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# Flying in the face of nutritional variability: modeling the origins and consequences of phenotypic plasticity using *Drosophila melanogaster*

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# ABSTRACT

This thesis employs the genetic model organism, *Drosophila melanogaster* and the Geometric Framework for nutrition (GF) to explore origins, consequences and molecular underpinnings of expressed phenotypic plasticity. The first two studies investigate the influence natural genetic polymorphism of the *foraging* gene can have on population level phenotypic plasticity in response to variable larval nutritional environments. The first of these *foraging* gene studies, Chapter 2, shows that allelic variants of *foraging* differ subtly in their larval life history and phenotypic plasticity, yet these subtle differences correspond to rover *for*$^{R}$ allelic variants displaying 'nutrient generalist' feeding strategies and sitter *for*$^{s}$ 'nutrient specialist'. The second *foraging* gene study, Chapter 3, demonstrates that the *foraging* gene can act as a 'plasticity gene', meaning that each allelic variant is capable of expressing alternate, phenotypic plasticity patterns in response to common nutritional environments. This study indicates that natural populations of *D. melanogaster* which contain alternate *foraging* genotypes are capable of expressing two discrete modes of phenotypic plasticity, which could facilitate their future evolution under nutritional environment change. Chapter 4 analyses the influence variable larval nutrition environments have on the expression of the male *D melanogaster* secondary sexual trait, the sex comb. By comparing the quality of sexual traits between flies raised across a broad range of food environments, this study demonstrates that nutrition plays a critical role in determining trait variability, suggesting nutritional environments can have a direct influence on microevolution via sexual selection. The final data chapter of this thesis characterises the gene expression changes that occur within individuals of a population that has become adapted to a recent extreme shift from a balanced protein to carbohydrate food environment, to a very high-protein one. The findings of Chapter 5 indicate that in order to evolve a carnivore-like phenotypic tolerance to utilising high-protein food as a principle source of calories, major changes in the expression of proteolysis and immune and stress response genes are required – supporting the 'multiplex stress response' hypothesis of ageing.

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# Chapter 1.

## General Introduction

# General Introduction

*Nutritional environment variability and the origins of the phenotype*

The environments within which animal species develop and reproduce are variable across space and time. Of all the components that comprise an animal's environment, sources of nutrition are arguably the most variable (Raubenheimer, 2010). Across generations of evolution in variable nutritional environments, animal species have evolved behaviours and physiological adaptations that support Darwinian fitness (Schluter, 2000; Kawecki & Ebert, 2004). The expression of behaviour and physiology in the living generation of animal species is influenced simultaneously the species' experience of these past nutritional environments and the present, realised environment (Jablonka & Lamb, 2005). The physiological and behavioural traits expressed by animals in response to the environment are termed, 'phenotypes' (West-Eberhard, 1989; 2003).

Species' experiences of past nutritional environments are manifested in their genomes and epigenomes (hereafter, 'genomes'). Species' genomes can be conceptualised as a series of predictive 'instructions' for how individuals can best optimise their fitness, given which phenotypes performed best (and were thus selected for) across the sum total of all previously encountered nutritional environments. Across an animal's lifespan, and especially during early development, these instructions interact with the animal's experienced environment, generating the animal's realised (expressed) phenotypes (Pilgiucci, 2001; Jablonka & Lamb, 2005). The character of these past environments – including their patterns of variability – will influence how the present generation of animals express their phenotypes. The genomes of species which have experienced comparatively stable environments, both within and between generations, are theorised to contain less capacity for the generation of variable phenotypes than the genomes of species that have experienced a greater degree of environment variability across time (Schmalhausen, 1949; Falconer, 1990; Stearns & Kawecki, 1994).

'Phenotypic plasticity' describes the capacity of a genotype or entire genome, to generate alternate forms of a phenotype in response to environmental variation (West-Eberhard, 1989; 2003; Suter *et al.*, 2013). Genomes of species that have

encountered more variable environments across evolutionary time are thought to better buffer the performance of individuals living in variable environments due to their greater expression of phenotypic plasticity (Pilgiucci, 2001). As in the case of any iteratively acquired 'ability', greater experience of different and perhaps stressful environments results in better performance the 'next time' a similar stressful situation is encountered. To extend this idea, species who have encountered less environmental variability across evolutionary time will possess greater genomic 'näivety' and a lowered capacity for adaptive phenotypic plasticity.

Species whose genomes display greater capacity for phenotypic plasticity are likely to be more 'evolvable' than those which express less plastic phenotypes. Phenotypic plasticity is one means by which variation – the 'raw material' which selection relies upon (Darwin, 1859) – is generated (West-Eberhard, 2003; Jablonka & Lamb, 2005). Expressing greater phenotypic variation in response to novel environments will result in a higher likelihood of one or more of these phenotypes acting as a performance protecting 'exaptation' (Gould & Vrba, 1982) if the environment were to shift (Chevin *et al*., 2010).

Species with greater capacity for plasticity are also less likely to be prone to expressing 'mal-adaptive' phenotypes under mismatch of evolved *vs.* actual environments (Raubenheimer *et al*., 2012). Such a mismatch is thought to be central to our own species present 'obesity epidemic' (Stöger, 2008). The hypothesis follows that *Homo sapiens* evolved famine resistant metabolic physiology to maximise fitness under unreliable Paeleolithic nutritional environments. This evolved propensity to store calories from food as fat, or 'metabolic thrift' is believed to now be at odds with our calorie-abundant 'westernised' nutritional environments (Stöger, 2008; Power & Schulkin, 2009).

The mechanisms by which phenotypic plasticity arises in living individuals through the interaction of their genes and the environment are poorly understood (Pigliucci, 2001; Chevin, 2010; Danchin, 2013). We do not fully understand what preserves the capacity for the expression of phenotypic plasticity across time, or what molecular mechanisms underpin its expression in the present (Pigliucci, 2001; West-Eberhard, 2003; Chevin *et al*., 2010). We also do not know if phenotypic plasticity

can be considered a ‘trait’, itself subject to selection (Chevin *et al*., 2010), even though this seems self-evident.

*Aim and structure of the thesis*

This thesis explores the origins, underlying molecular mechanisms, and consequences of phenotypic plasticity expressed in response to environmental variability. I quantify phenotypic plasticity expressed by the genetic model organism *Drosophila melanogaster* across explicitly defined model nutritional environments. As a proximate ‘origin’ of phenotypic plasticity, Chapters Two and Three investigate gene by environment interactions (Via & Lande, 1985) attributable to naturally occurring allelic variation (polymorphism) of the *D. melanogaster foraging* gene (Sokolowki, 2001; Hughson, 2014). As ‘consequence’ Chapter Four examines how early-life nutrition can drive the plastic expression and quality of an important secondary sexual phenotype in male *D. melanogaster*, the ‘sex comb’. By using mRNA-sequencing, Chapter Five explores how trans-generational change in gene expression contributes to the acquisition of a protective metabolic phenotype following a substantial and sustained shift in the nutritional environment. I have written these data chapters as four separate manuscripts, each with their own Introduction and Discussion. To acknowledge the contributions made by my supervisors and other collaborators in the designing and conducting of my experiments, I use the plural pronouns, ‘our’ and ‘we’ throughout my data chapters.

*How phenotypic plasticity is conceptualised throughout this thesis*

Within this thesis I regard phenotypes as the physical, physiological and behavioural traits (including life-history traits) possessed by individuals of a species that contribute to performance and ultimately Darwinian fitness. I maintain that all phenotypes of an individual, including those whose expression is not directly attributable to underlying genes (alternatively conceptualised as quantitative trait loci ([Lynch & Walsh, 1998]), must interact and ultimately contribute to an individual’s fitness.

Phenotypic plasticity describes the capacity of a phenotype to vary in its expression (e.g. its size, colour, shape or behaviour) between individuals of the same species or genus, or throughout the lifetime of an individual organism. Phenotypic

plasticity can be measured at the intra-specific, intra-genetic, intra-sexual or inter-sexual level. Phenotypic plasticity may also be measured at the intra-individual level when the same phenotype is measured at intervals across an individual's life. The degree of phenotypic plasticity within any one of these sub-groups can be quantified as the measured variation in the expression of the phenotype between individuals within the group, or in the case of intra-individual phenotypic plasticity, across the organism's lifetime.

Intra-specific phenotypic plasticity is the most convenient grouping to use in explaining my understanding of phenotypic plasticity. For example, I understand that the range in phenotype expression achievable amongst individuals of a species, *e.g.* horn length amongst a population of male *Onthophagus acuminatus* rhinoceros beetles, is attributable to either/or genetic and environmental variation (Emlen, 1994). Any perceivable variation between males in the expression of their horn length across different environments (even subtly different environments) I consider an example of inter-individual 'plasticity'. Even if only two individuals in a given natural population differed in their horn length (and even if they posses identical alleles of a hypothetical gene 'for' horn length), it cannot be claimed that horn length is not 'plastic'. Indeed the phenotype may be minimally plastic (perhaps due to cannalisation of the trait over generations of selection) but it is still variable and thus a plastic phenotype. The definition of phenotypic plasticity used in this thesis therefore describes the phenomenon of phenotypic variability. I feel this is the appropriate way to define 'phenotypic plasticity' in studies of evolution via natural or sexual selection, as any variation among reproductive members of a species might contribute to intra-specific variation in fitness.

My definition of phenotypic plasticity therefore follows the work of Pigliucci & Müller (2010), Jablonka and Lamb (2005) and West-Eberhard (2005), rather than that of quantitative geneticists who conceptualise phenotypic plasticity and the evolution of 'quantitative traits' from the Modern Synthesis frame (Huxley, 1952). Quantitative geneticists maintain that the contribution of the environment to phenotype variation accumulated within the lifetime of an individual is not heritable across generations, as it is not directly or indirectly genetic in origin. Thus lacking a mechanism for inheritance, quantitative geneticists do not consider environmental contributions to trait variability relevant to evolution via natural or sexual selection.

Quantitative genetics reserves the use of 'phenotypic plasticity' to refer to cases of gene by environment interaction (GEI). According to quantitative genetics theory, GEI and thus 'phenotypic plasticity' occurs when a quantitative trait locus (QTL) differs among members of a species both in the allelic construction of the QTL (i.e. differs in 'genotype') and how individuals with each version of the QTL differ in their mean phenotypic response to an environmental gradient (Falconer & Mackay, 1996; Lynch & Walsh, 1998). The influence and importance of the environment on 'phenotypic plasticity' to a quantitative geneticists is therefore always pegged to established heritable units – QTLs or genes.

This view of phenotype evolution and associated definition of phenotypic plasticity restricts and reduces the evolutionary importance of the environment. My conceptualisation of 'phenotypic plasticity' allows the environment to have a meaningful contribution to evolution via natural or sexual selection without an insistence that environment-mediated phenotype variation be dependent upon and heritable through genes, or indeed heritable at all. Environments experienced across organism's lifetime can have a measurable influence on reproductive success of an individual (without any modification to the structure of genes across generations), as environments experienced have the capacity to influence the performance phenotypes of reproductive individuals. Environmentally conferred alterations to an individual's reproductive success are an important component of evolution that must be analysed and understood, rather than considered 'noise'. An individual's suite of phenotypes is what is selected for or against, not underlying genotypes.

This thesis explores the interactive relationships that exist between the phenotypes expressed by individuals across their lives, the environments individuals experience, and individual's genes. This thesis takes a explicitly quantitative approach to the environment – allowing for the important contribution of environmental variation to phenotypic plasticity and evolutionary processes to be identified and robustly analysed.

Drosophila melanogaster *as a model organism*

*Drosophila melanogaster* is a tractable model species for addressing questions about phenotypic plasticity, indeed it was one of the first used (Morgan *et al*., 1925). *Drosophila* is a particularly amenable model due to the species' short generation time

and the ease with which populations can be cultured in the laboratory. Due to the species' long-standing model-status, *Drosophila melanogaster* genetics are well understood (Sokolowski, 2001; Edgar, 2006; Leopold & Perrimon, 2007; Baker & Thummel, 2007; Smith *et al*., 2014). As for our own species, *Homo sapiens*, the *D. melanogaster* genome is 'annotated'. This means that we understand what the protein products of the majority of the organism's genes are, what molecular processes these encoded proteins are associated with, and where the genes are 'located' along the long sequence of nucleotides that comprise the animal's genome (Encode Project Consortium, 2011). Extensive work over the last 25 years characterising the genes and genetic pathways underlying *D. melanogaster* metabolic physiology has demonstrated that up to 70% of the fly's metabolic genes have mammalian homologues (Pandey & Nichols, 2011). This means that despite hundreds of millions of years of divergent evolution (Nei *et al*., 2001; Peterson *et al*., 2004) the genes and genetic pathways controlling animal metabolism have been evolutionary conserved. Thus findings made when working with *Drosophila* are generally applicable to how other species, including our own, have become adapted to their nutritional environments.

*Defining experimental nutritional environments using the Geometric Framework for nutrition*

For experimental studies of phenotypic plasticity to yield logical results, the variable environments in which animal phenotypes are expressed must be explicitly defined. Failing to do so can result in un-quantified factors contributing to expressed phenotypic plasticity and spurious conclusions being drawn. All experiments detailed in this thesis either directly use, or are informed by, the Geometric Framework for nutrition (GF) (Simpson & Raubenheimer, 2012). The GF is a state-space modeling system, which allows for the single and interactive effects on the phenotype of multiple nutrients and other dietary properties (such as energy content) to be explored and quantified.. The GF employs the concept of nutrient space, a geometric coordinate space wherein axes (typically *x* and *y*) are represented by nutrient and other constituents of foods and diets. In this thesis, I utilise a nutrient space delimited by increasing concentrations of dietary protein on the *x* axis, and carbohydrate on the *y* axis, these being the two macronutrients most relevant to growing *D. melanogaster* (Behmer, 2009) Chapters 2, 3, and 4 measure *D. melanogaster* phenotypic plasticity expressed in response to diets that have been systematically sampled across protein –

carbohydrate nutrient space. These phenotypes are then mapped against nutrient space and statistically analysed following Lee *et al*. (2008), Dussutour *et al*. (2010) and South *et al*. (2011) for the influence protein, carbohydrate and total calories have on phenotype performance. Please refer to Figure 1 for more details on how I have used the GF in this thesis.

*Genetic polymorphism as an origin of phenotypic plasticity*

Natural genetic polymorphisms, or allelic variants, are one means through which the capacity to express phenotypic plasticity is believed to be retained by species over generations (Via *et al*, 1995; Nijhout, 2003; Gorur *et al*., 2005). The *foraging* gene of *Drosophila melanogaster* encodes a cyclic guanosine monosphosphate (cGMP)-dependent protein kinase (PKG) (Osborne *et al*., 1997). Two natural allelic variants exist, $for^{R}$ 'rovers' and $for^{s}$ 'sitters' (Sokolowski, 1980; Hughson *et al*., 2014). Rovers and sitters differ both as larvae and adults in a range of food-search related behaviours, and in the physiological phenotypes they express in response to common food environments (Kaun *et al*., 2007; Kent *et al*., 2009; Burn *et al*, 2012; Hughson *et al*., 2014). These rover *vs*. sitter phenotypic differences are attributable to each allelic variant expressing different levels of PKG in response to the same nutritional environment stimulus. *foraging* gene expression is also known to influence the expression of other genes (Kent *et al*., 2009), which are thought to further contribute to the diversity of phenotypes the genetic variants yield in response to food (Kent *et al*., 2009; Hughson *et al*., 2014).

