al. 2008) whereas in this experiment the protein content of the diets was the same although the carbohydrate was much greater in the W diet.

Two diets with mixed sucrose and yeast may have presented a different choice than the same constituents offered separately. To the flies, the P and W diets may have appeared more as a choice between some sugar and more sugar, for the same effort and so they may have maximised energy intake rather than regulated macronutrient ratios in this case. This did change their intake compared to the self-selected target (Lee et al. 2008), but not in response to diet experience. The main adaptive effect of eating the W diet, decreased time to eclosion, was detected in the grand-offspring rather than offspring generation (Chapter 3) and so it is not likely that this was a reason for the observed feeding choices.

4.4.4 Oviposition preferences

Whether flies had been cultured on the P or W diets, the fitness of the offspring would be enhanced by laying eggs on the P diet as, in the multigenerational diet experiment (Chapter 3), these eggs would develop to eclosion faster, have a larger thorax, larger wings and a higher dry weight, although at the expense of a slightly lower survival rate and lower lipid content than those laid on the W diet (Chapter 3). This is particularly true for the flies cultured on the P diet, but the flies from the W diet would also enhance the fitness of their potential offspring by laying eggs on the P substrate.

The W diet had an osmolality almost twice that of the P diet (Chapter 2, section 2.3.5), but this is not thought to have influenced oviposition decisions (Yang et al. 2008). Yang et al. (2008) experimentally lowered the sensitivity of the sweet-taste receptor (*Gr5a*) neurons and found that this was enough to significantly increase the egg-laying rate on a sucrose medium, and also that the flies chose lower concentrations of sugar over higher, and that the osmolality of the diet did not influence oviposition (Yang et al. 2008), suggesting that flies respond to sucrose and not some other aspect of the medium. The fitness benefits for the eggs laid on the P diet are increased size of the offspring, and decreased time to eclosion (Chapter 3) and potentially greater lifetime egg production (Lee et al. 2008), which may explain why flies preferred oviposition on the lower carbohydrate diet.

4.4.5 Correlation between feeding and oviposition preferences

The contrast between oviposition and feeding preferences may be due to differing needs for oviposition and adult feeding. Flies can show a similar differential feeding and oviposition preferences when presented with a different pair of food types. When given a choice between foods containing acetic acid or plain fly food, flies preferred to oviposit on the food containing acetic acid, but also avoided this food when not laying eggs (Joseph et al. 2009). However in Joseph's study it was concluded that the positional avoidance effect of acetic acid was due to olfaction rather than gustation (Joseph et al. 2009), which was not an issue in the experiments presented here as the diets differed only in the amount of sucrose present.

There was no correlation between oviposition and feeding preferences. While oviposition was found to be significantly different between isofemale lines and feeding was not, it was still possible that the two preferences might be linked. A strong positive or negative correlation would have shown that they might be linked, even though flies preferred one for oviposition (P) and the other (W) for feeding. However an individual isofemale line's feeding preferences could not have been predicted by their oviposition preferences and so different criteria, one set for adult and another for offspring fitness, may have been involved in their selection

4.4.6 Variation in oviposition preference between isofemale lines

Different isofemale lines had significantly different oviposition preferences, ranging between 42.5 ± 11.36 % (line 2) and 97.8 ± 1.23 % (line 12) of eggs laid on the

low-carbohydrate substrate. However, despite this wide variation, no isofemale line laid significantly more eggs on the W diet than on the P diet. Individual isofemale lines did not vary in their choice of oviposition substrate depending on their previous diet experience and this between-isofemale line variation is indicative of genetic variation controlling oviposition preferences (Hoffmann and Parsons 1988; Sheeba et al. 1998; David et al. 2004). In view of the effect of altering the sensitivity of the sweet-taste detecting neurons (Yang et al. 2008), this might be where some of this variation stems from.

4.4.7 Conclusion

Although flies did not alter either oviposition or feeding preferences to match their previous experience, they did make choices that would maximise their offspring's fitness (oviposition on P) and chose the more energy-dense of the two foods offered. This may not have maximised their lifetime egg-laying rate but may have been the best choice for the short-term as it would maximise energy intake for minimum effort while maintaining protein intake, if eaten in the same quantity as the alternative lower energy-density diet. Even if flies (or humans) are phenotypically adapted to a less energy-dense diet, they may still prioritise energy intake when given a free choice.

4.4.8 Acknowledgements

RS was assisted by Holly Pink (HP), a University of Southampton undergraduate student from the School of Biological Sciences, as part of her third-year project. HP carried out the first two repeats of the feeding preference experiments and the first three repeats of the oviposition experiments, and used the data for her own project. RS designed the experiments, carried out the remainder of the repeats of both experiments and all the analysis presented in this chapter.

Chapter Five

The response of *Drosophila melanogaster* to altered dietary protein: carbohydrate ratio and calorific content

5.1 Introduction

The nutritional causes of the epidemics of diet-related disease, including obesity, diabetes and cardiovascular disease, are not yet clear, with various candidates put forward as the main driver, including the energy density of modern diets (Bray and Popkin 1998; Rolls 2000; Prentice and Jebb 2003), the relative proportion of protein amongst other macronutrients (Wade et al. 1981; Theall et al. 1984; Eaton et al. 1997; Simpson et al. 2003; Eaton 2006; Warbrick-Smith et al. 2006; Brooks et al. 2010), lack of exercise causing an altered energy balance (Prentice and Jebb 1995; Eaton and Cordain 1997; Towler and Hardie 2007; Johnson et al. 2010), cultural and psychological factors (Elrick et al. 2002; Cameron and Doucet 2007; Brooks et al. 2010), and the predictive adaptive response (PAR) hypothesis (Gluckman and Hanson 2004; Hanson 2005). Leaving aside the energy balance of diet and exercise, cultural factors and, for the moment, PARs, the relative roles of diet quality (taken to mean the proportion of protein) and diet quantity (the total calories ingested) can be analysed using a model animal and an array of diets using a technique known as the geometric framework (Simpson and Raubenheimer 1993, 1995).

5.1.1 The Geometric framework

In the geometric framework, an array of diets are distributed within a two (or more) dimensional phase space where each dimension represents one aspect of a diet, for instance, a macronutrient (Simpson and Raubenheimer 1995). The other diet components (for example micronutrients) are held at an optimal level for that animal. This allows a response to the diets (for example physical size, fitness measures or lifespan) to be mapped onto this two-dimensional space in a way that allows the effect of individual components of the diet to be considered both singly and in interaction with each other and the total amount of macronutrients in the diets (Simpson and Raubenheimer 1995). It also allows an estimation of a baseline diet (or target) for a model organism (Simpson and Raubenheimer 1995; Lee et al. 2008). In the case of *Drosophila melanogaster* this is otherwise difficult to obtain, as although its nutritional needs in the laboratory are well-characterised (Sang 1959; Markow and O'Grady 2005), the diet

composition of wild *Drosophila melanogaster* is not fully understood (Lachaise et al. 1988). *Drosophila melanogaster* is a human commensal and will breed on a wide variety of rotting fruit and vegetation, eating both the fruit itself and moulds, especially yeast, growing on it. In its natural East African range it is known to favour bananas, but also mangoes, papaya and guava. These sources vary in their carbohydrate and protein content and decay also alters the macronutrient content of the fruit (Lachaise et al. 1988; Keller 2007; Stephan and Li 2007).

To measure the macronutrient intake target for an organism, in terms of the ratio of protein to carbohydrate (P:C ratio) and total amounts of macronutrients in the diet, it is offered a choice of at least two diets of varying macronutrient composition and the amounts ingested are measured over a period of time (Simpson and Raubenheimer 1995). A recent study established a self-selected intake target for *D. melanogaster* adults (Lee et al. 2008). The flies were given a choice of one solution containing sucrose and a suspension of yeast in water in capillary tubes, and the amount of each macronutrient each fly ingested was measured. The self-selected target was found to be a P:C ratio of 1:4 (approximately 30µg of protein d⁻¹ to 120µg carbohydrate d⁻¹) (Lee et al. 2008).

5.1.2 Recent insect nutrition studies using the geometric framework

A strength of the geometric framework approach is that it allows researchers to test competing hypotheses to explain a particular response (Simpson and Raubenheimer 1995; Simpson et al. 2010; Cotter et al. 2011). The extension of lifespan by dietary restriction was first thought to be a result of caloric restriction, and the phenomenon was reported in rodents (McCay et al. 1935; Weindruch and Walford 1982; Heilbronn and Ravussin 2003), yeast (Kaeberlein et al. 2007), *D. melanogaster* (Chapman and Partridge 1996; Partridge et al. 2005), *C. elegans* (Klass 1977) and in humans (Willcox et al. 2006; Roberts and Schoeller 2007; Willcox et al. 2007). Recent studies have pointed to both reduced caloric input (Min et al. 2007) and low protein (Mair et al. 2005; Min and Tatar 2006) being important in increasing lifespan. Using the geometric framework approach, Lee *et al* (2008) looked at both reproduction and lifespan in *D. melanogaster*. Lifespan and reproduction are often thought to be subject to a trade-off due to resource limitation (Williams 1966; Skorupa et al. 2008; Flatt 2011), although a recent study involving ablation of the germ line in female *D. melanogaster* did not find any resultant increase in lifespan (Barnes et al. 2006). Lee et al. (2008), using an array of diets of different P:C ratios (termed a “nutritional rail”) and with different caloric contents, showed that the

dietary restriction effect in *D. melanogaster* is not purely caused by either caloric or protein restriction, but also by the relative proportions of protein to carbohydrate in the diet of the flies, whose lifespan was at a maximum on diets of 1:16 P:C. This study also showed that lifetime egg-production was maximised on a ratio of 1:4 P:C and that the number of eggs laid per day was maximised on a ratio of 1:2 P:C (Lee et al. 2008). This provides some limited evidence for a trade-off between reproduction and ageing, although the responses to diet (lifespan and egg-production) do not trade-off exactly as the slope of each response is different (Lee et al. 2008; Tatar 2011). In a parallel experiment using the Queensland fly *Bactocera tryoni*, varying P:C ratios and calorific content of the diets produced a similar pattern, except that the P:C ratio for maximum lifespan was 1:21 and that for lifetime egg production was 1:3 (Fanson et al. 2009). Here, flies displayed compensatory feeding on the lower calorie diets, particularly when the P:C ratio was very low, and this did alter the observed patterns of the responses compared to an analysis without measuring intake (Fanson et al. 2009).

Skorupa *et al.* (2008) set up a range of no-choice diets, using *D. melanogaster*, to investigate lifespan, triglyceride and protein content, fecundity and intake. Although the results for lifespan and fecundity were broadly similar to those obtained by Lee et al. (2008), there were differences in the position of the peak fecundity (lower yeast: sugar ratio) and lifespan, where the maximum lifespan occurred at a lower yeast: sugar ratio than in Lee et al. (2008). Triglyceride content was found to be primarily linked to carbohydrate intake and increased with age, while protein content was linked to the yeast concentration in the diet (Skorupa et al. 2008). The different P:C values of the peaks are unlikely to be due to compensatory feeding as this would not alter their position relative to the yeast: sugar diet rails, but would only alter the distance from the origin along a rail. Skorupa et al. (2008) speculate that this difference may be caused by housing flies individually in Lee et al. (2008).

The trade-off between reproduction and lifespan was also the subject of an investigation in the tephritid fruit fly *Anastrepha ludens*, using a geometric framework of diet ratios of yeast: sugar of 0:1, 1:24, 1:9 and 1:3 and five dilutions (Carey et al. 2008). The results were very similar to those obtained by Lee et al. (2008), Skorupa et al. (2008) and Fanson et al. (2009), in that both lifespan and egg production were primarily controlled by the yeast: sugar ratio, and peaked at different points in the nutrient space (Carey et al. 2008). This study has been criticised for not measuring actual amounts ingested (Fanson et al. 2009), which could distort the nutrient phase-space if

compensatory feeding occurred, but it should also be noted that the conclusions in Lee et al. (2008) were similar whether food actually ingested, or food composition was used as a factor.

The immune response of *Spodoptera littoralis* has recently been examined using the geometric framework (Cotter et al. 2011). Dietary restriction had previously been shown to affect immune function, either positively in *D. melanogaster* (Ayres and Schneider 2009) and mice (Oarada et al. 2009) or negatively, regarding protein but not calories in mice infected with salmonella (Peck et al. 1992), and in the mealworm beetle *Tenebrio molitor* (Siva-Jothy and Thompson 2002). Cotter *et al.* (2011) used an array of diets varying in calorific content and P:C ratio to determine the most important nutritional factors underlying immune response indicators. They found that although some traits could be explained using a single dietary factor – for example haemolymph protein was controlled by the amount of protein ingested – overall larval performance was better explained by a combination of factors, having a peak value at a point along the 1:1 P:C diet rail (Cotter et al. 2011). *S. littoralis* larvae increased the amount they ate on the both the low protein and the low calorie diets, although they did not compensate by enough to match the consumption by larvae on more concentrated diets (Cotter et al. 2011).

These studies, together with others using the geometric framework to examine reproduction and lifespan in the cricket *Teleogryllus commodus* (Maklakov et al. 2008), the mexfly *Anastrepha ludens* (Kulminski et al. 2009), *Spodoptera exigua*, using temperature and P:C ratio (Lee and Roh 2010), and the Western Cherry fruit fly *Rhagoletis indifferens* (Yee 2010), which all found that the P:C ratio was the most important factor determining reproduction and lifespan, demonstrate that this technique can provide a more detailed and comprehensive view of how macronutrients affect the traits under investigation than can be achieved by varying a single factor. Using the geometric framework approach allows the interaction of different factors to be examined in a way not possible when only one factor is varied at a time and has highlighted the importance of nutrient ratios in determining fitness and longevity (K. P. Lee 2008; Fanson et al. 2009; Simpson and Raubenheimer 2009; Cotter et al. 2011; Tatar 2011).

5.1.3 Metabolic signalling mechanisms

One possible mechanism that could help explain the emerging importance of the P:C ratio is the complementary insulin signalling and target of rapamycin (TOR) pathways, which are linked and together help to control growth and differentiation (Bateman and McNeill 2004; Léopold 2004; Layalle et al. 2008; Simpson and Raubenheimer 2009). Down-regulation of TOR signalling during development increases the time to pupariation and decreases the final size of the larvae in *Drosophila* (Layalle et al. 2008). Experiments involving inhibition of the expression of dTOR (*Drosophila* target of rapamycin) extended lifespan in a similar way to dietary restriction, suggesting that this is a controlling mechanism (Kapahi et al. 2004). It was also found that this inhibition was only effective in extending lifespan if it was expressed in fat and muscle tissue (Kapahi et al. 2004). The fat body appears to function as a nutrient sensor, monitoring amino acid levels and directing the overall growth of the organism (Colombani et al. 2003).

The ability of AMPK (AMP-activated protein kinase) signalling to repress the TOR pathway in response to low nutrient levels acts as a brake on growth (Towler and Hardie 2007). Activation of the TOR pathway encourages growth while activation of AMPK inhibits growth and lipid deposition (Towler and Hardie 2007; Simpson and Raubenheimer 2009). In *Drosophila*, reduced AMPK function simulates starvation, shortening lifespan, increasing feeding rates and causing lowered growth and lipid levels (Johnson et al. 2010). Treatment with rapamycin (so inhibiting TOR) partially alleviated these effects (Johnson et al. 2010).

The diet phase-space is simplified in *Drosophila* and flies are available in sufficient numbers to allow a spread of diets. The metabolic responses to diet, for instance the Insulin and TOR pathways, are similar in flies and humans (Zhang et al. 2000; Kapahi et al. 2004), a nutritional target has already been uncovered (Lee et al. 2008) and although the mechanism controlling appetite and satiation in humans is not definitively known (Prentice 1998; Rolls 2000; Prentice and Jebb 2003; Simpson et al. 2003; Sorensen et al. 2003), it is thought that flies regulate both protein and carbohydrate intakes (Lee et al. 2008).

5.1.4 Experimental design and hypotheses

In the multigenerational diet experiment (Chapter 3), flies' responses to two diets simulating the transition between human Palaeolithic and modern Western were measured as a series of fitness indicators. What was not known was how these indicators vary with protein: carbohydrate ratio and calorific content over a wider range of diets. This information will allow the fitness indicators measured in the multigenerational diet experiment to be compared with the fecundity and longevity data from Lee et al. (2008). More generally, knowing how fitness indicators vary with protein and carbohydrate ratio and calorific content will indicate what dietary factors control traits linked to fitness.

This experiment is designed to look at the nutritional basis of several traits of interest in measuring fitness in *Drosophila*, and also to examine what macronutrient factors are most important in the deposition of lipids. In contrast to many of the above studies, flies will be kept on the experimental diets throughout their lives, from hatching to 3-day old adults at the start of their peak of fecundity (Robertson and Sang 1944; Novoseltsev et al. 2002). This is necessary for two reasons; one is that it hoped this will provide information useful to further PAR experiments, which will require the same conditions, and secondly, due to the importance of diet during larval development, when the final size of the fly is largely set (Bakker 1959; De Moed et al. 1999).

Specifically, the hypotheses to be tested are that:

1. The P:C ratio and calorific content of the diets will control different traits, and that traits taken as indicating fitness (survival, time to eclosion, thorax size, dry weight) should be maximal on the P:C 1:4 ratio as this corresponds to the intake target uncovered by Lee *et al.* (2008). Lipid content, while it is a fitness trait linked with starvation resistance, is expected to be controlled by the calorific content of the diets. The null hypothesis for each trait is that the response to diet does not depend on the isocaloric or P:C ratio of the diet, sex of the fly (except for survival, where sex of the egg was unknown) or any of their interactions.

2. Flies will compensate for low quality (low P:C) and low quantity (low calorie content) diets by eating more of them, but not to the extent that complete compensation is achieved. An experiment will look at the rate at which larvae consume food, by measuring gut residence time. This will check for complete compensation and indicate whether dietary quality or energy density is the most important in determining feeding

rate. The null hypothesis is that the response to diet does not depend on the isocaloric or P:C ratio of the diet or their interaction

Neurogenesis will also be measured using this experimental set-up, but the results are analysed separately in Chapter 6.

5.1.5 Diet range

The P:C ratios chosen for the four nutritional rails of the diets were 1:2, 1:4, 1:9 and 1:16 (Fig. 5.1). These are the same as used by Lee et al. (2008) with two exceptions. Lee et al. (2008) also used a 1:1 ratio, which was not used in this experiment, and a 1:8 ratio which was replaced by 1:9, as this was the P:C ratio previously used for the Western diet (W diet, see Chapter 2). The P:C ratio of 1:4 was the same as that used for the Palaeolithic diet (P diet, see Chapter 2).

The choice of the four levels of macronutrient concentrations (hereafter referred to as an “isocaloric”) was determined by extending the existing P and W diets. The P diet had a macronutrient concentration of 100 g l⁻¹ combined protein (20 g) and carbohydrate (80 g), while the W diet had 20 g l⁻¹ protein and 180 g l⁻¹ carbohydrate to give a combined total of 200 g l⁻¹. The new isocalorics were set at half the value of the P diet and twice the level of the W diet, to give four isocalorics of 50 g l⁻¹, 100 g l⁻¹, 200 g l⁻¹ and 400 g l⁻¹ (Fig. 5.1). Protein and carbohydrate have an almost identical energy content of 16.9 kJ g⁻¹ (Merrill and Watt 1973). The equivalent energy values of the isocalorics are 845 kJ l⁻¹, 1690 kJ l⁻¹, 3380 kJ l⁻¹ and 6760 kJ l⁻¹.

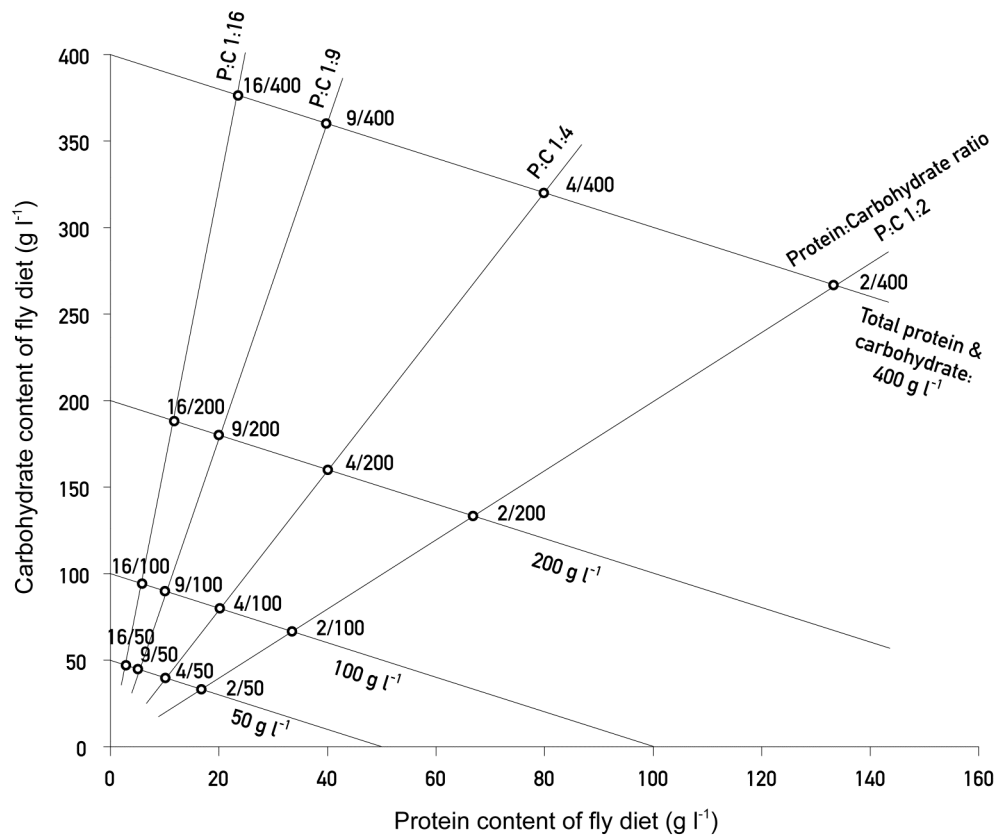


Figure 5.1: Nutrient space diagram showing the 16 diets used in experiments in this chapter. The x-axis represents the total protein (dry weight, g l^{-1}) in one litre of made-up fly diet, while the y-axis represents the total carbohydrate content (dry weight, g l^{-1}) derived from yeast and sucrose, in one litre of made-up fly diet. Lines radiating from the origin are nutritional rails and each represents possible diets with an identical protein:carbohydrate ratio. Isocaloric lines connect equal values of carbohydrate and protein content on the two axes. These represent possible diets with equal total carbohydrate and protein content (and equal calorific value). The intersections of the nutritional rails and isocaloric lines represent each of the 16 diet combinations used in this chapter. Diet 4/100 is the same as the “Palaeolithic” diet previously used (Chapter 3), and diet 9/200 the same as the previous “Western” diet.

5.2 Methods

5.2.1 Diet composition

The diet range experiment was factorial, with four levels of isocaloric and four levels of P:C ratio, making 16 diets in total. Having determined the amounts of protein and carbohydrate that needed to be present in each of the 16 diets, the quantities of yeast and sucrose necessary were calculated, taking the macronutrient composition of dried yeast to be 47% protein and 24% carbohydrate by weight (Caballero-Córdoba and Sgarbieri 2000; Yamada and Sgarbieri 2005). These quantities were then adjusted to take into account the change in concentration due to the contraction of agar (at a concentration of 20 g l⁻¹), using the procedure detailed in Chapter 2 (Section 2.3.4). The quantities of yeast and sucrose used in each diet are shown below in Table 5.1. Micronutrient quantities were the same as used for the P and W diets (Chapter 2, Table 2.2), as was the amount of added agar (Acros Organics, via Fisher Scientific, Loughborough) and propionic acid (Fisher Scientific, Loughborough) as an anti-fungal agent. Diets are designated by their P:C ratio and isocaloric, and so 2/400 stands for a P:C ratio of 1:2 and a total macronutrient content of 400 g l⁻¹.

The diets were made up as described in Chapter 2. For each laying chamber, 2.5 ml was poured into a 55 mm Petri dish (Fisher Scientific, Loughborough) and for culture tubes, 7 ml was poured into 75 mm by 25 mm soda glass specimen tubes (Fisher Scientific, Loughborough). These were kept in a cold room at 4° C for 24 h to allow condensation to clear.

Isocaloric (g l ⁻¹) (combined protein and carbohydrate)	P:C ratio 1:2		1:4		1:9		1:16	
	Protein / yeast (g l ⁻¹)	Carb / sucrose (g l ⁻¹)	Protein / yeast (g l ⁻¹)	Carb / sucrose (g l ⁻¹)	Protein / yeast (g l ⁻¹)	Carb / sucrose (g l ⁻¹)	Protein / yeast (g l ⁻¹)	Carb / sucrose (g l ⁻¹)
400	133.3	266.7	80.0	320.0	40.0	360.0	23.5	376.5
	283.7	198.6	170.2	279.1	85.1	339.8	50.1	364.5
	269.7	188.6	161.2	264.1	80.3	320.4	47.2	343.4
200	66.7	133.3	40.0	160.0	20.0	180.0	11.8	188.2
	141.8	99.3	85.1	139.6	42.6	169.8	25.0	182.2
	132.7	92.8	79.5	130.3	39.7	158.2	23.3	169.7
100	33.3	66.7	20.0	80.0	10.0	90.0	5.9	94.1
	70.9	49.6	42.6	69.8	21.3	84.9	12.5	91.1
	65.8	46.1	39.5	64.7	19.7	78.6	11.6	84.4
50	16.7	33.3	10.0	40.0	5.0	45.0	2.9	47.1
	35.5	24.8	21.3	34.9	10.6	42.4	6.3	45.6
	32.8	22.9	19.7	32.2	9.8	39.2	5.8	42.1

Table 5.1: The protein and carbohydrate constituents of the 16 experimental diets.

For each Protein: Carbohydrate (P:C) ratio there are two columns representing: on the top line of each group of three lines, the total amount of protein (left) or carbohydrate (right) required; on the middle line, the quantities of dried yeast or sucrose needed to achieve these quantities; and on the bottom line, the quantities of yeast or sucrose required after taking into account the contraction due to agar (at 20 g l⁻¹). Each group of three rows represents a different isocaloric. All values are in g l⁻¹.

5.2.2 Fly culturing

Flies to be used in the diet range experiment were taken from 12 isofemale lines (lines 2, 4, 5, 7, 9, 10, 16, 17, 18, 22, 31 and 32) that had been randomly selected from the 25 isofemale lines derived from the AV culture, maintained on the Standard diet (see Chapter 2). All lines were included in survival and time to eclosion measurements, but line 22 was not used for dry weight or lipid measurements, and lines 5, 18 and 22 were not used for thorax measurements. Each selected line was expanded from one to two culture bottles in the generation prior to being used for laying, to ensure an adequate supply of mated females. The emerged flies were kept in their culture bottles for 3 d in an incubator (LMS Ltd., Kent) at 20° C, under a light: dark regime of 16:8 h, with lights on at approximately 8 am (hereafter “the 20°C incubator”). Although humidity was not controlled, an open tray of water was placed at the bottom of the incubator to prevent drying out.

Flies were lightly anaesthetised using CO₂ and from each isofemale line, five females placed in an upturned tricorn beaker (Fisher Scientific, Loughborough). An inverted Petri dish containing one of the 16 diets was secured on top with masking tape.

Once the flies had recovered from the CO₂, the laying chamber was placed on its side on a tray in the 20°C incubator. This was repeated for each of the 16 diets.

After 24 h, the flies were removed from the laying chambers and 25 eggs (if 25 could be obtained), were cut from the diet using a scalpel, under an Olympus SZX7 dissecting microscope (Olympus Imaging and Audio Ltd., Southend-on-Sea), and placed in a specimen tube containing 7 ml of the same diet. The tube was secured using a polyfoam bung (Fisher Scientific, Loughborough) and placed in one of two metal racks in the 20° C incubator. Each rack was rotated through 90° daily, and alternated between two shelves in the incubator.

After 10 d, each tube was checked for eclosed flies daily at 10 am. Eclosed flies were sexed, their numbers recorded and placed in a specimen tube containing the same diet, in an incubator (LMS Ltd., Kent) at 8° C. When all flies had emerged, this was placed back in the 20° C incubator for 3 d. After this time all flies from each isofemale line / diet combination were placed in 1.5 ml eppendorf tubes (Fisher Scientific, Loughborough), flash-frozen in liquid nitrogen, and stored at -18° C until measurements could be taken. Several isofemale line / diet combinations did not produce enough eggs and so had to be restarted with the next generation of flies, using the same procedure as above.

5.2.3 Measurements

Flies were first sexed under a dissecting microscope. Five males and five females from each isofemale line / diet combination were selected randomly, if five were available. These were placed one at a time under stage microscopes, either a Leica DM LS2 or LB2 (Leica Microsystems UK Ltd., Milton Keynes) or a Nikon Eclipse E400 (Nikon Instruments Europe B.V., Kingston, Surrey) and illuminated from above using a light source. The Leica microscopes were connected to Leica DFC280 cameras (Leica Microsystems UK Ltd., Milton Keynes) controlled by computers running Adobe PhotoShop CS (Adobe Systems Inc, San Jose.), which was used to capture the images. The Nikon was connected to a Photometrics Coolsnap HQ camera (Photometrics, Tucson, AZ) controlled by MetaMorph version 6.1r4 software (Molecular Devices, Sunnyvale, CA) to capture the images. All images taken with the Nikon were at a resolution of 1392 by 1040 pixels, while those with the Leicas were 1280 by 1024 pixels. At the same time as the fly images were being captured, and on the same microscope, a

calibration image of a stage graticule (Pyser-SGI, Kent), was also captured for each magnification.

The photomicrographs were taken by RS (lines 2, 7, 16, 32) using the Nikon, and LC (line 4), GC (line 9), TF (line 10), KH (line 17), and JM (line 31) using the Leica cameras (see acknowledgments, Section 5.4.6).



Figure 5.2: Photomicrograph of a female *Drosophila melanogaster* taken using a 4x objective. The larger white bar represents the distance measured, from the anterior end of the thorax to the posterior tip of the scutellum. The smaller scale bar (bottom right) represents 0.1 mm.

Thorax images were captured using a 4x objective. Eye and ommatidia images were also captured at the same time using a 10x and 20x objective and are analysed separately in Chapter 6. The thorax was measured from the anterior end of the first thoracic segment to the posterior tip of the scutellum (Fig. 5.2).

Image distances were converted to actual measurements by measuring the distance in pixels of 1 mm on the graticule images using Adobe PhotoShop CS (Adobe Systems Inc, San Jose.), and comparing this with the length in pixels of the thorax in each fly image.

After thorax and eye measurements, samples were transferred to 2 ml glass vials (Fisher Scientific, Loughborough) and dried uncovered for 24 hours in an oven (Agar Scientific N6C, Kent) at 60° C. The dry weight was obtained by weighing the sample flies

five at a time using a Sartorius balance (Sartorius AG, Goettingen) capable of weighing 10µg. They were next soaked in 1 ml of a mixture of 1 part anhydrous ethanol (Fisher Scientific, Loughborough) to 2 parts ethyl acetate (Fisher Scientific, Loughborough) for 24 h, with the cap of the tube in place, to extract their lipids (Lin et al. 2004; Warbrick-Smith et al. 2006). The vials were kept on a rotator at 4 r.p.m. during this procedure. After 24 h the solvent was pipetted off, and the remainder allowed to evaporate uncovered for another 24 h. Once no solvent remained, the samples were re-dried for 1 h at 60°C before being re-weighed to obtain the lipid-free dry weight (lfdw). The proportion of the flies' dry weight which was accounted for by lipids was calculated for each diet treatment, isofemale line and sex as $(\text{dry weight} - \text{lfdw}) / \text{dry weight}$.

Flies used in the dry weight and lipid content assays were first selected, sexed and used to take photomicrographs by RS (lines 2, 7, 16, 32), TB (line 5), LC (line 4), GC (line 9), TF (line 10), KH (line 17), NJ (line 18) and JM (line 31) (see acknowledgements, Section 5.4.6). RS checked the selected flies again prior to drying. The dry weight and lipid assays were carried out by RS.

5.2.4 Gut residence time

Larvae to be used in the gut residence time experiment were taken from the same 12 isofemale lines as for the diet range experiment and each line had been maintained on four diets (2/400, 2/50, 9/400 and 9/50, Fig. 5.1) for seven generations. Ten mated female flies were taken from each isofemale line / diet combination and introduced to laying chambers as described above for the diet range experiment, and placed in the 20° C incubator for 24 h. Each day, females from four isofemale lines from each diet were placed into laying chambers, and the laying times staggered with 4 min between each and were arranged in two blocks, one each day. The order in which they were introduced to the laying chambers is shown below in Table 5.2.

Block 1

Isofemale line	Diet			
1	2/400	2/50	9/400	9/50
2	2/50	9/400	9/50	2/400
3	9/400	9/50	2/400	2/50
4	9/50	2/400	2/50	9/400
5	2/400	2/50	9/400	9/50
8	2/50	9/400	9/50	2/400

Block 2

Isofemale line	Diet			
9	9/400	9/50	2/400	2/50
16	9/50	2/400	2/50	9/400
17	2/400	2/50	9/400	9/50
22	2/50	9/400	9/50	2/400
28	2/400	2/50	9/400	9/50
31	2/50	9/400	9/50	2/400

Table 5.2: arrangement of the two blocks of isofemale lines into laying chambers for the gut clearance time experiment. Each Isofemale line / diet combination was separated by 4 min, starting from line 1 2/400 and moving left to right. Block 2 was put in to lay one day after Block 1.