Chapters 2 and 3 investigate the capacity these natural *foraging* allelic variants have for expressing phenotypic plasticity across a broad range of experimentally defined nutritional environments. The principal aim of Chapters 2 and 3 is to use the GF to characterise the phenotype reactions norms expressed by each *foraging* genotype – a novel application of the GF. Chapter 2 concentrates on larval life history traits, while the focus is extended to phenotypes relevant to adult fitness in Chapter 3.

The methodology used for quantifying phenotypic plasticity in these Chapters represents a substantial advance in reaction norm quantification. A genotype's range of phenotypic reaction norms are typically quantified in response to a one dimensional environmental gradient, thus limiting the measures' ecological and evolutionary relevance (Chevin *et al*., 2010). Chapters 2 and 3 employ the GF to quantify the

expression of multiple phenotypes, per *foraging* genotype, in response to multiple nutritional dimensions. Characterisation of multidimensional reaction norms supports the formulation of more informed hypotheses regarding the adaptive relevance of expressed phenotypic plasticity and the role past environments have played in the selection for each genotype's suite of phenotypic reaction norms (Chevin *et al*., 2010; Danchin, 2013). Through using *foraging* gene as a model system, Chapters 2 and 3 address a fundamental question of evolutionary biology – are genetic polymorphisms a proximate origin of phenotypic plasticity?

*Nutritional environments contribute to the quality of secondary sexual traits – therefore nutrition influences evolution via sexual selection*

Phenotypic plasticity generated through the interaction of a species' genome with the nutritional environment is not exclusively relevant to evolution via natural selection. Sexual selection also proceeds on the basis of variability in the expression of phenotypes (Darwin, 1859; Rowe & Houle, 1996). In *Drosophila* species, including *D. melanogaster*, males possess secondary sexual characters on the fore-legs, called sex combs (Kopp & True, 2002; Polak & Tomkins, 2012). Sex combs are important to male mating success due to their role in intrasexual competitive behaviours and intersexual courtship (Chen *et al*., 2002; Hoyer *et al*, 2008; Hurtado-Gonzales *et al*., in prep). In Chapter 4, I take advantage of this aspect of *D. melanogaster* sexual ecology to model how the quality of the experienced nutritional environment influences the expression and quality of secondary sexual traits. Chapter 4 utilises artificial diets formulated using principles of nutritional geometry to quantitatively control male fly nutrition-dependent condition, and to then map and analyse trait quality across nutrient space. Through this use of the GF, Chapter 4 reveals a central role for nutrition in determining sexual trait quality and thus contributing to the maintenance of variability of secondary sexual traits across generations of sexual selection. Chapter 4 thereby highlights the direct consequences that environmentally induced trait variation can have on future evolution via sexual selection.

*How does gene expression contribute to phenotype expression?*

A species' nutritional environment can shift markedly in nature, for example due to severe climatic events, a population undergoing dispersal, or due to intense intra-specific competition under localised population increase. Possessing a phenotypically plastic genome can 'offer' species adaptive capacity in the wake of such environmental change through the expression of exaptations (Gould & Vrba, 1982). Chapter 5 uses a laboratory selection experiment to first model how the number of individuals in a population expressing a performance-protective phenotype can increase across generations, and next to examine changes in gene expression associated with acquisition of the protective phenotype. In the experiment, a population of *D. melanogaster* are exposed to a nutritional environment change – from a stable ancestral food environment, to one limited in dietary carbohydrate yet abundant in dietary protein. Diets containing a high ratio of protein to carbohydrate have been associated with reduced lifespan in *Drosophila* (Lee *et al*., 2008), yet a minority of flies in such experiments display a lifespan protecting, high-P:C resistant phenotype. The expression of this trait is selected for over five generations, and using Illumina Hi-seq mRNA-sequencing, all *D. melanogaster* genes that change in expression with the acquisition of the protective phenotype are identified, and have their expression quantified. By tightly correlating phenotype expression to gene expression, Chapter 5 addresses the question, what molecular processes are required for an adaptive phenotype to be expressed? Findings from chapter 5 suggest that changes in the expression of complex networks of genes, rather than individual loci are required in the evolution of targeted, environment-specific adaptations.

*Relevance of the work*

Taken together, the findings of my thesis contribute to our limited understanding of how phenotypes arise in response to environmental variation and how the capacity for phenotype plasticity is inherited. The thesis also provides a quantitative demonstration of the role nutritional environments can play in shaping micro-evolutionary processes. The results also perhaps serve as a cautionary note to proponents of therapeutic genetics and genetic counseling for human health. This thesis demonstrates that nucleotide variation at a single locus can lead to unpredictable phenotype expression. The results also indicate that in expressing

alternate phenotypes, individuals of the same species are likely undergoing concurrent changes in the expression of hundreds of other genes. Artificial modification to gene expression in humans, via RNAi therapies for example (see Hagiwara *et al*., 2014; Roberts, 2014), even when tissue specific (Poehlmann, *et al*., 2014) could disrupt genetic pathways, whose expression has been 'honed' for performance through thousands of generations of selection, resulting in the expression of non-target, 'fitness-threatening' phenotypes.

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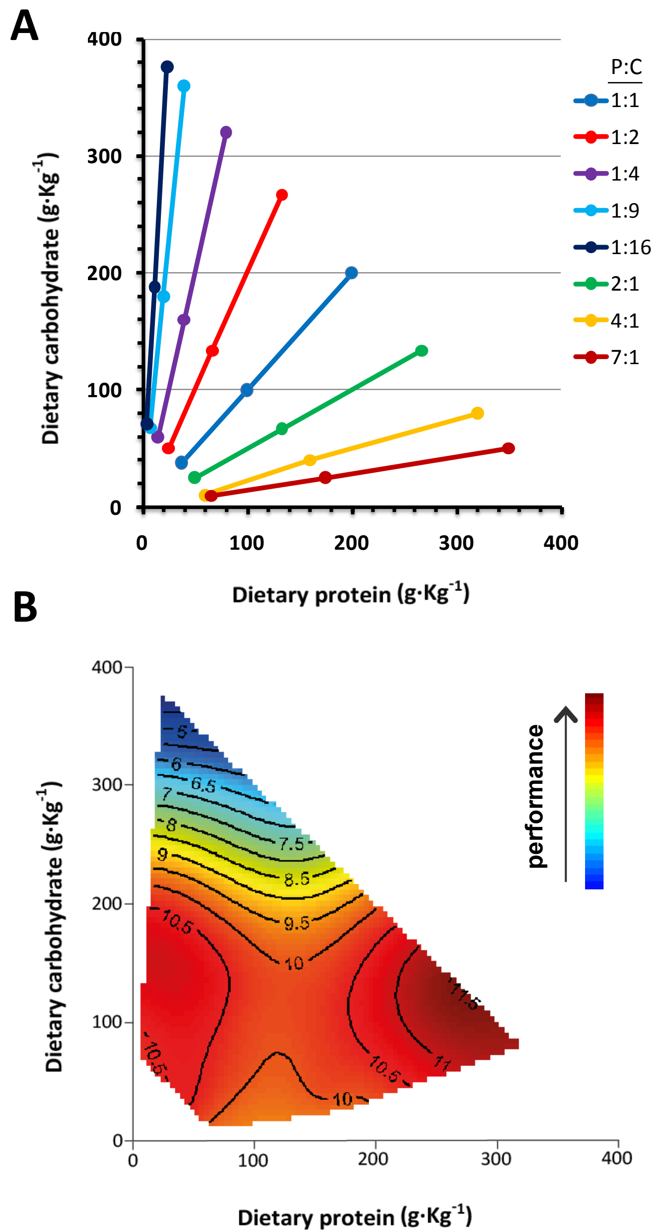


Figure 1. This figure demonstrate how the Geometric Framework for nutrition has been used to characterise phenotype performance in response to food throughout this thesis (using examples from Chapter 4). A (top panel) shows the position of the experimental *Drosophila melanogaster* rearing diets used in Chapter 4 in protein-carbohydrate (P-C) nutrient space. Coloured lines radiating from the origin indicate the position of the P-C diet 'rails'. Along each rail are three filled circles. These represent low-, medium- and high-energy formulations of experimental diets of the same P:C ratio. In Chapter 4, we raise flies on low-, medium-, and high-energy diets which fall along each of the eight P:C ratio diet rails indicated on panel A – a total of 24 diets (a similar approach is taken in Chapters 2 and 3) We measure phenotype performance in response to these diets. B (bottom panel) shows a thin plate spline (TPS) phenotype response surface fitted over nutrient space. The TPS allows for interpolation of fly performance between the points on nutrient space for which we did not formulate fly diets. Thus, the TPS is a 'surface of best fit', with points on the TPS of equal performance value being linked via contour lines. On these TPS surfaces, deep red areas indicate performance optima (or maxima), while deep blue indicates performance minima.

# Chapter 2.

**Natural allelic variation drives alternate feeding strategies in *Drosophila melanogaster***

## Abstract

This study shows that allelic variants of the *foraging* gene differ subtly in their larval life history and phenotypic plasticity to variable nutritional environments. We found that natural *for*$^{R}$ rover larvae more tightly regulate their protein (P) and carbohydrate (C) intake targets than *for*$^{s}$ and *for*$^{s2}$ sitter larvae. These difference in P-C intake appear to control the expression of other larval traits. Rovers, presumably through their active maintenance of P and C intake, develop more quickly than do sitter larvae. When confined to ‘extreme’ larval rearing diets (very high-protein and very-high carbohydrate diets) rover larvae also show higher survivorship than sitters. The ability of rovers to maintain faster development than sitters on very high-carbohydrate diets is however associated with rovers becoming ‘fatter’ than sitters. On high carbohydrate diets, rover *for*$^{R}$ larvae develop and store more triglycerides per unit body protein than do *for*$^{s}$ sitter larvae. These rover *vs.* sitter patterns correspond closely to those observed previously in nutrient ‘generalist’ *vs.* nutrient ‘specialist’ larval locusts and caterpillars. When confined to a non-target P-C diet, generalist larval insects consume sufficient quantities of the food in order to maintain their intake target for less abundant nutrient. In doing so, they consume above-target excesses of the more abundant nutrient, which can incur performance costs. In generalist locust nymphs and in caterpillars, the ‘cost’ of maintaining P intake targets when restricted to fixed high-C:P diets is increased lipid storage. ‘Specialist’ insect larvae in contrast do not so tightly maintain their nutrient targets, and as a result develop more slowly, and accumulate less lipid. We propose that rovers follow a ‘generalist’ and sitters a ‘specialist’ feeding strategy and that these differences are associated with their ‘roving’ between food patches *vs*. ‘sitting’ within patches foraging behaviours.

## Introduction

Understanding the evolutionary origins and influence of phenotypic plasticity in adaptation is an important aim of evolutionary biology. The role of the environment in shaping phenotypic variation in a living organism has both a past and present component. Stored within an organism’s genome is a legacy of environments past. Organisms possess inherited instructions accumulated over the many preceding generations experienced within past environments. These instructions encompass how

best to develop, survive and reproduce (West-Eberhard, 2003; Jablonka & Lamb, 2005; Danchin, 2013). How these instructions play out as phenotypes during development is influenced greatly by the organism's realised environment. The 'instructions' interact with the realised environment at every stage of an organism's life – and even before conception - generating each and every individual's suite of phenotypes.

Environments experienced by organism, past and present, are inherently multivariate. For animals, their nutritional environments are among the most temporally unreliable and complex. Evolving capacity for optimal phenotypic performance in the face of ever-changing nutritional environments is an extraordinary achievement of evolution. The Geometric Framework for Nutrition (GF) is a state-space modeling framework developed for studying the complexity of nutrition. The GF provides quantitative tools for teasing out the univariate entities – the nutrients and calories comprising multivariate nutritional environments – so that their individual and interacting influence on trait expression can be measured (Archer *et al*., 2009; Simpson & Raubenheimer, 1993; 1995; 2004; 2012). When combined with knowledge of discrete variation in the 'evolved instructions' possessed by animals, the GF can provide a powerful means by which to explore how past environments interact with those present in the generation of phenotypes.

The GF achieves employs an *n*-dimensional Cartesian geometric space called 'nutrient space' by which foods are represented as lines (or 'rails') radiating from the origin. The angle of individual food rails to the axes of nutrient space is determined by the relative amount of nutrients the food contains. Moving up a rail represents an increase in intake of the food by an animal. Foods that comprise nutrient space in GF experiments may be natural or synthetic; however, yielding logical interpretations requires that their composition be precisely known. Measures of organismal states or performance measures in response to given multidimensional nutrient intakes or diet compositions can then be mapped as response surfaces onto nutrient space, enabling identification of organisms' nutritional 'intake targets' and phenotypic performance optima (Simpson and Raubenheimer, 1995, 2004, 2012; Lee *et al.,* 2008). These GF surfaces are akin to fitness landscapes (Wright, 1988; Orr, 2005), but with species' fitness or performance traits mapped in response to environmental variation, as opposed to inherent genetic variation.

Evolutionary theory predicts that species evolve adaptations that maximise their Darwinian fitness in response to environments experienced across time (Schluter, 2000; Kawecki & Ebert, 2004). Logically, natural selection should have calibrated the animal's nutrient intake target, nutritional-balancing priorities and post-ingestive allocation strategies to maximise Darwinian fitness for genotype, sex, life stage and physiological state (Simpson & Raubenheimer, 1993; Simpson & Raubenheimer, 2012). Indeed, a growing body of recent comparative GF studies from insect species verifies that organisms regulate their nutrient intake to achieve increased fitness outcomes. For example, adult females of the fruit fly species, *Drosophila melanogaster* and *Bactrocera tryoni*, self-select diets that support maximal lifetime egg-production (Lee *et al.,* 2008; Fanson *et al.,* 2009). Similarly, female predatory ground beetles, *Anchomenus dorsalis*, mix a diet that promotes maximal egg production (Jensen *et al.,* 2012). Nutritional intake targets of adult male *Teleogryllus commondus* field crickets correspond with their maximal delivery of courtship song (Maklakov *et al.,* 2008) and male *Nauphoeta cinera* cockroaches will navigate to a nutritional intake target that promotes their production of female-attracting sex pheromones (South *et al.* 2011). Larval Lepidoptera will feed to maximise their growth rate (Simpson *et al.,* 2004) and even non-animal amoeboid multinucleate slime-moulds demonstrate innate nutritional 'wisdom' by self-selecting a diet that supports maximal expansion rates (Dussutour *et al.,* 2010).

Important to studies of phenotypic plasticity and adaptation, these species-level GF studies demonstrate quantitatively that phenotypic performance decreases in a graded manner away from the intake target across nutrient space (*i.e.* presumably away from the mean nutritional environment of generations past). Rather than dropping away dramatically from the intake target, phenotypic performance is buffered by phenotypic plasticity. For example, the response surface for female *D. melanogaster* lifetime egg production measured by Lee *et al*. (2008) shows that moving across nutrient space away from the performance maxima located over the 1P:4C rail, to an isocaloric diet located on the 1P:1C rail, results in a 50% reduction in performance, but not a 100% reduction. Even if a fly moves yet further from the optimum to an isocaloric 2P:1C diet, she can still generate eggs, albeit at 25% of maximum capacity.

Buffering of performance by phenotypic plasticity is made possible by the legacy component of the environment. Adequate instructions for survival in sub-optimal and fringe nutritional environments are retained by species, allowing compromise phenotypes to be expressed under nutritional stress. These instructions, coded by genes and their epigenetic markers, are expressed and interact with the environment in real time throughout development (West-Eberhard, 2003; Jablonka & Lamb, 2005). As no two realised environments are temporally or spatially identical, this interaction will always be unique. In turn, the compromise phenotypes generated are always novel, in their detail at least. This process therefore leads to both the generation of a 'best fit' phenotype for the current situation (as informed by past environments) and the generation of new phenotypic variation for future natural selection to act upon (Schlichting & Pigliucci, 1998; West-Eberhard, 2003). How exactly phenotypic plasticity arises from interactions between the epi/genome and new food environments, and whether it does so with characteristic patterns, remains relatively obscure (Via *et al.,* 1995; Kent *et al.,* 2009; Danchin, 2013).

One means through which species are hypothesised to retain the capacity for adaptive plastic phenotypic expression is through genetic polymorphisms (Via & Lande, 1985; Via *et al.,*1995; Sgrò & Hoffmann, 2004; Gorur *et al.,* 2005). Routinely these are conceptualised as extending species' niche breadth (Levene, 1953) by providing two or more genetically discrete phenotypic 'strategies' for delivery of higher fitness in alternate environments and whose relative frequency within a population is entrained by cyclical environmental fluctuation (Schradin, 2013). Through a GF lens, we might hypothesise that these different allelic variants provide performance buffering through possession of alternate intake targets. Via *et al.* (1995) proposed that genetic variants may take on more specific adaptive roles, theorising that one genotype provides the optimal phenotype in the mean ancestral environment, while a number of others specialise in delivery of adventitious plasticity (Via *et al.,* 1995). Nijhout (2003) reprised these ideas and postulated that within populations, species retain 'exploitational' genotypes, which, due to their exceptional sensitivity to novel environments, upon exposure to environment stress, yield relatively more potentially-adaptive plasticity than 'mean environment' genotypes.

*Drosophila melanogaster* populations exhibit genetic polymorphism of the *foraging* (*for*) gene, hypothesised to provide adaptation to variability to nutritional

environments (Sokolowski, 1985; Rodriguez *et al*., 1992; Sokolowski *et al*., 1997; Fitzpatrick *et al*., 2007; Kaun *et al*., 2007a; Kaun & Sokolowski, 2008). Two naturally-occurring allelic variants of *for* exist, $for^{R}$ 'rovers', and $for^{s}$ 'sitters' (de Bell & Sokolwoski, 1989; de Belle *et al*., 1989; Sokolowski, 2001). *for* encodes a cGMP-kinase, PKG (Osborne *et al*., 1997), with $for^{R}$ larvae having higher nervous system PKG (Osborne *et* al., 1997; Kaun *et al*., 2007a,b) and adult $for^{R}$ flies expressing higher brain PKG than sitters of either life stage (Osborne *et al*., 1997; Belay *et al*., 2007). Rovers and sitters also differ phenotypically as larvae and adults in their feeding and foraging behaviour (Sokolowski, 1980; Sokolowski, 2001; Burns *et al*., 2012), learning and memory (Mery *et al*., 2007; Kaun *et al*., 2007b), metabolite storage patterns (Kaun *et al*., 2008) and nutrient absorption efficiencies (Kaun, *et al*., 2007a). These differences all emerge as gene-by-environment interactions (GEI) in response to the food environment (Kent *et al*., 2009). PKG expression itself has been demonstrated to be responsive to nutrition and likely mediates *foraging* GEIs (Kaun *et al*., 2007a; Kent *et al*., 2009). When rover larvae are food deprived, PKG expression decreases and the change in PKG expression is correlated with an increase in food intake. Sitter larvae do not show a change in PKG expression under food deprivation and maintain consistent food intake levels (Kaun *et al*., 2007a).

Previously, these rover/sitter phenotype expression differences have been measured in response to a limited range of food environments; either the presence/absence of food, or high *vs*. low calorie formulations of an equivalent protein:carbohydrate (P:C) ratio food. In this study we empirically explore differences in phenotypic plasticity profiles due to *foraging* genotype in response to systematically-manipulated nutritional environments. Using a GF approach, we compare feeding behaviour and life-history phenotypes expressed by larval *D. melanogaster* that differ in their *for* allele across a comprehensive P-C nutrient space. In response to larval rearing nutrition, we measure survivorship to pupation, developmental rate, and total body trigylcerides (TAG). We also investigate how natural allelic variation in *foraging* influences the P:C targets selected by larvae, and how medium and long term disruption in self-regulation of macronutrient intake influences later P:C target self-selection and TAG accumulation.

This study represents a shift in focus from using the GF to ask how feeding behaviours and environments interact to promote maximal fitness between different

species and the sexes, to using the GF to examining the gradations of phenotype plasticity that radiate out around GF response surface performance optima *within species*. Conceptually, we are dividing species level phenotype GF response surfaces at the level of the gene, generating genotype-specific reaction norms (Schlichting & Pigliucci, 1998). Through doing so we ask, what role does natural genetic variation within a species play in shaping phenotype plasticity profiles and thus potentially contribute to performance maintenance across nutritional environments and generations?

## Materials and Methods

### *Fly stocks*

Three different *foraging* genetic strains were used in this study: natural rover (*for*$^{R}$), sitter (*for*$^{s}$) and a sitter mutant (*for*$^{s2}$). These strains share a common isogenic third chromosome from *for*$^{R}$ and a common X chromosome (de Belle & Sokolowski, 1987; de Belle *et al.,* 1989; Pereira & Sokolowski, 1993). The sitter mutant *for*$^{s2}$ strain was generated from a *for*$^{R}$ rover genetic background using gamma radiation (de Belle *et al*., 1993; Pereira & Sokolowski, 1993). *for*$^{s2}$ larval and adult flies display behaviour and PKG expression levels that do not differ from those of natural *for*$^{s}$ sitter larvae and adults (Osborne *et al*., 1997; Osborne *et al*., 2001; Belay *et al*., 2007; Fitzpatrick *et al*, 2007; Kaun *et al*., 2007a; Kaun *et al*., 2007b; Kaun *et al*., 2008). Given its rover genetic background, yet sitter phenotypes, *for*$^{s2}$ is typically used as a control for the involvement of *foraging*-dependent PKG in phenotype mediation. We now know that *for*$^{s}$ and *for*$^{s2}$ *foraging* sequence differs across a number of sites (A. Allen & M. B. Sokolowski, unpublished data). Notwithstanding this, we have included *for*$^{s2}$ in our experiments to facilitate cogent comparison between studies and to model the contribution naturally-arising mutant allelic variants might make to species-level phenotypic plasticity in nature.

Prior to experiments, all strains were maintained for hundreds of generations on a standard laboratory diet comprising 50 g dry yeast, 100 g sucrose, and 16 g agar per L of distilled water. The culture diet and subsequent experimental diets also contained 8 g of $KNaC_4H_4O_6$, 1 g of $KH_2PO_4$, 0.5 g of $CaCl_2$, 0.5 g of $MgCl_2$, 0.5 g of $Fe_2(SO_4)_3$ and 5 mL of propionic acid per L of distilled water. Culture rearing and

experiments were conducted at 25°C, under 12L:12D light cycle, with lights on at 08:00 h.

*No-choice experiments: experimental diets and rearing protocol*

These experiments were staggered over three rounds. For each round, larvae from each genotype were sourced from three replicate parental vials containing approximately 100 male and 100 female adult flies that were ≤ five days post-eclosion. Mated adults were then moved to three fresh vials closed with a Petri dish containing oviposition medium topped with ~ 0.5 g fresh yeast paste. Oviposition medium was 1.8 g agar, 50 mL distilled $H_2O$ and 45 mL dilute grape juice. The medium mix was boiled then allowed to cool and set. After 24 h the oviposition medium and yeast were replaced, and after another 12 h all hatchlings were removed from the Petri dish. After an additional 3 h, hatchlings were introduced to the experiment. This process ensured experimental larvae were all within 3 h of hatch.

Larvae were reared from egg hatch to pupation on one of 24 diets varying in protein to carbohydrate ratio (P:C) and total energy density, providing comprehensive sampling of protein and carbohydrate nutrient space. Diet P:C ratios (i.e. diet 'rails') were 1:1, 1:2, 1:4, 1:9, 1:16, 2:1, 1:16, 2:1, 4:1, and 7:1, and each ratio was formulated at three energy densities (i.e. three positions along each diet 'rail'); 75, 150 and 300 $g.kg^{-1}$ of P+C. Diet macronutrient sources were: thermolysed baker's yeast (containing 47.4% P, 24% digestible C, Lowan Whole Foods), sucrose, and casein (95.7% P, 0.8% C, Sigma-Aldrich). Agar (93.9% C) was used to gel diets (comprising < 2 $g.kg^{-1}$ of any experimental diet) and, following Lee *et al.* (2008), distilled water was used to dilute P:C mixes to the required energy density.

We poured 1 mL of each diet into a separate well in a sterile, 24-well tissue culture plate (Falcon, # 08-772-1). Once set, a flame-sterilised probe was used to score diet surfaces to facilitate feeding. A single larva was then introduced to each well. Larvae were enclosed within wells using 1.5 x 2.5 cm, snuggly-fitting, cylindrical foam caps. As *D. melanogaster* exhibit larval cannibalism, larvae were raised individually (Vijendravarma, *et al.,* 2013). Diets were maintained at ~100% relative humidity by placing each well plate into a 15 x 20 cm plastic container fitted with a perforated lid. A total of 32 plates were set up per genotype, resulting in 768 rover, sitter and mutant larvae entering the experiment. Development was checked

daily from day 3. All 2304 larvae from each of the three experimental rounds were monitored to score development time and survivorship. Surviving larvae from experiment rounds one and two were collected as pupae (≤ 24 h post-pupariation) for total body TAG (per unit total body protein) analysis. Survivors from round 3 were followed through to eclosion for measurement of adult traits (See Chapter 3, this thesis).

*Spectrophotometric analysis of total body triglycerides and protein*

The method of Kaun *et al*. (2008) was modified to prepare samples of TAG and protein for spectrophotometirc analysis, as follows; individual pupae were placed within sterile 1.5 mL centrifuge tubes and homogenised for 40 s over ice in 300 µL of 0.1% Tween-20 solution (a surfactant) using sterile plastic pestles and a hand-held motorised tissue grinder. Samples were then shaken down briefly and placed in a 70°C water bath for 5 min to denature endogenous enzymes. Samples were then chilled on ice for 2 min. After cooling, samples were spun down for 2 min at 10 000 *g*. Approximately 250 µL of the supernatant was removed and transferred to a fresh, sterile 1.5 mL centrifuge tube. Samples were vortexed for 20 s and then dispensed in 50 µL duplicates to each of two sterile 96-well spectrophotometric microplates (Thermo Scientific, EW-01930-13).

*Total body TAG measurement*

TAG concentrations were measured using the Infinity Triglycerides reagent (Sigma, T2449). Briefly, 200 µL of reagent was added to each 50 µL of sample, and incubated for 20 min. TAG concentrations were determined at 20 min by comparing absorbance at 540 nm against standard curves. Standard curves were formulated using with serial dilutions of a 200 $\mu g.mL^{-1}$ Sigma TAG analytical standard (Sigma 17811-1AMP).

*Total body protein measurement*

Protein levels were measured using the bicinchoninic acid (BCA) reaction method. 200 µL of Pierce BCA Protein Assay reagent (Thermo Scientific, 23227) was added to each 50 µL of sample and tubes were incubated for 30 min. Protein concentrations were measured against standard curves constructed from serial

dilutions of a 200 μg.mL$^{-1}$ bovine serum albumin analytical standard (provided in the BCA kit). Absorbance between samples and standards was compared at 562 nm.

*Phenotype response surface visualisation and statistical analysis*

Phenotype performance response surfaces were plotted against nutrient space 'rails' for survivorship to pupation, developmental rate (plotted as the inverse of number of days to pupation) and total body TAG. Traits were fitted against P-C using the fields package (ver. 6.8) in R (ver. 3.0.2). Fields allows for data visualisation by fitting 2D response surfaces as thin plate splines (TPSs). These TPSs interpolate response variable values for the regions of nutrient space which occur 'between' the rails and display points of equal *z* value as the same colour and connect regions of equivalent value with isobar contour lines. TPS surface smoothness is determined by varying λ, the TPS tuning parameter. For consistency of interpretation, when creating TPSs for each phenotype we maintained the same λ value of 0.05.

*Trait and phenotype response surface statistical analysis: generalised linear modeling*

Phenotype response surfaces were statistically analysed using a Lande & Arnold generalised linear modeling (GLM) approach (following Lee *et al*., 2008 and Dussutour *et al*., 2010). This approach allows for positive and negative influences of the linear terms, the linear terms' cross product, and *n*th order polynomial terms of a model to be statistically analysed. Terms were protein (*x*) and carbohydrate (*y*). Models were built using a Taylor series function to predict the shape of our trait response surfaces SPSS (ver. 21). We used a backwards entry model-building approach whereby all terms were forcibly entered into the first iteration of the model. To test for inter-genotype differences in response surface shape, we incorporated genotype as a factor. We calculated the probability of our terms significantly contributing to the shape of phenotype response surface using maximum likelihood. Non-significant terms are removed from successive iterations of the model in order of their non-significant contribute to fit. Model iterations ceased once an optimally-fitting model was built. If a model showed that response variable surface differed due to genotype, we built three comparative pairwise models to determine which genotypes differed. These were $for^{R}$ *vs*. $for^{s}$, $for^{R}$ *vs*. $for^{s2}$, and $for^{s}$ *vs*. $for^{s2}$. The

relative positive or negative contribution of each model terms to the surface was evaluated through the term's β coefficient and through $p$. We used α of 0.05. Here we report final, best-fitting versions of each model.

*Comparing developmental rate and survivorship of natural foraging strains in extreme nutritional environments*

Visualisation of response surfaces indicated that gene-by-environment differences in phenotype response due to natural *foraging* genotypes were most pronounced on the edges of nutrient space. Results from Kaun *et al.* (2007a) also indicated that natural rovers and sitters may differ in their developmental trajectories on 'extreme' diets. To investigate these patterns further, we decomposed our development rate and survivorship response surfaces and plotted survival curves comparing inter-genotype differences from the 1P:9C – 1P:16C and 4P:1C – 7P:1C diet rails, predicting that $for^R$ curves would be more similar to one another than $for^s$ curves.

*Choice experiments*

*Protein:carbohydrate dynamic target: binary choice experiments*

We forced larvae of each genotype to demonstrate regulation of P:C intake (to 'defend' their P:C targets, *sensu* Simpson & Raubenheimer, 1993) in a two-phase experiment. First, we reared individuals of each genotype from hatch to 60 h old on one of three pre-treatment diet compositions, before, in the second phase, allowing the larvae the opportunity to redress any imbalance experienced in the pretreatment period by offering simultaneous choices of complementary foods.

The rationale of the first phase was to assess whether larvae would differ with genetic strain in their capacity to redress macronutrient deficits (Simpson & Raubenheimer, 2012) and also to control the potential influence of habituation and/or neo-phobia on subsequent food choices. To this aim, one pre-experiment diet was carbohydrate biased, one protein biased and one provided equal parts protein to carbohydrate. All pre-treatment diets were of a 100 g P+C. $Kg^{-1}$ energy density, and were formulated to P:C ratios of 1:4, 1:1 and 4:1 using ingredients listed above.

The pre-treatment rearing was conducted as follows: nine 375 mL plastic vials were setup for each pre-treatment group. Nine 1P:4C vials, nine 1P:1C, and nine 4P:1C vials. Next we organised the vials into genotype groups such that there were three 1:4, three 1:1 and three 4:1 vials allocated to rovers, three of each to sitters, and three of each to mutant sitters. We then introduced 100 adult female and 100 adult male flies of the appropriate *foraging* genotype to their assigned vials. To age standardise experimental larvae, we fitted a Petri dish containing a 3 mm deep layer of non-nutritive agar, topped with approximately 2 g of pure yeast paste across the opening of each vial. We allowed parent flies to mate and females to oviposit onto the yeast paste for 24 h. After 24h, each Petri dish was then replaced with a new Petri dish containing a 3 mm deep layer of pre-treatment diet. As stated above, within each genotype's set of nine vials, three vials were fitted with Petri dishes containing the 1:4 pre-treatment diet, three were fitted with 1:1 dishes, and three the 4:1 diet dishes.