The food medium on which eggs were laid had been coloured with SuperCook red food dye (Dr. Oetker UK Ltd., Leeds) at a concentration of 10 ml l⁻¹. A small scale trial using this dye had indicated that flies would accept this as a laying substrate, larvae developing on it would develop into adults and that it was possible to observe the gut contents of larvae reared on this medium under a dissecting microscope.

After the females had been removed from the laying chambers, the covered Petri dishes were returned to the 20° C incubator for a further 48 hours. Following the order in which females had been placed in the laying chambers, and with 4 min between each isofemale line / diet combination, 10 randomly selected larvae from each Petri dish were removed from the coloured diet using a paintbrush, briefly washed in distilled water and placed on a fresh Petri dish containing the same, but uncoloured, diet before being replaced in the 20° C incubator. These were then checked once every hour under an Olympus SZX7 dissecting microscope (Olympus Imaging and Audio Ltd., Southend-on-Sea) and the colour of their gut contents recorded until no trace of the red diet remained (Burnet et al. 1977).

5.2.6 Statistical analysis

The mean of the response variable of each treatment, isofemale line and sex combination was used as the replicate in statistical analysis. All response variables except for lipid proportion and survival were tested for a normal distribution using a Kolmogorov-Smirnov test in IBM SPSS Statistics 19 (SPSS Inc, Chicago). Thorax length and dry weight all conformed to a normal distribution. Proportion data do not follow a normal distribution as they are bounded by 0 and 1 (Crawley 2007). Therefore the lipid proportion data were first transformed using the arcsine of the square root (Crawley 2007). After this transformation the data conformed to a normal distribution, confirmed by a Kolmogorov-Smirnov test in SPSS Statistics 19 (SPSS Inc., Chicago). Survival data were treated as a two-vector response variable in a generalised linear model with binomial errors in R 2.8.1 (R Foundation for Statistical Computing, Vienna, Austria). This approach allows the numbers of dead and surviving flies to be taken into account, rather than just the proportion surviving, and does not require transformation of the original data (Crawley 2007).

The data were then analysed using a Generalized Linear Model in R 2.1.8 (R Development Core Team 2008), using a model building approach where factors or interactions were removed from the full model if their removal did not significantly increase the residual variance. Terms were removed from each full model in the order of highest-order interaction and least significant first, assessed by an ANOVA procedure on each model. The effect of removing a term was assessed by comparing the total deviance explained by the models with the term included and without, using an analysis of variance and a Chi-squared test. If the change in deviance was not significant, the term was left out, or if significant it was retained. Once the minimal adequate model was arrived at, the significance of each term was then assessed by building the model up, using isofemale line in all models (and for lipid and weight measurements, batch process date), and then sequentially adding the other factors in the minimal adequate model in the order: sex, isocaloric and P:C ratio (except for survival and gut-residence time, where sex was not known). Interactions between factors were added only after the factors themselves had been added. Each data point (mean of isofemale line/treatment/sex) was weighted by the number of flies used to obtain the data point (except for in the survival analysis).

Contour plots of each response variable against the protein and carbohydrate content of the experimental diets were produced using SigmaPlot 11 (Systat Software Inc, San Jose, California). This procedure interpolates values as contour lines and shading between the 16 data points for each diet. If the interpolated values were above or below the four surrounding data points, the resulting graphs were edited to show the same colour values as the actual data points, although the interpolated contour lines were left in place for reference.

5.3 Results

5.3.1 Survival

To examine whether macronutrient content or macronutrient ratio was the most important factor determining the survival of fly eggs to eclosion, flies were allowed to lay eggs on 16 diets, each composed of one of four total macronutrient contents (isocalorics) and one of four P:C ratios, and the survival to eclosion of the eggs was recorded.

Both the isocaloric (change in deviance 58.14, 3 d.f., $p < 0.001$) and the P:C ratio (change in deviance 97.56, 3 d.f., $p < 0.001$) of the diets were significant factors determining survival (Fig. 5.3 and Table 5.3). Survival increased as the total macronutrient content (Fig. 5.3b) and the P:C ratio of the diets increased (Fig. 5.3c).

There was also a significant interaction between the macronutrient content and the P:C ratio (change in deviance 35.26, 9 d.f., $p < 0.001$) as survival was much lower when both macronutrient content and P:C ratio was low (16/50 diet: $36.5 \pm 9.4\%$ survival to eclosion) and also was higher when both were high (2/400 diet: $77.2 \pm 3.6\%$ survival to eclosion). The majority (9 out of 16) of the diets had a survival rate of between 60 and 70% and when represented as a contour plot (Figure 5.4) it is apparent that there is a gradient running from a low percentage survival at low P:C ratios / low isocaloric to a high percentage at high P:C / high isocaloric diets. On the low P:C ratio diets the isocaloric level had a greater influence on survival than when the P:C ratio was higher, with a greater difference between 16/50 and 16/400 (24.14%) than between 9/50 and 9/400 (16.93%), 4/50 and 4/400 (4.07%) or 2/50 and 2/400 (12.95%).

Different proportions of eggs survived to eclosion depending on which isofemale line they were from (change in deviance 87.75, 11 d.f., $p < 0.001$).

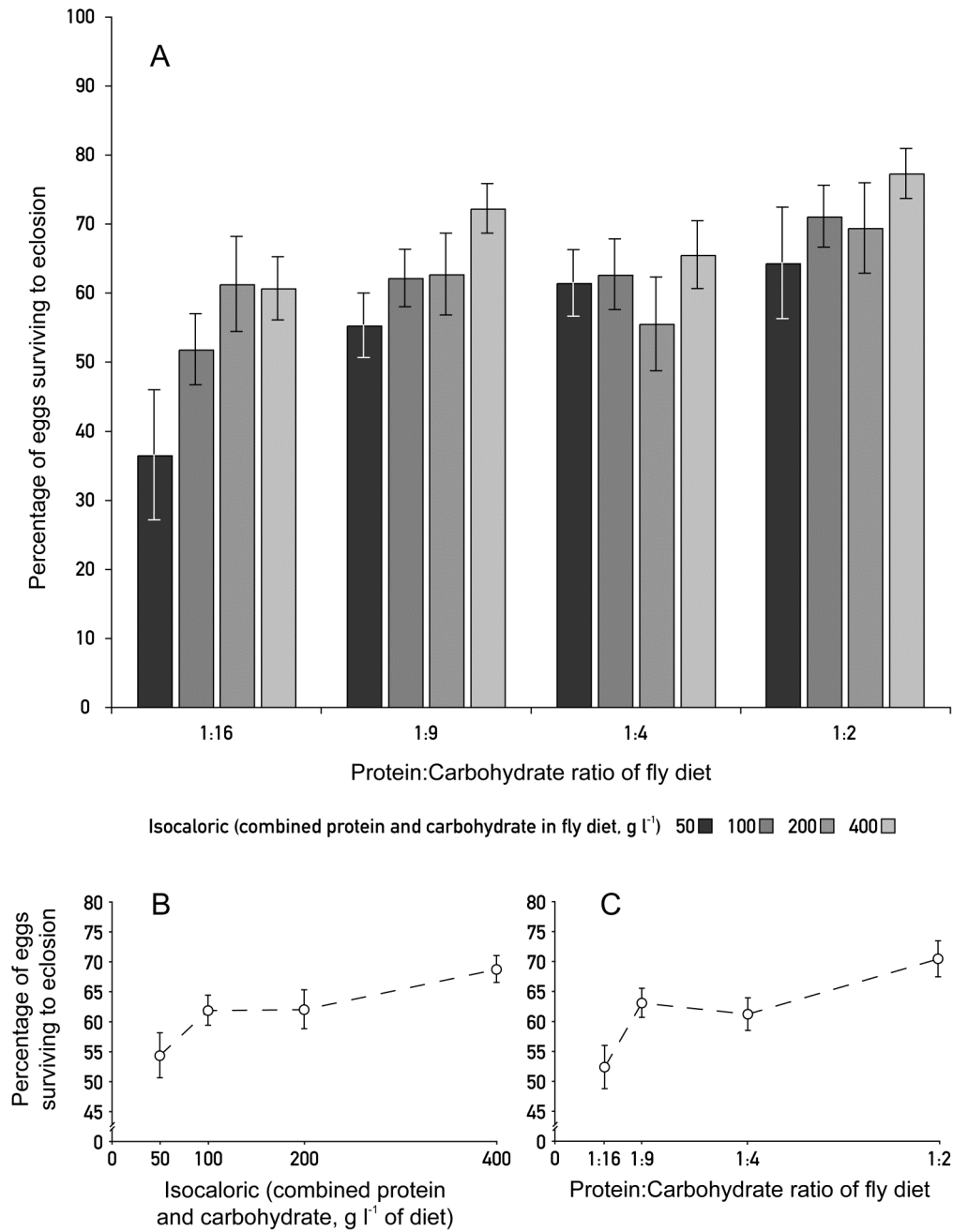


Figure 5.3: Mean percentage of eggs surviving to eclosion. **A:** each bar represents the mean survival percentage of 12 isofemale lines of flies raised on 16 differing diets. They are grouped on the x-axis by the P:C ratio of their diets and differently coloured bars represent diets from different isocalorics (combined protein and carbohydrate, g l⁻¹). **B:** Mean survival to eclosion grouped by isocaloric of fly diet. **C:** Mean survival to eclosion grouped by P:C ratio of fly diet. All error bars represent ± 1 standard error of the mean.

Minimal adequate model for survival

Factor	Res. dev.	-dev.	Res. d.f.	d.f.	<i>p</i>	sig.
Null	1024.89		187			
Iso	937.14	87.75	176	11	< 0.001	***
Cal	878.99	58.14	173	3	< 0.001	***
PC	781.43	97.56	170	3	< 0.001	***
Cal:PC	746.17	35.26	161	9	< 0.001	***

Table 5.3: The minimal adequate model for proportion of eggs surviving to eclosion. The significance of each factor was assessed by adding factors in turn to the model (iso) and assessing the reduction (– dev) in residual deviance (res. dev.). Key to factors: Iso – isofemale line; Cal: total macronutrient content (g l⁻¹); PC – protein:carbohydrate ratio of diet.

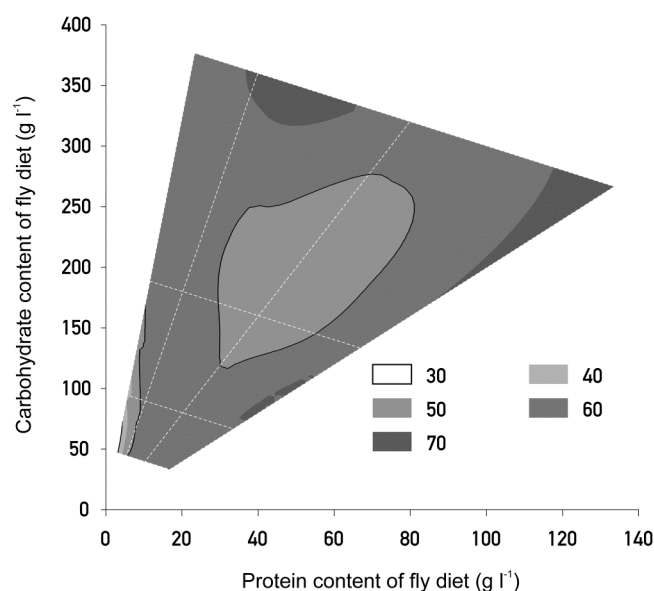


Figure 5.4: Mean percentage of eggs surviving to eclosion. The x-axis represents the protein content of the 16 experimental diets, and the y-axis represents the carbohydrate content. The solid shading on the surface indicates mean percentage survival to eclosion (see key). The dotted white lines (and the boundaries of the shaded area) represent the four P:C ratios and the four isocalorics used to construct the diets. Each intersection or corner represents one of the 16 diets.

5.3.2 Time to eclosion

The time taken for a fly egg to develop through the larval and pupal stages to eclosion as an adult is an important indicator of fly fitness, as a faster development means that the fly can begin to breed sooner. The time to eclosion is altered by factors including temperature and diet. To answer the question of whether the total macronutrient content of the diet or the proportion of protein to carbohydrate controls the speed of development, flies from 12 isofemale lines were allowed to lay eggs on 16 diets, each of one of four isocaloric values and one of four P:C ratios and the time taken for the resulting adults to eclose was recorded.

The largest influence on time to eclosion was the P:C ratio (change in deviance 10010.5, 3 d.f., $p < 0.001$), as the time taken significantly decreased at higher P:C ratios compared to lower ones (Fig. 5.5, Fig. 5.6). The mean time to eclosion was 4.8 d faster when flies developed on a P:C 1:2 diet (15.2 ± 0.1 d) than on a P:C 1:16 diet (20.0 ± 0.3 d). The total quantity of macronutrients was also significant (change in deviance 537.4, 3 d.f., $p < 0.01$), but the reaction norm was a different shape to that for P:C ratio (Fig 5.5 b and c). An intermediate value (200g l^{-1}) produced the fastest-eclosing flies overall, although this did vary according to the P:C ratio as there was a significant interaction between macronutrient content and P:C ratio (change in deviance 508.6, 9 d.f., $p < 0.001$), particularly for the lowest P:C ratio, 1:16. Time to eclosion was less affected by the isocaloric at the high P:C ratios (Fig. 5.5, Fig. 5.6).

The sex of the fly was not a significant factor either in itself or as an interaction with other factors and so these terms were removed from the model (Table 5.4), although isofemale lines were significantly different in their time to eclosion (change in deviance 3809, 11 d.f., $p < 0.001$).

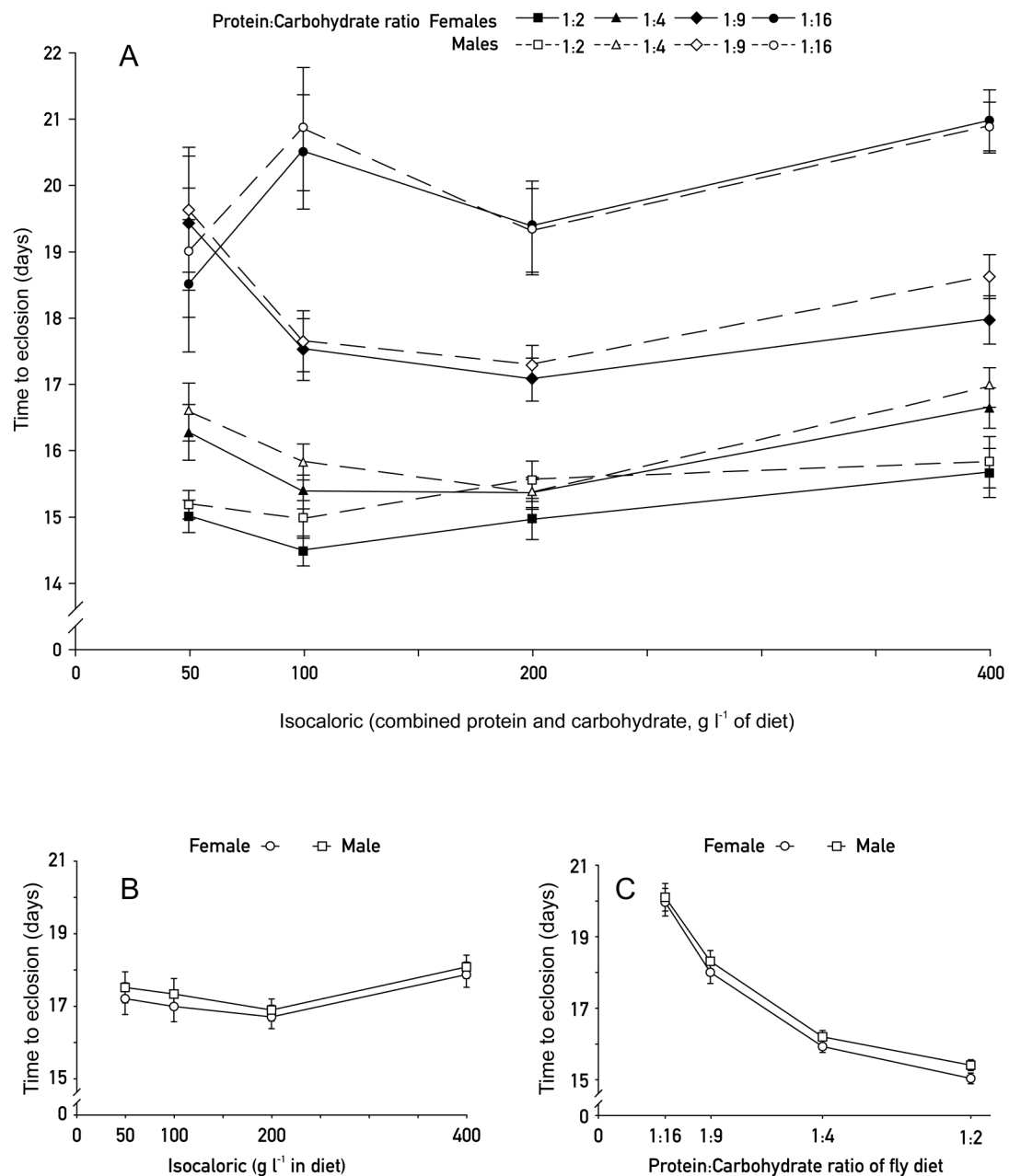


Figure 5.5: Mean time to eclosion for flies on differing diets. **A:** each point represents the mean time to eclosion of male of females from 12 isofemale lines of flies raised on 16 differing diets. The x-axis represents the isocaloric (combined protein and carbohydrate, g l⁻¹) of their diet and each series of points represents diets of the same protein:carbohydrate (P:C) ratio. **B:** Mean time to eclosion, grouped by isocaloric of fly diet and sex. **C:** Mean time to eclosion, grouped by P:C ratio of fly diet and sex. All error bars represent ± 1 standard error of the mean.

Minimal adequate model for time to eclosion (d)

Factor	Res. dev.	-dev.	Res. d.f.	d.f.	p	sig.
Null	19790		375			
Iso	15980	3809	364	11	< 0.001	***
Cal	15443	537.4	361	3	< 0.01	**
PC	5432.5	10010.5	358	3	< 0.001	***
Cal:PC	4923.9	508.6	349	9	< 0.001	***

Table 5.4: The minimal adequate model for time to eclosion. The significance of each factor was assessed by adding factors in turn to the model (iso) and assessing the reduction (– dev) in residual deviance (res. dev.). Key to factors: Iso – isofemale line; Cal: total macronutrient content (g l^{-1}); PC – protein:carbohydrate ratio of diet.

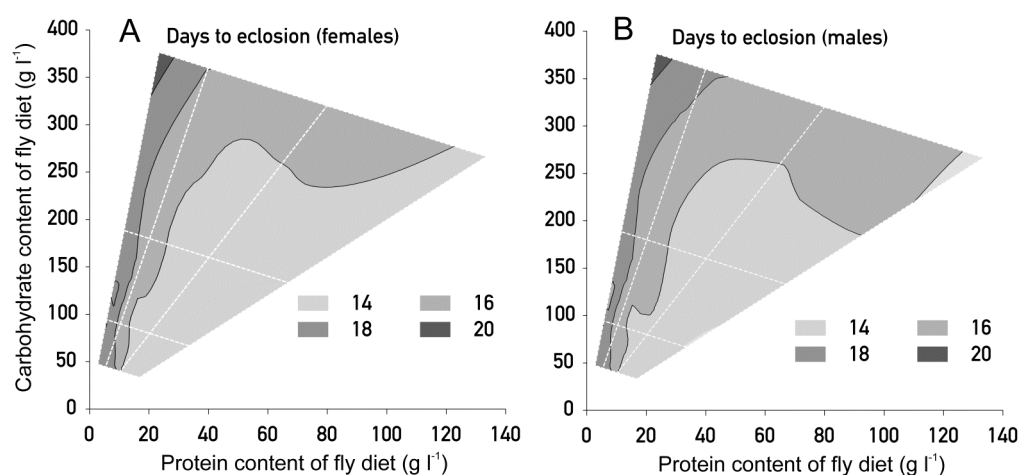


Figure 5.6: Mean time to eclosion (d) for **A:** female flies and **B:** male flies, represented as contour plots. The x-axis represents the protein content of the 16 experimental diets and the y-axis represents the carbohydrate content. The solid shading on the surface indicates the mean or interpolated value. The dotted white lines (and the boundaries of the shaded area) represent the four P:C ratios and the four isocalorics used to construct the diets. Each intersection or corner represents one of the 16 diets.

5.3.3 Thorax length

Thorax length is an important fitness indicator in flies as in females it reflects fecundity and in males it is associated with greater mating success (Partridge et al. 1987). Whether the size of the thorax, and so potential fitness, was affected by diet quality (the P:C ratio) or by diet quantity (the total concentration of macronutrients) was tested by allowing flies from nine isofemale lines to lay eggs on 16 diets, each composed of one of four macronutrient concentrations (50, 100, 200 and 400 g l^{-1} protein and carbohydrate

combined) and one of four P:C ratios (1:2, 1:4, 1:9 and 1:16). The length of the thorax of the resulting adults was measured after allowing the flies to develop on these diets from laying until 3 d after eclosion.

Females had a greater mean thorax length (Fig 5.7) than male flies (change in deviance 3.6251, 1 d.f., $p < 0.001$). The average female, at $1.079 \text{ mm} \pm 0.004$, had a 0.111 mm longer thorax than the average male ($0.968 \text{ mm} \pm 0.003$).

Thorax length significantly decreased on higher isocaloric diets (change in deviance 0.2226, 3 d.f., $p < 0.001$, Fig. 5.7, Fig. 5.8). The mean female value for the 400 g l^{-1} diets was $1.059 \text{ mm} \pm 0.007$, 0.026 mm less than the mean for the 50 g l^{-1} diets ($1.085 \text{ mm} \pm 0.008$). Males responded in the same way as females, with flies on the 400 g l^{-1} diets ($0.942 \text{ mm} \pm 0.007$) having a thorax shorter by 0.040 mm than those on 50 g l^{-1} diets ($0.984 \text{ mm} \pm 0.007$).

Flies developing on diets with higher P:C ratios had significantly larger thoraxes (change in deviance 0.3396, 3 d.f., $p < 0.001$, Fig. 5.7, Fig. 5.8). Males and females responded in the same way to altered P:C ratios with the mean length for the P:C 1:2 diets (female: $1.102 \text{ mm} \pm 0.006$, male: $0.991 \text{ mm} \pm 0.005$) being greater than for the P:C 1:16 diets (female: $1.048 \text{ mm} \pm 0.009$, male: $0.946 \text{ mm} \pm 0.008$). The effect on thorax length of the P:C ratio was not significantly different at different isocalorics and so the P:C ratio x isocaloric interaction term was removed from the statistical model (Table 5.5). Isofemale lines responded differently to the 16 diets (change in deviance 0.4334, 8 d.f., $p < 0.01$).

Minimal adequate model for thorax length (mm)

Factor	Res. dev.	-dev.	Res. d.f.	d.f.	<i>p</i>	sig.
Null	5.6281		268			
Iso	5.1947	0.4334	260	8	< 0.01	**
Sex	1.5696	3.6251	259	1	< 0.001	***
Cal	1.3470	0.2226	256	3	< 0.001	***
Pc	1.0075	0.3396	253	3	< 0.001	***

Table 5.5: The minimal adequate model for thorax length. The minimal adequate model was arrived at by removing non-significant terms from the full model. The significance of each factor was assessed by adding factors in turn to the model (iso) and assessing the reduction (– dev) in residual deviance (res. dev.). Key to factors: Iso – isofemale line; Sex – sex of fly; Cal: total macronutrient content (g l^{-1}); PC – protein:carbohydrate ratio of diet.

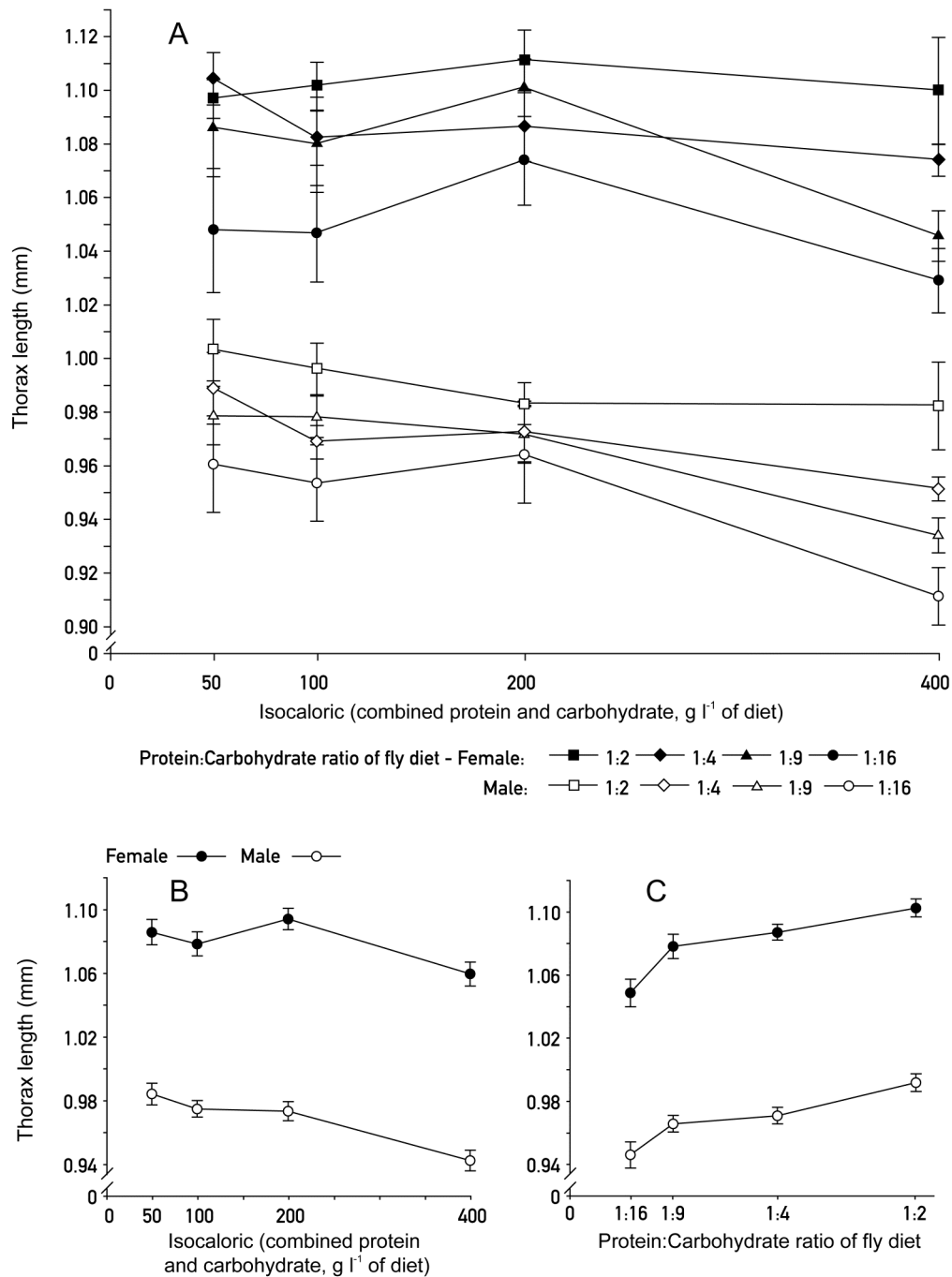


Figure 5.7: Mean thorax length of flies on differing diets. **A:** each point represents the mean thorax length of nine isofemale lines of flies raised on the 16 experimental diets. The x-axis represents the isocaloric (combined protein and carbohydrate, g l⁻¹) of their diet and each series of points represents diets of the same protein:carbohydrate (P:C) ratio. **B:** Mean thorax length, grouped by isocaloric of fly diet and sex. **C:** Mean thorax length, grouped by P:C ratio of fly diet and sex. All error bars represent ± 1 standard error of the mean.

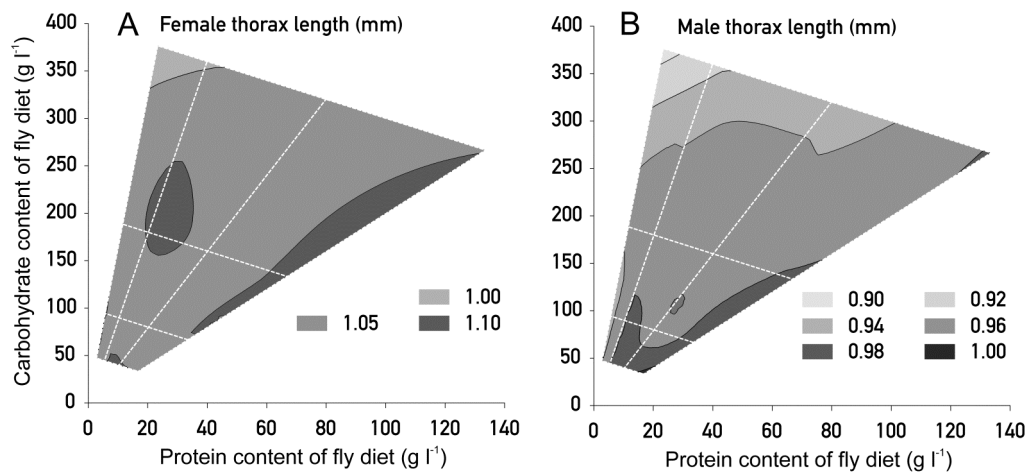


Figure 5.8: Mean thorax length (mm) for **A:** female flies and **B:** male flies, represented as contour plots. The x-axis represents the protein content of the 16 experimental diets and the y-axis represents the carbohydrate content. The solid shading on the surface indicates the mean or interpolated value. The dotted white lines (and the boundaries of the shaded area) represent the four P:C ratios and the four isocalorics used to construct the diets. Each intersection or corner represents one of the 16 diets.

5.3.4 Dry weight

Dry weight is related to the size of an adult fly and to fecundity and mating success, and is thought to be largely determined during the larval stages of the fly lifecycle (Bakker 1959). Dry weight was not significantly altered as a result of offspring diet in the multigenerational diet experiment (Chapter 3). However, if dry weight does change in response to diet, it is important to know whether this is in response to diet quality (the P:C ratio) or to diet quantity (the total concentration of macronutrients). This was tested by allowing flies from eleven isofemale lines to lay eggs on 16 diets, each composed of one of four macronutrient concentrations (50, 100, 200 and 400 g l⁻¹ protein and carbohydrate combined) and one of four P:C ratios (1:2, 1:4, 1:9 and 1:16). The dry weight of the resulting 3 d old adults was measured. The null hypotheses were that neither sex, P:C ratio, isocaloric nor any of their interactions would affect the flies' dry weight.

The main determinant of dry weight was sex (Fig 5.9, Fig. 5.10) as females were significantly larger than males (change in deviance 3706487, 1 d.f., $p < 0.001$). Both the P:C ratio (change in deviance 208338, 3 d.f., $p < 0.001$, Fig 5.10c) and the isocaloric (change in deviance 439725, 3 d.f., $p < 0.001$, Fig. 5.10b) of the diet affected dry weight, which increased with higher calorific content and higher P:C ratio (Fig. 5.10).

However, the interactions of sex with dietary factors were also significant (Table 5.6, Fig. 5.9, 5.10). Males responded less strongly to either the isocaloric of the diet (Fig. 5.10b) or the P:C ratio (Fig. 5.10c) than females. The interaction of the two dietary factors was also different in each sex (sex:cal:pc interaction in Table 5.6, change in deviance 207039, 9 d.f., $p < 0.001$), as females tended to have increased dry weight with both isocaloric and P:C, while males responded more strongly to the P:C ratio (Fig. 5.9, Fig. 5.10). Isofemale lines also had significantly different responses (change in deviance 592192, 10 d.f., $p < 0.01$).

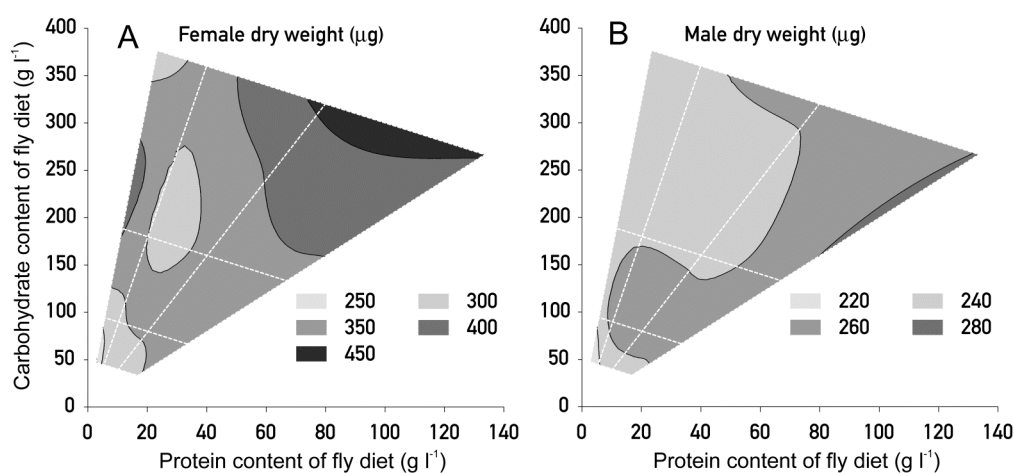


Figure 5.9: Mean dry weight (μg) for **A:** female flies and **B:** male flies, represented as contour plots. The x-axis represents the protein content of the 16 experimental diets and the y-axis represents the carbohydrate content. The solid shading on the surface indicates the mean or interpolated value. The dotted white lines (and the boundaries of the shaded area) represent the four P:C ratios and the four isocalorics used to construct the diets. Each intersection or corner represents one of the 16 diets.