Adult flies were again left to mate and oviposit, this time into the pre-treatment diet. We standardised the age range of larvae entering the experiment by removing the adults from the vials after 24 h. We then cleared all 27 Petri dishes of hatchlings. Following another 3 h, we again cleared all hatchings. Larvae were then left to develop in the media until they were 60 h (± 3 h) post-hatch, at which age they were entered into the choice experiment.

The second phase of the experiment tested whether larvae could redress the previous nutritional perturbation and defend a P:C target. We achieved this through using four separate binary choice diet pairings. We designed four binary choice 'arenas', all of which contained a more protein-biased diet on the arena left-hand side and a carbohydrate-biased diet on the right-hand side (following Dussutour *et al.,* 2010). The arena pairings were (P:C) 1:1 *vs.* 1:4, 2:1 *vs.* 1:9, 4:1 *vs.* 1:16 and 7:1 *vs.* 1:2. Diets were formulated at a fixed energy density of 100 g P+C.kg$^{-1}$ using the ingredients listed above.

Next, 90 replicates of each diet pair were prepared in 25 mm diameter Petri dish 'arenas'. One mm depth of each diet was poured into either half of the dish. Intermixing of diet across the two halves of the dish was prevented by a dividing acetate strip (later removed). Ten third-stadium larvae from each genotype, and from each of the pre-treatment diets, were then allocated to diet pairs. This set-up resulted

in *n* of ten Petri dishes for each diet pair within each pre-treatment diet background, from each *for* strain (total *n* of 360 dishes and 3600 larvae). The ten larvae were selected haphazardly from any of the appropriate three pre-treatment diet vials and placed together in the centre of the dish. Their position (left or right half) was recorded after 4, 20 and 44 h.

At each time point, the position of an individual larva on either the left or right-hand side of the dish was counted. To generate a feeding preference index, we counted the position of a single larvae on either side as representing ten 'units' of the diet selected. For example, consider the ten larvae placed within the 2:1 *vs.* 1:9 diet Petri dish. If at a given time point four of these larvae were on the left-hand side, and six on the right-hand side, then we counted 40 units of the 2:1 diet had been selected, and 60 units of the 1:9 diet selected. To allow for visual representation of the larvae's dynamic P-C preferences, we represented these 'units' as grams (g) on subsequent figures. After counting, the total units of P and C selected for each dish were calculated as a P+C value of 100 'g' for that time point. Repeated measures MANOVA in SPSS (ver. 21) was then used to test for P and C selection differences due to genotype and pre-treatment diet and time.

To demonstrate that the P-C selection patterns demonstrated by larvae were non-random, we generated a null model group of ten larvae for each diet pair, from each pre-treatment diet group and each *foraging* genetic strain. The null model larvae were generated using the random function in Microsoft Excel (ver 14.4.1). Using Sigma Plot (ver 11.2) we created boxplots showing the left- or right-hand side position of the null model larvae *vs*. the position of the real larvae measured at the second experimental time point time (20 h). (Refer to Figure 8a, b and c for these results.)

*Protein:carbohydrate and energy density dynamic intake target: four-way choice experiments*

Our previous choice experiment identified that all larval P:C intake targets lay between the 1:1-1:2 P:C rails. However, as experimental diets were fixed at 100 g P+C.kg$^{-1}$ we could not determine if energy requirements differed with genotype. To complete our measures we tested diet selection by larvae across four diets of either 1:1 or 1:2 P:C at two complementary energy densities, 75 g P+C.kg$^{-1}$ and 400 g

P+C.kg$^{-1}$. Diets were prepared as described above, but with each occupying one quadrant of the Petri dish arena. Following the above breeding and age-staging procedures, three vials of larvae from each genotype (nine in total) were raised to 60 h old on a 1P:1.5C 100 g P+C.kg$^{-1}$ pre-treatment diet. As above, larvae were allocated in groups of ten, and had their quadrant location recorded after 4, 20 and 44 h. As above, the location of a single larva was taken to represent a 10 'g' unit of diet intake to allow for relative differences between genotypes to be analysed and visually represented. There were 36 dishes for each genotype and data were analysed by repeated measures MANOVA in SPSS (ver. 21).

To determine whether experimental larvae were actively regulating their P-C intake during our four-diet choice experiment, we compared the position of real larvae *vs.* null model larvae in the four-diet choice arenas using the above described method. (Refer to Figure 9 for these results.)

*Total body TAG levels achieved under self-regulation of protein-carbohydrate intake over the final larval stadium vs. the entire larval period*

We utilised the above experimental design to determine whether TAG levels differed with *for* genotype under self-regulation of macronutrient intake from the third stadium to pupae, and from hatchling to pupae. To examine TAG differences due to genotype under macronutrient intake self-regulation across the third larval stadium, we collected one pupa (≤ 24 h post-purariation) from each of ten of the above experimental Petri dishes (*n* = 10 per pupa per genotype). We also wanted to analyse whether TAG levels differed with *for* genotype when larvae were given the opportunity to self-select their diet across the entire larval period. We therefore set up an additional 30 Petri dishes containing the above combination of diets. We placed ten recently-hatched (aged to ≤ 3 h as above) larvae together into the centre of each dish, with ten replicate dishes per genotype. One pupa (≤ 24 h post-purariation) was collected from each dish. Total body TAG and protein levels were determined for all pupae using the above-described methods. TAG per unit body protein levels were statistically analysed using univariate ANOVA and Bonferroni-corrected multiple comparisons in SPSS (ver. 21).

## Results

*Survivorship to pupation*

Generalised linear modeling (GLM) of the percentage of larvae from each *foraging* genetic strain to reach pupation showed there was no statistical difference between the *for*$^R$, *for*$^s$ and *for*$^{s2}$ survivorship response surfaces (Wald $\chi^2 = 0.134$, d.f. = 2, $p = 0.935$, Figure 1). Thin plate splines representing each *foraging* strain's survivorship showed a common maximum of greater than 95% survived located over the mid-energy, 1P:1C region of nutrient space, with the *for*$^s$ maximum appearing comparatively narrower and more peaked (Figure 1b). In natural *for*$^R$ rovers (Figure 1a) and especially mutant *for*$^{s2}$ larvae (Figure 1c), the survivorship maxima extend across nutrient space toward higher-protein rearing diets. Despite this trend, the linear term of dietary protein only approached significance in its contribution to survivorship (Wald $\chi^2 = 3.686$, d.f. = 1, $p = 0.055$). For all *foraging* genotypes, the linear term of carbohydrate was associated with survivorship (Wald $\chi^2 = 7.358$, d.f. = 1, $p = 0.007$), though TPS surfaces indicate that above 150 g $C.kg^{-1}$ larval survivorship steadily declined. This influence of high carbohydrate causing lowered survivorship was supported by the GLM. The quadratic term of dietary carbohydrate was significantly associated with lower survivorship for all *foraging* genotypes (Wald $\chi^2 = 14.563$, d.f. = 1, $p < 0.001$, negative $\beta$ coefficient, Table 1).

*Developmental rate to pupation*

TPS response surfaces of larval development showed that 1P:1C-1P:2C diets promoted the fastest time to pupation for all *foraging* genotypes (Figure 2). This trend was reflected in GLM analysis, which found the linear term of protein was significantly associated with faster development for all larvae (Wald $\chi^2 = 36.731$, d.f. = 1, $p < 0.001$, positive $\beta$ coefficient, Table 2a). TPS surfaces also showed that developmental rate for larvae of all *foraging* genotypes declined with increase in dietary carbohydrate above 100 g $C.kg^{-1}$. Rearing diets that offered very high-P:C were also associated with marginally-slower larval development. These TPS trends were corroborated by GLM analysis of surface shape, which found both the quadratic terms of carbohydrate and protein were significantly associated with slower development for larvae of all *foraging* strains (*carbohydrate*$^2$, Wald $\chi^2 = 29.208$, d.f. =

1, $p < 0.001$, negative β coefficient, Table 2a; *protein²,* Wald $\chi^2 = 38.531$, d.f. = 1, $p < 0.001$, negative β coefficient, Table 2a). GLM also showed that developmental rate to pupation differed according to *foraging* genotype (Wald $\chi^2 = 15.884$, d.f. = 2, $p < 0.001$). Follow-up between genotype GLM found this was due to the mutant $for^{s2}$ sitter larvae developing marginally faster than natural *foraging* variants in the mid-energy 1P:2C and 1P:1C-2P:1C regions of nutrient space ($for^{R}$ vs. $for^{s2}$ *genotype*, Wald $\chi^2 = 5.662$, d.f. = 1, $p < 0.05$, Table 2c; $for^{s}$ vs. $for^{s2}$ *genotype*, Wald $\chi^2 = 14.459$, d.f. = 1, $p < 0.001$, Table 2d). Despite a TPS trend that showed $for^{s}$ larvae developed more slowly than $for^{R}$ in high carbohydrate regions of nutrient space, GLM showed no significant difference between the developmental rate surfaces of natural *foraging* allelic variants (Wald $\chi^2 = 3.017$, d.f. = 1, $p = 0.082$, Table 2b).

We further investigated differences in development rate to pupation between the natural *foraging* genotypes when reared on 'extreme' diets, by plotting curves representing the percentage of larvae that successfully reached pupation against the number of days it took larvae to reach pupation (Figure 3). Curves show that in response to rearing in either higher P:C, or C:P, nutritional environments, rover $for^{R}$ larval developmental trajectories appear more canalised than $for^{s}$ across diet treatments.

*Whole body triglycerides*

Generalised linear modelling showed that very high-carbohydrate diets were associated with high levels of larval triglyceride per unit body protein (TAG) for all *foraging* genotypes (*carbohydrate²*, Wald $\chi^2 = 130.727$, d.f. = 1, $p < 0.001$, positive β coefficient, Table 3a, Figure 4), while linear increase in dietary protein was associated with lower TAG for all larvae (*protein*, Wald $\chi^2 = 13.477$, d.f. = 1, $p < 0.05$, negative β coefficient, Table 3a). Inter-genotype GLM found that mutant sitter $for^{s2}$ larvae had the lowest TAG levels overall ($for^{R}$ vs. $for^{s2}$ *genotype*, Wald $\chi^2 = 55.561$, d.f. = 1, $p < 0.001$; $for^{s}$ vs. $for^{s2}$ *genotype*, Wald $\chi^2 = 48.282$, d.f. = 1, $p < 0.001$). TPS response surfaces show $for^{s2}$ larvae store much lower TAG in response to all of P-C rearing nutritional environments than the natural *foraging* genotypes, apart from the very highest-energy, high-C:P diets (Figure 4c). Natural *foraging* genotype larvae showed comparable TAG levels across rearing diet nutrient space up to the 1P:2C food rail. Increase in dietary C concentration relative to P beyond this nutritional rail resulted in

*for*$^R$ larvae storing significantly more TAG than their *for*$^s$ counterparts (*for*$^R$ vs. *for*$^s$ *genotype*, Wald $\chi^2 = 3.868$, d.f. = 1, $p < 0.05$, cf. Figures 4a & b, Table 3e).

*Protein:carbohydrate dynamic intake targets for larvae from each foraging genotype in response to pre-rearing on either a 1P:4C, 1P:1C or 4P:1C diet*

The between-subjects multivariate component of our repeated measures MANOVA showed that pre-rearing larvae of each *foraging* genotype for 60 h on either a 1P:4C, 1P:1C, or 4P:1C 'pre-treatment' diet did not influence their P-C selections (*pre-treatment*, Wilks' $\lambda = 0.974$, $F_{(4, 646.0)} = 2.119$, $p = 0.077$, Table 4). Within-subjects multivariate tests also showed that pre-treatment feeding did not influence the P-C selections made by larvae over the 44 h duration of the choice experiment either (*pre-treatment∗time*, Wilks' $\lambda = 0.971$, $F_{(8, 642.0)} = 1.169$, $p = 0.315$, Table 4, Figure 5).

The between-subjects component of our repeated measures MANOVA analysis also showed that, across the entire experiment, P:C selection significantly differed due to *foraging* genotype (*genotype*, Wilks' $\lambda = 0.964$, $F_{(4, 646.0)} = 3.025$, $p < 0.05$, Table 4). *Post-hoc* univariate multiple comparisons showed *for*$^R$ rover larvae selected significantly more C- and less P-biased food than *for*$^s$ larvae (*protein for*$^R$ vs. *for*$^s$, $p = 0.014$; *carbohydrate for*$^R$ vs. *for*$^s$, $p = 0.014$, Table 4) but not than *for*$^{s2}$ larvae (*protein for*$^R$ vs. *for*$^{s2}$, $p = 0.119$; *carbohydrate for*$^R$ *vs. for*$^{s2}$, $p = 0.118$, Table 4). Sitter larvae, *for*$^s$ and *for*$^{s2}$, did not significantly differ in their P or C selection across the entire experiment (*protein for*$^s$ *vs. for*$^{s2}$, $p = 1.0$; *carbohydrate for*$^s$ *vs. for*$^{s2}$, $p = 1.0$, Table 4). The *for*$^R$ tendency for selection of higher C food is visible in Figure 5, panels a-c. Rover P:C intake 'trajectories' (blue lines and circles) are consistently located in marginally-higher C to P regions of nutrient space than the sitter larval trajectories.

To further characterise P-C selection differences expressed between *foraging* strains within each pre-treatment group, we conducted individual repeated measures MANOVAs on each group. For the 1P:4C pre-treatment group, repeated measures MANOVA multivariate tests showed that when P and C intake are considered together, *foraging* genetic strains do not differ in their diet selection (*genotype* Wilks' $\lambda = 0.942$, $F_{(2, 196.0)} = 1.494$, $p = 0.205$, Table 4a). Univariate tests showed a

significant interaction between *foraging* strain and P:C intake over time (*protein*: *time∗genotype,* $F_{(3.974, 196.735)} = 3.920$, $p < 0.05$, *carbohydrate*: *time∗genotype,* $F_{(3.978, 196.908)} = 3.438$, $p < 0.05$, Table 4a), however, post-hoc multiple comparisons failed to confirm this, showing no statistically significant differences between P or C intake by larvae of each strain across the experiment (*protein* $for^R$ *vs.* $for^s$, $p = 0.416$; *carbohydrate* $for^R$ *vs.* $for^s$, $p = 0.416$; *protein* $for^R$ *vs.* $for^{s2}$, $p = 1.0$; *carbohydrate* $for^R$ *vs.* $for^{s2}$, $p = 1.0$; *protein* $for^s$ *vs.* $for^{s2}$, $p = 0.820$; *carbohydrate* $for^s$ *vs.* $for^{s2}$, $p = 0.824$, Table 4a, Figure 5a).

Multivariate tests from the 1P:1C pre-treatment group repeated-measures MANOVA showed significant difference in diet selection between larvae of each *foraging* strain over the experiment (*genotype* Wilks' $\lambda = 0.870$, $F_{(4, 220.0)} = 3.972$, $p < 0.001$, Table 4b). Univariate tests showed that both protein and carbohydrate selection differed over time due to genotype (*protein*: *time∗genotype,* $F_{(3.653, 202.765)} = 3.755$, $p < 0.05$, *carbohydrate*: *time∗genotype,* $F_{(3.653, 202.765)} = 3.755$, $p < 0.05$, Table 4b). *Post-hoc* comparisons showed differences in $for^R$ *vs.* $for^s$, and $for^s$ *vs.* $for^{s2}$ P and C selection drove these significant patterns (*protein* $for^R$ *vs.* $for^s$, $p < 0.05$; *carbohydrate* $for^R$ *vs.* $for^s$, $p < 0.05$; *protein* $for^s$ *vs.* $for^{s2}$, $p < 0.05$; *carbohydrate* $for^s$ *vs.* $for^{s2}$, $p < 0.05$, Table 4b) with mutant $for^{s2}$ sitter larvae resembling $for^R$ rovers in their P and C selection over time (*protein* $for^R$ *vs.* $for^{s2}$, $p = 1.0$; *carbohydrate* $for^R$ *vs.* $for^{s2}$, $p = 1.0$, Table 4b, Figure 5b).

Repeated measures MANOVA on the P and C selection across time by larvae pre-reared on the 4P:1C diet showed *foraging* genotypes only approached significantly differing from one another (*genotype* Wilks' $\lambda = 0.928$, $F_{(4, 226.0)} = 2.149$, $p = 0.076$, Table 4c). *Post-hoc* multiple comparisons showed these near-significant differences were due to the non-significant trend apparent on Figure 5c, which shows $for^{s2}$ larvae selecting higher P:C food over time than natural *foraging* larvae (*protein* $for^{s2}$ *vs.* $for^R$, $p = 0.093$; *carbohydrate* $for^{s2}$ *vs.* $for^R$, $p = 0.092$, *protein* $for^{s2}$ *vs.* $for^s$, $p = 0.057$; *carbohydrate* $for^{s2}$ *vs.* $for^s$, $p = 0.057$; *protein* $for^R$ *vs.* $for^s$, $p = 0.1$; *carbohydrate* $for^R$ *vs.* $for^s$, $p = 0.1$ Table 4c).

*Protein:carbohydrate dynamic intake targets when larvae can regulate both dietary macronutrient ratio and energy density*

The multivariate tests component of the repeated measures MANOVA showed that larval selection of dietary P:C ratio and concentration from within the 1P:2C-1P:1C region of nutrient space did not differ due to *foraging* genotype (*genotype*, Wilks' $\lambda = 0.854$, $F_{(4, 82)} = 1.689$, $p = 0.160$, Table 5a, Figure 6). Univariate tests showed that when P and C intake by larvae of all *foraging* genetic strains are analysed simultaneously, both P and C intake levels increased between each time point over the duration of the experiment, *i.e.* from one time point to the next, all larvae selected higher-calorie food. This pattern is evident on Figure 6. Within each genotype's intake 'trajectory', the distance in nutrient space between larvae's P:C selections increased over time (*protein*: *time*, $F_{(1.897, 79.658)} = 3.158$, $p = 0.05$; *carbohydrate*: *time*, $F_{(1.980,83.171)} = 2.385$, $p < 0.01$, Table 5b). Figure 6 also shows a non-statistically significant trend, first identified in the previous set of intake experiments, whereby natural *for*$^{R}$ larvae (blue circles) chose marginally-higher C diets than sitters.

*Rover vs. sitter differences in P:C intake target*

As an alternative means to investigate inter-genotypic differences in P:C selection by larvae of each *foraging* strain across all our choice experiments, we tabulated the mean P:C intake target displayed by *for*$^{R}$, *for*$^{s}$ and *for*$^{s2}$ in each experiment. Rover P:C targets are more alike both across and within experiments than the *for*$^{s}$ and *for*$^{s2}$ larvae P:C targets (Table 7). Rover larvae consistently chose a P:C target of 1:1.2 across the first of our three choice experiments and a P:C of 1:1.16 in choice experiments wherein larvae could regulate caloric and P:C intake simultaneously. Contrastingly across the four choice experiments, *for*$^{s}$ larvae selected mean P:C targets of 1:1.13, 1:0.9, 1:1.22 and 1:1.5. Similarly, *for*$^{s2}$ selections were inconsistent, with average P:C target selections of 1:1.32, 1:1.23, 1:1.1 and 1:1.46 (Table 7).

*Body triglyceride levels attained by larvae self-selecting their dietary macronutrient ratio and energy density for the entire larval period vs. third larval stadia only*

Univariate ANOVA showed there was no main effect of *foraging* genotype on larval TAG levels (*genotype*, $F_{(2, 54)} = 2.230$, $p = 0.117$, Table 6a); however, there was

a significant genotype-by-feeding regime interaction (*genotype*∗*feeding regime*, $F_{(3, 54)} = 3.818$, $p < 0.05$, Table 6a). *Post-hoc* within-*foraging* genotype comparisons showed the $for^R$ allelic variant drove this significant interaction. Rover larvae allowed to self-regulate their P:C intake for the entire larval period accumulated significantly less TAG (~2.5 $mg.mL^{-1}$/protein $mg.mL^{-1}$ less) than those permitted to self-regulate P:C intake in the 3rd larval stadia only ($for^R$ 3rd to pupa *vs.* $for^R$ entire, $F_{(1, 19)} = 9.031$, $p < 0.01$, Figure 7). Within sitter genotype *post-hoc* tests showed natural $for^s$ and mutant $for^{s2}$ sitters did not differ significantly in the TAG levels they accumulated when allowed to self-regulate their P:C intake from hatch to pupation *vs.* from only the 3rd stadia ($for^s$ 3rd to pup. *vs.* $for^s$ entire, $F_{(1,19)} = 0.383$ , $p = 0.544$ ; $for^{s2}$ 3rd to pup. *vs.* $for^{s2}$ entire, $F_{(1, 19)} = 2778$ , $p = 0.113$, Table 6b, Figure 7).

## Discussion

This study shows that allelic variants of the *foraging* gene do differ, albeit subtly, in their phenotype responses to variable nutritional environments. The mode of phenotypic plasticity yielded by each *foraging* genetic strain results in alternate life history outcomes, which in nature could influence microevolution. Overall, $for^R$ rovers show greater canalisation of their nutrient selection behaviour and developmental trajectories, and more plasticity in their triglyceride (TAG) storage patterns, than $for^s$ and $for^{s2}$ sitters. Both natural and mutant sitter larvae showed greater canalisation of TAG levels stored but greater plasticity in their nutrient intake behaviour and developmental timing. Perhaps unsurprisingly, given our experimental subjects were of the same species and life stage, we identified gross similarity in the diet-dependent survivorship and developmental timing of larvae from each *foraging* genetic strain across 'mean' nutritional environments.

Contrary to patterns displayed in caterpillars, juvenile locusts (Simpson *et al.*, 1988) and adult *D. melanogaster* (Ribeiro & Dickson, 2010; Vargas *et al.*, 2010), rover and sitter *D. melanogaster* larvae showed no evidence of behaviourally redressing putative nutritional imbalances incurred through prior rearing on either P- or C-biased diets. Instead, our choice experiments indicated that rover larvae maintain a consistent P:C intake target of ~1P:1.2C regardless of the quality of nutritional environments they have previously experienced. This finding corresponds with previous work by Kaun *et al.* (2007a), which detailed variation between larval rovers

and sitters in their total food intake behaviour. These authors showed that when rover larvae are exposed to nutrient environments very low in available energy, they respond by increasing their total food intake. This increase in food intake is concomitant with a decrease in circulating PKG (Kaun *et al.*, 2007a), the product of *foraging* gene expression (Obsourne *et al*, 1997). Sitters, contrastingly, do not show a change in PKG expression under these circumstances and maintain consistent food intake regardless of food caloric content (Kaun *et al.*, 2007a). Similar behavioural adjustments by rovers, mediated by *foraging* expression, in response to food quality likely facilitate their adherence to the 1P:1.2C target identified here.

The propensity for rover larvae to maintain their macronutrient target may actually drive plasticity in the other traits that displayed measurable rover *vs.* sitter differences: development trajectories and TAG levels. The survival curves of natural $for^{R}$ *vs.* $for^{s}$ larvae confined to rearing diets within the P and C 'extremes' of nutrient space show rover trajectories are less variable across rearing diets than sitter trajectories. These more-canalised development rates could be due to rover larvae maintaining their P:C intake and absorption across development, thus maintaining a consistent supply of nutrients to growing tissues. Rover larvae absorb more glucose from their food, per unit intake, than sitter larvae (Kaun *et al.*, 2007). When restricted to feeding on a P-biased diet, this ability would allow rover larvae to more closely maintain their C target. If the larval rover capacity to increase their total food intake in response to low calorie food extends to low-P:C food, then simply increasing intake of a high-C:P food would allow rover larvae to maintain their P target. Indeed, this observation by Kaun *et al*. (2007), whereby rovers respond to decrease in food energy density by increasing their intake, could in part be driven by rovers regulating their P intake. In making this observation, Kaun *et al*. (2007) exposed larvae to foods of decreasing total energy concentration, but of the equivalent ~1:3 P:C ratio. Below we discuss an experimental approach that can resolve this potential confounding, and resolve whether rover *vs.* sitter differences in P:C and total energy intake regulation is important in the regulation of the plasticity of other phenotypes.

Regulation of P-C intake and absorption by $for^{R}$ rover larvae may not only maintain their developmental trajectories across variable food environments: $for^{R}$ regulation of dietary P and C likely also influences the levels of TAG they accumulate. We have shown that when rover larvae are confined to very high-C:P

diets, they develop marginally higher levels of whole body TAG than *for*$^{s}$ or *for*$^{s2}$ sitters. Our experiment in which larvae were allowed to self-regulate their P-C intake across the entire larval period, versus the third stadium only, demonstrates that *for*$^{R}$ larvae also accumulate more TAG than either sitter *foraging* variant when self regulation of P-C intake is restricted to the third larval stadium. This higher *for*$^{R}$ propensity for 'fatness' when restricted to non-target foods during the larval period (even foods very close to their 1P:1.2C target) could be driven by 'macronutrient leverage' (Simpson & Raubenheimer, 2012). As mentioned above, to maintain their P target when restricted to feeding on low P:C foods, larval rovers likely increase their total food intake. This behaviour, common in other juvenile insects (Simpson & Raubenheimer, 2000; Lee *et al.*, 2004; Raubenheimer *et al.*, 2005), results in consumption of dietary carbohydrate beyond requirement and the storage of excess energy consumed as lipids (Lee *et al.*, 2004). Possibly maintenance of C, or an as-yet uncharacterised essential micronutrient, may account for higher levels of *for*$^{R}$ rover TAG between the population of rovers allowed to self regulate their diet for the entire larval period *vs.* only the third larval stadium.

Larvae of the natural *for*$^{s}$ sitter genotype show opposite plasticity patterns to those of *for*$^{R}$ rovers. While sitter larvae did regulate their P:C intake targets to typically fall between the 1P:1C and 1P:2C diet rails, neither the *for*$^{s}$ and *for*$^{s2}$ allelic variants displayed rover-like consistency in the level of P and C selected across choice experiments. Sitter larvae demonstrated less canalisation of their food selection behaviour than rovers. Again, these differences we have observed correspond with the feeding behaviour differences observed by Kaun *et al.* (2007). Unlike rover larvae, sitter larvae do not adjust their food intake behaviour in response to a drop in food total calorie content. Regardless of food quality, sitter larvae maintain equivalent food intake levels (Kaun *et al.*, 2007). Sitters also 'sit'. Prior experiments on both larval and adult sitter flies demonstrate they move less frequently between alternate patches of food than *for*$^{R}$ rovers, are less likely to leave a food source once it is encountered, and show lower rates of 'food exploration' behaviour than rover flies (Sokolowski, 1980; de Bell & Sokolwoski, 1989; de Belle *et al.*, 1989; Sokolowski, 2001; Belay *et al.*, 2007; Kent *et al.*, 2009; Burns *et al.*, 2012), which provides evidence that with respect to their food intake, sitters are less sensitive and responsive to food composition. Indeed, Kent *et al.* (2009) working with adult *foraging* gene allelic

variants have demonstrated that individuals of the *for*$^{s2}$ allele show lower transcriptional responsiveness to nine alternate foods than adult *for*$^{R}$ flies. Using Affymetrix chips, they compared the *for*$^{s2}$ *vs. for*$^{R}$ expression levels of >1000 genes important in *D. melanogaster* metabolism from flies maintained on each of these nine foods. Of all the genes that significantly changed in expression across the experiment (*i.e.* those that were significantly up- or down-regulated in both rover and sitter flies), in 77% of cases the significant change occurred in individuals of the *for*$^{R}$ allelic variant (Kent *et al*., 2009).

Inverse to the pattern displayed by rovers, the less 'exacting' P:C intake regulation of sitter larvae appears to result in their expression of greater plasticity than rovers in developmental trajectories when reared on P- and C-biased foods. The lower TAG levels measured for sitter *vs.* rover larvae when restricted to imbalanced diets provides indirect evidence that *for*$^{s}$ sitter larvae do not regulate their macronutrient intake as tightly as rovers. Instead of accumulating lipids, due to the ingesting of excesses of one macronutrient when regulating the intake of the other to maintain development, sitter larvae develop more slowly. As a result, sitters express more canalised TAG levels across varying larval food environments and more plastic developmental trajectories.

These rover *vs.* sitter P:C intakes, and resultant phenotypic plasticity differences, are reminiscent of intra-specific macronutrient regulation and phenotype patterns displayed by nymphs of the desert locusts *Schistocerca gregaria* and caterpillars of the noctuid moth *Spodoptera exempta*. Desert locusts display a form of behavioural phenotypic plasticity, phase polyphenism, whose expression is entrained by individual experience of local population density fluctuations (Simpson & Sword, 2009). Under situations of high local population density, nymphs express a 'gregarious' behavioural phase, characterised by intraspecific attraction and the undertaking of long-distance migrations *en masse*. Conversely, individuals of the 'solitarious' phenotypic phase, expressed by nymphs from low-density populations, display behavioural repulsion to conspecifics and move only over short distances (Simpson & Sword, 2009). Simpson *et al.* (2002) used the GF to compare the P:C intake of nymphs of the gregarious *vs.* solitarious phase. These authors found locust of both phases shared a common P:C intake target but differed in their P:C intake regulation behaviour when confined to imbalanced P:C foods, in accordance with

their behavioural ecology. The gregarious form's marked propensity for dispersal results in their exposure to, and consumption of, a wide variety of food plants. Across a solitarious nymph's development, due to their tendency for localised movement only, they encounter a comparatively-limited range of food plants. Similar to what we hypothesise occurs in rovers, Simpson *et al*. (2002) found that when gregarious phase desert locust nymphs are confined to imbalanced P:C foods, they eat sufficient quantities to maintain their P:C target, with a consequence of excess consumption of the more abundant, non-target macronutrient. Contrastingly, solitarious nymphs restrict their intake of imbalanced P:C foods, thus reducing the amount of excess relative to target macronutrient consumption. Perhaps a similar tendency to reduce 'over' consumption of P or C above target levels accounts for observations we made from sitters in the present study. Associated with these patterns of intake were differences in lipid storage, with gregarious locusts having higher body lipid levels, and perhaps consequently suffering reduced survivorship, on high-carbohydrate, low-protein diets than did solitarious locusts (Simpson *et al*., 2002).

Lee *et al*. (2004) described remarkably similar P-C intake regulation patterns to those displayed by desert locusts in 'solitarious' *vs.* 'gregarious' in *S. exempta* caterpillars. As in desert locusts, these lepidopterans display population density-dependent phase polyphenism whereby gregarious individuals aggregate and migrate, while solitarious caterpillars forage locally and do not aggregate. Lee *et al*. (2004) used the GF to test the macronutrient intake behaviour and total body lipid levels of *S. exempta* caterpillars raised in crowded *vs.* isolated laboratory conditions; a treatment sufficient to induce the expression of either behavioural phase in this species. As in locusts, gregarious phase individuals maintained their P-C target across imbalanced diets. Those raised as 'solitaries', as in desert locusts, ate quantities of each imbalanced P-C such that their target intake for the more-abundant nutrient in the diet was met but not exceeded to a marked degree, thereby leaving a substantial shortfall in the deficient nutrient relative to its target. Lee *et al*. (2004) demonstrated the consequence of these differences in P:C intake was phase-specific variation in total body lipid deposition. Due to higher total intake of calories by gregarious caterpillars on imbalanced diets, they amassed greater lipid stores across development as compared to solitarious caterpillars (Lee *et al*., 2004).