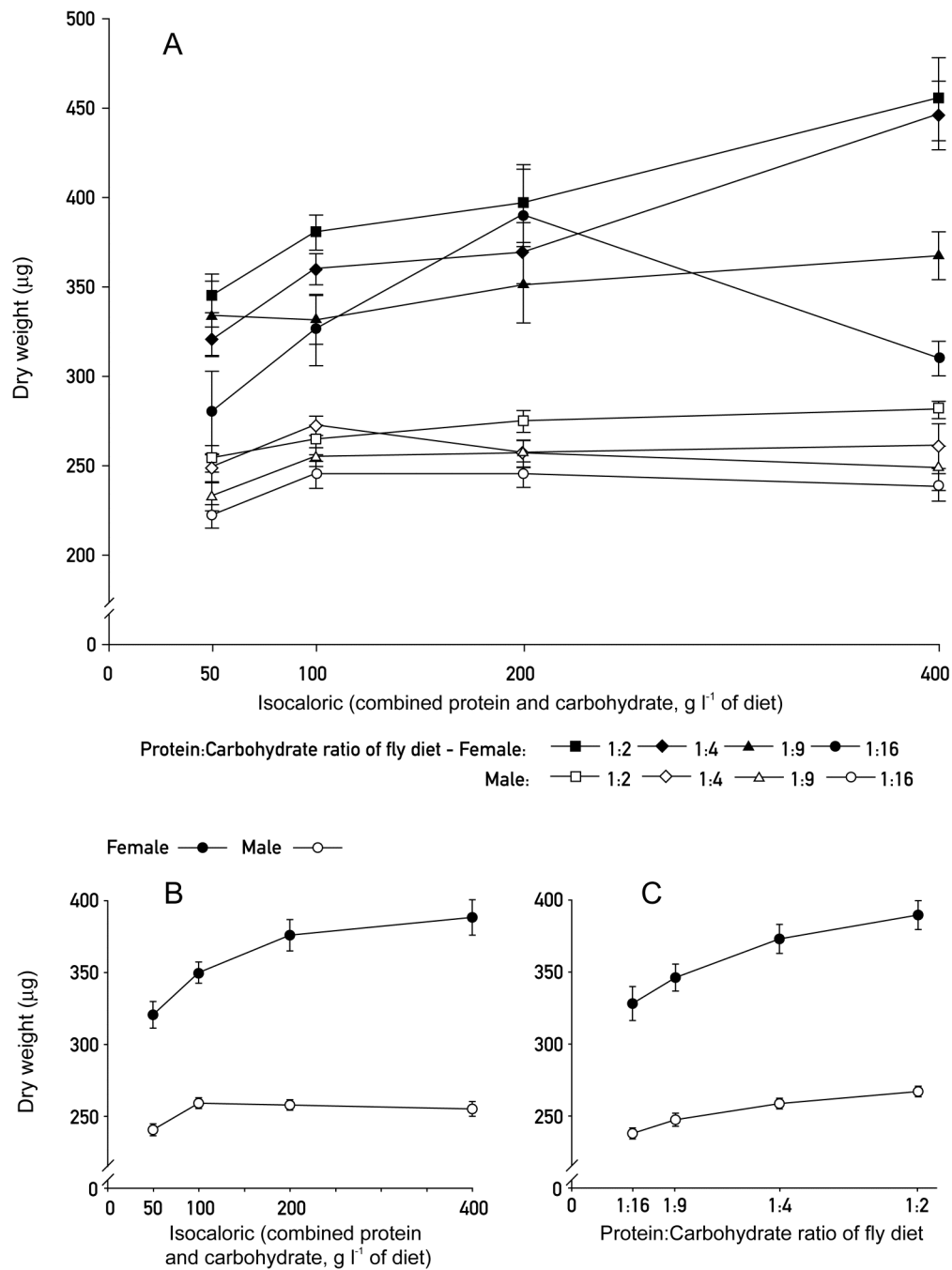


Figure 5.10: Mean dry weight (μg) of flies on differing diets. **A:** each point represents the mean dry weight of 11 isofemale lines of flies raised on the 16 experimental diets. The x-axis represents the isocaloric (combined protein and carbohydrate, g l^{-1}) of their diet and each series of points represents diets of the same Protein:Carbohydrate (P:C) ratio. **B:** Mean dry weight, grouped by isocaloric of fly diet and sex. **C:** Mean dry weight, grouped by P:C ratio of fly diet and sex. All error bars represent ± 1 standard error of the mean.

Minimal adequate model for dry weight (μg)

Factor	Res. dev.	- dev	Res. d.f.	d.f.	<i>p</i>	sig.
Null	7589442		310			
Batch	6919597	669845	294	16	< 0.05	*
Iso	6327405	592192	284	10	< 0.01	**
Sex	2620918	3706487	283	1	< 0.001	***
Cal	2190193	439725	280	3	< 0.001	***
Sex:Cal	2024719	165473	277	3	< 0.001	***
PC	1816382	208338	274	3	< 0.001	***
Sex:PC	1759555	56827	271	3	< 0.05	*
Cal:PC	1470622	288933	262	9	< 0.001	***
Sex:Cal:PC	1263583	207039	253	9	< 0.001	***

Table 5.6: The minimal adequate model for dry weight. The minimal adequate model was arrived at by removing non-significant terms from the full model. The significance of each factor was assessed by adding factors in turn to the model (iso) and assessing the reduction (– dev) in residual deviance (res. dev.). Key to factors: Batch – processing batch; Iso – Isofemale line; Sex – sex of fly; Cal: total macronutrient content (g l^{-1}); PC – protein:carbohydrate ratio of diet.

5.3.5 Lipid content of flies

The lipid content of a fly can relate to fitness as it may indicate a greater resistance to starvation (Chippindale et al. 1996; Baldal et al. 2006) but it is also of interest as flies, like humans, store excess energy from the diet as lipids (Musselman et al. 2011). However it is also of interest whether the lipid content is increased by the total calorific content of the diet or by the ratio of one component to the other. This was tested by allowing flies from eleven isofemale lines to lay eggs on 16 diets, each composed of one of four macronutrient concentrations (50, 100, 200 and 400 g l^{-1} protein and carbohydrate combined) and one of four P:C ratios (1:2, 1:4, 1:9 and 1:16). The lipid content as a proportion of dry weight of the resulting 3 d old adults was then measured. The null hypotheses were that neither sex, P:C ratio, isocaloric nor any of their interactions would affect the flies' lipid content.

Female flies had a higher mean lipid content overall (change in deviance 0.2146, 1 d.f., $p < 0.01$, Table 5.7), with a mean lipid content of $18.21 \% \pm 0.51$ compared to the $16.64 \% \pm 0.51$ of males (Fig 5.11, Fig. 5.12). The isocaloric of the diet had a significant influence on lipid content (change in deviance 1.7045, 3 d.f., $p < 0.001$), with those flies on the higher isocalorics having higher lipid content than those on lower isocalorics (Fig 5.11, Fig. 5.12c).

The P:C ratio of the diets had no significant influence either as a main effect (change in deviance 0.1017, 3 d.f., $p = 0.077$), or in interaction with sex or the isocaloric of the diet (Fig. 5.11, Fig. 5.12). Male and female flies did not have a significantly different response to the P:C ratio or to the isocaloric of the diets, and individual isofemale lines did not respond differently to the diets (change in deviance 0.3197, 10 d.f., $p = 0.139$).

Minimal adequate model for lipid content (%)

Factor	Res. dev.	-dev.	Res. d.f.	d.f.	p	sig.
null	9.1230		310			
batch	6.4516	2.6715	294	16	< 0.001	***
iso	6.1318	0.3197	284	10	0.139	
sex	5.9173	0.2146	283	1	< 0.01	**
cal	4.2128	1.7045	280	3	< 0.001	***
pc	4.1111	0.1017	277	3	0.077	

Table 5.7: The minimal adequate model for lipid content, using arcsine square-root transformed proportions. The minimal adequate model was arrived at by removing non-significant terms from the full model. The significance of each factor was assessed by adding factors in turn to the model (iso) and assessing the reduction (– dev) in residual deviance (res. dev.). Key to factors: Batch – processing batch; Iso – Isofemale line; Sex – sex of fly; Cal: total macronutrient content (g l^{-1}); PC – protein:carbohydrate ratio of diet.

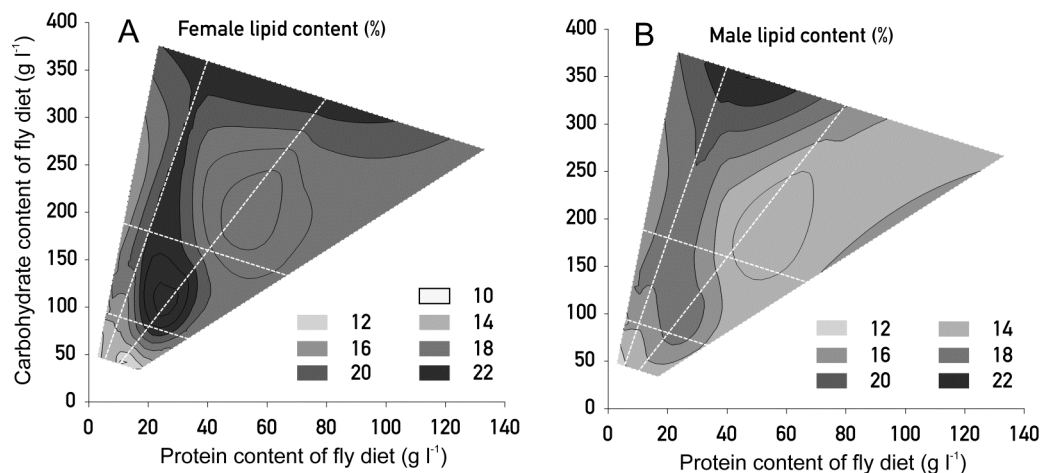


Figure 5.11: Mean lipid content (%) for **A:** female flies and **B:** Male flies, represented as contour plots. The x-axis represents the protein content of the 16 experimental diets and the y-axis represents the carbohydrate content. The solid shading on the surface indicates the mean or interpolated value. The dotted white lines (and the boundaries of the shaded area) represent the four P:C ratios and the four isocalorics used to construct the diets. Each intersection or corner represents one of the 16 diets.

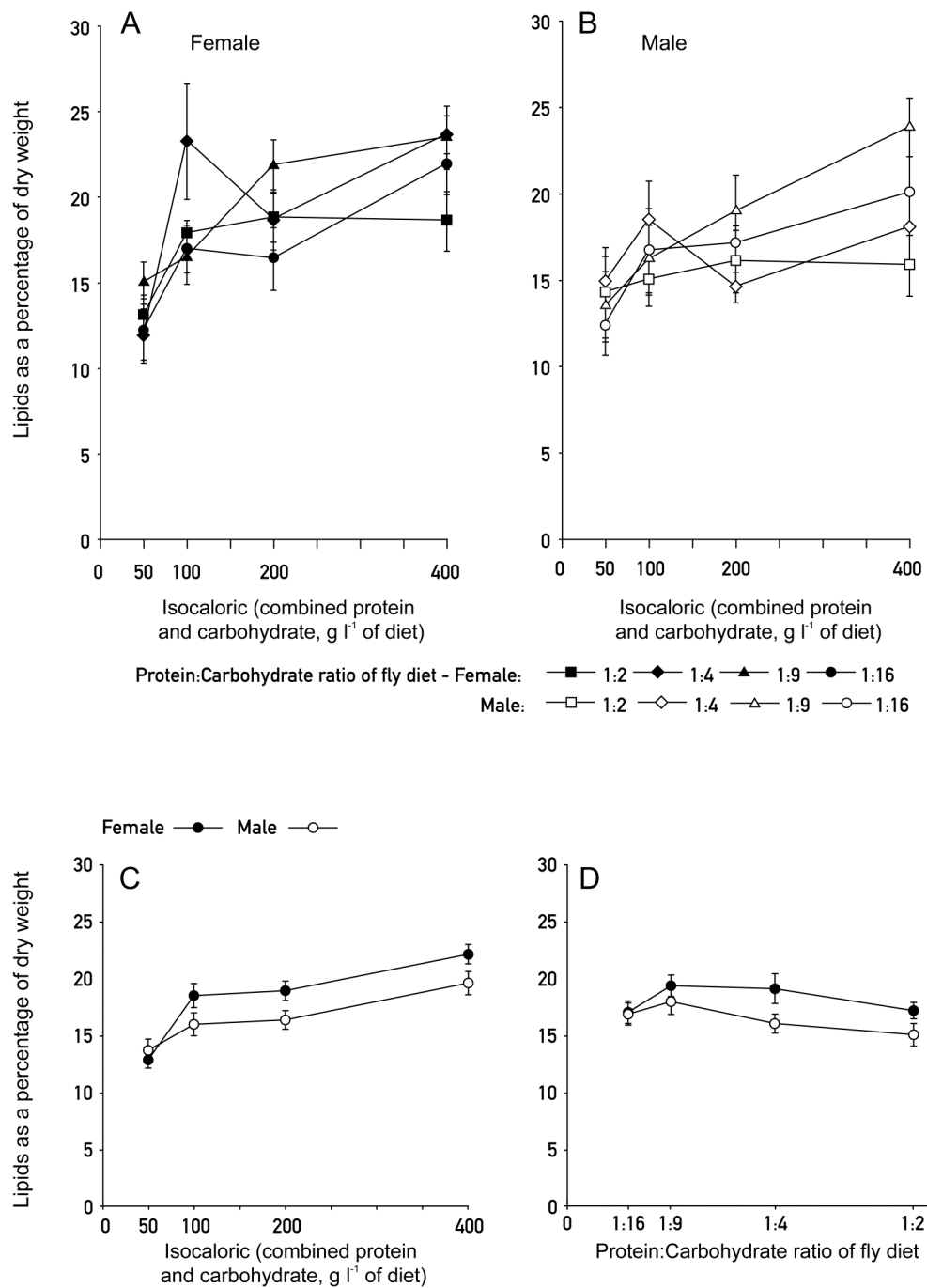


Figure 5.12: Mean lipid content of flies on differing diets, as a percentage of dry body weight. Each point represents the mean thorax length of 11 isofemale lines of flies raised on the 16 experimental diets, grouped by **A:** female flies and **B:** male flies **C:** isocaloric of fly diet and sex. **D:** Protein:Carbohydrate ratio of diet and sex. Error bars represent ± 1 standard error of the mean.

5.3.6 Gut residence time

Larvae may feed at different rates on different diets depending on the calorific content or on the P:C ratio of the diet. If they do, this may be detected as a faster or slower gut-clearance rate. To check for this, larvae from 12 isofemale lines were allowed to develop on four dyed fly diets, each composed of one of two levels of isocaloric and two P:C ratios and then the larvae placed on un-dyed diet. The length of time each larva took to replace its gut contents with un-dyed food was recorded. The null hypothesis was that neither the isocaloric of the diet nor the P:C ratio would affect gut residence time.

In two of the diet treatments, there were very low numbers of surviving larvae (2/50 – 7 isofemale lines survived out of 12; 9/50 – 4 lines out of 12). There were two missing lines from 2/400 and one from 9/400. Consequently the degrees of freedom in the generalized linear model were lower than if all combinations had been present.

Flies provided with the low-isocaloric diets had a significantly longer gut residence time of 6.0 ± 0.3 h (Fig. 5.13, Table 5.8) than the 3.8 ± 0.2 h taken by flies on the high-isocaloric diets (change in deviance = 203.3, 1 d.f., $p < 0.001$). A higher P:C ratio of the diet shortened gut residence time by a smaller amount, at 4.3 ± 0.4 h compared to 4.8 ± 0.3 h for the lower P:C ratio diets (change in deviance = 38.5, 1 d.f., $p < 0.01$). This effect was more apparent on the high-isocaloric diets, indicating that the effect of P:C ratio varied with calorific content (change in deviance = 18.87, 1 d.f., $p < 0.05$). Gut residence time was not significantly different across isofemale lines (change in deviance = 66.63, 10 d.f., $p = 0.94$).

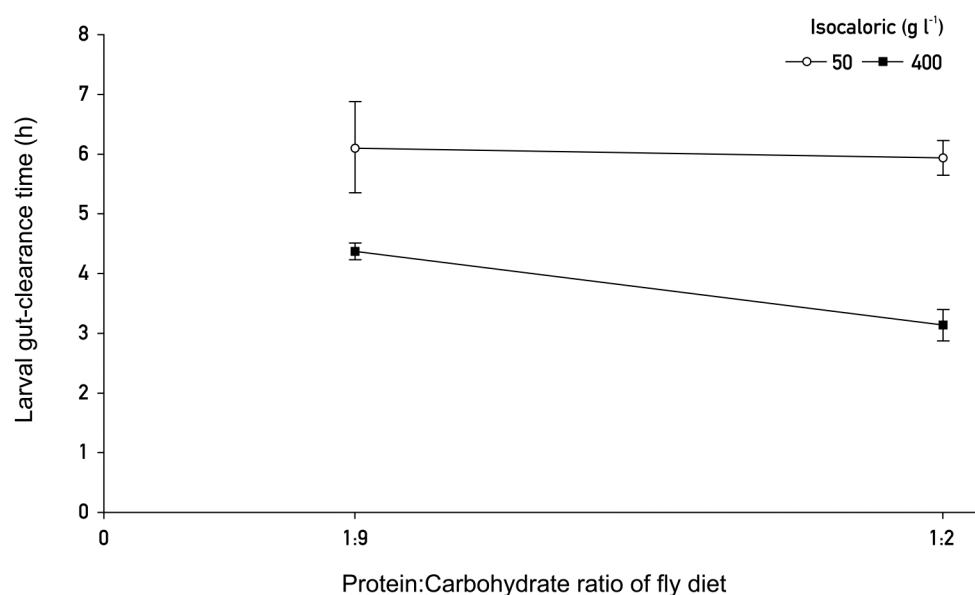


Figure 5.13: Mean gut-clearance time (h) of L3 fly larvae from 12 isofemale lines, raised on four different diets of differing calorific content and protein:carbohydrate (P:C) ratio. Diets contained either 50 g l⁻¹ (open circles) or 400 g l⁻¹ (closed squares) of combined protein and carbohydrate and with a P:C ratio of either 1:9 or 1:2. Error bars represent ± 1 standard error of the mean.

Minimal adequate model for gut clearance time

Factor	Res. dev.	-dev.	Res. d.f.	d.f.	p	sig.
Null	388.88		31			
Block	387.07	1.81	30	1	0.71	
Iso	320.44	66.63	20	10	0.94	
Cal	117.15	203.30	19	1	< 0.001	***
PC	78.64	38.51	18	1	< 0.01	**
Cal:PC	59.77	18.87	17	1	< 0.05	*

Table 5.8 The minimal adequate model for gut-clearance time was arrived at by removing non-significant factors and interactions from the full model. The significance of each factor was then assessed by adding factors in turn to the model (Block+iso) and assessing the reduction (– dev) in residual deviance (res. dev.). Key to factors: Block – block; Iso – isofemale line; Cal – total macronutrient content (g l⁻¹); PC – P:C ratio of diet.

5.4 Discussion

5.4.1 Were flies' responses determined by the protein: carbohydrate ratio or by the isocaloric of their diets?

This experiment measured how the P:C ratio and macronutrient content of fly diets influence morphological fitness indicators and so casts light on what dietary factors are most important for flies' fitness.

The amount of diet actually consumed by the flies in the diet range experiment was not measured, as tracking the daily amounts eaten from the hatching of the eggs to 3-day old adults would have been impractical at this time. Lee *et al.* (2008) also ran a parallel experiment where diet was manipulated but intake was not measured, and found the same patterns as when flies were housed singly and intake was measured (Simpson and Raubenheimer 2009).

The majority of the diet treatments had an egg to adult survival rate of between 60 and 70 % (Fig. 5.3 and 5.4). However survival was strongly affected by extremes of diet, as flies on low isocaloric and low P:C ratio diets had a dramatic drop in survival rate to just over 30%, while for those on high isocaloric diets with high P:C ratios, survival rate rose.

Dry weight followed a similar pattern, although the increase with isocaloric and P:C ratio was more linear (Fig. 5.9, Fig. 5.10). The exception to this was male flies, which did not respond to increasing calorific content as strongly.

Flies on low isocaloric diets generally had large thoraxes and low lipid levels, and this was especially true when a low isocaloric was combined with a high P:C ratio, or when a high isocaloric was paired with a low P:C ratio (Fig. 5.7 and Fig. 5.11). For females, thorax length was less dependent on the isocaloric of the diet than it was for males (Fig. 5.7, Fig. 5.8).

The time to eclosion was most strongly affected by the P:C ratio of the diets, with a short development time on high P:C ratios and a long development time on low P:C ratio diets. The isocaloric of the diet did have a smaller but still significant effect, but here the minimum time to eclosion was in flies developing on the intermediate isocaloric diets (Fig. 5.5, Fig 5.6).

The P:C ratio of the flies' diet was a significant factor in the responses of survival percentage, time to eclosion, thorax size and dry weight, all relating to fitness (Partridge et al. 1994; Chippindale et al. 1996; Prasad and Joshi 2003; Skorupa et al. 2008). These all increase the chances of leaving offspring in the next generation, whether by surviving to adulthood, reaching adulthood sooner, or increasing mating success or egg-laying ability (Partridge et al. 1987; Partridge and Fowler 1993). However, all of the traits influenced by the P:C ratio reached their maximum values (in terms of fitness) at the highest P:C ratio, 1:2, rather than the ratio of 1:4 associated with lifetime egg production (LEP) by Lee et al. (2008). The P:C 1:2 ratio was the ratio at which flies had the highest rate of egg-production; but also a shorter lifespan (Lee et al. 2008). It is debatable whether lifetime egg production or egg-laying rate is most important to a wild *D. melanogaster*, as their lifespan in the wild is thought to be in the region of 1.3 to 6.2 d (Rosewell and Shorrocks 1987). This could mean that although their potential lifespan is reduced on a higher P:C diet than the ratio which produced the highest lifetime egg production over a period of between 8 and 12 days (Lee et al. 2008), they may be capable of producing more eggs in their likely lifespan. Flies on the lowest P:C ratio were much slower to eclose than those on higher ratios, a pattern is also seen in the lifespan data from Lee et al. (2008) and Fanson et al. (2008). An exception to this was the 16/50 diet, where development time was fast relative to other diets on its isocaloric (50 g l⁻¹) or P:C ratio (1:16). This may have been caused by variation between isofemale lines, as larvae in some lines (isofemale lines 2, 5, 9, 16, 18 and 32) appeared to be developing slowly, but then mostly died, while those isofemale lines which could survive in this low-nutrient environment developed quickly. It may be that either the critical size for pupariation was not reached, or that having been reached, nutritional resources were not sufficient to take the larvae through to pupariation in those isofemale lines.

Flies on the higher isocalorics had unexpectedly smaller thoraxes than those on the lower isocalorics, and this was true for both males and females. Faster developmental time has been linked with larger size in flies (De Jong and Bochdanovits 2003; Layalle et al. 2008) despite selection experiments showing the opposite (Robertson 1960, 1963; Partridge and Fowler 1993). Similarly, there are observations that developmental time negatively correlates with thorax size along latitudinal clines (De Jong and Bochdanovits 2003) and when food quality was varied (Valtonen et al. 2011). Layalle et al. (2008) propose this to be the result of a higher growth rate during the larval stages. A higher growth rate means the larva reaches the trigger point for pupariation sooner, and in the

remaining time to pupariation gains more weight than those with a slower growth rate (Layalle et al. 2008).

Chippindale et al. (1996) selected flies for starvation resistance and found that two of the traits that also changed in the selected flies were increased lipid content and a longer time to eclosion. In this study lipid content was more closely associated with the isocaloric of the diet than the P:C ratio (which was associated with eclosion), although there was some overlap between the two as slower-eclosing flies were also found to be those on higher isocalorics, with correspondingly higher lipid content. Skorupa et al. (2008) found that flies on high carbohydrate diets had the highest triglyceride content, rather than those only on high isocalorics.

5.4.2 Feeding rate

Larvae on the higher isocalorics were processing more food than those on lower isocalorics, as their gut residence times were around two-thirds of those on the lower isocalorics (Fig. 5.13). The shorter gut-residence time in the high isocaloric larvae could be a result of the rate of absorption of nutrients depending on concentration, leading to a shorter residence time (Raubenheimer and Simpson 1997). This could also explain the longer residence time seen in the low-nutrient isocalorics, to extract as much as possible out of the food ingested (Raubenheimer and Simpson 1997). However, Skorupa et al. (2008) also found an increase in feeding rates in adult flies at higher isocalorics in the same range of P:C ratios used in these diets. When considered over the full range of their diets, carbohydrate appeared to be the main factor increasing the feeding rate and protein suppressed feeding (Skorupa et al. 2008).

Wong et al. (2008) contend that dietary restricted adult flies have a larger gut capacity than normally fed flies and therefore attempts to measure their food intake by measuring a labelled (by dye or radioactively) amount of diet ingested will be biased by this. Although the food intake by larvae was measured in this study, the results, that the flies on the higher isocaloric had a residence time of about 63% of those on lower isocalorics, and that the fastest of all were also on a higher P:C ratio, fit well with their observation that dietary restricted flies had a crop size 45% larger than unrestricted flies (Wong et al. 2008). In future, measurements should be limited to 30 min on the novel food, and intake measured by colorimetry (Wong et al. 2008).

5.4.3 Ecological Significance

Drosophila melanogaster adults may only have a life expectancy of between 1 and 6 d in the wild, and their food sources may be widely dispersed (Spencer 1950; Rosewell and Shorrocks 1987; Markow and O'Grady 2008). During this time, they will need to not only locate a food source, but also mate and for the females, locate a suitable oviposition site (Markow and O'Grady 2008). The preferred site is usually ripe or just rotting fruit (Lachaise and Silvain 2004), which has a relatively high sugar content compared with that same fruit several days later, as the yeasts which decompose the fruit ferment the sugars to alcohol (Spencer 1950). In temperate climates their preferred sites may not be available at all times of the year, but late summer and autumn provide the highest abundance of ripe fruit (Spencer 1950).

In terms of the proportions of sugars to proteins, while the yeast will ferment sugar, this may lead to a higher ratio of protein to carbohydrate. Rotting cactus, a host for the related *D. pachea*, does not contain significantly less nitrogen than undamaged cactus (Markow et al. 1999). So a fall in the level of carbohydrate may be a signal to the larvae that the host plant or fruit is running out of resources. In this situation, it may be advantageous to not only develop quickly but also to be able to disperse to find a suitable new host.

If this is the case then the traits that are maximised at a high P:C ratio are those which would promote an ability to emerge and disperse. A shorter time to eclosion, higher dry weight, especially in females where this is likely to be linked to fecundity, and a larger thorax would enable larvae to develop and leave the food source quickly and dispersal ability would be increased by a larger body size (Roff 1977).

5.4.4 PARs in relation to larval diet

Although larval to adult PARs were not explicitly tested, one aspect of the phenotypic response to diet was similar to that found in the butterfly *B. anynana*, where it is thought to be a response to poor larval food conditions and directed at dispersal ability (Saastamoinen et al. 2010).

In the experiments described in this chapter, thorax size decreased with increased calorific content of the diet in males and females (Fig 5.7, Fig. 5.8) while dry weight increased with increased calories, in females especially (Fig. 5.9, Fig. 5.10), and so the ratio of thorax size to the remainder of the fly increased on the lower isocaloric diets. In

males, the effect is more that dry weight declined at very low calories (Fig 5.10b). Although not measured, the abdomen of flies on lower isocaloric diets is likely to be much smaller, and conversely that well-fed larvae produce adults with comparatively large abdomens and smaller thoraxes. This effect was also seen in relation to larval food restriction in the tropical butterfly *B. anynana* (Saastamoinen et al. 2010). This was linked to dispersal ability, and a similar reason may be why the absolute size of the thorax of the flies increases at low isocalorics. It would be adaptive for a larva developing on a low-calorie diet to develop dispersal ability at the expense of fecundity so that a better food source may be found for its own offspring (Saastamoinen et al. 2010). This will require further testing.

5.4.5 Conclusions and future work

Using the geometric framework technique with *Drosophila melanogaster* has allowed the macronutrient factors which produce different phenotypic effects to be separated from the quantities of macronutrients provided in the diets (Simpson and Raubenheimer 1995). Males and females responded in broadly similar ways and from all the responses there emerges two distinct phenotypes, not dissimilar to those described when selecting for starvation resistance (Chippindale et al. 1996). The first, when the development diet (and for the first three days of adulthood) has a high proportion of protein compared to carbohydrate (20% or over) is characterised by fast development and eclosion, a larger thorax, larger dry mass and slightly lower lipid, while the second has slow development and eclosion, higher lipids, smaller thorax and less dry mass, and occurs when the diet during development (and the first three days of adulthood) has a low proportion of protein compared to carbohydrate (10% or less). The quantity or concentration of macronutrients in the diet appears to be less important, accounting for less variation than does diet quality, except in lipid content, where it is the most important factor. The effect of diet on survival is less certain, as although both P:C ratio and isocaloric are important, it may be that the absolute quantity of protein in the diet is the most important factor, particularly at low levels.

This approach could be used to investigate PARs in relation to larval diets, especially if coupled to a behavioural assay, for example testing the stress induced by flight as this may indicate whether dispersal ability is enhanced, or at least prioritised, when larval food resources are low, as found by Saastamoinen et al. (2010). There were gradients in the responses of survival, time to eclosion, thorax size, dry weight and lipid

content in relation to both of the manipulated dietary parameters, calorific content and P:C ratio, in the experiments in this chapter. Fecundity has been measured in relation to adult diets using a geometric framework (Lee et al. 2008; Skorupa et al. 2008), but it could be usefully used as a response in relation to larval diet, which would highlight whether the phenotypic changes observed here have fitness consequences. This need not be a lifetime fecundity measure, but could be early-life fecundity as this may be more ecologically relevant to *D. melanogaster* (Rosewell and Shorrock 1987). It could also be helpful if gene expression levels were measured, for instance those concerned with the InR/Tor or AMPK pathways, and comparison made with how these match the phenotypic responses seen in this study. These responses appear to be phenotypically similar to those seen when either the TOR pathway or the AMPK pathways are activated, either accelerating or suppressing growth (Layalle et al. 2008; Johnson et al. 2010). The flies on the lower P:C ratios may have a higher activation of the AMPK pathway due to lower nutritional quality (Simpson and Raubenheimer 2009; Johnson et al. 2010).

The factors potentially controlling human obesity include not only the actual composition of the diet (Wade et al. 1981; Theall et al. 1984; Eaton et al. 1997; Simpson et al. 2003; Eaton 2006; Warbrick-Smith et al. 2006; Brooks et al. 2010) but also cultural and behavioural factors (Prentice and Jebb 1995; Eaton and Cordain 1997; Towler and Hardie 2007; Johnson et al. 2010) and the PAR hypothesis (Gluckman and Hanson 2004; Hanson 2005). The response of the flies in this experiment does show clearly that macronutrients on their own may not have different effects on the accumulation of lipids, but that when placed on a no-choice diet, a higher calorific content produces flies with a higher lipid content. This is especially so as larvae developing on the higher isocaloric diets appear to process a higher volume of food than those on lower isocaloric diets (Fig. 5.13).

The effect of the P:C ratio on the flies' body size and fitness indicators was significant and controlled speed of development and size, in the same way but over a larger range of diets than in the multigenerational diet experiment (Chapter 3). In terms of fly fitness, a higher P:C ratio produced the highest fitness indicators overall, and this did vary with the isocaloric of the diet as well. These higher P:C diets correspond to what is thought to be similar to a human ancestral diet (Eaton 2006). However, as noted above, flies in the wild are short lived and may prioritise reproduction. In future, using a range of diets in an array to measure fitness indicators could be combined with measures

of fly health to provide insights into the effect of dietary components on aspects of their reproductive and dispersal abilities and their health.

5.4.6 Acknowledgements

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Chapter Six

The effect of diet on neurogenesis in *Drosophila melanogaster*

6.1 Introduction

6.1.1 Nutrition and brain development in humans and other mammals

The quality of periconceptual and perinatal nutrition is thought to alter the phenotype of humans and other mammals including rats and mice, in anticipation of the continuation of the maternal nutritional environment (Gluckman and Hanson 2004; Burdge et al. 2007b; Watkins et al. 2008). One aspect of an altered phenotype may be the development of the brain, which has been shown to be affected by nutrition during pregnancy and post-natally (Molteni et al. 2002; Gornez-Pinilla and Vaynman 2005; Isaacs et al. 2008; Dauncey 2009). The size of the human brain is unusual in a primate and it accounts for 25% of the resting metabolic rate, in contrast to 10% for most other primates (Aiello and Wheeler 1995; Leonard et al. 2007) so it might be expected that adverse maternal nutrition during pregnancy would result in some changes in its size or structure.