The manner in which gregarious phases of locusts and caterpillars encounter their nutritional environments resembles that of rover larvae in nature. Like these migratory insects, a rover larva feeding on an imbalanced food would be more likely than a sitter to encounter a complementary P-C food at a later time point, due to their genetically-determined 'roving' between patch foraging (Sokolowski, 1980; Sokolowski, 2001). Raubenheimer and Simpson (2003) hypothesise the reason gregarious phase locusts and caterpillars (in our case rovers) 'tolerate' the putative costs of consuming excess of a more-abundant macronutrient when restricted to feeding on imbalanced food, is due to the higher likelihood migratory individuals (or rovers) have of encountering a complementary P:C food – a 'nutritional antidote' (Simpson & Raubenheimer, 2012) – in the near future. Simpson and Raubenheimer refer to this nutrient intake strategy as 'nutrient generalist' foraging, and the pattern of macronutrient intake behaviour displayed by non-migratory, solitarious phase insects as a 'nutrient specialist' strategy (Simpson *et al*., 2002; Raubenheimer & Simpson, 2003; Simpson & Raubenheimer, 2012). Therefore, in our experiments wherein rover and sitter larvae were restricted to fixed, imbalanced rearing diets, the rover's putative 'generalist' food intake strategy may have caused $for^R$ larvae to maintain their P-C target while concurrently ingesting quantities of the more-abundant macronutrient in excess of the target – under the evolved 'expectation' (due to roving) that a complementary food source would be soon encountered. Meanwhile, 'costs' of the excess macronutrient ingested accumulated. As in the nutrient-generalist gregarious *vs.* nutrient-specialist solitarious *S. exempta* caterpillars of Lee *et al*. (2004), our $for^R$ larvae accumulated more lipid reserves when maintained on non-target P:C foods than our putative nutrient-specialist sitters. While the performance consequences of these subtle rover *vs.* sitter TAG accumulation differences are not known (we explore this further below) perhaps avoidance of sub-optimal TAG levels has led to, or re-enforced, the expression of sitter type P-C intake behaviour (or that of nutrient specialists more generally).

To verify that macronutrient intake regulation differences drive the $for^R$ rover *vs.* $for^s$ sitter phenotypic plasticity expression patterns we have observed, more precise characterisation of feeding behaviour of larvae from each *foraging* genetic strain is required. To achieve this, we need to accurately quantify intake of larvae, ideally for their entire larval period, across P-C nutrient space. Following the

approach of Simpson *et al*. (2002), larvae would be maintained from hatch to pupation on P- through to C-biased diets of a fixed energy density. Phenotypes we measured in the present study would then be re-recorded and mapped across nutrient space in response to P:C *intake* as opposed to rearing-diet composition. Such a study could also be used to test our hypothesis that rover larvae behave as nutritional generalist and sitters, specialists. Using the experimental design of Simpson *et al*. (2002) would allow for the characterisation of rover and sitter larvae's 'intake arrays' relative to their P-C intake target. Typically, animals following generalist feeding strategies display linear intake arrays resulting from their tendency, when restricted to feeding along an imbalanced P:C food rail, to consume excesses amounts of the non-target nutrient in order to approach their intake target of the more-limiting nutrient. 'Specialist' animals display arc-shaped intake arrays. This is due to the specialist's tendency, when feeding on imbalanced-P:C foods, to consume sufficient quantities of the more-abundant nutrient to the target, but to minimise its intake beyond the target (Raubenheimer & Simpson, 1999). We predict that across P-C nutrient space, *for*$^{R}$ larvae will produce a linear intake array, and *for*$^{s}$ and *for*$^{s2}$, arced.

We have identified that natural allelic variants of the *foraging* gene differ sufficiently in the responses to variable larval nutritional environments as to affect their life histories. While we do need to confirm its relationship with macronutrient intake, *for*$^{R}$ rover larvae are more prone to accumulating TAG than natural *for*$^{s}$, and especially mutant *for*$^{s2}$, sitter larvae. Kaun *et al*. (2008) correctly predicted that rovers may store more TAG than sitters, which these authors postulated rovers utilise to sustain 'roving'. Kaun *et al*.(2008) also demonstrated that rovers are able to mobilise TAG more quickly during starvation than either sitter *foraging* genetic strains. Together these results suggest that for rovers at least, rather being a costly 'side effect' of maintaining their P intake when confined to high-C:P foods, TAG accumulation as larvae could support larval and adult *for*$^{R}$ flies as 'roving' fuel, and possibly guard against starvation resistance if their movement resulted in encounter with a poor-quality nutritional environment (Simmons & Bradley, 1997; Aguila *et al*., 2007; Ballard *et al*., 2008). Supporting this idea are findings from Behmer *et al*. (2003). These authors compared lipid levels of *Locusta migratoria* nymphs maintained over four days in either large arenas with distantly-spaced complementary P:C diet dishes, or smaller arenas, which required locusts to move less when feeding

between dishes. Paradoxically, locusts that moved more accumulated more lipids, leading the authors to conclude that the lipid accumulation was due to a predictive 'feedforward' behaviour-physiology response (Behmer *et al*., 2003) the expression of which may have evolved to facilitate future, long-distance, between-food site movements. The subtle *for*$^{R}$ vs *for*$^{s}$ differences in developmental rate to pupation we noted here were maintained by the cohort of round three flies we followed through to eclosion in an accompanying study. (See Chapter Three for a discussion of the life history implications of rover *vs.* sitter differences in developmental rate.)

This study suggests that within a single species, alternate, genotype-specific maintenance of macronutrient intake can generate plasticity in a range of separate phenotypes. This result poses some very interesting questions. Across *D. melanogaster* evolution in variable nutritional environments, which 'trait' conferred by the *foraging* gene, has been selected for; the rover *vs.* sitter P-C intake behaviour differences, or the rover *vs.* sitter differences in developmental plasticity that these P-C intake behaviours yield? Or both? Resolving this may not be possible; however, laboratory selection experiments could potentially reveal differences in the adaptive capacity of *for*$^{R}$- *vs. for*$^{s}$-mediated plasticity. As one example, separate homozygous populations of each *foraging* allelic variant could be established, and then exposed to a variety of defined, variable nutritional environments over many generations. Some experiments would see rover *vs.* sitter populations exposed to nutritional environments that vary over long time frames (many generations) and others short (within generations, or change every second or third generation). At different evolutionary intervals, for example, every four generations, rover *vs.* sitter performance could be evaluated. Another approach could be to expose populations comprising different ratios of *for*$^{R}$:*for*$^{s}$ individuals and, across variable environments similar to those described above, monitor which genotype increases or decreases in incidence over time (following a similar approach to that of Sokolowski *et al*., 1997).

A final, related question arising from this study is: has the (putative) nutrient generalist macronutrient intake strategy of rovers evolved as a consequence of 'roving' itself, or has roving behaviour evolved to support a generalist foraging strategy, necessitated by fluctuating food environments? While logic suggests the influence of the environment 'comes first' in a sequence of evolutionary events, Clark *et al*. (2012) have begun to quantitatively investigate these questions using the

‘nutrient specialist’ locust species, *Locusta migratoria* (Raubenheimer & Simpson, 2003) as a model. Clark *et al*. systematically trained *L. migratoria* nymphs to ‘generalist’ and ‘specialist’ feeding regimes. In the generalist regime, nymphs were exposed sequentially to highly P-biased, and then highly C-biased foods. In the specialist training, locusts were exposed to a sequence of foods with a P-C composition close to the *Locusta* P:C intake target. They subsequently compared the P-C intake regulation behaviour of trained locusts and demonstrated that individuals from the ‘generalist’ environment displayed linear intake arrays similar to those of true generalist insects (Simpson *et al*., 2002; Lee *et al*, 2004), while those maintained as specialists retained an arc-shaped intake array, typical of *Locusta* and other specialists (Raubenheimer & Simpson, 2003). The work of Clark *et al*. certainly suggests that variability in the nutritional environment is what initiates and establishes behavioural responses. It would be very interesting to monitor *Locusta* trained in this fashion for any substantial changes in behaviour toward that characteristic of a ‘gregarious’ phase migratory locust. Other evidence we have suggestive that roving evolved as a direct response to the nutritional environment, rather than somehow spontaneously is that *for*$^{s}$ larvae begin to ‘rove’, indistinguishably from *for*$^{R}$ larvae, in the total absence of food (Sokolowski, 1980).

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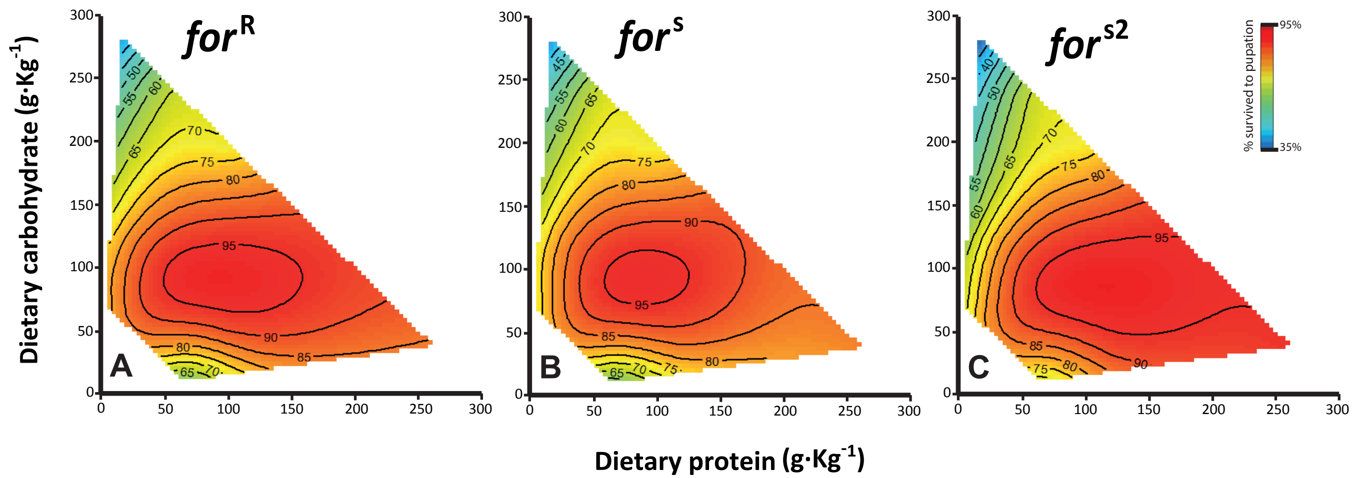



Figure 1. A) – C) Thin plate spline (TPS) response surfaces showing the percentage of larvae from each *foraging* genetic strain to survive to pupation. Surfaces grade across nutrient space from red areas denoting high survivorship to regions of pale blue showing lower survivorship. Generalised linear modeling (GLM) of surface shape indicated there were no differences in larval survivorship due to *foraging* genotype (Wald $\chi^2$ = 0.134, d.f. = 2, $p$ = 0.935). The linear term of dietary carbohydrate (C) was associated with higher survivorship for each genetic strain (Wald $\chi^2$ = 7.358, d.f. = 1, $p$ = 0.007). TPS surfaces for each *foraging* genotype indicated that linear increase in dietary protein (P) also contributed to higher survivorship, however GLM showed this relationship approached significance only (Wald $\chi^2$ = 3.686, d.f. = 1, $p$ = 0.055). Very low and very high levels of dietary C were associated with lower survivorship for all allelic variants, with GLM showing a significant relationship between the quadratic term of dietary C and lower survivorship (Wald $\chi^2$ = 14.563, d.f. = 1, $p$ < 0.001). Larvae from each *foraging* allelic variant survived best on mid-energy rearing diets between the 1P:1C and 2P:1C food rails.

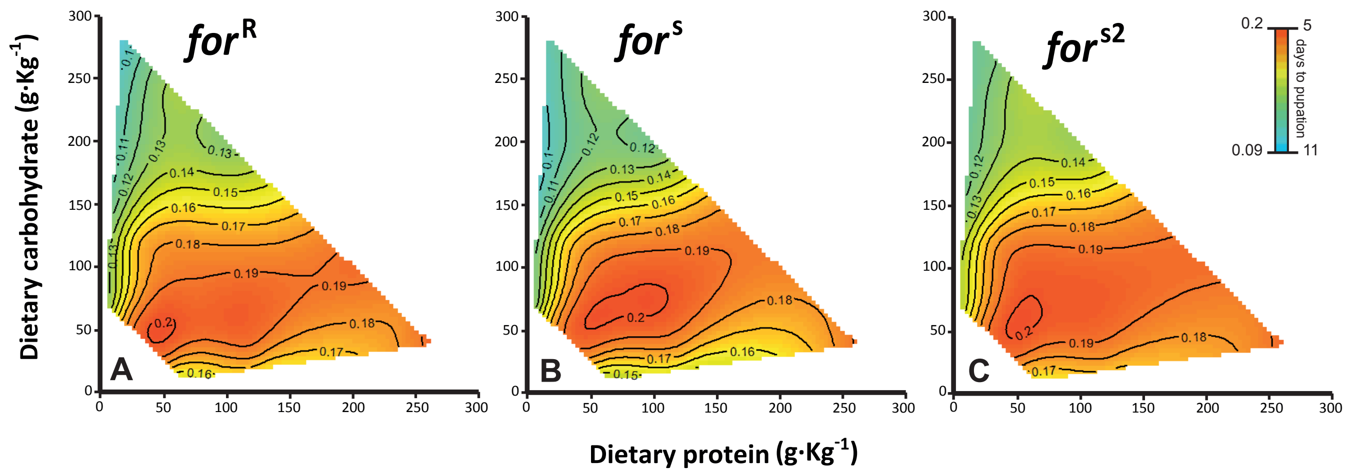



Figure 2. A) – C) Thin plate spline (TPS) response surfaces showing diet-dependent development rate of larvae from each *foraging* genetic strain. The most rapid development time to pupation is indicated by deep orange, while the slowest development is represented by pale blue. Generalised linear modeling (GLM) showed that *foraging* genotype was significantly associated with differences in surface shape (Wald $\chi^2 = 15.884$, d.f. = 2, $p < 0.001$). Subsequent genotype-by-genotype GLM indicated mutant *for*$^{s2}$ larvae developed faster than the two natural *foraging* genotypes (*for*$^{R}$ vs. *for*$^{s2}$ *genotype*, Wald $\chi^2 = 5.662$, d.f. = 1, $p < 0.05$; *for*$^{s}$ vs. *for*$^{s2}$ *genotype*, Wald $\chi^2 = 14.459$, d.f. = 1, $p < 0.001$), which did not significantly differ from one another (*for*$^{R}$ vs. *for*$^{s}$ *genotype*, Wald $\chi^2 = 3.017$, d.f. = 1, $p = 0.082$). The *for*$^{s2}$ TPS (panel C) shows the mutant larvae reach pupation earlier than the natural *foraging* larvae in both the very high-protein (P) and carbohydrate (C) regions of nutrient space. GLM showed that the linear term of P was significantly associated with faster development for all *foraging* genotypes (Wald $\chi^2 = 36.731$, d.f. = 1, $p < 0.001$). Both quadratic terms of P and C were also significantly associated with development time (*protein*$^2$, Wald $\chi^2 = 38.531$, d.f. = 1, $p < 0.001$; *carbohydrate*$^2$, Wald $\chi^2 = 29.208$, d.f. = 1, $p < 0.001$).