Experimental evidence in other mammals indicates that nutrition in pregnancy does affect brain development. The effect of energy restriction on pregnant rats was tested by Atallah et al., (1977), who found that when rats were restricted to half of the normal feed, brain and body mass were significantly lower in the 8 week-old pups. However, when some of this restricted diet was replaced with casein, body size was still restricted, but brain mass was the same as the unrestricted controls (Atallah et al. 1977). This indicates that protein was being preferentially diverted from metabolism and overall growth to development of vital organs (Atallah et al. 1977). More recently, similar effects have been seen in guinea pig pups which had been subjected to under-nutrition during gestation (Mallard et al. 2000). The restricted-nutrition pups did not show as great a reduction in brain volume (16%) as they did in total body weight (42%), indicating that as in the rats, resources were diverted towards brain development (Mallard et al. 2000).

Fetal intra-uterine growth restriction in pre-term infants has been shown to reduce the volume of the hippocampus, a structure of the brain important in learning and memory, although the authors could not be certain that this reduction was not part of a global reduction in growth caused by under-nutrition (Lodygensky et al. 2008). A similar reduction in volume has been observed in the cortex following nutritional restriction in

the womb, although here grey matter was reduced more than white matter in intra-uterine restricted growth infants (Tolsa et al. 2004).

In contrast to the above studies showing a detrimental effect of poor nutrition, others suggest that over-nutrition can cause memory, brain development and mental health problems in adults (Newcomer 2007; Jurdak et al. 2008; Jacka et al. 2010; Kanoski and Davidson 2011).

A “Western” diet has been linked to depression in Australian women, although no single dietary factor was found to be close to significant, and other factors including smoking and activity levels were included in the analysis (Jacka et al. 2010). In another study on the links between mental health and metabolic syndrome, there was no clear distinction between an association or a cause, making it difficult to be certain whether metabolic syndrome is a cause of mental illness or a symptom (Newcomer 2007).

However, some animal studies have demonstrated that a diet high in refined carbohydrates or fats can cause memory problems (Molteni et al. 2002; Jurdak et al. 2008; Kanoski and Davidson 2011). When rats were kept on a high-fat simple carbohydrate diet, their spatial memory was adversely affected, and expression of BDNF (Brain-derived neurotrophic factor, which helps to maintain neurons and is involved in neurogenesis) was lower than in controls (Molteni et al. 2002). The effects of cardiovascular disease or hypertension on the brain were ruled out as the diet produced no symptoms of these at the time of the experiment (Molteni et al. 2002), although another study found evidence of impairment of memory in diabetes-induced rats (Stranahan et al. 2008). In a follow-on study, the effects of a Western diet were reversed by doses of the antioxidant Vitamin E, suggesting that free radicals may be the mechanism by which high-fat, high-carbohydrate diet causes low BDNF and memory impairment in rats (Wu et al. 2004). In another study, rats fed a control diet were found to perform significantly better on a spatial memory task than rats who were fed a diet high in simple carbohydrates (Jurdak et al. 2008). These studies have been suggested to indicate that the Western diet (that is, one high in saturated fat and simple carbohydrate, and with a high energy density), may even initiate a vicious cycle if impairment of the hippocampus by diet weakens appetitive control and so promotes weight gain and further memory impairment (Kanoski and Davidson 2011)

6.1.2 Ommatidia and neurogenesis in *Drosophila melanogaster*

When *Drosophila* are raised on a poor diet, they do not perform as well as controls on tasks related to memory and learning ability (Guo et al. 1996; Xia et al. 1997; Kolss and Kawecki 2008). This is thought to be the result of a trade-off between learning ability and development in an unsuitable environment as the poor nutritional environment also resulted in smaller flies, although in this study, brain size was not measured (Kolss and Kawecki 2008).

Visual acuity is an important trait in the ecology of *Drosophila* and plays an important part in courtship (Willmund and Ewing 1982; Schöffel and Willmund 1985). Males follow females by visually tracking them, and males with mutations affecting vision are unable to do this (Willmund and Ewing 1982; Schöffel and Willmund 1985). Females assess visually not only the courtship dance of males, but also the males' eyes, although they prefer males with no eyes to those with white eyes (Willmund and Ewing 1982; Schöffel and Willmund 1985). The visual system of *Drosophila* is also important in flight control, as visual cues are used to assess and control the speed of flight, largely independently of wind speed (Fry et al. 2009).

The size of the optic lobe in *Drosophila* can be assessed by the number of ommatidia (facets) in the compound eye as each ommatidia is associated with a column of cells in the lamina of the optic lobe (Mast et al. 2006). The *Drosophila* compound eye consists of around 750 ommatidia, each innervated by eight photoreceptors (R cells), and the number of ommatidia is closely related to the size of the optic lobe (Rein et al. 1999). Females have an optic lobe around 6% larger than males, and in wild-type flies kept in similar environments, the variation in number of ommatidia and the corresponding size of the optic lobe is small (Rein et al. 1999). The size of the different regions of the *Drosophila* brain can be altered during adulthood by environmental and social conditions, but the number of projections from the retina to the lamina of the optic lobe (and therefore the number of ommatidia) do not change (Heisenberg et al. 1995).

The outer R cells, R 1-6, are sensitive to green light and innervate the lamina (Mast et al. 2006). The inner two cells, R7 and R8, are sensitive to ultraviolet or blue green light and innervate the medulla (Mast et al. 2006). Axons from each ommatidium enter the brain as a single fascicle, but then split up so that axons from ommatidia which face a common direction are directed at a single target region (Mast et al. 2006). During development, the already formed R8 axons direct the formation of the lamina, in the

same sequence (rows from posterior to anterior) in which they differentiated in the retina, causing the differentiation of the lamina precursor cells (Mast et al. 2006). This process matches the number of cells of the lamina to the number of ommatidia.

Differentiation of the R8 cells occurs in the eye imaginal disk of the third instar larva and is promoted by Ato expression at the anterior side of a morphogenic furrow travelling posterior to anterior (Lim and Choi 2003). The spacing of the R8 cells is determined by inhibition of Ato in the neighbouring cells (Baonza et al. 2001; Frankfort and Mardon 2002). This mechanism also ensures that the each row of R8 cells is staggered with respect to the previous row and creates a regularly spaced array of R8 cells (Baonza et al. 2001). The size of the imaginal disk is controlled by a histone deacetylation protein HDAC3, which controls gene suppression and apoptosis (Zhu et al. 2008).

Eye size in the developing larva is linked to nutrient availability as the insulin signalling (InR) pathway and the TOR pathway are linked and together help to control growth and differentiation (Bateman and McNeill 2004; Léopold 2004). TSC (a repressor of TOR) is not necessary for the differentiation of R8 cells in the eye imaginal disk, but it does have an influence on the timing of their formation and coordinates with the other neurons of the ommatidial complex (Bateman and McNeill 2004). TSC is itself repressed by AKT, a component of the InR pathway (Bateman and McNeill 2004), linking the two pathways, with TOR responding to amino acids (Colombani et al. 2003) and the InR pathway to carbohydrates (Rulifson et al. 2002). Therefore it is expected that diet, especially larval diet, will have an influence on eye size and the size of the optic lobe.

6.1.3 Experimental aims

A number of studies have shown a detrimental effect of under-nutrition (Atallah et al. 1977; Mallard et al. 2000; Lodygensky et al. 2008) or over-nutrition (Molteni et al. 2002; Wu et al. 2004; Jurdak et al. 2008; Stranahan et al. 2008) on learning or brain structure in humans and in other animals. If *Drosophila melanogaster* show an effect in relation to the diet of previous generations, this may indicate that resources are diverted from brain development in response to environmental conditions, in a PAR in anticipation of the adult environment. It will also be useful to know how different dietary

components of the larval and early adult diet affect the brain and whether diet quality or quantity change the relative resources put into neurogenesis.

Measurement of the number of ommatidia per eye, scaled to body size in *Drosophila*, is a way of assessing the proportion of resources devoted to neurogenesis during development. This can be carried out on a large number of subjects in a relatively short time compared to using techniques involving measurement of the whole brain. The number of ommatidia is set during the final larval instar (Mast et al. 2006) and so should reflect the larval dietary environment rather than conditions encountered as an adult, which can affect the size of different regions of the brain (Heisenberg et al. 1995).

Competition for limited nutritional resources is an important factor determining the relative size of body parts (Nijhout 2003) although on the other hand poor larval nutrition may adaptively increase the size of some brain regions in anticipation of having to find a new environment as an adult (Heisenberg et al. 1995). Although this was proposed in relation to the relative size of the mushroom bodies (relating to memory), it is not unreasonable to assume that the visual system might also benefit from an adaptive effect, as it is important in the control of flight (Fry et al. 2009).

In the first experiment presented here, data were collected on eye size, ommatidia size and ommatidia number from flies from the multigenerational diet experiment (Chapter 3). As the low-carbohydrate P diet and W diet had different effects on offspring fitness, it was hypothesised that flies might divert resources to or from neurogenesis in anticipation of an adult environment that was a continuation of the parental or grandparental one. Males and females may also have differing reactions to the diets, as for instance, the demands of courtship on the visual systems of males and females are different (Willmund and Ewing 1982; Schöffel and Willmund 1985; Heisenberg et al. 1995). Therefore the null hypothesis was that neither sex nor any generations' diet or interaction of diets would affect the number of ommatidia per eye, relative to body size, taken as thorax length.

In the second experiment, ommatidia measurements were taken from flies in the diet range experiment (Chapter 5) and the effect of varying macronutrient content and the P:C ratio was explored. If low energy content or a low P:C ratio acted as a constraint on eye development, then we should expect a higher number of ommatidia in relation to thorax length on richer diets. Alternatively, if poor diets induce an adaptive effect, for example directed at dispersal ability, the opposite may be true. The null hypothesis was

that neither the energy content of the diet (isocaloric), the P:C ratio of the diet nor sex of the fly should affect the ommatidia number relative to the length of the thorax. For both of the above, eye size, ommatidia size and absolute number of ommatidia were also examined with similar null hypotheses.

6.2 Methods

6.2.1 Response to parental and grandparental diets

Samples from the multigenerational diet experiment (Chapter 3) were used for all measurements. The flies used for the eye and ommatidia measurements were the same flies as used for thorax, wing length, dry weight and lipid percentage in Chapter 3. Samples had been stored at -18 °C, sorted by isofemale line and diet group. Five female and five males were chosen randomly from each sample, if five flies were available. Measurement of eye and ommatidia size took place at the same time as eye and wing, using a stage microscope (Nikon Eclipse E400) with camera lucida attachment (Nikon Y-1DT) and a cold light source (Schott KL1500), using 10x objective for the eye measurement and 20x for the ommatidia. Measurements were calibrated using a 1 mm stage graticule (Pyser-SGI, Kent) at the start of each measurement session (1 mm for the 10x measurements and 0.1 mm for the 20x). The measurements from the tracings were then converted to millimetres.

Eye size was measured only as eye length (Fig. 6.1), due to the difficulty of keeping the whole of the rim of the eye in a plane parallel to the plane of focus. The width of the eye was obtained from multiplying length by a factor of 0.7638 (from measurement of 12 eyes photographed parallel to the microscope), and its area determined by the formula $\text{area} = (\frac{1}{2} \text{ length} \times \frac{1}{2} \text{ width}) \times \pi$. Ommatidia area was measured by measuring the combined diameter of eight ommatidia, dividing this by eight and then the area of one ommatidia was calculated using the formula of the area of a regular hexagon ($\text{area} = ((3 \times \sqrt{3})/2) \times (\frac{1}{2} \text{ diameter})^2$). The number of ommatidia per eye was then obtained by dividing eye area by ommatidia area. Responses derived from more than one measurement, for instance the number of ommatidia per eye, were calculated for individual flies before a mean for the isofemale line was calculated. Data for thorax length was obtained from the same flies as were used to collect data for eye and ommatidia size (see Chapter 3 for thorax length analysis).



Figure 6.1: Photomicrograph of the eye of a male *Drosophila melanogaster*, taken using a 10x objective. The larger white bar represents the distance measured along the long axis of the eye. The smaller scale bar (bottom right) represents 0.2 mm.

6.2.2 Diet range

Samples from the diet range experiment were used for all measurements and these were taken from the same flies as the thorax, dry weight and lipid percentage measurements (see Chapter 5 for details). Five flies of each sex were selected randomly from each isofemale line / diet combination and photomicrographs were taken on stage microscopes (see Chapter 5 Methods for full details). The photomicrographs were taken by RS (lines 2, 7, 16, 32), LC (line 4), GC (line 9), TF (line 10), KH (line 17) and JM (line 31) (see Section 6.4.4 Acknowledgments). They were measured using Adobe PhotoShop CS (Adobe Systems Inc., San Jose, USA) by RS, when thorax and ommatidia measurements in pixels were converted to millimetres by calibrating with the photomicrographs of the graticules taken at the start of each measurement session (Psyer-SGI, Kent).

Tonal balance was altered for clarity when necessary but no other editing of the photomicrographs was carried out. Eye size, ommatidia size, the number of ommatidia and the number of ommatidia scaled to thorax were calculated in the same way as were the data from the multigenerational diet experiment.

6.2.3 Statistical analysis

Response variables were measured on individual flies, but the mean from each treatment, isofemale line and sex combination was used as the replicate in the statistical analysis. All response variables were tested for a normal distribution using a Kolmogorov-Smirnov test, and equality of variance between sex and diet groups using Levene's test in IBM SPSS Statistics 19 (SPSS Inc, Chicago). Responses were not significantly different from a normal distribution, and the variances were not significantly different between treatments in either the multigenerational diet experiment or the diet range experiment. The data were then analysed using a Generalized Linear Model in R 2.1.8 (R Development Core Team 2008), using a model building approach where factors or interactions were removed from the full model if their removal did not significantly increase the residual variance, assessed by analysis of variance and a chi-squared test. Factors and interactions were tested in the sequence of highest order of interaction first, and then least significant interaction or factor, assessed by analysis of variance.

The factors "day of egg-laying" (in the multigenerational diet experiment) and "isofemale line" were kept in models regardless of significance and none of their interactions with diet or sex were included. Day of egg laying, in the multigenerational diet experiment, varied according to how quickly previous treatment / isofemale line combinations were ready to lay eggs for the F2 generation, and was included in case of unquantified environmental factors not otherwise accounted for. Isofemale line represents the genetic variability in this model, with the 25 isofemale lines in the multigenerational diet experiment and the nine isofemale lines in the diet range experiment being randomly-selected samples from the original AV population (see Chapter 2). Each data point (mean of isofemale line/treatment/sex) was weighted in the analysis (but not in the figures) by the number of flies used to obtain that data point, so that if less than five flies were available, that data point would not have a disproportionate influence on the statistical model.

6.3 Results

6.3.1 Multigenerational diet experiment – eye area, ommatidia number and ommatidia area

The area of the flies' eyes, area of ommatidia, the number of ommatidia per eye and the number of ommatidia per eye relative to the size of the fly might change in response to different grandparental, parental or offspring diets. To test this, 25 isofemale lines over three generations of flies were raised on two diets P or W, and each generation half the flies on each diet were swapped to the opposite diet. The null hypotheses were that for each of the response variables, there would be no effect of grandparental, parental or offspring diet, or of the sex of the fly, or any interaction between these factors.

The area of the eye and the number of ommatidia per eye responded in a similar way to diet, although sex was the major determinant of eye size (change in deviance 0.1292, 1 d.f., $p < 0.001$, Fig. 6.2a and b, and Table 6.1) and ommatidia number (change in deviance 1158739, 1 d.f., $p < 0.001$, Fig. 6.2e and f and Table 6.3). Females had larger eyes overall, and the highest number of ommatidia. Males and females responded differently to the parental diet, as females had a smaller eye area if their parents had developed on the W diet, while males had a smaller eye area if their parents developed on the P diet (change in deviance 0.0009, 1 d.f., $p < 0.05$). The same effect was true for the number of ommatidia per eye (change in deviance 62248, 1 d.f., $p < 0.05$). In both eye size (change in deviance 0.0008, 1 d.f., $p < 0.05$) and the number of ommatidia per eye (change in deviance 55664, 1 d.f., $p < 0.05$) flies developing on the high-carbohydrate W diet had significantly smaller eyes and fewer ommatidia per eye.

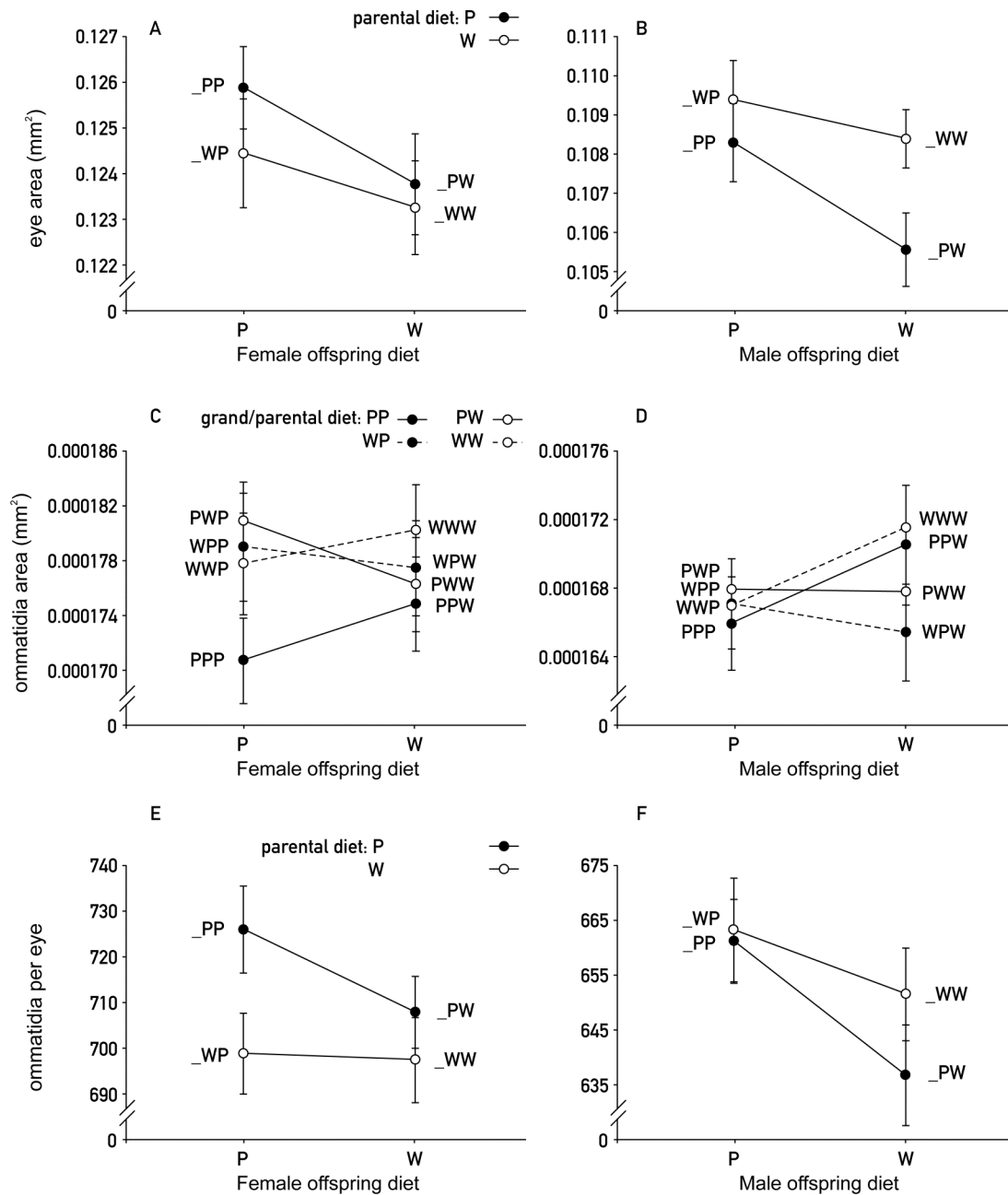


Figure 6.2: The main factors controlling **A:** females' eye area (mm²); **B:** males' eye area (mm²); **C:** females' ommatidia area (mm²); **D:** males' ommatidia area (mm²); **E:** the number of ommatidia per eye for females and **F:** the number of ommatidia per eye for males, in an experiment to test the influence of offspring, parental and grandparental diet on flies. P is the normal-carbohydrate diet and W the high-carbohydrate. All error bars are ± 1 standard error of the mean.

The area of an individual ommatidium was significantly larger in females than males (change in deviance 5.09×10^{-8} , 1 d.f., $p < 0.001$, Fig. 6.2c and d and Table 6.2), and the response of males and females to parental and grandparental diet was also different (change in deviance 2.70×10^{-9} , 1 d.f., $p < 0.05$). In females, flies whose parents

and grandparents had both developed on the P diet had smaller ommatidia than other groups, while in males there was no clear pattern of parental or grandparental influence.

Factor	Res. dev.	– dev.	Res. d.f.	d.f.	<i>P</i>	Sig.
Null	0.1947		367			
Day	0.1929	0.0018	357	8	0.9704	
Iso	0.1827	0.0604	333	1	0.7701	
Sex	0.0637	0.1292	332	1	< 0.0001	***
Offspring	0.0629	0.0008	331	1	< 0.05	*
Parental	0.0629	0.0000	330	1	0.6437	
Sex: Parental	0.0620	0.0009	329	1	< 0.05	*

Table 6.1: The minimal adequate model for mean eye area (mm²). The minimal adequate model was arrived at by removing non-significant terms from the full model. The significance of each factor was assessed by adding factors in turn to the model (Day + Iso) and assessing the reduction (– dev) in residual deviance (res. dev.). Key to factors: Day – day of laying; Iso – Isofemale line; Sex – sex of fly; Offspring – offspring diet; Parental – parental diet.

Factor	Res. dev.	– dev.	Res. d.f.	d.f.	<i>P</i>	
Null	3.99E-07		367			
Day	3.87E-07	1.28E-08	357	10	0.2980	
Iso	2.46E-07	1.40E-07	333	24	< 0.001	***
Sex	1.95E-07	5.09E-08	332	1	< 0.001	***
Parental	1.94E-07	1.03E-09	331	1	0.1838	
Grandparental	1.94E-07	1.00E-11	330	1	0.8929	
Sex: Parental	1.94E-07	1.31E-10	329	1	0.6366	
Sex: Grandparental	1.94E-07	5.70E-10	328	1	0.3239	
Parental: Grandparental	1.93E-08	3.30E-11	327	1	0.8114	
Sex: Grandparental: Parental	2.00E-07	2.70E-09	326	1	< 0.05	*

Table 6.2 The minimal adequate model for mean ommatidia area (mm²). The minimal adequate model was arrived at by removing non-significant terms from the full model. The significance of each factor was assessed by adding factors in turn to the model (Day + Iso) and assessing the reduction (– dev) in residual deviance (res. dev.). Key to factors: Day – day of laying; Iso – Isofemale line; Sex – sex of fly; Offspring – offspring diet; Parental – parental diet; Grandparental – grandparental diet.

Factor	Res. dev.	– dev.	Res. d.f.	d.f.	<i>p</i>	
Null	7036211		367			
Day	6893789	142422	357	10	0.9999	
Iso	5289719	3914222	333	24	< 0.0001	***
Sex	4130980	1158739	332	1	< 0.0001	***
Offspring	4075316	55664	331	1	< 0.05	*
Parental	4070456	4860	330	1	0.9999	
Sex: Parental	4008208	62248	329	1	< 0.05	*

Table 6.3 The minimal adequate model for mean ommatidia per eye .The minimal adequate model was arrived at by removing non-significant terms from the full model. The significance of each factor was assessed by adding factors in turn to the model (Day + Iso) and assessing the reduction (– dev) in residual deviance (res. dev.). Key to factors: Day – day of laying; Iso – Isofemale line; Sex – sex of fly; Offspring – offspring diet; Parental – parental diet.

6.3.2 Multigenerational diet experiment – ommatidia number relative to thorax length

To test whether relative resources devoted to neurogenesis changed with parental, grandparental or offspring diet, the number of ommatidia, with thorax length as a covariate, was used as the response variable. The null hypothesis tested was that flies would not vary the resources being devoted to neurogenesis during development, according to the offspring diet, parental diet or grandparental diet.

No diet was close to significant on its own and the factor most strongly affecting the proportion of resources put into neurogenesis was the sex of the fly was sex (change in deviance 49703, 1 d.f., $p < 0.05$, Fig. 6.3 and Table 6.4). Male flies, although they had lower numbers of ommatidia overall, had more ommatidia when scaled to body size than did females.

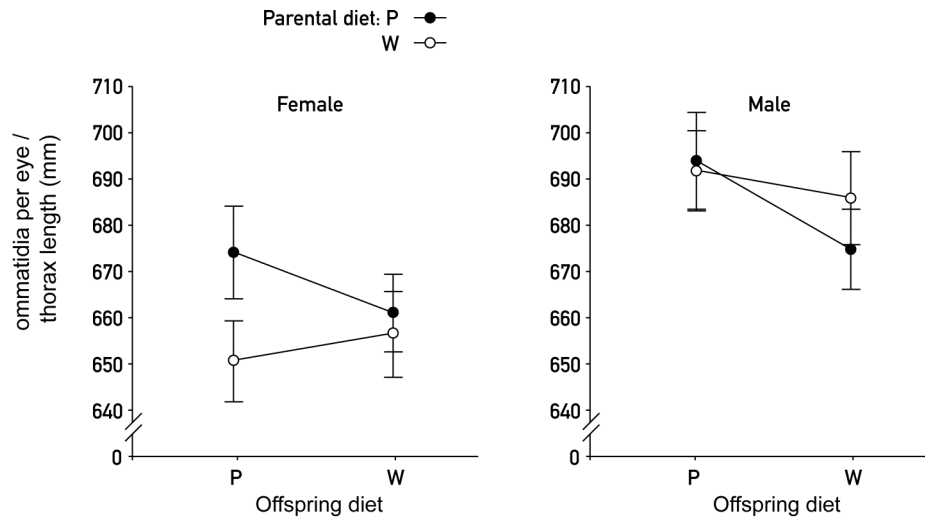


Figure 6.3: The number of ommatidia per eye divided by the length of the thorax, grouped by sex, parental diet and offspring diet of flies. Closed circles: parental diet P, open circles: parental diet W. P is the normal-carbohydrate diet and W the high-carbohydrate. All error bars are ± 1 standard error of the mean.

The parental diet did have a significant interaction with the sex of the fly (change in deviance 52919, 1 d.f., $p < 0.05$). Female flies with P parents had higher numbers of ommatidia per eye than did those with W parents, and for male flies the opposite was true (Fig 6.3., Table 6.4).

Factor	Res. dev.	– dev.	Res. d.f.	d.f.	p
Null	7035810		367		
Day	6893553	142257	357	10	0.999
Iso	5285117	1608435	333	24	< 0.0001 ***
Thorax	4147962	1137155	332	1	< 0.0001 ***
Sex	4098259	49703	331	1	< 0.05 *
Parental	4096039	2220	330	1	0.999
Sex: Parental	4043120	52919	329	1	< 0.05 *

Table 6.4: Minimal adequate model of number of ommatidia with thorax length as a covariant. The minimal adequate model was arrived at by removing non-significant terms from the full model. The significance of each factor was assessed by adding factors in turn to the model (Day + Iso) and assessing the reduction (– dev) in residual deviance (res. dev.). Key to factors: Day – day of laying; Iso – Isofemale line; Sex – sex of fly; Parental – parental diet.

6.3.3 Diet range experiment - eye area, ommatidia number and ommatidia area

The diet range experiment was designed to test the effects of total amount of macronutrients (and so calorific value of the diets) and of the protein: carbohydrate (P:C) ratio in the diets, which flies were kept on throughout their lives. The hypothesis was that the relative resources devoted to neurogenesis would change according to the diet of the flies. There were four levels of total macronutrients (50, 100, 200 and 400 g l⁻¹ protein and carbohydrate combined) and four P:C ratios (1:16, 1:9, 1:4, 1:2), giving a total of 16 diets (e.g. 2-100 had a P:C ratio of 1:2 and a total macronutrient content of 200 g l⁻¹).

Eye size, ommatidia size and the number of ommatidia per eye followed similar patterns, with females having larger eyes (change in deviance 0.0610, 1 d.f., $p < 0.001$, Fig. 6.4a and b and Table 6.5), larger ommatidia (change in deviance 5.55×10^{-8} , 1 d.f., $p < 0.001$, Fig. 6.4c and d and Table 6.6), and more ommatidia per eye (change in deviance 409599, 1 d.f., $p < 0.001$, Fig. 6.4e and f and Table 6.7) than males.

Factor	Res. dev.	– dev.	Res. d.f.	d.f.	p	Sig.
Null	0.1330		265			
Iso	0.1268	0.0062	257	8	0.1271	
Sex	0.0658	0.0610	256	1	< 0.0001	***
Calories	0.0575	0.0083	253	3	< 0.0001	***
PC	0.0526	0.0049	250	3	< 0.0001	***

Table 6.5: The minimal adequate model for mean eye area (mm²) in the diet range experiment. The minimal adequate model was arrived at by removing non-significant terms from the full model. The significance of each factor was assessed by adding factors in turn to the model (Iso) and assessing the reduction (– dev) in residual deviance (res. dev.). Key to factors: Iso – Isofemale line; Sex – sex of fly; Calories: total macronutrient content (g l⁻¹); PC: protein to carbohydrate ratio of macronutrients.

Factor	Res. dev.	– dev.	Res. d.f.	d.f.	p	Sig.
Null	2.29E-07		269			
Iso	1.73E-07	5.55E-08	261	8	< 0.0001	***
Sex	1.26E-07	4.74E-08	260	1	< 0.0001	***
Calories	1.22E-07	2.02E-09	257	3	< 0.05	*

Table 6.6: The minimal adequate model for mean ommatidia area (mm²) in the diet range experiment. The minimal adequate model was arrived at by removing non-significant terms from the full model. The significance of each factor was assessed by adding factors in turn to the model (Iso) and assessing the reduction (– dev) in residual deviance (res. dev.). Key to factors: Iso – Isofemale line; Sex – sex of fly; Calories: total macronutrient content (g l⁻¹).

Factor	Res. dev.	– dev.	Res. d.f.	d.f.	<i>p</i>	Sig.
Null	3579237		265			
Iso	2989428	589809	257	8	< 0.0001	***
Sex	2579829	409599	256	1	< 0.0001	***
Calories	2433085	146744	253	3	< 0.01	**

Table 6.7: The minimal adequate model for mean number of ommatidia per eye in the diet range experiment. The minimal adequate model was arrived at by removing non-significant terms from the full model. The significance of each factor was assessed by adding factors in turn to the model (Iso) and assessing the reduction (– dev) in residual deviance (res. dev.). Key to factors: Iso – Isofemale line; Sex – sex of fly; Calories: total macronutrient content (g l⁻¹).

The effect of higher macronutrient content in the diet was to reduce eye size (change in deviance 0.0083, 3 d.f., $p < 0.001$, Fig. 6.4a and Table 6.5), ommatidia size (change in deviance 2.02×10^{-9} , 3 d.f., $p < 0.05$, Fig. 6.4c and Table 6.6) and the number of ommatidia (change in deviance 146744, 3 d.f., $p < 0.01$, Fig. 6.4e and f and Table 6.7), in males and females. The P:C ratio was a significant factor determining eye size, as flies developing on diets with higher P:C ratios had a larger eye area than those on diets with lower ratios (change in deviance 0.0049, 3 d.f., $p < 0.001$, Fig. 6.4b and Table 6.5).

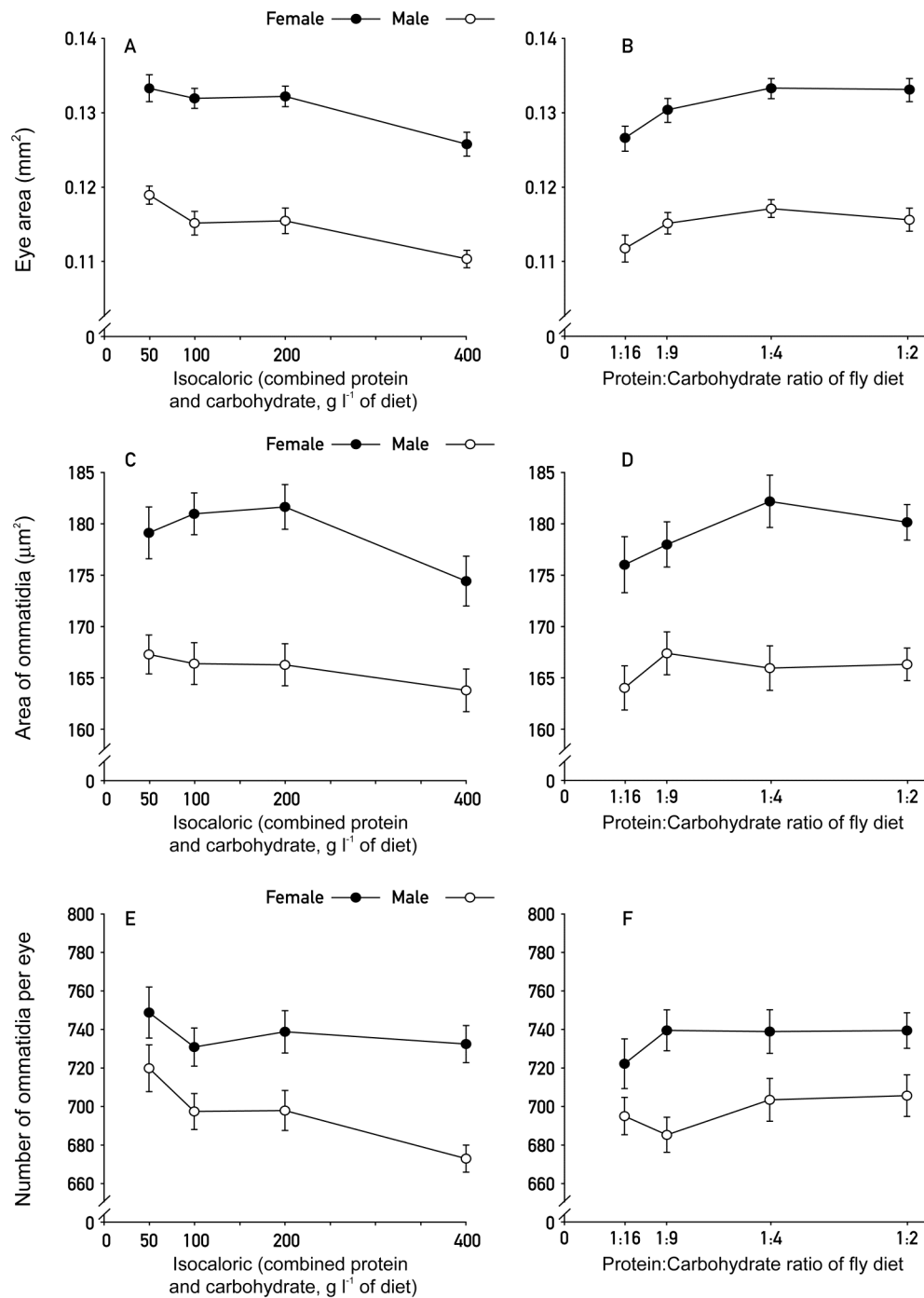


Figure 6.4: The effect of macronutrient content and Protein:Carbohydrate (P:C) ratio on three characteristics of the fly eye. Each point represents the mean from nine isofemale lines raised on the 16 experimental diets. **Eye area** (mm²) grouped by **A**: isocaloric of fly diet and sex and **B**: P:C ratio and sex. **Ommatidia area** (μm²) grouped by **C**: isocaloric of fly diet and sex and **D**: by P:C ratio and sex. **Number of ommatidia** per eye grouped by **E**: isocaloric of fly diet and sex and **F**: by P:C ratio and sex. All error bars represent ± 1 standard error of the mean.

6.3.4 Diet range experiment – ommatidia number relative to thorax length

When the relative effort put into neurogenesis was tested using thorax length as a covariate, neither the sex of the fly, nor any dietary factor or interaction had a significant effect on the relative amount of resources dedicated to neurogenesis (Fig. 6.5, Table 6.8).

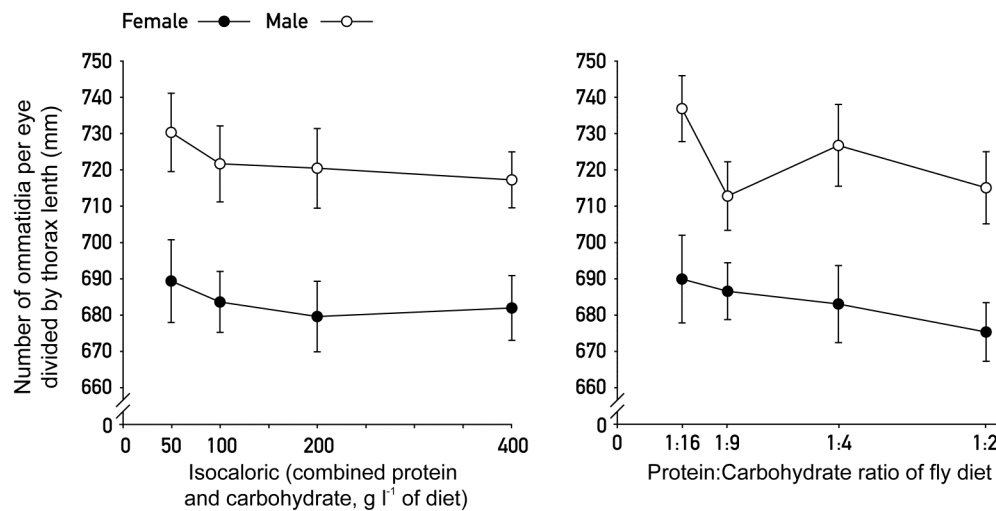


Figure 6.5: Mean number of ommatidia per eye divided by thorax length (mm) (omm/thor) of flies on differing diets. Each data point represents the mean omm/thor of nine isofemale lines of flies raised on the 16 experimental diets, grouped by isocaloric of fly diet and sex (left) or by Protein:Carbohydrate (P:C) ratio of the diet and sex (right). All error bars represent ± 1 standard error of the mean.

Factor	Res. dev.	- dev	Res. d.f.	- d.f.	p	sig.
Null	3380399		264			
Iso	2758112	622287	256	8	< 0.0001	***
Thorax	2113169	644943	255	1	< 0.0001	***
Full	1975406	137763	224	31	0.9999	

Table 6.8: Minimal adequate model of ommatidia number per eye in the diet range experiment, with thorax length as a covariate. The minimal adequate model was arrived at by removing non-significant factors and interactions from the full model. The significance of each factor was then assessed by adding factors in turn to the model (Iso) and assessing the reduction (- dev) in residual deviance (Res. dev.). Key to factors: iso – isofemale line; Thorax – length of thorax; Full – full model of main effects and interactions of Sex, Calories and P:C ratio. No effect was significant, so all were removed from the model.

6.4 Discussion

6.4.1 PARs in relation to diet and ommatidia number

In the multigenerational diet experiment, females and flies on P diet as offspring had a larger thorax and more ommatidia per eye than males and flies on the W diet, while parental diet had opposite effects depending on sex. In females, a P diet in parents produced offspring with larger eyes and more ommatidia, while males whose parents had developed on P-diet had smaller eyes and fewer ommatidia. This indicates that in females, without taking the size of the fly into account, parental diets acted as a constraint, while in males flies of the offspring generation showed an adaptive response in their thorax and eye size to the less suitable W-diet of their parents (Heisenberg et al. 1995; Huey et al. 1999; Jasienska et al. 2006). This could relate to the requirements of mating, as larger males and males with good vision are better able to compete for females during courtship (Schäffel and Willmund 1985; Partridge et al. 1987).

The number of ommatidia per eye was largely determined by the size of the eye, as the size of the ommatidia themselves was relatively consistent, except for a small effect in females where ommatidia were smaller (that is, the ommatidia density of the retina was higher) when both parents and grandparents had developed on the P-diet.

When the number of ommatidia was considered in relation to the size of the fly, the effect of parental diet was significant, but very small. As the offspring diet was not significant when thorax was included as a covariate, it is difficult to state whether the effects of parental diet were constraint (Model 5, Fig 1.4) or adaptation (Model 9, Fig. 1.4). The number of ommatidia appears to be controlled by the same factors that control the overall size of the fly, and almost all of the effect of diet comes from eye size, which varies in the same way as thorax size with some contribution from the sex of the fly, at least on these diets.

6.4.2 Macronutrient quantity, P:C ratio and ommatidia

Flies' responses showed a similar pattern in the diet range experiment, in that eye size and absolute number of ommatidia had largely the same significant factors as controlled the size of the thorax (Chapter 5). Here, the significant factors were sex (females larger with more ommatidia per eye and larger eyes), the isocaloric of the diet (higher isocalorics produced smaller eyes, fewer ommatidia and smaller thoraxes) and the

P:C ratio (larger thorax and eye). The exception was that flies developing on diets with higher macronutrient content had slightly smaller ommatidia. This may indicate that a smaller cell size plays a part in reducing the size of flies on these diets, rather than cell number, as was the case with wing size in *D. melanogaster* in response to temperature (Partridge et al. 1994).

Again though, when the number of ommatidia was considered in relation to thorax size, there was no effect of diet, indicating that the flies did not change the proportion of resources devoted to the development of the eye according to diet. The only significant effect was the sex of the fly. In both the multigenerational diet and the diet range experiment, males had more ommatidia relative to thorax length than did females, by 5.7% in the diet range experiment and 3.9% in the multigenerational diet, which contrasts with the greater absolute number of ommatidia found in females (8.3% more in the multigenerational diet and 5.5% in the diet range experiment). This may reflect the importance of vision to males, in tracking females during courtship (Schäffel and Willmund 1985). The greater absolute amount of ommatidia in females agrees well with the observation by Rein (1999) that the female optic lobe was 6% larger than in males.

If the optic lobe of the flies stays in the same proportion to other regions of the brain, this would indicate that neurogenesis in general in *D. melanogaster* remains proportional to body size, at least in regard to diet. The diet range experiment produced large changes in other responses, including thorax size and developmental time, so brain development does seem to be a characteristic important enough to be defended against adverse dietary conditions. On the other hand, the number of ommatidia per eye may not entirely reflect the size of the whole brain. Regions of the brain have been shown to be highly plastic in response to social stimuli during adulthood (Heisenberg et al. 1995), and the lamina of the optic lobe is itself capable of changing in response to environmental stimuli as an adult (Barth et al. 1997). If the number of ommatidia in relation to thorax length had changed in response to diet, this may have provided an insight into how the development of the brain responds, as the number of ommatidia remain fixed from the L3 larval stage.

Diet may have an effect on neurogenesis in *Drosophila*, but detecting any effect will require a different technique to assess the brain, as the number of ommatidia only seem to correspond to the size and sex of the fly. Another technique which may provide an indication of the impact of diet on *D. melanogaster* is a behavioural approach, looking at

memory and learning with respect to diet. This would also have the advantage of linking diet directly to ecologically significant effects, such as the ability to find a suitable habitat or to avoid unpleasant stimuli.

The nutritional environment of flies has been shown to severely affect their memory and ability to learn, and this effect also appears to be a constraint that gradually increases over several generations (Guo et al. 1996; Xia et al. 1997; Kolss and Kawecki 2008). This effect occurred between two diets which were much less dissimilar than those in the diet range experiment, which showed no effect of diet on neurogenesis regarding the retina and optic lobe. If this learning effect has a morphological counterpart, it may be in the size or structure of the mushroom bodies. Guo et al. (1996) did not measure the number of ommatidia or the size of the flies on the two diets, as the difference in learning ability was an unintended consequence, so there is no information on possible physical correlates of the difference in learning ability between the two groups of flies. Kolss et al. (2008) did measure dry body mass, and found that the poor food selection lines were lighter than controls, and eclosed faster on the poor food. Although these lines were selected for survival and fast development on the poor food, it is a possibility that flies showing an adaptive response to poor parental food, such as those whose grandparents developed on the W diet in Chapter 3, would also demonstrate a similar learning ability impairment.

6.4.3 Conclusion

In humans and other mammals, the size of regions of the brain have been shown to be defended against adverse nutritional conditions, as a poor environment affects the brain less than it affects other more general morphological characteristics. In flies, changes in the number of ommatidia were almost completely controlled by eye area, which mirrored changes in the overall size of the fly, and so this effect did not seem to occur, provided it is assumed that ommatidia number truly reflects the overall size of the *Drosophila* brain. However, in *Drosophila* learning and memory has been shown to be affected by diet over more than one generation and so future experiments on PARs regarding neurogenesis in *Drosophila* should concentrate on learning ability in relation to intergenerational diet effects. Additionally these may have morphological counterparts in specific brain regions connected with memory, such as the mushroom bodies.

6.4.4 Acknowledgements

RS was assisted by the following University of Southampton undergraduate students from the School of Biological Sciences as part of their third-year projects - Louella Clark (LC), Gillian Cuthbert (GC), Tanya Fennessy (TF), Kate Higgins (KH), and Jason Munson (JM), who took photomicrographs using frozen samples from isofemale lines 4, 9, 10, 17, and 31 from the diet range experiment, using the data collected from their isofemale lines for their own projects. RS designed and carried out the diet range experiment, took the photomicrographs for isofemale lines 2, 7, 16 and 32, measured all the photomicrographs and carried out all the analysis presented in this chapter.

Chapter Seven

Variation between isofemale lines in response to diet

7.1 Introduction

7.1.1 Variation between genotypes and the cost of plasticity

Diet does not only influence human health at the population level, as gene by environment interaction also plays an important part in susceptibility to obesity, diabetes and cardiovascular disease, the components of the metabolic syndrome (Schaefer et al. 1995a; Schaefer et al. 1995b; Ordovas and Shen 2008; Andreassi 2009). Dietary interventions aimed at lowering LDL cholesterol found significant between subject differences in response, from +5% LDL after intervention to -40%, although there was a reduction at the population level. Genetic variability between ethnic groups can also make diagnosis difficult, for instance the criteria used to diagnose metabolic syndrome do not work equally well in all groups (Haffner 2006). One genetic variant that is known to influence BMI and insulin sensitivity is PPAR γ 2, which can have an alanine substituted for a proline at one point, significantly lowering the risk of developing diabetes and of becoming obese (Deeb et al. 1998). As expression of this gene in rats has also been shown to be altered by maternal diet (Burdge et al. 2007b) it seems reasonable that in humans, there could also be significant interaction between genes and the maternal environment.

In *D. melanogaster*, one study found that gene by environment interactions on the reaction norm of temperature against developmental time accounted for 52% of the variance, with a substantial proportion of lines being faster than controls in one environment and slower in the other. Kent et al. (2009) looked at the reaction norms between fed and food-deprived conditions in two sets of flies who differed only in having either rover or sitter alleles of the *for* gene, and found that the rovers showed significantly more plasticity in their response to the food environment. The differences attributed to the *for* gene and its interaction with the genetic background included lipid and carbohydrate storage, foraging behaviour and protein synthesis (Kent et al. 2009).

In the parental diet experiments (Chapter 3 and 6), when flies' parents or grandparents developed on either P or W diets, the effects on the phenotype of the offspring generation could be significant, and could have a significant adaptive effect; for example when grandparents developed on the W (high carbohydrate) diet, flies in the

offspring generation developed faster than those whose grandparents had developed on the P (low-carbohydrate diet) (Chapter 3).

For a particular phenotypic characteristic, if each isofemale line displayed an adaptive or compensatory effect resulting from a parental or grandparental diet, this could indicate that adaptive phenotypic plasticity had been strongly selected for in the past and was an important characteristic of all flies from the AV culture. Alternatively, if all isofemale lines do not behave in the same way, some may have an adaptive response to a grandparental diet, while others may have a neutral or even a response that indicates a constraining influence from the grandparental diet. This would be an example of a gene by environment interaction, which in this case would be represented in the statistical models as an interaction between isofemale line and diet (either offspring, parental or grandparental). These gene by environment interactions are common in plasticity studies (Pigliucci 2005; van Buskirk and Steiner 2009). They indicate that different genotypes within a population respond to their environment, whether presence / absence of predators, temperature or dietary regime, to different degrees or in different ways and so provide raw material for selection to occur (Via 1993; van Buskirk and Steiner 2009). Some examples are found in the expression of heat shock proteins in response to thermal stress in *D. melanogaster* (Krebs and Feder 1997), the size and age at maturity in the frog *Rana temporaria* in response to water availability (Merilä et al. 2004) and in the energetic content of the Trinidadian guppies *Poecilia reticulata* in combinations of low- or high food-availability environments between maternal and offspring generations (Bashey 2006).

This variability in response could reflect an environment-independent cost in either maintaining the capacity for adaptive plasticity in that phenotypic characteristic, for instance incurred by the capacity to sense a relevant aspect of the environment (DeWitt 1998; Auld et al. 2010). Alternatively it could be the result an environment-dependant cost of actually implementing that plasticity, either because of resources diverted from other aspects of development, or because if the actual environment encountered by the offspring is different to that predicted by the adaptation, plasticity may be maladaptive (DeWitt 1998; Hughes et al. 2003; Gluckman and Hanson 2006b; Auld et al. 2010). Even if the prediction is wrong however the cost of being able to respond might not, on average, be more than if an organism did not display plasticity (Auld et al. 2010).

An example of the potential costs of phenotypic plasticity is provided by a study of the freshwater snail *Physa heterostrophha* (DeWitt 1998), which alters its shell morphology in response to predators. This plasticity is easily recognised as adaptive, as the changes in shell morphology are related to the ways that each predator attacks; in response to the shell-crushing tactics of sunfish it becomes rounder, and in response to crayfish, which hook out the snail through the shell opening, the shell becomes longer (DeWitt 1998). Plasticity was defined as the absolute difference in phenotypes between environments containing one or the other predator. There was no difference in energetic cost between producing either shell, but families of snails displaying most plasticity also had a slower growth rate in one of the environments. There were also significant differences in plasticity between families in reaction to the two different predator environments (DeWitt 1998).

In a recent meta-analysis of 27 studies spanning plants and animals, the fitness costs of phenotypic plasticity were found to be weak and similar to the costs of canalisation (van Buskirk and Steiner 2009). Selection for the ability to respond plastically to the environment will tend to increase the slope of a reaction norm, while selection for developmental canalisation will tend to flatten the slope, and both may have costs (van Buskirk and Steiner 2009).

If the isofemale lines in the between-generation diets experiments (Chapters 3 and 6) display different amounts and kinds of plasticity in relation to previous generations' environments, this may also mean that in the flies some non-significant dietary effects, when the gene by environment effect is considered, may have significant but opposing responses in different isofemale lines. This variation between genotypes could act as the raw variation for selection of adaptive plasticity (Pigliucci 2005). The ability to respond to an adverse environment can lead to a greater tolerance for varied environments, and so slow down selection by shielding individual genotypes from selective pressures (Ghalambor et al. 2007).

The ability to make a phenotypic response not only to the immediate environment, but also to influence the offspring or grand-offspring's phenotype has been suggested as a better strategy than either an immediate response or no plasticity (Jablonka et al. 1995; Ghalambor et al. 2007). This is in contrast to a situation when the plasticity is induced in the same generation as the environmental cue is detected, as here a long time lag may result in a fitness cost (Padilla and Adolph 1996). Individual isofemale lines of flies in the

experiments described in Chapter 3 that display greater plasticity as a result of the grandparental or parental diet than others, may therefore have an advantage if the environment is predictable. If some isofemale lines are more plastic than others this may be evidence of different strategies to deal with environmental change.

7.1.2 Coordinated phenotypic responses

If adaptive phenotypic plasticity is a general characteristic of an organism then it might be expected that this plasticity extended over several phenotypic characteristics. This could mean that several traits change at once in response to diets, and it would be expected that they change in the same way, that is either adaptively or by being constrained by diet. An alternative is that each trait may vary independently in each isofemale line, so that it would not be possible to predict whether an adaptive response to diet in wing length, for instance, would also indicate an adaptive response in thorax length in that isofemale line. This may be complicated by correlations between morphological traits, so that it may be difficult to distinguish a co-varying trait from two independent instances of phenotypic plasticity (DeWitt 1998).

Taking a wider view than examining the detailed responses of individual isofemale lines, grandparental, parental and offspring diets may have effects that are too subtle to be detected when only measuring one phenotypic trait in the offspring generation. If the difference in any one trait is quite small, so that it is insignificant on its own, it is still a possibility that many small changes in several traits may amount to a distinctive phenotype.

This may also be true of traits that are significant in themselves, but when considered together may together constitute something similar to the collection of traits seen in response to under-nutrition in humans, the “survival phenotype” (Gluckman and Hanson 2006a; Joglekar et al. 2007). In this, inadequate nutrition during pregnancy is thought to cause lower birth weight, reduced muscle mass, and a propensity to gain weight in later life, leading to obesity, cardiovascular disease and diabetes (Gluckman and Hanson 2006a; Joglekar et al. 2007).

Principal Component Analysis is one method to take multiple phenotypic characters and examine which of them, if any, vary together most strongly (Quinn and Keough 2002). This may show that the diet experienced by previous generations has a stronger effect on the phenotype of the offspring than is possible by looking at only one

trait at a time. Noach et al. (1996) used this technique to distinguish the kind of phenotypic plasticity found in two populations of *D. melanogaster* in response to temperature. The Tanzanian population varied in overall size, that is that all traits varied together, while the French population changed shape as well as changing size (Noach et al. 1996). Noach attributed this to the greater range of temperatures found in France, whereas the Tanzanian population had had no need to evolve plasticity in respect to temperature variation (Noach et al. 1996).

Similar methods were employed to investigate the changing shape of wings due to temperature differences in *D. simulans* (Matta and Bitner-Mathe 2004), significant environmental effects on the phenotype of corals (Bruno and Edmunds 1997), separating out phenotypes of water weed in different environments (Geng et al. 2007) and the changing shape of the suite of mouthparts in *Rana sylvatica* tadpoles in the presence of predators (Relyea and Auld 2005).

7.1.3 Experimental aims

In the previous chapters, where experiments have been described which examined the effect of previous generations' diets on fitness indicators, each indicator was considered singly, and each diet was considered only regarding its population-level effect on the 25 isofemale lines (Chapters 3 and 6). In this approach the isofemale line was considered as a replicate, rather than including the possibility that isofemale lines may respond differently to grandparental or parental diet, due to different genotypes, or that phenotypic change may occur in several characteristics at the same time to produce a distinct phenotypic 'syndrome' in response to previous generations' diets or combinations of diets. Also considered here is the possibility that some isofemale lines had greater overall plasticity, and that there may be a fitness cost to plasticity.

Isofemale line interactions with diet

The aim of these analyses is to take the data gathered in the grandparental and parental diet experiments (Chapters 3 and 6) and, by adding isofemale line: diet interactions terms, asking whether the phenotypic plasticity in different traits due to parental or grandparental diets is a purely population-level characteristic (interaction not significant) or whether adaptive plasticity is also dependant on genotype. This may show distinct groups of isofemale lines that respond in the same way as each other. The null

hypothesis tested in each case, in addition to that already tested in Chapters 3 and 6, is that the phenotypic response to diet is not dependant on the isofemale line.

This depended on whether a higher or lower value of the variable was considered to indicate higher fitness, and how the offspring generation responded to developing on P or W diets. For example, for time to eclosion, flies developing on the offspring P diet eclosed faster than flies developing on W (Chapter 3), and a faster eclosion time is associated with higher fitness. In this case, those whose grandparents developed on the W diet eclosed faster than those whose grandparents developed on the P diet (Chapter 3), and so this response was considered adaptive. This means that for time to eclosion in the offspring generation, having a parental or grandparental diet of P (P d1/d0) results a greater time to eclosion than a W d1/d0, and so for each isofemale line, the more positive the difference between P d1/d0 and W d1/d0, the more adaptive was that isofemale line's response to a W diet in the previous generation.

Correlation of phenotypic responses to diet

Two approaches will be used to investigate whether the different phenotypic traits associated with fitness are correlated with each other or vary independently. Firstly, the phenotypic responses of each isofemale line to previous generations' diets will be ranked in order of adaptiveness, and then tested for correlation between the rank order in each characteristic. The null hypotheses are that for both grandparental and for parental diets, the response in one fitness trait in an isofemale line in the offspring generation is not correlated with that isofemale line's response to a different fitness trait.

Secondly, to investigate whether phenotypic responses are correlated, all the phenotypic responses will be pooled and PCA carried out. If there are distinct groupings this may point to a previously hidden combination of diets or isofemale lines that behave differently.

Cost of phenotypic plasticity

Thorax length is a determinate characteristic related to fitness, fixed in larval stages of development, and has been shown to be plastic with respect to the parental diet and sex (Chapter 3). If there is a cost to the maintenance of phenotypic plasticity in this characteristic, this may be paid in another phenotypic characteristic associated with fitness. Therefore the plasticity of thorax length was tested to find if those lines which displayed the largest absolute (either larger or smaller in response) regarding parental diet

paid a cost in decreased survival or longer development time. The null hypothesis tested was that the mean absolute difference in thorax size for each isofemale line between the two parental diets P and W (degree of plasticity for that isofemale line) was not related to the mean development time for that isofemale line.

7.2 Methods

7.2.1 Response of isofemale lines to grandparental and parental diets

Data from the multigenerational diet experiments testing the influence of grandparental and parental diets on offspring fitness indicators (Chapters 3 and 6) were further analysed to examine whether individual isofemale lines responded to the diets differently. In the multigenerational diet experiment, the measured responses were the proportion of eggs surviving to eclosion, time from egg-laying to eclosion, thorax and wing length, the number of ommatidia per eye (using thorax length as a covariate), dry weight and lipid content. These responses were analysed using generalized linear models in a model-building approach in R 2.8.1 (R Foundation for Statistical Computing, Vienna, Austria), using the methods described in Chapter 3. In that analysis, isofemale line was used as a factor in all models as the 25 isofemale lines were the replicates in the experiment, but no interaction of isofemale line with sex or any diet was included in the full model. Here, the full models included all the interactions between isofemale line, diet and sex. Non-significant terms were removed using stepwise deletion, assessed by an analysis of variance and a chi-squared test, followed by sequentially adding significant terms back to the null model, as described in Chapter 3. As one isofemale line from P and one from W were missing from the grandparental generation, there are no data for plasticity for lines 16 and 22 for grandparental diets, and consequently interactions with the grandparental diet in the statistical models have two less degrees of freedom than for parental or offspring diets.

7.2.2 Similarity of response of isofemale lines across measured variables

The response of each of the measured variables to the grandparental and parental diets was assessed to determine whether a smaller value for having a grandparent or parent developing on P rather than W was adaptive or showed a constraint, using the criteria explained above in Section 7.1.3.

Each response variable was assessed in this way and the adaptive response to previous diet was considered to be when for each isofemale line, the response to having a parent or grandparent from a P diet was a higher figure than having a parent or grandparent from W for survival, time to eclosion and lipid content, and if the value for groups with P parents or grandparents was smaller than those with W parents or grandparents for thorax length, wing length, dry weight and ommatidia number. For each

response variable, the difference in the offspring generation between P and W grandparental and parental diets was calculated separately, and isofemale lines were ranked by how adaptive each response had been to grandparental or parental diets, with isofemale lines displaying the most adaptive response having the highest rank for that variable. An ANOVA on ranks could not be carried out as the data were significantly different from a normal distribution using a Kolmogorov-Smirnov test, and so two Kruskal-Wallis tests, one for the responses to grandparental diet and one for the responses to parental diet, were used to assess if isofemale lines behaved in a consistently adaptive manner to previous generations' diets across different response variables. All statistical tests were carried out using SPSS 19.0 (SPSS Inc., Chicago).

7.2.3 Principal Component Analysis

The measured responses to diets were compiled into a single data set in which each grandparental diet / parental diet / offspring diet / isofemale line / sex combination had six associated response variables: time to eclosion, thorax length, wing length, ommatidia number (scaled to thorax length), dry weight and lipid content (percentage by weight, arcsine square-root transformed). Survival was not included as the sex of the eggs laid was unknown, whereas sex was a factor in all the other response variables. Cases were excluded if any one of the six response variables was missing for that case. Each response variable was then re-scaled to have a mean value of zero, and divided by the standard deviation for the re-scaled response variable, so that all response variables had an equal mean and a standard deviation of one.

A principal component analysis was then carried out, with the output as the correlation coefficients between the standardised response variables and a series of principal component values associated with each case. This was first carried out using the data for both sexes combined and then again for males and females separately. All statistical analysis was carried out using SPSS 19.0 (SPSS Inc., Chicago).

7.2.4 Costs of phenotypic plasticity

The degree of plasticity was calculated for each isofemale line as the absolute difference in thorax length (mm) between offspring whose parents had developed on the P diet and those whose parents had developed on the W diet. This was done separately for females and males as they had shown a significantly different response to the parental diet (Chapter 3). For each of the four combinations of two offspring environments (P

and W) and two sexes, the degree of plasticity and the associated time to eclosion for each isofemale line were analysed using linear regression. All statistical tests were carried out in SPSS 19.0 (SPSS Inc., Chicago).

7.3 Results

7.3.1 Survival

This experiment was designed to test whether there was any influence of previous generation's diet on the survival of the offspring generation, and also to test whether this influence was adaptive. These effects have been dealt with as the mean of all isofemale lines in previous analyses, but here the effects due to individual isofemale line variation are tested. In addition to diet's main effects on survival (flies developing on W had a higher survival rate than those on P, and flies with W parents had a lower survival on P than those with P parents), there was significant variation in survival between isofemale lines in relation to diet (Fig. 7.1, Table 7.1). In addition to the significant variation between survival in different isofemale lines in interaction with the offspring diet (change in deviance 119.4, 24 d.f., $p < 0.0001$) there were also equally significant interactions between isofemale lines and parental and grandparental diets (Fig. 7.1, Table 7.1). For each isofemale line, these are presented in Figure 7.1 as the mean survival percentage, for each group with P parental or grandparental diets, minus the percentage for those with W. A negative figure means that those flies with W parents or grandparents had a higher survival rate than those with P in that isofemale line. As offspring on W diets had a higher mean survival rate than those on P diets, a positive percentage in Figure 7.1 indicates an adaptive response to the P diet in the next generations, and a negative one indicates constraint. When isofemale line was included in the model, neither the parental nor the grandparental diet had any significance on its own (Table 7.1).

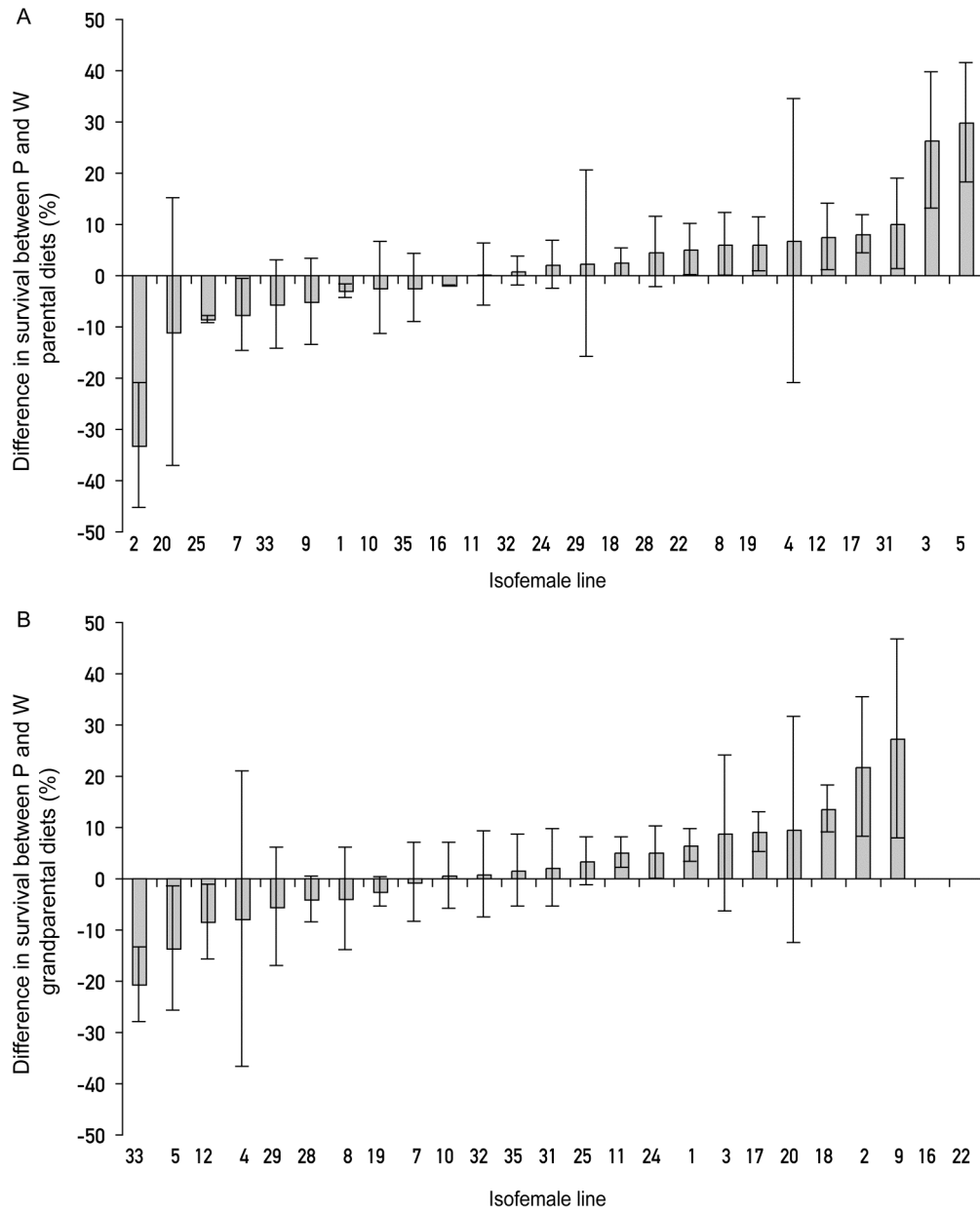


Figure 7.1: A: The difference in percentage of eggs surviving to eclosion in each isofemale line in the offspring generation between diet groups ($n = 4$) with parental diets P and W. **B:** The difference in percentage of eggs surviving to eclosion in each isofemale line in the offspring generation between diet groups ($n = 4$) with grandparental diets P and W. A negative value indicates that survival was higher for that isofemale line when the parental or grandparental diet was W. All error bars represent ± 1 standard error of the mean.