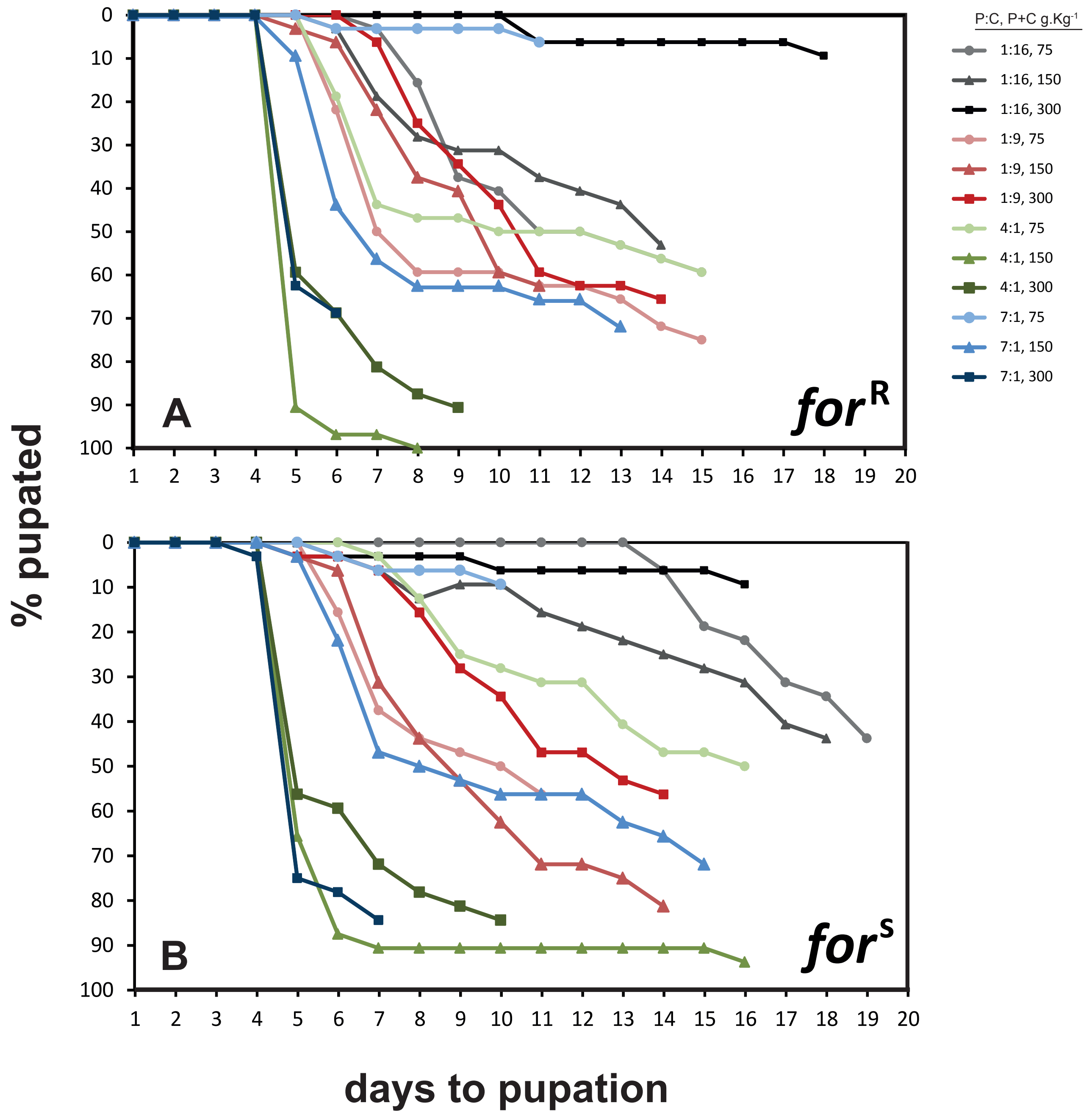



Figure 3. Curves showing the percentage of larvae from the natural *foraging* genotypes that survived to pupation over the number of days to reach pupation when reared on 'extreme' diets. When raised from hatchling to pupa on imbalanced protein (P) to carbohydrate (C) foods, *for*$^{R}$ larvae (panel A) reach pupation more quickly and survive to pupariation in greater number sitter *for*$^{s}$ larvae (panel B).

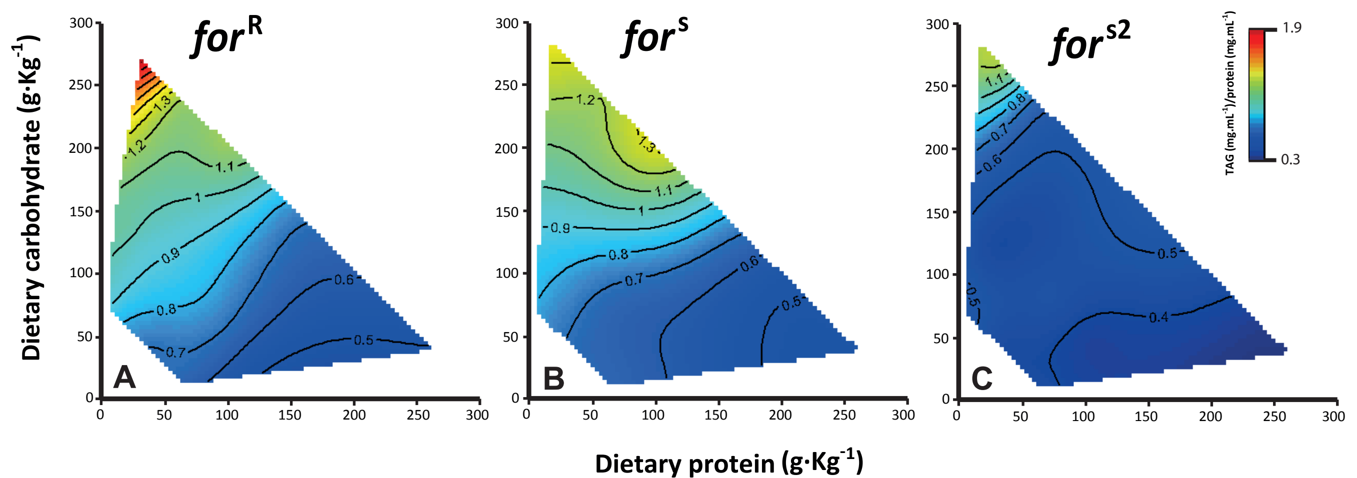



Figure 4. A) – C) Thin plate spline (TPS) response surfaces of whole body triglyceride levels (TAG), per unit body protein, for larvae from three *foraging* genetic strains. The highest TAG levels are represented on surfaces as deep red. Gradation from red to deep blue indicates decrease in TAG. TPS surfaces show very high-carbohydrate (C) diets are associated with greater TAG storage in larvae of each *foraging* genetic strain. Generalised linear modeling (GLM) showed that the quadratic term of C was significantly associated with increase in TAG for all *foraging* genotypes (*carbohydrate²*, Wald $\chi^2 = 130.727$, d.f. = 1, $p < 0.001$), while the linear term of dietary protein was associated with lower body TAG (*protein*, Wald $\chi^2 = 13.477$, d.f. = 1, $p < 0.05$). Between *foraging* genotype GLM showed the $for^{s2}$ TAG surface significantly differed in profile to those of the natural *foraging* genotypes, with the $for^{s2}$ TPS (panel C) showing mutant sitters have more uniform TAG levels, and far less body TAG than either $for^{s}$ or $for^{R}$ larvae ($for^{R}$ vs. $for^{s2}$ *genotype*, Wald $\chi^2 = 55.561$, d.f. = 1, $p < 0.001$; $for^{s}$ vs. $for^{s2}$ *genotype*, Wald $\chi^2 = 48.282$, d.f. = 1, $p < 0.001$). GLM comparing the TAG levels attained by natural *foraging* larvae following rearing on high-C diets showed that rover $for^{R}$ larvae store more TAG than $for^{s}$ sitters ($for^{R}$ vs. $for^{s}$ *genotype*, Wald $\chi^2 = 3.868$, d.f. = 1, $p < 0.05$).

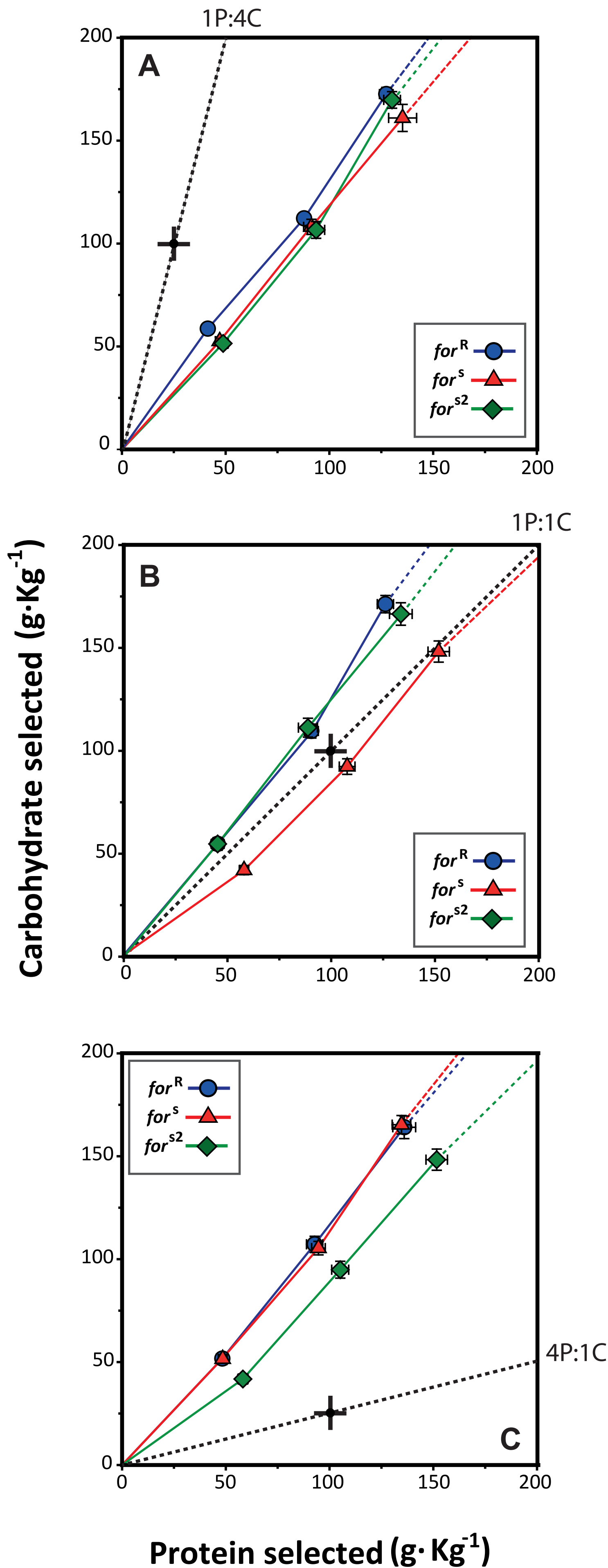



Figure 5. Panels A) – C) show the mean (± s.e) dietary protein (P) to carbohydrate (C) ratio selected by 3rd stadia larvae of each *foraging* genotype measured across three time points over 44 h. Larvae had been raised for the previous two larval stadia on either: a 1P:4C, 100 g P+C.Kg$^{-1}$ (panel A), a 1P:1C, 100 g P+C.Kg$^{-1}$ (panel B), or a 4P:1C, 100 g P+C.Kg$^{-1}$ diet (panel C). Black crosshairs show the location of each pre-rearing diet (hereafter, pre-treatment) in nutrient space.

To visualise the dynamic nature of P-C selection by larvae, on each panel larval P-C selections are represented as cumulative 'intake' trajectories, with each genotype's 'time 4 h' selection appearing at the base of the trajectory, 'time 20 h' in the middle and 'time 44 h' at the top. Dashed lines emanating from the 44 h measure are intended to help visualise the position of each genetic strain's P:C intake target.

Caption continues on the following page.

Figure 5. (Continued from previous page.) Repeated measured MANOVA showed that pre-treatment diet did not significantly influence P-C selection by larvae (*pre-treatment*, Wilks' λ = 0.974, $F_{(4, 646.0)}$= 2.119, $p$ = 0.08), although P-C selection did significantly differ due to *foraging* genotype (*genotype*, Wilks' λ = 0.964, $F_{(4, 646.0)}$= 3.025, $p < 0.05$), with post-hoc multiple comparisons showing $for^R$ rover larvae selecting significantly more C- and less P-biased food across the entire experiment than $for^s$ larvae (*protein*, $for^R$ vs. $for^s$, $p = 0.014$; *carbohydrate*, $for^R$ vs. $for^s$, $p = 0.014$).
Panel A) Individual MANOVA on larval P and C selection after pre-treatment on a 1P:4C diet showed despite $for^R$ larvae appearing to select higher C diets over time that *foraging* allelic variants did not statistically differ in their diet choice over time (Wilks' λ = 0.942, $F_{(2, 196.0)}$ = 1.494, $p = 0.205$). Panel B) Repeated measures MANOVA identified that larvae pre-reared on the 1P:1C diet differed significantly in their P-C selection across the experiment (Wilks' λ = 0.870, $F_{(4, 220.0)}$ = 3.972, $p < 0.001$). Post-hoc tests showed $for^s$ larvae were selecting significantly lower C diets over time than either $for^R$ and $for^{s2}$ larvae ($p < 0.05$ for all $for^s$ vs. $for^R$ & $for^s$ vs. $for^{s2}$ *protein* & *carbohydrate* selection comparisons). Panel C) despite $for^{s2}$ larvae appearing to select lower C:P diets across the 44 h experiment, MANOVA identified no statistical differences in P:C selection between *foraging* genotypes (Wilks' λ = 0.928, $F_{(4, 226.0)}$= 2.149, $p = 0.076$).