Factor	Res. dev.	– dev	Res. d.f.	– d.f.	<i>p</i>
Null	1007.6		180		
Day	902.1	105.5	170	10	< 0.0001 ***
Iso	657.1	245.0	146	24	< 0.0001 ***
Offspring	634.2	22.9	145	1	< 0.0001 ***
Iso: offspring	514.8	119.4	121	24	< 0.0001 ***
Parental	511.0	3.8	120	1	0.5
Iso: parental	387.2	123.8	96	24	< 0.0001 ***
Grandparental	387.0	0.2	95	1	0.66
iso: Grandparental	266.3	120.7	73	22	< 0.0001 ***

Table 7.1: Minimal adequate model for survival to eclosion, including the interactions of isofemale line (iso) with offspring diet (Offspring), parental diet (Parental) and grandparental diet (Grandparental). The minimal adequate model was arrived at by removing non-significant factors and interactions from the full model. The significance of each was then assessed by adding terms sequentially to the null model and assessing the reduction in deviance (– dev.) relative to the residual deviance (res. dev.) and reduction in degrees of freedom (– d.f.).

7.3.2 Time to eclosion

A faster time to eclosion enables flies to begin breeding earlier, and flies on the offspring P diet were significantly faster to eclose than those on the offspring W diet. The effects of parental diet indicated a constraint (see Chapter 3) as those whose parents had developed on P eclosed faster than those on W, while the effects of grandparental diet indicated an adaptive effect operating to accelerate development in those flies whose grandparental diet had been W (Fig. 3.7). However, there was significant variation in time to eclosion between the isofemale lines with regard to diet, including variation in the response to offspring diet (change in deviance 0.1161, 24 d.f., $p < 0.001$). The response to previous generation's diet was also significantly different between isofemale lines (Table 7.2). Although the difference between eclosion time between flies whose parental diet was P was overall faster than for offspring of W parents, for some lines the opposite was true. These lines showed a faster eclosion time when their parents had developed on W (shown by a positive response in Fig. 7.2a). Similarly, although most lines showed a positive difference (adaptive) in eclosion time regarding grandparental diet (Figure 7.2b), some were slower to eclose if their grandparents had developed on the W diet (left hand lines showing a negative difference in Fig. 7.2b).

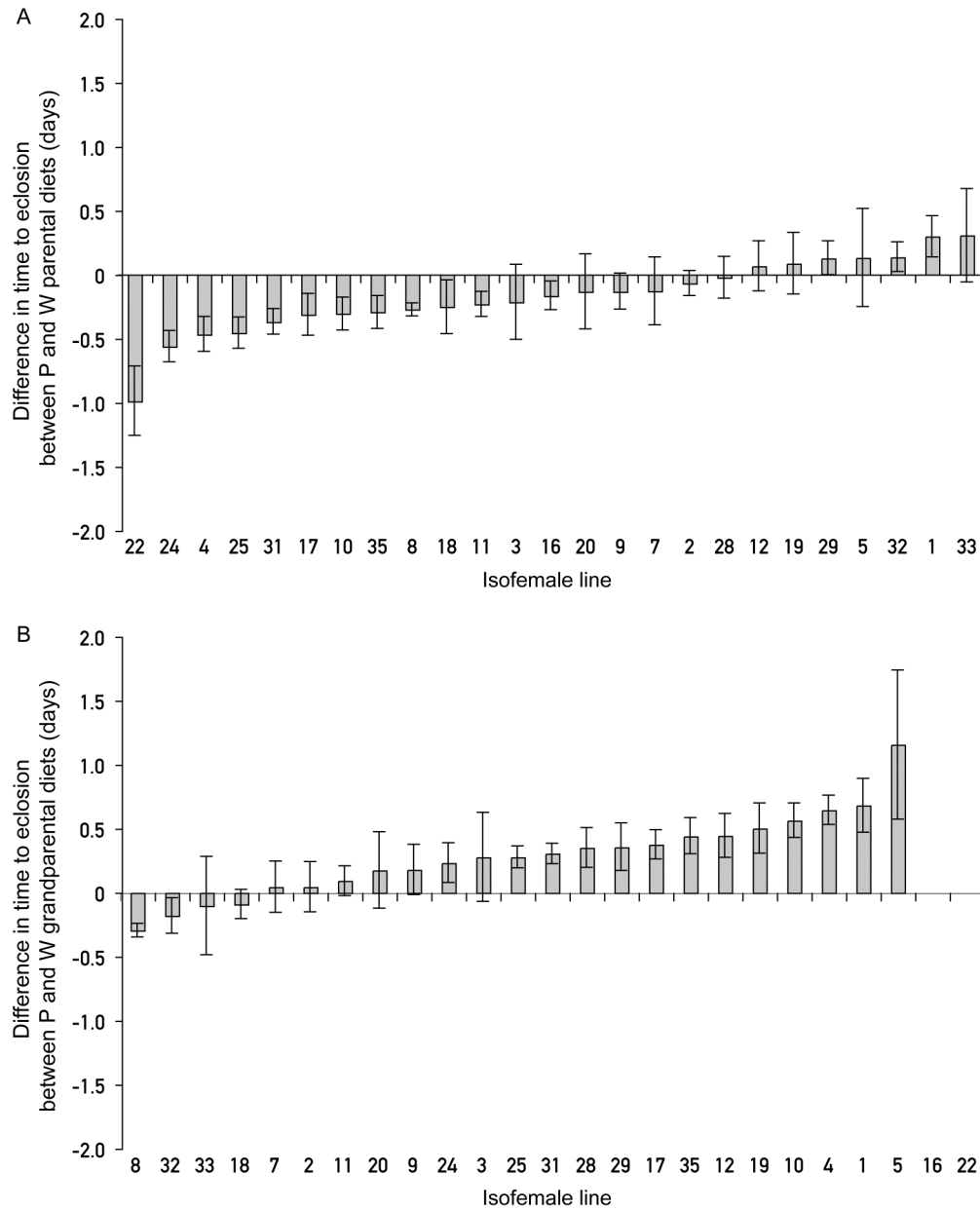


Figure 7.2: A: The difference in time to eclosion in each isofemale line in the offspring generation between flies whose parental diets were P or W ($n = 4$). **B:** The difference in time to eclosion in each isofemale line in the offspring generation between flies whose grandparental diets were P or W ($n = 4$). A negative value indicates that time to eclosion was greater for that isofemale line when the parental or grandparental diet was W. All error bars represent ± 1 standard error of the mean.

Factor	Res dev	– dev	Res d.f.	– d.f.	<i>p</i>	
Null	4.4995		378			
Day	4.1894	0.3101	368	10	< 0.01	**
Iso	3.7699	0.4195	344	24	0.033	*
Sex	3.6596	0.1103	343	1	< 0.01	**
Offspring	0.8301	2.8295	342	1	< 0.0001	***
Sex:Offspring	0.8222	0.0080	341	1	0.069	
iso: Offspring	0.7061	0.1161	317	24	< 0.001	***
Parental	0.6952	0.0109	316	1	0.026	*
iso: Paental	0.5905	0.1047	292	24	< 0.001	***
Offspring: Parental	0.5893	0.0010	291	1	0.451	
iso: Offspring: Parental	0.5206	0.0680	267	24	0.065	
Grandparental	0.3879	0.1327	266	1	< 0.0001	***
iso: Grandparental	0.2463	0.1416	244	22	< 0.0001	***
Offspring: Grandparental	0.2443	0.0020	243	1	0.163	
Parental: Grandparental	0.2410	0.0033	242	1	0.067	
iso: Offspring: Grandparental	0.1863	0.0547	220	22	< 0.0001	***
iso: Parental: Grandparental	0.1314	0.0549	198	22	< 0.0001	***

Table 7.2: Minimal adequate model for time to eclosion, including the interactions of isofemale line (iso) with offspring diet, parental diet and grandparental diet. The minimal adequate model was arrived at by removing non-significant factors and interactions from the full model. The significance of each was then assessed by adding terms sequentially to the null model and assessing the reduction in deviance (– dev.) relative to the residual deviance (res. dev.) and reduction in degrees of freedom (– d.f.).

7.3.3 Thorax length

A larger thorax indicates greater potential for mating success in both males and females, and flies on the P offspring diet had larger thoraxes than those on W. Parental diet had no significant effect on its own on thorax length, and neither did grandparental diet, although male and female flies responded differently to parental diets (Chapter 3).

However there was a significant variation in thorax length depending on the interaction of both parental diet and isofemale line (Table 7.3, change in deviance 0.12564, 24 d.f., $p < 0.001$) and between isofemale lines and grandparental diet (change in deviance 0.08728, 22 d.f., $p < 0.05$). For both parental and grandparental diets, a positive difference in thorax length between the offspring of P and W parents or grandparents would indicate a constraint from previous diets (descendants of W flies being smaller) whereas a negative difference would indicate an adaptive response (descendants of W flies being larger than those of P flies). The largest adaptive response to parental diet was a P-W difference of -0.043 mm (offspring of W flies being larger) while the largest

constraint from parental diet was 0.024 mm (Fig. 7.3a). For grandparental diets, the largest constraint from parental diet was 0.027 mm and the largest adaptive response was -0.026 mm (Fig. 7.3b). For comparison, the mean difference in thorax length between flies raised on P or W diets in the offspring generation was 0.01 mm (Chapter 3).

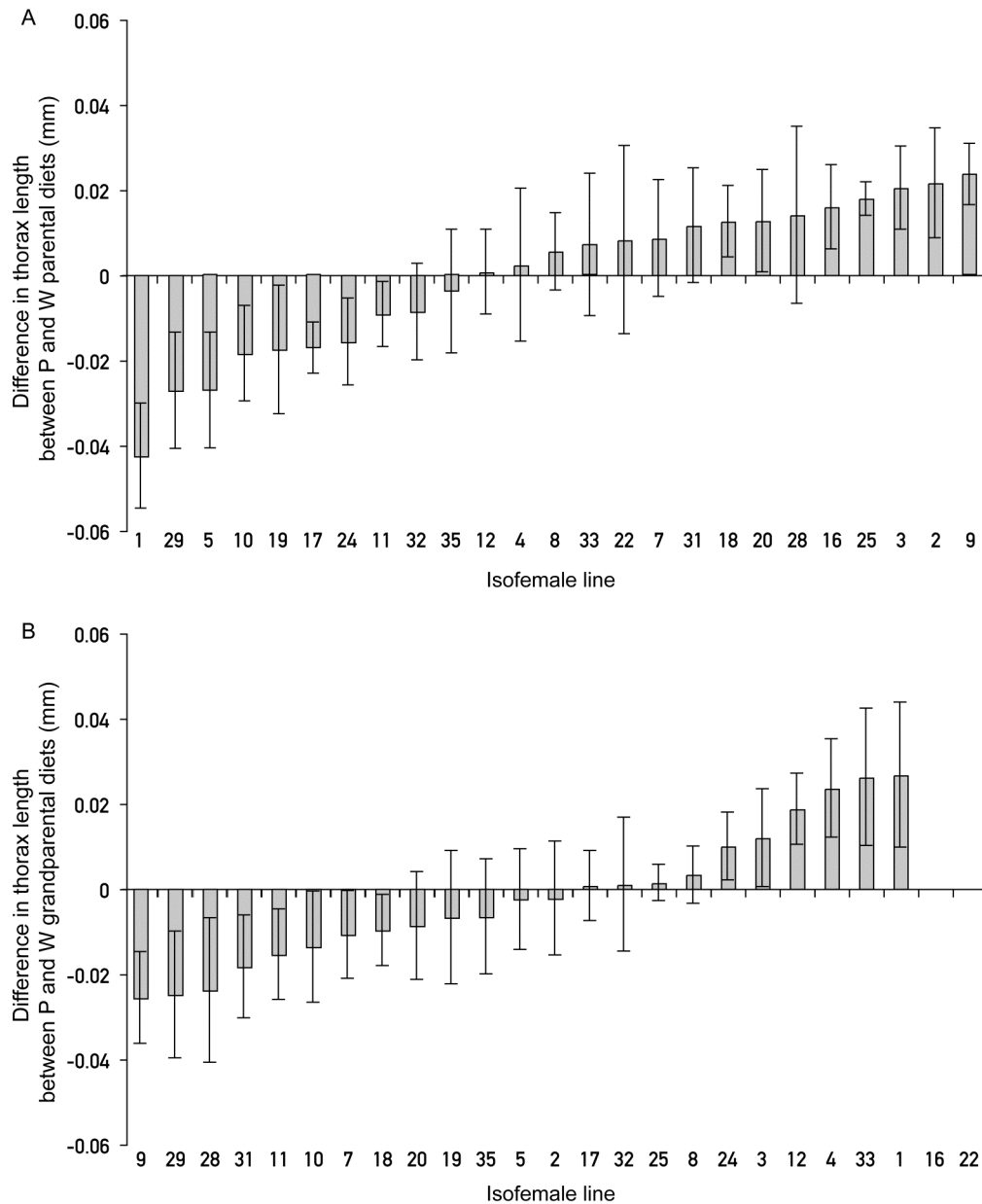


Figure 7.3: A: The difference in thorax length in each isofemale line in the offspring generation between groups having parental diets P and W. **B:** The difference in thorax length in each isofemale line in the offspring generation between groups having grandparental diets P and W. A negative value indicates that thorax length was greater for that isofemale line when the parental or grandparental diet was W. All error bars represent ± 1 standard error of the mean.

Factor	Res. dev.	– dev.	res d.f.	– d.f.	p	Sig
Null	7.60730		367			
Day	7.53450	0.07280	357	10	0.9688	
Iso	7.07020	0.46430	333	24	0.5871	
Sex	0.89940	6.17080	332	1	< 0.0001	***
Offspring	0.86013	0.03926	331	1	< 0.001	***
Parental	0.85606	0.00407	330	1	0.2105	
Grandparental	0.85535	0.00071	329	1	0.6004	
Iso: Offspring	0.78483	0.07052	305	24	0.2860	
Iso: Parental	0.65920	0.12564	281	24	< 0.001	***
Iso: Grandparental	0.57192	0.08728	259	22	0.0123	*
Sex: Parental	0.56217	0.00975	258	1	0.0344	*
Parental: Grandparental	0.54895	0.01322	257	1	0.0129	*

Table 7.3: Minimal adequate model for thorax size, including the interactions of isofemale line (iso) with offspring diet, parental diet and grandparental diet. The minimal adequate model was arrived at by removing non-significant factors and interactions from the full model. The significance of each was then assessed by adding terms sequentially to the null model and assessing the reduction in deviance (– dev.) relative to the residual deviance (res. dev.) and reduction in degrees of freedom (– d.f.).

7.3.4 Wing length

When isofemale line was included as a factor in interaction with diets in the full model for wing length, no interaction between isofemale line and diet or sex proved to be significant, and so all were discarded from the model. Therefore the minimal adequate model for wing length is no different when considering isofemale lines than when only considering diet and sex, and is the same as that analysed in Chapter 3.

7.3.5 Dry weight

The dry weight of a fly is associated with greater fecundity (Prasad and Joshi 2003) and this experiment was designed to test whether different isofemale lines responded differently to the influence of the two previous generations' diets on dry weight. The analysis including the interactions of isofemale line with diet was very similar to that without isofemale line interactions (Chapter 3), except that the interaction of parental and grandparental diet was no longer significant, while the interaction between isofemale line, parental and grandparental diet was (change in deviance 0.18692, 21 d.f., $p < 0.01$), meaning that isofemale lines responded differently to parental / grandparental diet combinations (Fig. 7.4, Table 7.4). The combined influence of grandparental and parental diet showed an adaptive response by those flies whose grandparents developed on the W

diet, meaning that if the dry weight of a fly whose grandparents or parents were on W diet was greater than that of a fly whose grandparents were on the P diet (a negative response in Fig. 7.4), the response is adaptive; the opposite shows a constraint by the parental or grandparental diet. This response could be in a different direction in the same isofemale line depending on the grandparental diet and parental diet combination (Fig. 7.4). For some lines, the response is opposite depending on whether grandparental diet was P (Fig. 7.4a) or W (Fig. 7.4b).

Factor	Res dev	– dev	Res df	– d.f.	<i>p</i>	sig.
Null	11.144		360			
Day	10.923	0.221	350	10	0.718	
Iso	10.314	0.610	326	24	0.738	
Process	9.834	0.480	313	13	0.291	
Sex	1.536	8.298	312	1	< 0.0001	***
Offspring	1.533	0.002	311	1	0.503	
Iso: Offspring	1.364	0.169	287	24	0.060	
Parental	1.358	0.006	286	1	0.280	
Iso: Parental	1.242	0.117	262	24	0.426	
Offspring: Parental	1.242	0.000	261	1	0.930	
Grandparental	1.204	0.037	260	1	< 0.01	**
Iso: Grandparental	1.096	0.109	238	22	0.367	
Offspring: Grandparental	1.095	0.000	237	1	0.749	
Parental: Grandparental	1.095	0.001	236	1	0.715	
Iso: Parental: Grandparental	0.909	0.186	215	21	< 0.01	**
Offspring: Parental: Grandparental	0.868	0.040	214	1	< 0.01	**

Table 7.4: Minimal adequate model for dry weight, including the interactions of isofemale line (iso) with offspring diet, parental diet and grandparental diet. The minimal adequate model was arrived at by removing non-significant factors and interactions from the full model. Day – day of egg laying; Process – lipid extraction batch. The significance of each was then assessed by adding terms sequentially to the null model and assessing the reduction in deviance (– dev.) relative to the residual deviance (res. dev.) and reduction in degrees of freedom (– d.f.).

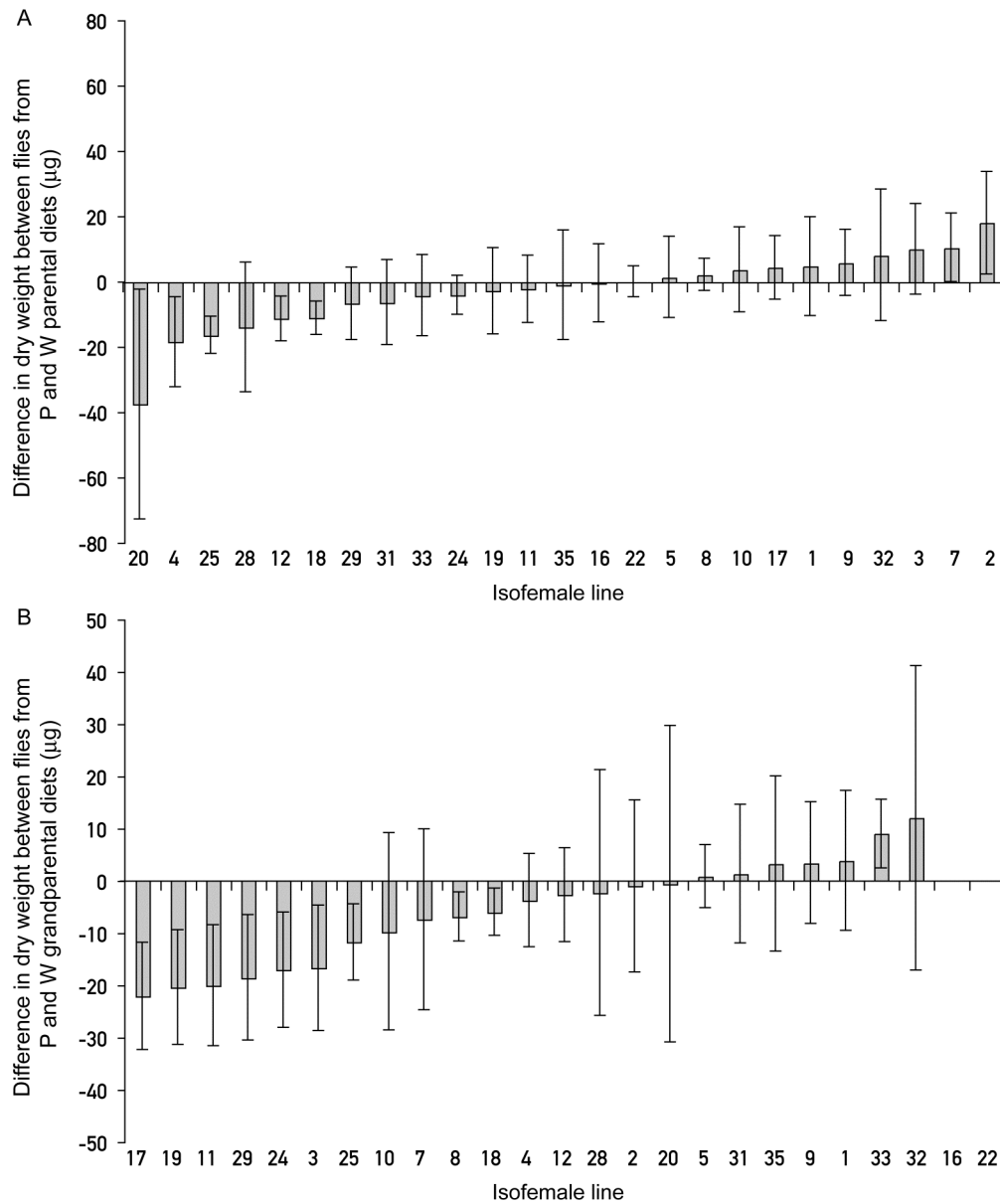


Figure 7.4: A: The difference in dry weight (μg) in each isofemale line in the offspring generation between groups with parental diets P and W; **A:** when grandparental diet was P (n = 2). **B:** When grandparental diet was W (n = 2). A negative value indicates that dry weight was higher for that isofemale line when the parental diet was W. All error bars represent ± 1 standard error of the mean.

7.3.6 Lipid content

The lipid content of flies on both offspring and grandparental W diet was higher than when developing on the P diet (Chapter 3). Isofemale lines responded differently to P or W diets in the offspring generation (change in deviance 0.463, 24 d.f., $p < 0.01$) and in the grandparental generation (change in deviance 0.406, 22 d.f., $p < 0.01$) (Fig. 7.5, Table 7.5).

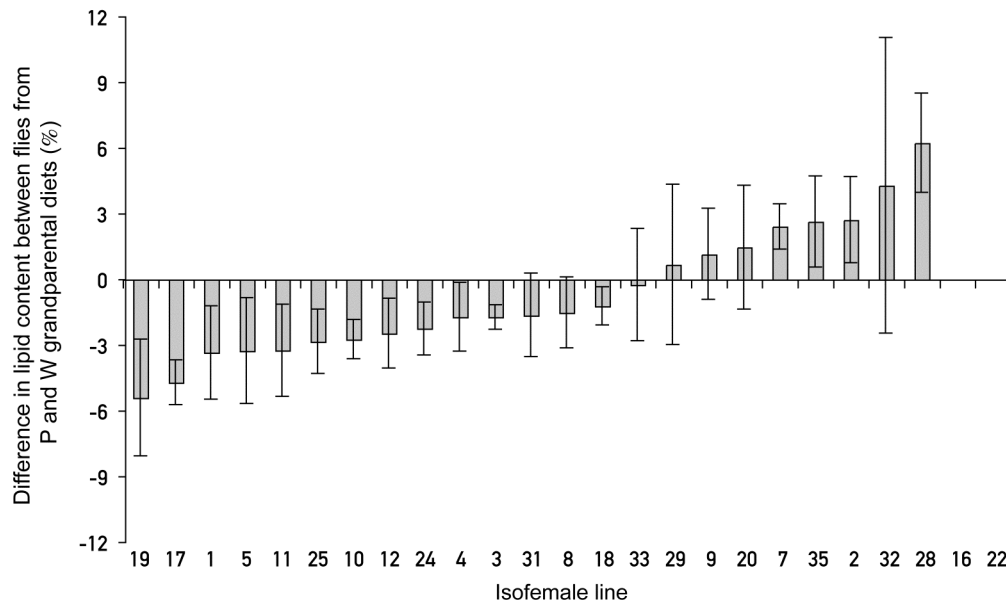


Figure 7.5: The difference in lipid content (%) in each isofemale line in the offspring generation between groups whose grandparental diets were P and W. A negative value indicates that lipid content was higher for that isofemale line when the grandparental diet was W. All error bars represent ± 1 standard error of the mean.

Factor	Res. dev.	– dev.	Res d.f.	– d.f.	P	sig.
Null	7.894		360			
Day	7.570	0.324	350	10	0.133	
Iso	4.200	3.370	326	24	< 0.0001	***
Process	3.475	3.475	313	13	< 0.0001	***
Offspring	3.384	0.091	312	1	< 0.01	**
iso: Offspring	2.921	0.463	288	24	< 0.01	**
Parental	2.919	0.002	287	1	0.693	
Iso: Parental	2.615	0.304	263	24	0.167	
Parental: Offspring	2.615	0.000	262	1	0.841	
Iso: Offspring: Parental	2.338	0.277	238	24	0.253	
Grandparental	2.272	0.066	237	1	< 0.01	**
iso: Grandparental	1.866	0.406	215	22	< 0.01	**
Grandparental: Offspring	1.854	0.012	214	1	0.243	
Grandparental: Parental	1.844	0.010	213	1	0.289	
Offspring: Parental: Grandparental	1.768	0.076	212	1	< 0.01	**

Table 7.5: Minimal adequate model for lipid content by percentage of dry weight, including the interactions of isofemale line (iso) with offspring diet, parental diet, and grandparental diet. Day – day of egg laying; Process – lipid extraction batch. The minimal adequate model was arrived at by removing non-significant factors and interactions from the full model. The significance of each was then assessed by adding terms sequentially to the null model and assessing the reduction in deviance (– dev.) relative to the residual deviance (res. dev.) and reduction in degrees of freedom (– d.f.).

7.3.7 Ommatidia number

When considered without any isofemale line interactions with diet, the only significant factors influencing the number of ommatidia relative to thorax size was sex and the interaction of sex with parental diet (Chapter 6). However with isofemale line included, there are significant differences in the response of different isofemale lines to the grandparental diet (change in deviance 470986, 22 d.f., $p < 0.01$). Some lines had significantly more ommatidia when their grandparents had developed on W diet rather than P, and for other lines the reverse was true (Fig. 7.6, Table 7.6). As a main factor, the grandparental diet was not significant (change in deviance 25015, 1 d.f., $p = 0.151$).

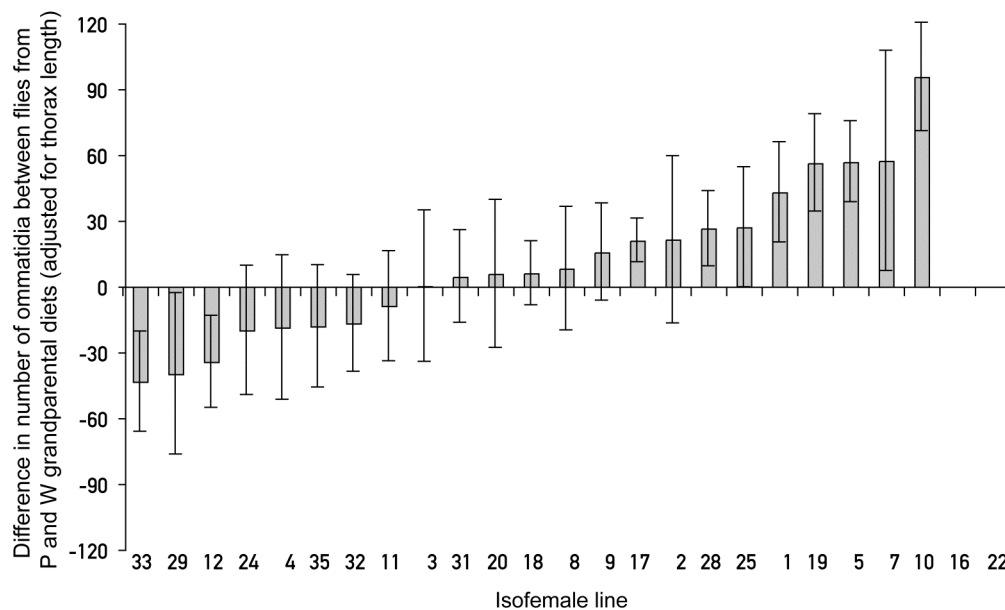


Figure 7.6: The difference in number of ommatidia divided by thorax length in each isofemale line in the offspring generation between grandparental diets P and W ($n = 4$). A negative value indicates that ommatidia number was higher for that isofemale line when the grandparental diet was W. All error bars represent ± 1 standard error of the mean.

Factor	Res. dev.	– dev.	Res d.f.	– d.f.	<i>P</i>	sig
Null	7035810		367			
Day	6893553	142257	357	10	0.999	
Iso	5285117	1608435	333	24	< 0.0001	***
Thorax	4147962	1137155	332	1	< 0.0001	***
Sex	4098259	49703	331	1	< 0.05	*
Offspring	4055594	42664	330	1	0.062	
Parental	4052088	3507	329	1	0.999	
Sex: Parental	3996592	55495	328	1	< 0.05	*
Grandparental	3971578	25015	327	1	0.151	
Iso: Grandparental	3500591	470986	305	22	< 0.01	**

Table 7.6: Minimal adequate model number of ommatidia with thorax length (Thorax) as a covariant, including the interactions of isofemale line (iso) with grandparental diet. The minimal adequate model was arrived at by removing non-significant factors and interactions from the full model including all interactions of isofemale line with sex, offspring diet, parental diet and grandparental diet. The significance of each was then assessed by adding terms sequentially to the null model and assessing the reduction in deviance (– dev.) relative to the residual deviance (res. dev.) and reduction in degrees of freedom (– d.f.).

7.3.8 Rank correlation between isofemale lines' responses to diet

In the above experiments designed to test whether different isofemale lines responded differently to parental or grandparental diets, some lines responded adaptively for some fitness indicators, whereas on those same indicators some responded as if constrained by the previous generations' diets. To test whether individual lines respond in a consistent way across different responses (for example, could an isofemale line's response of thorax length to grandparental diet be predicted from that line's response of eclosion time), each line's responses were ranked on "adaptiveness" separately for parental and grandparental diet before using the ranks as the basis for a Kruskal-Wallis test.

Isofemale lines did not show consistent adaptive or constrained responses to parental diet (Fig. 7.7), using survival, time to eclosion, thorax length, wing length, ommatidia number (scaled to thorax), lipid content and dry weight ($H = 33.056$, 24 d.f., $p = 0.103$). There was no consistent response to grandparental diet either ($H = 17.424$, 22 d.f., $p = 0.740$). Therefore, if an isofemale line performed adaptively when considering one response, this would not predict how that line would behave on a different response.

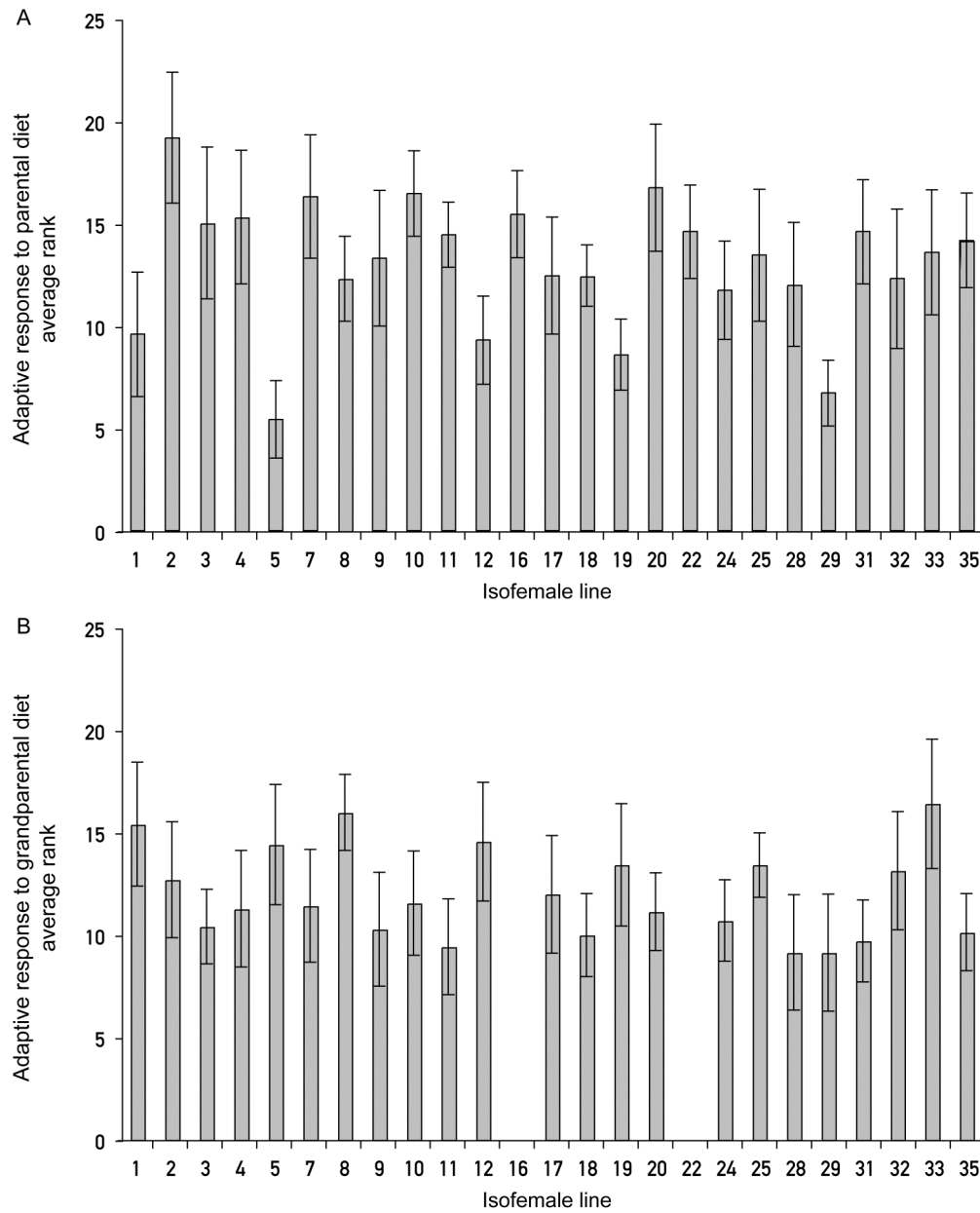


Figure 7.7: Average rank of each isofemale line, scored by adaptive response to **A:** parental diet; **B:** grandparental diet (higher rank is more adaptive). Diets P and W had a differential effect on fitness indicators in the offspring generation. If the response to the diet that produced the mean lowest response (in terms of fitness) in the offspring generation was compensatory when this diet was a parental or grandparental diet, this was scored as adaptive. Each isofemale line was ranked on survival rate, thorax length, wing length, dry weight, lipid content and ommatidia number. Error bars represent ± 1 s.e. of the mean rank.

7.3.9 Principal Component Analysis

Principal Component Analysis (PCA) may show underlying patterns in a data set consisting of multiple measured responses, which may not be readily discernible from a consideration of the individual responses. Here, data from the experiments designed to test for the influence of offspring, parental and grandparental diets were combined into a single data set and standardised so that each response had the same mean value and standard deviation, before PCA was carried out. If there were trends in the phenotypic response to diets, which spanned two or more responses, these should be apparent when the responses are considered together as a Principal Component (PC). Correlations between responses were also measured.

Considering the whole data set of male and female flies, the responses relating to the size of the fly (thorax length, wing length and dry weight) had high correlation values with each other, which was reflected in the composition of PC1 (Table 7.7), which accounted for 47.9% of the variance in the data set. There was little correlation between the number of ommatidia scaled to thorax size (omm/thor), lipid content and time to eclosion and any other response. The main positive contribution to PC2 was made by lipid content and a negative contribution by omm/thor, while PC2 accounted for 19.4% of the variance (Table 7.7). No further PCs were calculated.

Plotting PC1 against PC2 shows reveals two distinct groups (Fig. 7.8), separated mainly by differences in PC1. These groups correspond to male (negative PC1) and female (positive PC1) flies, and the difference in PC1 values reflects the smaller size of the males.

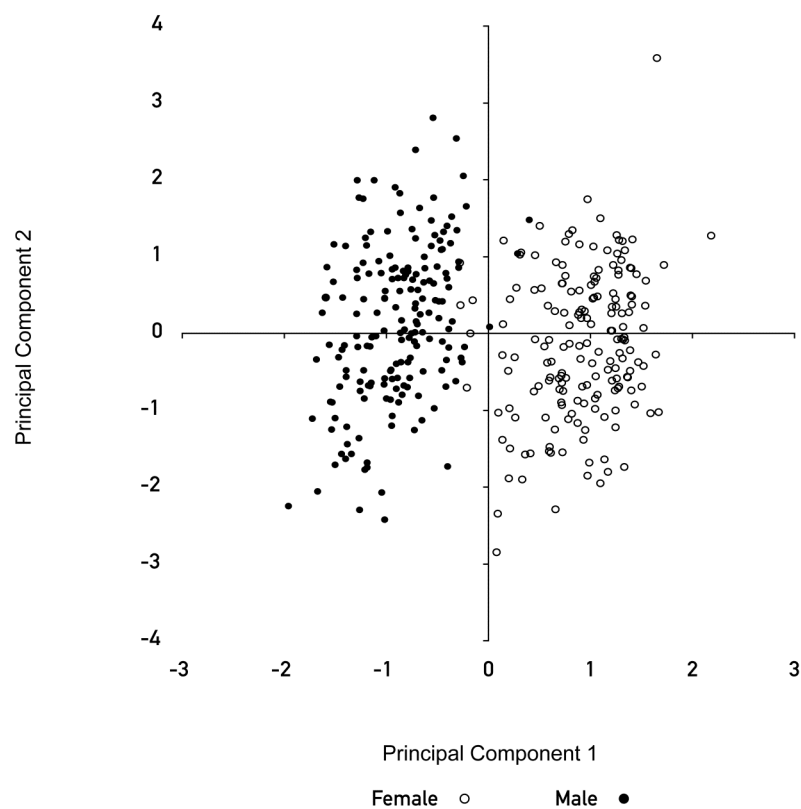


Figure 7.8: Principal Component 1 against Principal Component 2 from a principal component analysis using thorax length, dry weight, ommatidia number divided by thorax length, lipid content, wing length and time to eclosion as the responses to P or W as diets over three generations. Data points are split by sex (see legend). Principal components 1 and 2 were made up as set out in Table 7.7.

1. Correlation Matrix

	thorax	wing	omm/thor	dry weight	Lipid	eclosion
thorax	1					
wing	0.854	1				
omm/thor	-0.324	-0.218	1			
dry weight	0.848	0.823	-0.241	1		
lipid	0.022	-0.048	-0.199	0.209	1	
eclosion	-0.253	-0.199	0.024	-0.167	0.018	1

2. Variance

Component	Eigenvalue	% Var.	Cumul. %
1	2.875	47.914	47.914
2	1.164	19.394	67.308
3	0.914	15.238	82.546
4	0.785	13.087	95.633
5	0.143	2.380	98.013
6	0.119	1.987	100.000

3. Component make-up

	Component	
	1	2
Thorax	0.949	-0.096
Wing	0.915	-0.195
Omm/thor	-0.407	-0.563
Dry weight	0.925	0.044
Lipid	0.131	0.823
eclosion	-0.311	0.347

Table 7.7: Principal component analysis on all flies, using thorax length (thorax), wing length (wing), the number of ommatidia divided by the length of the thorax (omm/thor), dry weight, lipid content percentage (transformed by arcsin square root) and time to eclosion. **1**, correlation between standardised measured variables. **2**. Percentage of total variance accounted for by each principal component. **3**. Proportion of variance of each measured standardised variable included in principal components 1 and 2.

When female flies were considered separately, there was less correlation between thorax length, wing length and dry weight (Table 7.8), which was also reflected in the composition of PC1. The strongest correlation was between lipid content and dry weight, both major contributors to PC2 (Table 7.8). There were no distinct groupings when PC1 was plotted against PC2 for the females, but rather the points were spread out along both the PC1 and PC2 axes. When the offspring diet was considered, most of the W-diet females appeared in the positive PC2 region while most P-diet females were in

the negative PC2 region (Fig. 7.9a), corresponding to a generally greater lipid content and eclosion time for the W-diet flies. However, when labelled by parental diet (Fig 7.9b) and grandparental diet (Fig. 7.9c) there were no distinct patterns or groupings. Similarly, when labelled by diet combination over the three generations of the experiment (for example, PPP or WPP), there was no discernible pattern other than that most of the P offspring diet females appear in the negative region of PC2 (Fig. 7.10), as previously seen when only offspring diet was considered (Fig. 7.9a).

1. Correlation Matrix

	thorax	wing	omm/thor	dry weight	Lipid	eclosion
thorax	1					
Wing	0.340	1				
omm/thor	-0.367	-0.034	1			
dry weight	0.232	0.267	-0.104	1		
Lipid	-0.006	-0.212	-0.138	0.460	1	
eclosion	-0.154	-0.068	-0.047	0.028	0.055	1

2. Variance

Component	Eigenvalue	% Var.	Cumul. %
1	1.752	29.20	29.20
2	1.398	23.30	52.50
3	1.029	17.15	69.65
4	0.970	16.16	85.81
5	0.494	8.24	94.05
6	0.357	5.95	100.00

3. Component make-up

	Component	
	1	2
thorax	0.735	-0.371
Wing	0.513	-0.552
omm/thor	-0.542	-0.037
dry weight	0.709	0.386
Lipid	0.374	0.812
eclosion	-0.110	0.382

Table 7.8: Principal component analysis on female flies, using thorax length (thorax), wing length (wing), the number of ommatidia divided by the length of the thorax (omm/thor), dry weight, lipid content percentage (transformed by arcsin square root) and time to eclosion. **1**, correlation between standardised measured variables. **2**, Percentage of total variance accounted for by each principal component. **3**, Proportion of variance of each measured standardised variable included in principal components 1 and 2.

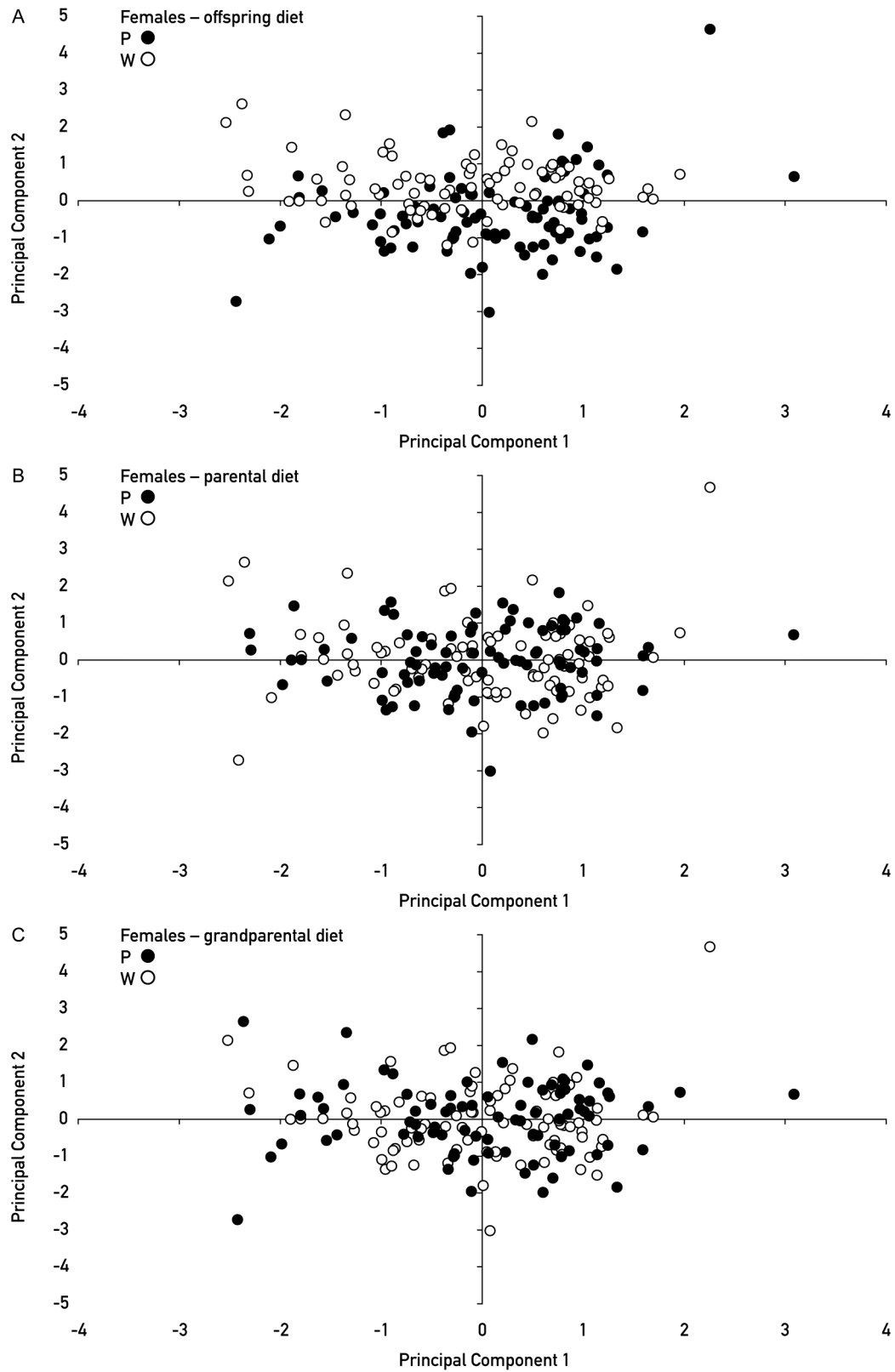


Figure 7.9: Principal Component 1 against Principal Component 2 from a principal component analysis using female flies' thorax length, dry weight, ommatidia number divided by thorax length, lipid content, wing length and time to eclosion as the responses to P or W as diets over three generations. Data points are split by **A:** offspring diet; **B:** parental diet; **C:** Grandparental diet. Principal components 1 and 2 were made up as set out in Table 7.8.

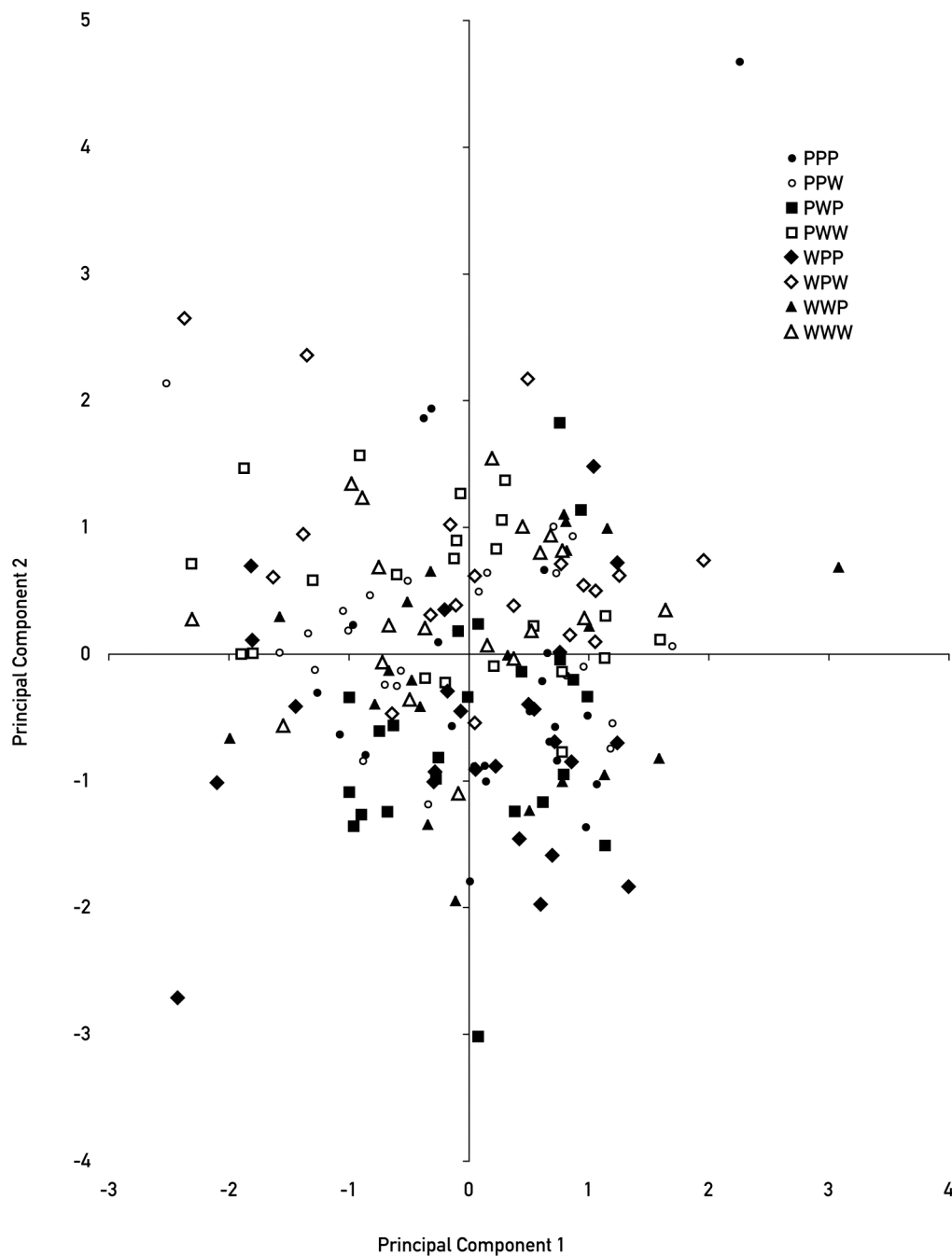


Figure 7.10: Principal Component 1 against Principal Component 2 from a principal component analysis using female flies' thorax length, dry weight, ommatidia number divided by thorax length, lipid content, wing length and time to eclosion as the responses to P or W as diets over three generations. Data points are split by diet combination over three generations (see legend). Principal components 1 and 2 were made up as set out in Table 7.8.

Considering only male flies, correlations between phenotypic characters were very similar to females (Table 7.9), which was reflected in the composition of both PC1 and PC2. There was a greater correlation between thorax length and dry weight than in females (Table 7.8 and 7.9). As before, there were no distinct groupings when PC1 was plotted against PC2 except for when data points were grouped by the offspring diet (Fig 7.11a), where flies were largely separated by PC2. There was very little pattern when PC1 and PC2 were grouped by parental diet (Fig. 7.11b) or grandparental diet (7.11c). Similarly, when labelled by diet combination over all generations of the multigenerational diet experiment there was no discernible pattern other than that most of the P offspring diet males appear in the negative region of PC2 (Fig. 7.12).

1. Correlation Matrix

	thorax	wing	omm/thor	dry weight	lipid	eclosion
thorax	1					
Wing	0.356	1				
omm/thor	-0.234	-0.091	1			
dry weight	0.384	0.294	-0.115	1		
Lipid	0.116	0.032	-0.259	0.419	1	
eclosion	-0.230	-0.070	0.011	-0.043	-0.017	1

2. Variance

Component	Eigenvalue	% Var.	Cumul. %
1	1.99	33.10	33.10
2	1.16	19.27	52.37
3	0.95	15.90	68.27
4	0.88	14.73	83.00
5	0.60	9.95	92.95
6	0.42	7.05	100.00

3. Component make-up

	Component	
	1	2
thorax	0.729	-0.352
Wing	0.565	-0.408
omm/thor	-0.458	-0.358
dry weight	0.749	0.165
Lipid	0.547	0.629
eclosion	-0.257	0.563

Table 7.9: Principal component analysis on male flies, using thorax length (thorax), wing length (wing), the number of ommatidia divided by the length of the thorax (omm/thor), dry weight, lipid content percentage (transformed by arcsin square root) and time to eclosion. **1.** correlation between standardised measured variables. **2.** Percentage of total variance accounted for by each principal component. **3.** Proportion of variance of each measured standardised variable included in principal components 1 and 2.

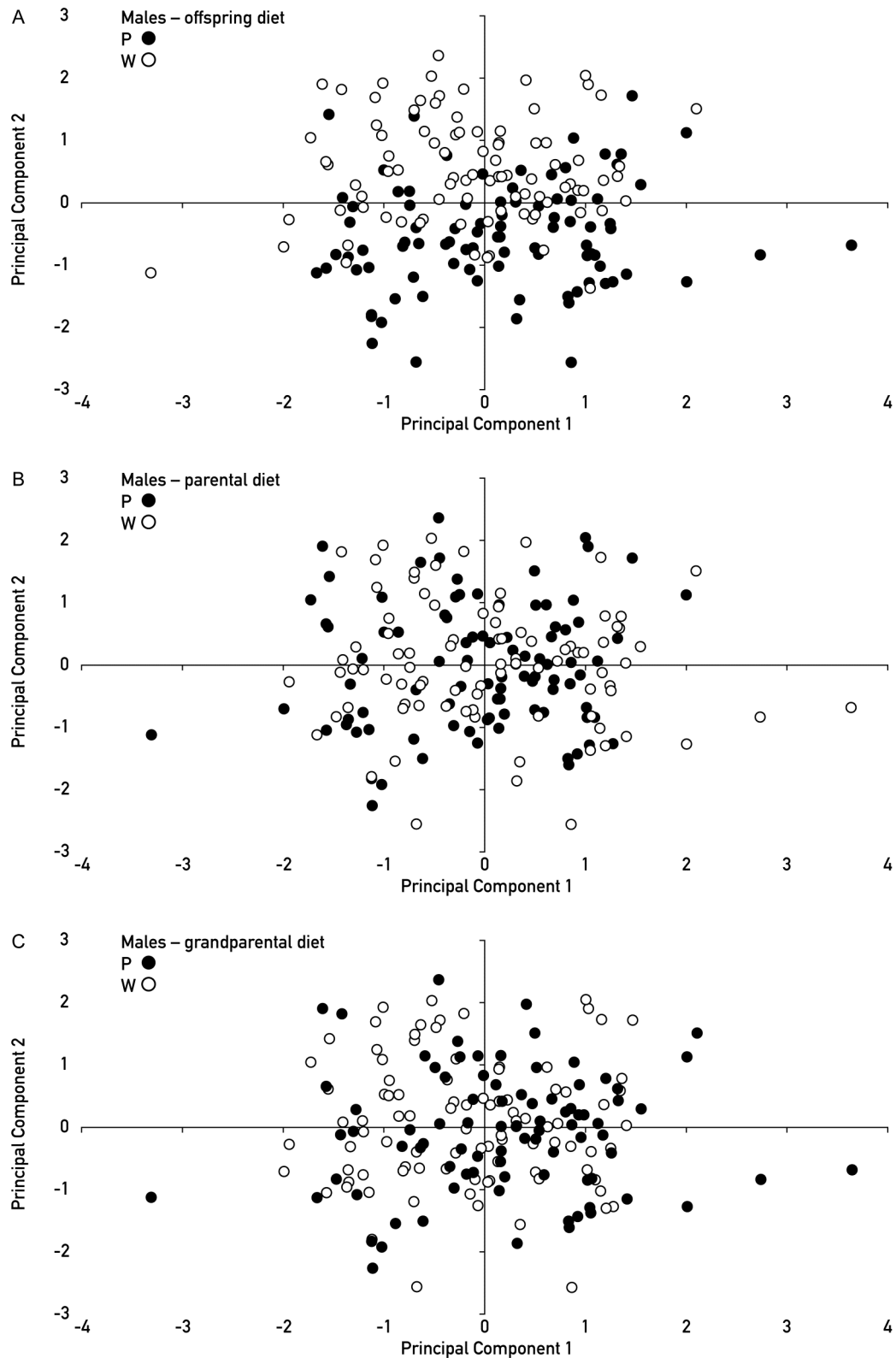


Figure 7.11: Principal Component 1 against Principal Component 2 from a principal component analysis using male flies' thorax length, dry weight, ommatidia number divided by thorax length, lipid content, wing length and time to eclosion as the responses to P or W as diets over three generations. Data points are split by **A:** offspring diet; **B:** parental diet; **C:** Grandparental diet. Principal components 1 and 2 were made up as set out in Table 7.9.

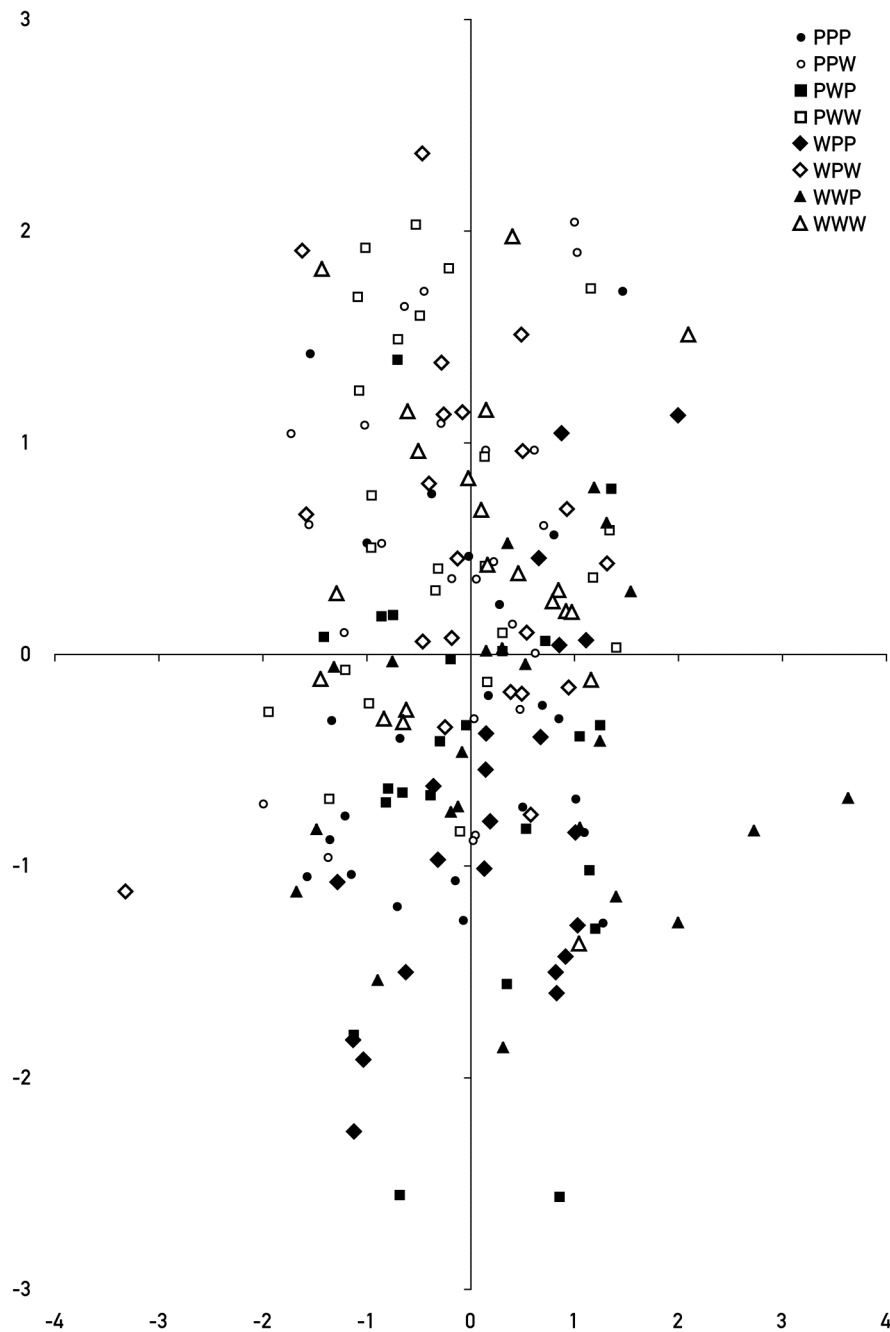


Figure 7.12: Principal component 1 against Principal Component 2 from a principal component analysis using data for thorax length, dry weight, ommatidia number divided by thorax length, lipid content, wing length and time to eclosion from male flies as the responses to P or W as diets over three generations. Data points are split by diet combination (see legend). Principal components are the same as for Figure 7.11.

7.3.10 Costs of plasticity

Retaining the capacity to make phenotypic changes in response to diet, whether in the offspring, parental or grandparental diet, may in itself have a cost (DeWitt 1998; Ghalambor et al. 2007; Auld et al. 2010). To test this, the absolute degree of plasticity of thorax length between the parental P and W diets displayed by each isofemale line was calculated, and its effect on time to eclosion was tested using four separate regressions, for each of the combinations of male, female, offspring diet P and offspring diet W. The null hypothesis was that for each sex/diet combination, the degree of plasticity shown would not affect the speed of development (time to eclosion).

The degree of plasticity did not significantly influence the time to eclosion in any of the four sex/environment combinations (Table 7.10). It is difficult to make out any pattern between the degree of plasticity and time to eclosion (Fig. 7.13 a-d). The null hypothesis was accepted and there is no cost, in time to eclosion, to maintaining thorax size plasticity.

Sex	Offspring diet	R squared	Beta	F	d.f.	p
Female	P	0.006	0.079	0.143	1, 23	0.709
	W	0.003	0.058	0.078	1, 23	0.783
Male	P	0.024	-0.253	1.578	1, 23	0.222
	W	0.020	-0.140	0.462	1, 23	0.504

Table 7.10: Regression analysis between time to eclosion for females and males developing on offspring diets P and W and degree of plasticity in the response of thorax length to the parental diets P and W. R squared refers to the proportion of variation in time to eclosion accounted for by the degree of plasticity, beta refers to the slope of the regression line.

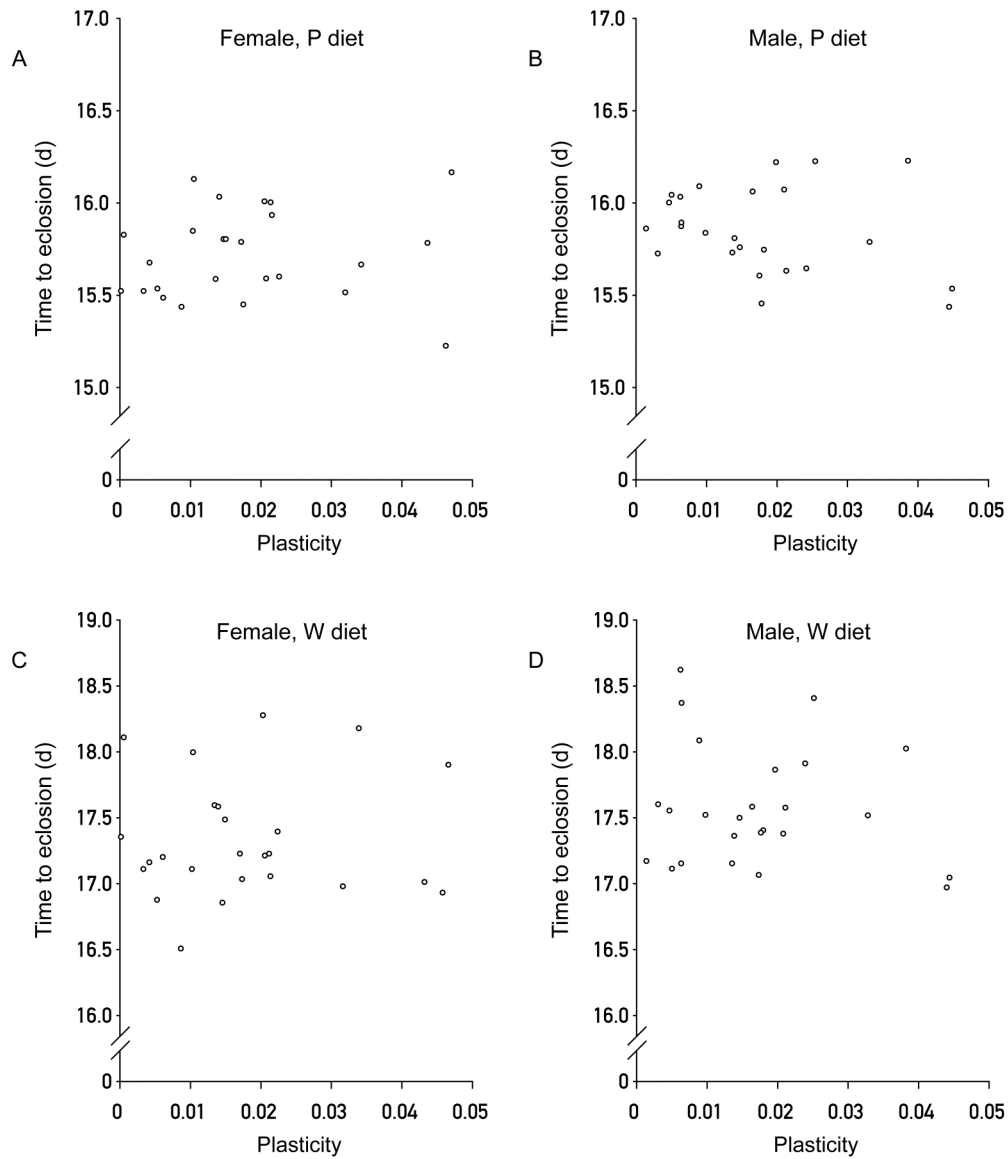


Figure 7.13: Time to eclosion (d) against plasticity between the parental P and W diets, measured as the absolute value of the difference in thorax length (mm) between offspring whose parents had developed on P or W diets. Each data point represents the mean value for each isofemale line in each of the four sex/offspring diet combinations. **A:** Female flies, developing in the P diet; **B:** Male flies, developing in the P diet; **C:** Female flies, developing in the W diet; **D:** Male flies, developing in the W diet.

The plasticity of the response of thorax length was also tested against a potential cost in the survival rate (Fig. 7.14 and Table 7.11). There was no significant effect of plasticity on the rate of survival (Table 7.11) and so the null hypothesis that the plasticity of the response of thorax length to parental diet did not affect the survival rate in the offspring generation was accepted.

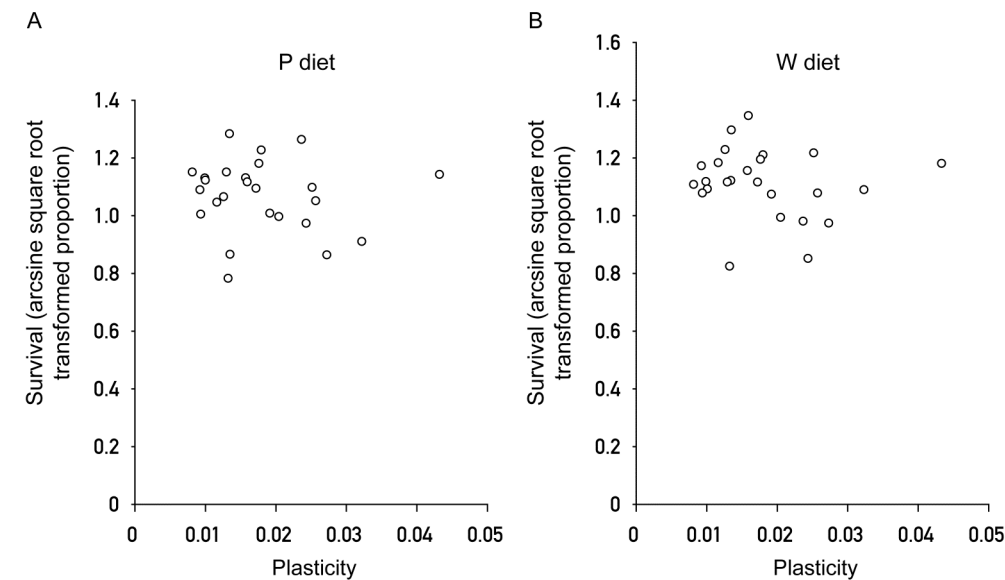


Figure 7.14: Survival rate (arcsine square-root transformed) against plasticity between the parental P and W diets, measured as the absolute value of the difference in thorax length (mm) between offspring whose parents had developed on P or W diets. Each data point represents the mean value for each isofemale line in each of the two offspring diets. **A:** flies developing on the P diet; **B:** flies developing on the W diet.

Offspring diet	R squared	Beta	F	d.f.	p
P	0.012	-0.108	0.270	1, 23	0.608
W	0.020	-0.142	0.476	1, 23	0.497

Table 7.11: Regression analysis between survival rate (arcsine square-root transformed) in offspring diets P and W and the degree of plasticity in the response of thorax length to the parental diets P and W. R squared refers to the proportion of variation in survival rate accounted for by the degree of plasticity, beta refers to the slope of the regression line.

7.4 Discussion

7.4.1 General discussion

Although not tested as a formal hypothesis, one expectation was that if isofemale line: diet interactions were significant, it would be regarding phenotypes where the main diet effect was not significant, so that some lines would respond in an adaptive direction and the others would respond in the opposite way, showing a maladaptive or constrained effect of the previous generations' diets. When this was borne out, there was no clear pattern in which some lines responded consistently to parental or grandparental diets adaptively or were constrained by them. For instance, in thorax length, line 1 offspring had a larger thorax when parental diet was W, but a smaller thorax when the grandparental diet was W, the opposite pattern to that of line 9. Others responded in the same way to both grandparental and parental diet (e.g. line 29). The pattern in survival to eclosion was similar, in that although the isofemale: diet interaction was significant there was no pattern to whether lines behaved consistently across generations.

This does show however that parental and grandparental diets did have a significant effect on survival and thorax length, and grandparental diet influenced ommatidia number. Their effect however depended on the isofemale line but with the significant variation in phenotype characteristics being balanced, so that the main effect (diet) was not significant. The way one isofemale line behaved regarding one character or diet did not predict how it would behave in another.

Some diet effect have no significant variation due to genotype, and the effect is purely environmental. This could indicate that these responses to diet had been strongly selected for in the past. The effect of offspring diet, the differential effects of sex on parental diet and the interaction of parental and grandparental diet do not have any significant variation associated with isofemale line, implying that each isofemale line behaves similarly. Some of these effects may be adaptive and so have been selected for, for instance the increase in thorax size when parental diet was W in males, whereas others, for instance the smaller size of the thorax when the offspring diet was W, may be beyond the limits of plasticity, and so show a constraint by diet.

In other characteristics, both the isofemale line interaction and the main diet effect were significant. There were significant isofemale line: diet interactions, with significant main effects of diet, for the offspring diet regarding survival, for all diets regarding time

to eclosion, and the offspring and grandparental diets regarding lipid content. So for these three characteristics, although the environmental effect was significant overall, it was modulated by genotype. Again though, there was no clear pattern of groups of isofemale lines behaving in the same way across generations or phenotypic characteristics, nor even of individual lines behaving in a consistent way.

While it may seem surprising that phenotypic plasticity comes without a fitness cost, this is not an uncommon result (van Buskirk and Steiner 2009). In the above experiments, the measure of cost used may not be ideal, as it would be preferable to measure the cost of plasticity in terms of fecundity. It is also possible that variation in plasticity represents different strategies to cope with changing diets over generations, and that the cost of different responses may be spread across the whole phenotype, so that for instance an adaptive (and so presumably costly) response in one characteristic may be balanced by less developmental effort going into another (DeWitt 1998). The cost of plasticity might also not be detectable in one environment, but might be apparent in an environment where flies had to compete for resources and mates post-eclosion. Given the large variations between isofemale lines' responses to the grandparental and parental diets, it seems unlikely that each isofemale line has a response that has already been selected to be cost-free, as suggested by DeWitt (1998). Costs may however be spread over several phenotypic characteristics, which may also go some way to explaining the lack of correlation between different isofemale lines' phenotypic responses.

The lack of a coordinated response across the different phenotypic characters within each isofemale line shows that different isofemale lines do not display the same 'suite' of responses to previous generations' diets. This may be the result of different strategies to maximise fitness on different diets. Some isofemale lines may develop a larger thorax while neglecting a different character, other may maximise fitness in different ways.

There was a very clear separation between male and female in the PCA, mainly due to size (PC1), and a less distinct separation by lipid content and eclosion (PC2) regarding offspring diet when only females were examined. No other groupings were apparent, whether based on generational diet or isofemale lines. Nor were any groups apparent when intergenerational diet groups (e.g. PWW) were examined. This means that there is no evidence of an ecotype-like suite of phenotypic responses in response to the diets of previous generations, adaptive or not, emerging from the correlation of isofemale

responses across phenotypic responses, or from the PCA. On the basis of the diets tested, there was no “survival phenotype” (Gluckman and Hanson 2006a, b) displayed by the flies in these experiments.

There were no distinct groupings of isofemale lines with similar reactions to the parental or diets, comparable to the difference between rovers and sitters in their plasticity (Kent et al. 2009). Rather, there was a continuum in the degree of plasticity shown regarding parental and grandparental diets, from lines which reacted adaptively to an adverse diet to those who were constrained by it. This suggests that rather than being the result of differences in one gene, as with for (Kent et al. 2009) or the variants of PPAR γ 2 in humans (Deeb et al. 1998), the response to previous diet is polygenic.

The pattern of how the isofemale lines behaved is similar in many ways to that found by David et al. (1994), for thorax and wing size in relation to temperature. Each isofemale line behaved differently, although following a similar reaction norm (David et al. 1994). If only the reaction norms between two temperatures in David et al. (1994) are considered, as only two diets are considered here, some lines are larger at one temperature and for others the opposite is true. This is exactly what was seen in these experiments when the isofemale: diet interaction term was significant, in many cases even when the main diet effect was also significant, for example in the time to eclosion response to parental and grandparental diets (Fig. 7.1). If a larger range of parental and grandparental diets were considered in a future experiment, these reaction norms might show a pattern more similar to that seen in David et al. (1994).

7.4.1 Conclusions

The significant effects of previous generations' diets that were detected were variable, both between phenotypic characteristics and isofemale lines. There was no evidence of a distinctive response to previous diets involving all of the phenotypic characteristics measured, and when variation between individual isofemale lines was significant, they behaved quite differently to each other. There appeared to be no cost involved in phenotypic plasticity of thorax length in relation to previous diets, although the measured plasticity in individual lines could be quite high compared to the mean difference between dietary treatments. The implications of the variability in response, although it is not possible to say from this study which genes are affected, is that in *D. melanogaster*, adaptive responses to parental diets (or, PARs) are likely to be highly dependant on individual genotypes, and this may be the case in humans as well.

Fecundity, measured by the production by the offspring generation of eggs or viable adults, may be a better way to judge the response to any diet, given that phenotypes (fitness indicators) and isofemale lines vary so much in response to changes in both offspring, parental and grandparental diets. Individual phenotypic characteristics are important, but their reaction norms may be better interpreted when measured together with the actual fecundity of flies in that environment. Assumptions on how phenotypes used as fitness indicators vary over different dietary environments may need to be measured against fecundity in each environment. It would be useful to conduct a series of experiments to determine selection on the reaction norms of phenotypic characteristics, in response not only to offspring diet but also parental and grandparental diets. This would show not only how parental and grandparental diets alter phenotypes, but whether the phenotypic response is adaptive, canalised or maladaptive.

Chapter Eight

Synthesis and conclusions

The experiments reported in this thesis were designed to investigate whether *Drosophila melanogaster* displays Predictive Adaptive Responses (PARs) (Gluckman et al. 2005d), in response to fly diets modelling human hunter-gatherer and modern Western diets, and the form that responses might take (hereafter, the “multigenerational diet experiment”). More generally and in support of the experiments on PARs, the response of *D. melanogaster* to diet was explored through the use of a nutrient space model (hereafter, the “diet range experiment”). This also extends the work of Lee et al. (2008) by linking their work on fecundity and longevity to phenotypic characteristics.

8.1 The Predictive Adaptive Response hypothesis

The Predictive Adaptive Response hypothesis was developed out of a synthesis of observations on the developmental origins of human “lifestyle” diseases and concepts of adaptive developmental plasticity and maternal effects from ecological studies (Gluckman and Hanson 2004; Godfrey et al. 2010). It seeks to explain diseases often thought to stem from lifestyle choices in humans, including the metabolic syndrome, obesity, cardiovascular disease and diabetes, which most often affect the middle aged and elderly, in terms of the evolutionary response to the periconceptual and developmental environment (Gluckman and Hanson 2004; Gluckman et al. 2005d; Gluckman et al. 2009a). The risk factors proposed for these diseases, whether dietary or genetic, had proved difficult to reconcile with the observed epidemiology (Barker 1997; Hales and Barker 2001; Gluckman et al. 2005d). Central to the PARs hypothesis (and to maternal effects), is that by sensing cues from the parental (or grandparental) environment, an offspring phenotype may be altered to be more optimised to an anticipated environment (Hales and Barker 2001; Gluckman and Hanson 2004; Gluckman et al. 2009a). Often this environment is anticipated to be a continuation of the parental environment, but some examples exist where the parental environment causes a different phenotype, for instance coat length in the meadow vole *Microtus pennsylvanicus* alters in response to melatonin and this is thought to be in anticipation of the season to come (Lee et al. 1989).

This phenotypic plasticity might be induced to maximise the chance of surviving to reproductive age, and consequently the chance of reproduction (Gluckman and Hanson

2006a). In humans, the greatest selection pressures are on the young, and so it is to be expected that surviving to maturity is the goal of development (Kuzawa 2007; Jones 2009). However, the trade-offs made in response to a poor maternal environment, including reduced muscle mass, thickened arteries and reduced sensitivity to insulin (Gluckman et al. 2005a; Gale et al. 2006; Drake and Reynolds 2010), which helped conserve energy and resources during development, may cause disease in later life once the individual has aged beyond the reproductive years (Gluckman et al. 2005d). This is especially possible if the predicted environment (for example, nutrition-limited) did not occur (Gluckman et al. 2005d; Godfrey et al. 2010).

The related “mismatch” paradigm also states that when the prediction and the reality do not coincide, disease states will be more likely (Godfrey et al. 2010). This applies to both a poor to rich and a rich to poor nutritional environment mismatch (Godfrey et al. 2010). This might not be true of fitness (in evolutionary terms) itself, as the outcomes of a mismatch are generally put in terms of health (Gluckman et al. 2009a; Godfrey et al. 2010), although the cause may be phenotypic plasticity acting to maximise fitness (Gluckman and Hanson 2005; Godfrey et al. 2010; Bateson and Gluckman 2011).

8.2 Summary of results

In the diet range experiment (Chapter 5), survival to eclosion increased with total calories and from low P:C ratio (1:16) to high (1:2). In the multigenerational diet experiment, eggs laid on the W diet had a higher survival rate. There was a small but significant effect of parental diet, having the effect of a PAR induced by parental P diet, as eggs laid by parents who developed on P had a higher survival rate than those on the W diet.

A higher P:C ratio decreased time to eclosion and although calories were important, the minimum eclosion time was in the middle of the calorie range of the diets, with the two extremes (50 g l⁻¹ and 400 g l⁻¹) being slower. This was similar in the multigenerational experiment, as the W offspring diet flies took longer to eclose than the P diet flies. Parental diet had the effect of constraining eclosion time, while grandparental diet showed a PAR, as W grandparents had faster-eclosing grand-offspring than P-diet grandparents.

Thorax length was greater in females, and a higher P:C ratio increased thorax length, while higher calories reduced length, as also happened in the multigenerational

diet experiment, as W diet offspring had smaller thoraxes than P diet offspring. Females showed a constraint from the parental diet, while males showed a PAR, being larger if their parents had developed on the W diet.

Wings were longer in P diet flies and there was a possible PAR resulting from the grandparental W diet. Wing length was not measured in the diet range experiment. Dry weight was greater in the P offspring flies, and there was a PAR on dry weight induced by the grandparental W diet, as the grand-offspring of W flies had a larger mass than those of P grandparents. In the diet range experiment, dry weight was higher at both high calorific content and at higher P:C ratios.

The lipid content of the flies was controlled by the total calorie content of the diets in the diet range experiment, with no overall influence of P:C ratio. This was reflected in the slight increase in lipid content on the higher-calorie W diet in the multigenerational diet experiment, although the intergenerational effects may have been largely driven by an unusually high value for one group (WPW). The number of ommatidia was not affected by diet composition, although the parental diet did have a different effect on males and females in the multigenerational experiment, as males had fewer ommatidia after correction for size if they and their parents developed on W, while females had more ommatidia relative to size if they and their parents developed on the P diet.

Overall, there were two examples of constraint by the parental diet (time to eclosion and female thorax), where parental diet acted in the same direction as offspring diet, and three examples of PARs induced by the parental diet (survival, male thorax and dry weight). Grandparental diet induced three examples of PARs (survival, eclosion and dry weight), where the effect of the grandparental diet acted in the opposite direction to that diet in the offspring generation. There were no examples of constraint from the grandparental diet. Additionally there were significant isofemale line interactions with parental diet for eclosion time, survival, thorax length and with grandparental diet for lipid content, survival, eclosion and thorax length.

No cost of phenotypic plasticity for thorax length was found, measured in either of time to eclosion or in survival, and no isofemale line displayed more overall plasticity in relation to previous diets than any other, despite their responses often being significantly different when any one character trait was considered. Principal component analysis revealed that the two main principal components (PCs) were associated with size and fast development (PC1), or lipid content and slow development (PC2). There were no

distinct phenotypic groupings when PC1 was plotted against PC2, except for sex and offspring diet.

When offered a choice, flies did not prefer to feed on the diet they had developed on, nor did they choose to oviposit on their own diet. Instead, the majority of flies from both diets laid eggs on the low-carbohydrate P diet and chose the high-carbohydrate W diet for feeding.

8.3 Responses, predictions and adaptive effects

8.3.1 Responses

Diet was chosen as the factor to be used to investigate PARs, as it has a profound impact on both human health (Roseboom et al. 2000; Gluckman and Hanson 2005; Gluckman et al. 2005d), and on fly fitness indicators (Buch et al. 2008; Lee et al. 2008; Skorupa et al. 2008; Kristensen et al. 2011). Other factors such as stress and activity levels might also produce PARs in *D. melanogaster*, and there have already been several investigations into the effect of temperature that showed phenotypic plasticity in relation to parental environment (Huey et al. 1995; Huey et al. 1999; Gilchrist and Huey 2001), although temperature is less relevant from a human health perspective.

The reasons for choosing the two diets P and W are set out in Chapter 2, but briefly, they were designed to represent the difference (in fly diet terms) between Palaeolithic, assumed to be the diet that humans evolved eating, (Eaton et al. 1997; Cordain et al. 2000b; Cordain et al. 2000c; Konner and Eaton 2010) and modern Western human diets, associated with “lifestyle” diseases (Prentice and Jebb 2003; Cordain et al. 2005; Simpson and Raubenheimer 2005). The Western diet was more energy dense and had a lower protein: carbohydrate ratio (1:9) than the P diet (1:4), which was chosen as it was the diet that flies had chosen when allowed to select the proportion of macronutrients, and also the diet on which they had displayed the highest lifetime egg-production (Lee et al. 2008).

As the Predictive Adaptive Response hypothesis is rooted in evolutionary theory (Gluckman et al. 2005d), testing both the hypothesis, and whether *D. melanogaster* displays PARs required that the response variables be connected with fitness, or, the likelihood of leaving viable descendants (van Buskirk and Steiner 2009). The most accurate way of

testing this would be to measure fecundity and the number of surviving grand-offspring. However, available resources did not permit this and so fitness was measured using phenotypic characteristics that are often used as fitness proxies: survival; time to eclosion; thorax length; dry weight; wing length; lipid content and the number of ommatidia per eye relative to thorax length.

The other side of the PAR hypothesis is the detrimental effect that phenotypic alterations directed towards survival and fitness may have on an organism's health, and so two additional responses were included that are relevant to human health and wellbeing, as well as being indicators of fly fitness. These were lipid content, which could shed light on the causes of obesity in relation to diet; and the number of ommatidia per eye, a measure of neurogenesis (Bateman and McNeill 2004).

As noted above, the first two principal components of the PCA on the results of the multigenerational diet experiment revealed that responses separated into two broad groups, one concerned with the size of the fly (thorax, wing and dry weight) and fast development, while the second corresponded to lipid content and slower development. The elements which varied together in PC1, the 'fitness' responses, were also the responses maximised at a high P:C ratio in the diet range experiment (although there was also an effect of total calories). Ommatidia number did not change significantly with diet.

One assumption that was made is that a "good" value of a fitness indicator in one environment corresponds to a good value in all the environments tested. Comparing these results from both sets of experiments to the fecundity of the flies in the nutritional geometry experiment of Lee et al. (2008) shows that the two sets of fitness measures correspond, with higher fitness at low P:C ratios, while longevity (only measured in Lee et al. 2008) was at a maximum at high P:C ratios. This correspondence of the two sets of fitness indicators to a similar array of diets (in effect a reaction norm in two dimensions) indicates that the fitness indicators measured in the multigenerational diet and diet range experiments do reflect real fitness across different dietary environments.

While longevity or the possible mechanisms involved in faster growth were not experimentally investigated in this thesis, one of the possible mechanisms causing the increase in growth and fitness indicators with a lower P:C ratio could be the differential activation of the InR / dTOR pathways, which are thought to respond in *D. melanogaster*, and in a similar way in humans, to carbohydrate and protein respectively (Zhang et al. 2000; Baker and Thummel 2007). These could accelerate or slow growth, and it is

possible that some epigenetic mechanism (in the broad sense) could be acting across generations to speed growth in a predicted dietary environment that is less than ideal.

In the diet range experiment and in the multigenerational diet experiment, lipid content of the flies increased with the total energy density of the diets (calorific content). Macronutrient content (P:C ratio) had no significant global effect. If human metabolism responds in a similar way, this does suggest that adiposity, in response to one generations' no-choice diet, may follow a very simple relationship of increasing energy density causing higher adiposity. There are also of course many issues relating to choice of foods, appetite, and levels of activity in humans, and their relation to obesity (Prentice and Jebb 1995; Cordain et al. 1998; Rolls 2000; Booth et al. 2002; Simpson and Raubenheimer 2005). For instance, although total calories may be important, some evidence points to different effects of macronutrients on the timing and intensity of the action of ghrelin, an appetite-stimulating hormone which is produced in response to hunger and is suppressed for longer by protein and lipid than by carbohydrate (Foster-Schubert et al. 2008). However, appetite aside, the data from the diet range experiment suggest that energy density of the diet is the main factor controlling lipid storage in flies.

Neurogenesis, at least as measured by the number of ommatidia and related to the size of the optic lobes (Mast et al. 2006), is not strongly affected by diet although there was a response in individual isofemale lines. Differences due to diet might be detected if rather than using the number of ommatidia as a response, the ability of flies to learn is tested in different diets.

The response of flies to the diet choice and oviposition substrate choice experiments revealed that flies did not choose diets based on their previous experience. They did however distinguish between the two diets offered and the choices made may increase their fitness. Eating the W diet as an adult would provide more energy which may have been attractive to flies as the other diet choice contained the same protein content with less carbohydrate. Oviposition on the P diet would maximise fitness whatever their previous diet, as eggs laid on this diet would develop faster and be potentially larger as adults.

8.3.2 Predictions, or the influence of past generations' diet

At the outset of the multigenerational diet experiment, a conservative expectation was that if parental or grandparental diets did alter the phenotype of the offspring

generation, their effects would be in the same direction as the effect of diet in the offspring generation, due perhaps to greater (or poorer) egg-provisioning. Eclosion time and female thorax length (and to some extent, male ommatidia number) did indeed respond this way, to the parental diet. However, most of the changes attributable to previous generations' diets were in the opposite direction, suggesting a compensatory mechanism (or, adaptive) that corrects for the effects of the parental or grandparental diet on fitness. So, grandparents developing on the W diet (slow eclosion) had grand-offspring whose development was accelerated compared to the grand-offspring of those on the P diet. The mechanism behind this is not known but it is noticeable that grand-offspring from the P diet grandparents do not seem to have this advantage as they eclosed more slowly when they developed on the W diet. Only the parental diet seemed consistently to be able to produce an effect in the same direction as the offspring diet, suggesting that this may be due to a different mechanism to that which induces a compensatory effect.

These compensatory effects, also seen in the response of dry weight, survival and the length of the male thorax, suggest that either the offspring alter their fitness using cues from the previous generation, or that the previous generation may actively enhance the offspring's fitness in response to diets. This also suggests that a cost will be paid, in some aspect of the flies' fitness. If not, then it seems likely that all flies would accelerate development on all diets, as fast development would give a competitive advantage over slower-developing flies.

No cost of phenotypic plasticity was detected, using thorax plasticity as the response and time to eclosion and survival as costs. Some other aspect of the flies' life history, not measured in these experiments, may be where the cost falls. It seems unlikely that the cost would fall on egg production in the offspring, as all the adaptive phenotypic responses to poor diet tended to move the flies' phenotype towards that associated with higher egg production in the diet range experiment and in Lee et al. (2008). It may be paid in other aspects of the flies phenotype which could for instance have an impact on longevity, where the cost would be paid in potential reproduction in the future. Given that a fly's life expectancy in the wild is between 1 and 6 d (Rosewell and Shorrocks 1987) this may be a price worth paying.

In humans, the mismatch paradigm, related to but not the same as the Predictive Adaptive Response hypothesis, suggests that the cost of maintaining fitness through

phenotypic plasticity is paid in disease later in life (Gluckman et al. 2005d; Godfrey et al. 2010). In flies, a cost may be paid in aspects of “health”, for instance impaired sugar metabolism (Rulifson et al. 2002; Colombani et al. 2003) or heart function (Birse et al. 2010). Either of these could have an impact on competitive ability or longevity. It is also possible that costs may be distributed in different ways in different isofemale lines. Within each isofemale line, plasticity regarding one response did not correlate with plasticity in other responses, so different lines may be making different trade-offs. Further work is necessary to determine if there are groups of isofemale lines which may trade off one fitness indicator for another, as the isofemale line: previous generations’ diet interaction was significant in survival, male thorax length, lipid content and time to eclosion. The implication for humans is that the response to parental or grandparental diets may be different depending on the individual as well as on the environment.

Neither of the responses that could also be considered health indicators, lipid content and neurogenesis, revealed much change in response to diet in the multigenerational diet experiment. The implication for human health is that if there is a phenotypic response to parental or grandparental diets, then it may be harder to predict how this will affect an individual’s phenotype and response to its adult environment, and how this translates into disease risk, if individuals make different trade-offs.

8.3.3 Adaptations: What do the predictive responses mean?

The effects of parental and grandparental diets on survival, eclosion, thorax size and dry weight were significant in the multigenerational diet experiment, but the question remained of whether these effects produce an adaptive advantage in the offspring, tailored to a predicted environment. There are essentially three different types of significant response that can be made to the grandparental or parental diet. In these examples diet A refers to a diet that produces a higher value of a fitness indicator than diet B, regardless of parental diet.

The first is that the effect of parental and offspring diet are additive, so that a poor parental diet reduces fitness indicators in the offspring, and the effects of a good parental diet boost the fitness indicators of the offspring. This may result from for instance, of better provisioning of eggs (Bonduriansky and Head 2007), or in mammals, maternal care (Weaver et al. 2004), and is similar to the “hot parents best” hypothesis (Gilchrist and Huey 2001). Statistically, there would be a significant effect of both offspring and

parental diet, and parental diet A would have higher fitness indicators associated with it in the offspring.

The second is that the effect of parental diet on the offspring is compensatory, so that in a dietary environment where fitness indicators would be lowered, their impact is lessened by a phenotypic adaptation which increases their potential fitness, in anticipation of the continuation of the adverse environment. This may result from various epigenetic mechanisms, including altered gene expression or physical provision of nutrients (Gluckman et al. 2005d; Bonduriansky and Day 2009), and is the equivalent of the “cold-parents best” (Gilchrist and Huey 2001) or Predictive Adaptive Response (Gluckman et al. 2005d) hypothesis. Statistically, there would be a significant effect of both offspring and parental diet, and parental diet B would have higher fitness indicators associated with it in the offspring.

Thirdly, there may be little or no difference in fitness between organisms developing in two environments, but when the offspring of parents from one develop in the other environment, their fitness is lower. This is the “mismatch” (Godfrey et al. 2010) or beneficial acclimation hypothesis (Leroi et al. 1994), which is often seen when there are two alternative morphs, for instance in the response of snails to the presence of one or other predator (DeWitt 1998), or alternative morphs which are most appropriate for the anticipated season which the organism will develop in (Lee et al. 1989). This has been extended to include health consequences of growing up in a different environment to that predicted (Godfrey et al. 2010), but unless the impact is severe these consequences might not affect fitness. Here, diets A and B may have equal fitness associated with them. Statistically, there would be a significant interaction term of offspring and parental diet, and offspring which developed on a different diet to their parents would have lowered fitness.

The responses to parental diets in the multigenerational diet experiment never took the form of a mismatch (using fitness as the outcome). The clear adaptive effects were always of the compensation or PAR type, as also seen in relation to diet in previous investigations (Prasad et al. 2003; Vijendravarma et al. 2010). This may be for two reasons. Firstly, when parental environment experiments have been conducted which found a significant mismatch effect these have been two alternative morphs (DeWitt 1998), in response to two greatly differing environments, or a seasonal response as in voles (Lee et al. 1989). Where the environments are continuous the compensatory

response is often found, for instance between two points on a food-quality gradient (Vijendravarma et al. 2010) or temperature gradient (Steigenga and Fischer 2007). Secondly, the mismatch as proposed by Gluckman and Hanson for humans is directed at the quality of life or health, not necessarily fitness in the biological sense; the adverse health effects stem from phenotypic changes directed at maintaining fitness in the face of an adverse environment (Godfrey et al. 2010). There may only be an effect on biological fitness at extremes, for instance in response to iodine deficiency in Nepal (Gluckman and Hanson 2005). The conditions it explains are often adult-onset, after the age of reproduction, and so the mismatch effect between poor and rich diets in humans might not have an effect on biological fitness. There were no effects of parental diets on the two “health” indicators in the multigenerational diet experiment, lipid content and neurogenesis, that would indicate they were adversely affected by any of the effects on fitness indicators.

All of the measured fitness indicators responded to diet and all but wing length had at least one clear response to parental diets (wing length did have a significant three-way interaction between all generations’ diets). With one exception (dry weight), these all had significant significant isofemale line: diet interactions associated with them. No one isofemale line was significantly more phenotypically plastic across all the indicators, and so it may be possible that plasticity in one feature is traded off against plasticity in another within each isofemale line. There was significant variation between isofemale lines for some traits which were not in themselves significant, including the response of thorax to grandparental diet. This may indicate that different strategies were being employed, with some lines compensating while others did not. This represents the kind of variation which could be selected for and result in the establishment of a population-wide parental effect. If the cost of maintaining adaptive plasticity was high compared to the benefit, the response to grandparental diet could move towards an additive parental effect (that is, grandparental and offspring diets work in the same way), or if the benefit outweighed the cost then a significant adaptive effect over all lines may evolve. If humans show a comparable range of variation in response to parental diets then this may need to be taken into account when looking at ways to ameliorate the effect of parental diets on health (Gluckman and Hanson 2005; Gluckman et al. 2009a).

8.4 Further work

Further work on the PAR hypothesis could be carried out using these experiments as its basis, to investigate the relative influence of different life-stages on PARs induced in the offspring generation. While this would be useful in terms of understanding how *D. melanogaster* responds to previous generations' diets, the developmental stages of a fly are of course very different to human development. It could however shed light on whether the environment which induces a PAR in the next generation(s) is the developmental or the adult environment of the parent. This could inform the study of how human environments affect the next generation. Additionally, none of the work in this thesis addressed whether the effect of parental diet was maternal or paternal. As there were differences in the response of males and females to previous diets it is possible that the diets of either of the two sexes may make a contribution to offspring fitness.

The trade-offs that may be made in maintaining phenotypic plasticity could also be further investigated. Although no cost of plasticity was found in these experiments, the large variation between isofemale lines suggests that there may be different strategies employed in different lines, and that there may be costs in fitness. This variation would also lend itself to selection experiments on the evolution of phenotypic plasticity, to select lines that display adaptive plasticity or are constrained by the diet of previous generations.

Measuring fitness indicators is a relatively convenient way of measuring the response to previous generations' diets, but having found significant responses in these, the effect on fecundity should be measured directly. Also, to simplify the analysis of dietary influence while also gaining information on the effects of a dietary characteristic (for instance carbohydrate, energy density or the P:C ratio), it may be possible to measure the reaction norm of parental (and grandparental) diet against an appropriate offspring diet using fitness indicators, lifetime fecundity and longevity as responses, although this offspring diet would have to be chosen carefully to be as "neutral" as possible in its effects on fitness. If appropriate, this could be extended using the techniques of the geometric framework (Simpson and Raubenheimer 1995; Lee et al. 2008) to compare reaction norms over a two-dimensional array of diets, with the reaction norms induced by parental and grandparental diets. This would lead to better understanding of whether and how the effects of previous generations' diets vary across a wide range of diets, while also eliminating the effect of varying offspring diet.

The range of responses measured may also be usefully extended. The PAR hypothesis in humans is used to explain observations regarding health outcomes, caused as a result of phenotypic plasticity induced by parental or grandparental environments and directed towards maintaining survival and fitness in the offspring (Gluckman and Hanson 2005). Epidemiological investigations are possible in humans, but using *D. melanogaster* it would also be possible to tie in the differences in fecundity and longevity in response to previous generations environments, to morphological fitness indicators and to the emerging models of diabetes (Broughton et al. 2005; Wolf et al. 2006; DiAngelo and Birnbaum 2009; Musselman et al. 2011), obesity (Skorupa et al. 2008; Musselman et al. 2011) and heart function (Wolf et al. 2006) now being explored in *D. melanogaster*. This could provide further insight into the causes and health effects of Predictive Adaptive Responses in humans.

8.5 Conclusions

The diet range experiment extended the work on fecundity and longevity by Lee et al. (2008) in relation to adult diets to the larval diet of flies and showed that the P:C ratio of the diet and the calorific content have different effects on phenotypic characteristics of the adult flies, matching the morphological fitness indicators with the fecundity measured previously measured. There may be a trade-off between thorax size and the size of the fly on poor diets, which may indicate an adaptive effect of poor larval diet. Although these were no-choice diets, the lipid content of the flies increased with the calorific content of the diets rather than any effect of higher carbohydrate content.

Predictive Adaptive Responses have been previously investigated in *Drosophila melanogaster* (Prasad et al. 2003; Vijendravarma et al. 2010) but this is the first time that they have been investigated using diets that model the difference between two human diets, one modelled on the diet that humans may have evolved to eat, and an average contemporary Western diet. This difference is hypothesised to be the cause of many of the “lifestyle” diseases including cardiovascular disease, diabetes and hypertension. The influence of both parental and grandparental generations was also considered, as PARs may extend over several generations, and this study highlighted that the grandparental and parental dietary environments influenced different aspects of the offspring generations’ phenotypes, and in different ways. Individual isofemale line responses to the parental and grandparental diets could be very different, and as the response from one phenotypic character could not predict the response of another in any one isofemale line, there may be different strategies of plasticity employed by different lines. This could potentially be a target for selection on phenotypic plasticity.

Some of the phenotypic characteristics altered by parental diets were not altered in an adaptive way but in the same direction as that diet influenced the offspring, while in others both the parental diet and grandparental diets produced a compensatory effect, or Predictive Adaptive Response, enhancing fitness of the offspring generation by increasing survival rate, thorax size in males, dry weight and accelerating development. Different kinds and directions of intergenerational effect are caused by the parental and grandparental environments, some reducing fitness and some compensating for a predicted diet which produced a less-fit phenotype as an offspring diet, by enhancing fitness through a Predictive Adaptive Response.

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