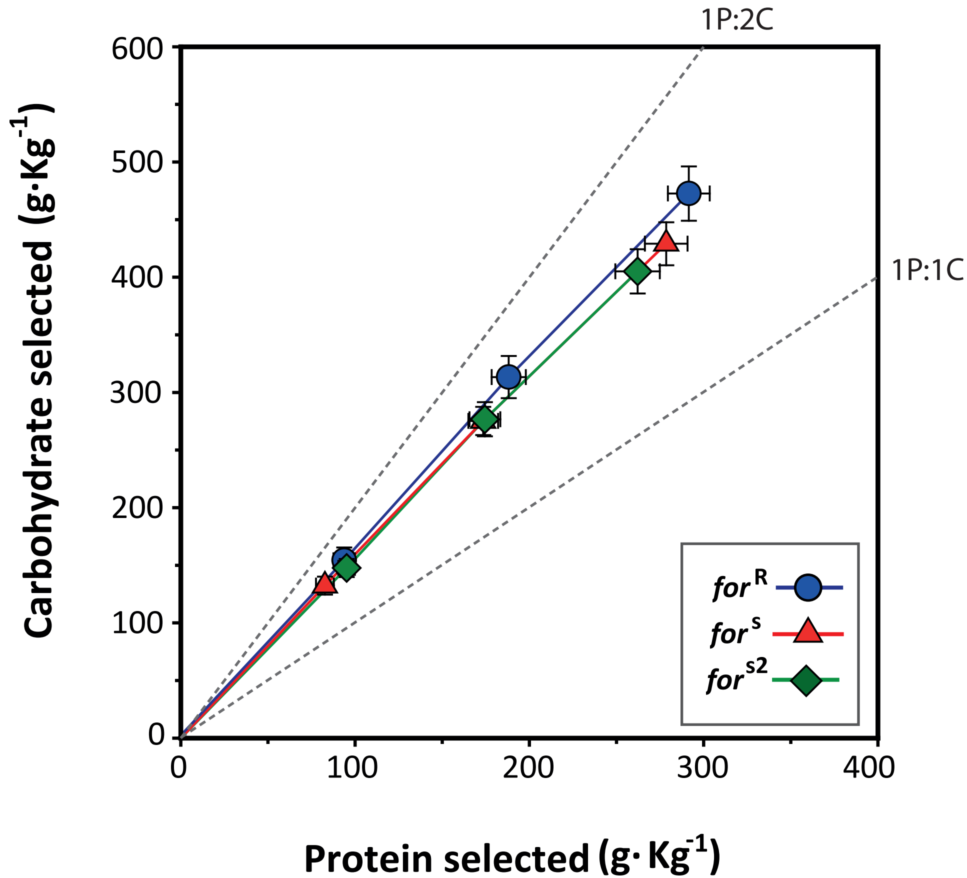



Figure 6. Mean (± s.e) dietary protein (P) to carbohydrate (C) ratio and diet energy density selections made by 3rd stadia larvae of each *foraging* genotype measured across three time points over 44 h. Larvae of each genotype had been raised for the previous two larval stadia on a 1P:1.5C 100 g P+C.Kg$^{-1}$ diet. To visualise changes in dietary P:C ratio and energy density selection by larvae over time, selections are represented as cumulative 'intake' trajectories. Each genotype's 'time 4 h' selection appears at the base of the trajectory, 'time 20 h' in the middle and 'time 44 h' at the top. Larvae were permitted to regulate their diet selections from between the 1P:1C and 1P:2C diet rails (indicated by dashed lines) as previous experiments indicated that the P:C intake targets for larvae of all *foraging* genetic strains fell within this range. Repeated measures MANOVA showed that when pre-reared on a common diet, close to their mutual P:C intake target, larvae from each *foraging* genetic strain did not significantly differ in their selection of dietary P:C ratio or concentration (*genotype*, Wilks' $\lambda = 0.854$, $F_{(4, 82)} = 1.689$, $p = 0.160$).

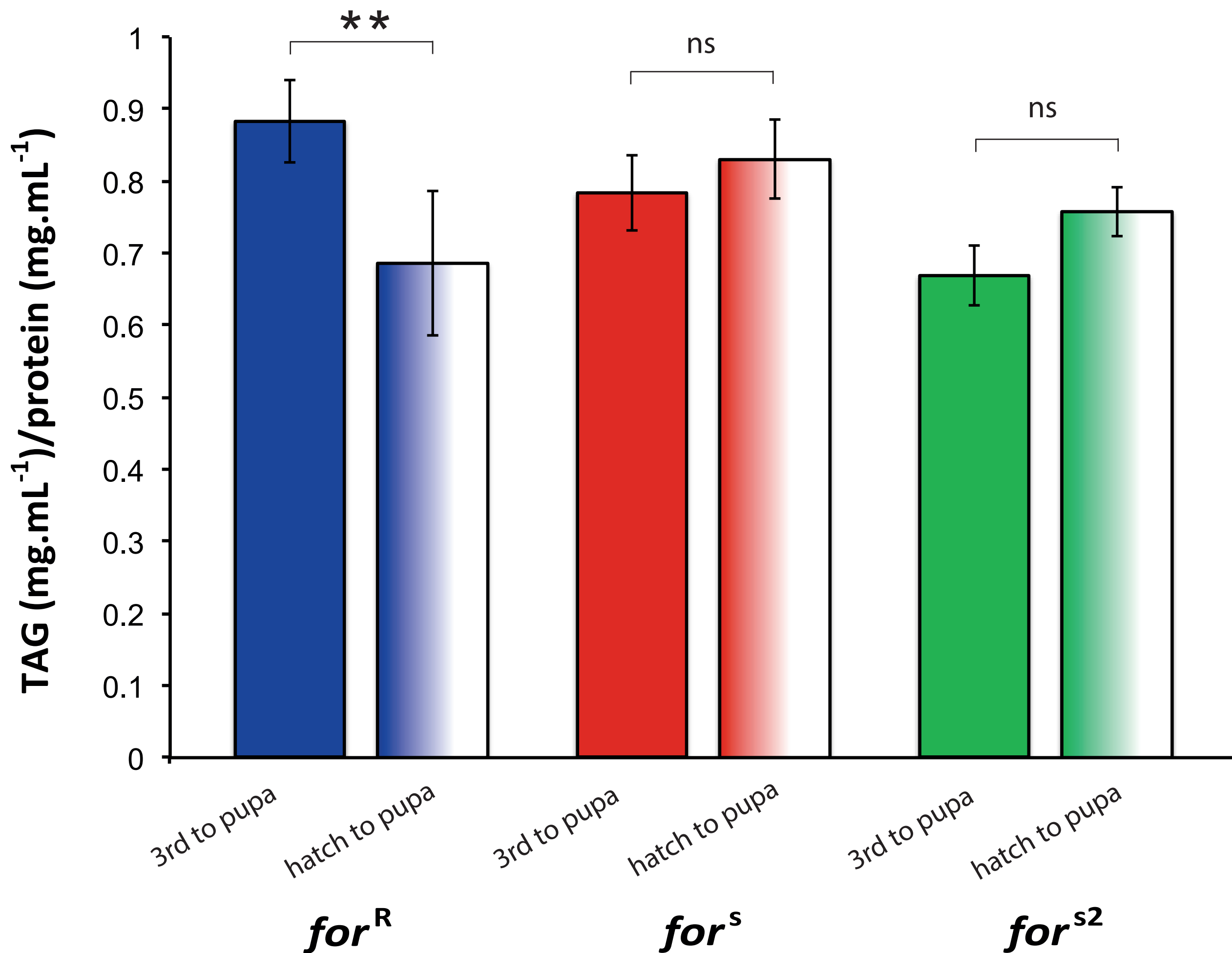
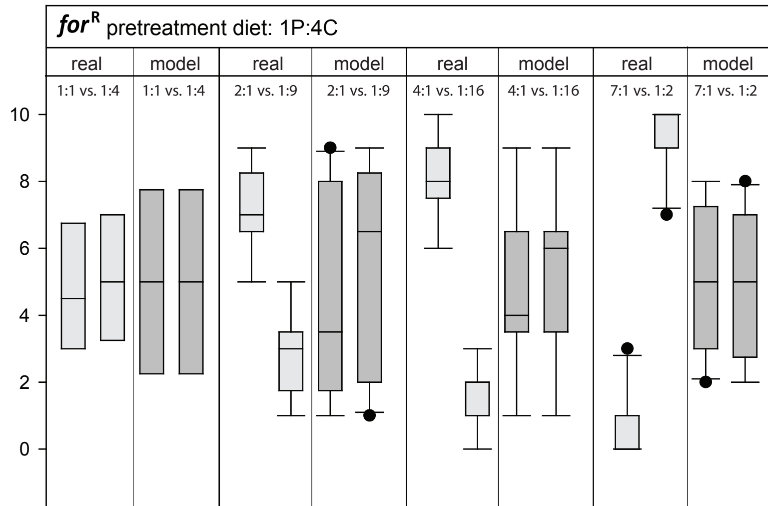
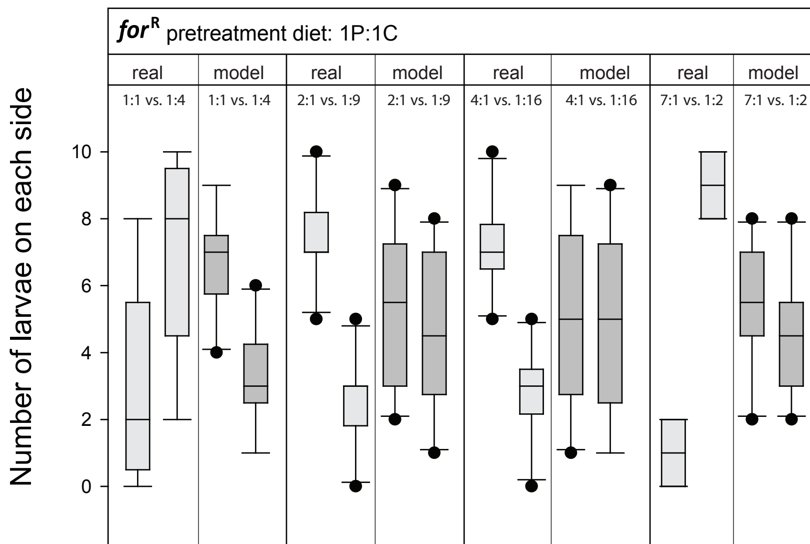



Figure 7. Mean (± s.e) whole body triglyceride levels (TAG) (per unit body protein) for larvae of each *foraging* genotype allowed to self-regulate their protein (P) to carbohydrate (C) intake for either the final larval stadia ('3rd to pupa') or for their entire larval period ('hatch to pupa'). ANOVA showed no main effect of genotype on TAG levels (*genotype*, $F_{(2, 54)} = 2.230$, $p = 0.117$). There was however a significant interaction between genotype and the period of time over which larvae were allowed to self-regulate their P-C intake (*genotype*feeding regime*, $F_{(3, 54)} = 3.818$, $p < 0.05$). Post-hoc within genotype comparisons showed this result was driven by the $for^{R}$ allelic variant. Rover larvae allowed to self regulate their P:C intake for the entire larval period accumulated significantly less TAG than those permitted to self-regulate P-C intake during the 3rd larval stadia only ($for^{R}$ *3rd to pupa* vs. $for^{R}$ *entire*, $F_{(1, 19)} = 9.031$, $p < 0.01$). ** indicates significance at the p < 0.001 level.



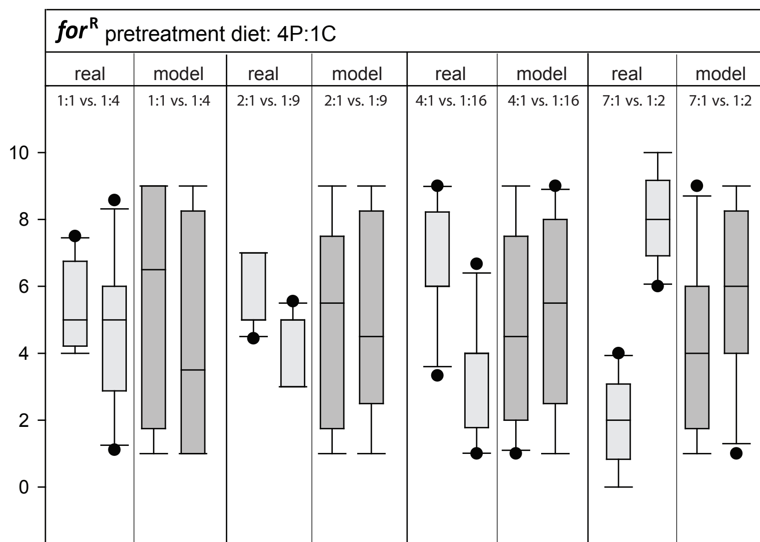




Figure 8. Panels demonstrating that experimental larvae actively regulated their protein (P) to carbohydrate (C) intake during binary-choice experiments. (Please see the methods section for an explanation of the experimental setup.)

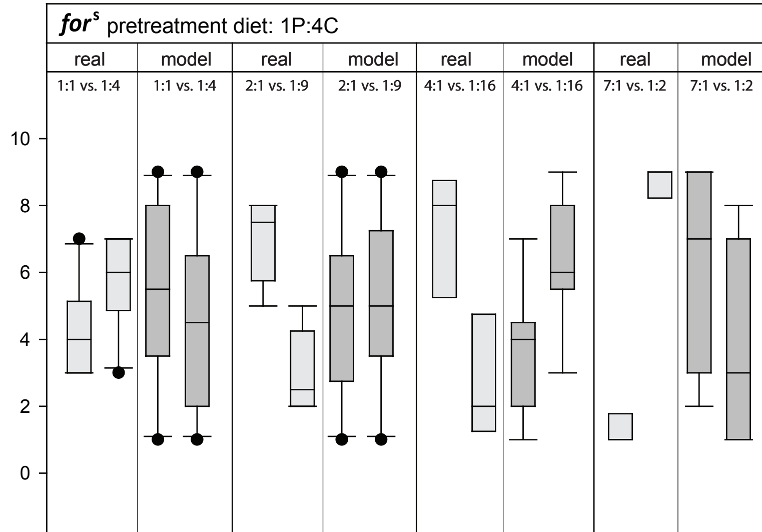
Each panel shows eight pairs of boxplots. Within each pair of pale grey boxplots, the left hand side plot shows the actual ('real') number of larvae counted on the higher P-C diet side of the Petri-dish at 22 h (time point chosen arbitrarily). The right hand pale grey boxplot within each pair shows the real number of larvae on the higher C-P side of the dish at 22 h.

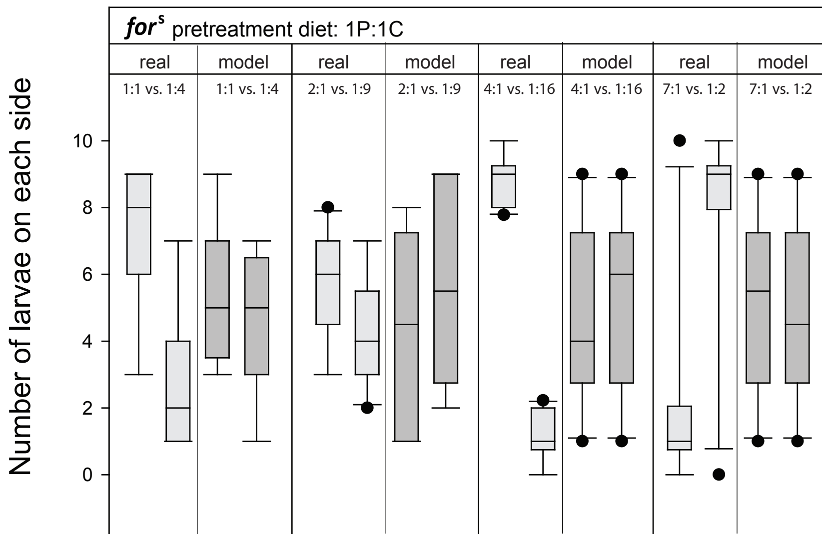
Adjacent to each pair of pale grey plots are dark grey boxplot pairs labeled 'model'. These plots show the number of larvae that would occur on either the left (high P) or right (high C) Petri-dish side if larvae were positioned due to random chance.

For both 'real' and null 'model' larvae, boxplots show the median, first and third quartiles, and as whiskers, the 9th and 91st percentile. Each boxplot represents an *N* of ten dishes.

Figure 8a (panels on left). Boxplots representing the number of real and null model *for*$^R$ larvae on the left (high protein) and right (high carbohydrate) sides of dishes containing pairs of experimental diets. Four different 'binary-choice' dishes were used, containing the following P:C diet pairs: 1:1 vs. 1:4, 2:1 vs. 1:9, 4:1 vs. 1:16 or 7:1 vs. 1:2.

The Top panel shows diet selections made by *for*$^R$ larvae pre-reared on a 1P:4C, 100 g P+C.Kg$^{-1}$ diet. The middle panel shows *for*$^R$ selections made after pre-rearing on 1P:4C, 100 g P+C.Kg$^{-1}$. The bottom panel shows selections of *for*$^R$ larvae made following pre-experimental rearing on a 4P:1C, 100 g P+C.Kg$^{-1}$ diet.



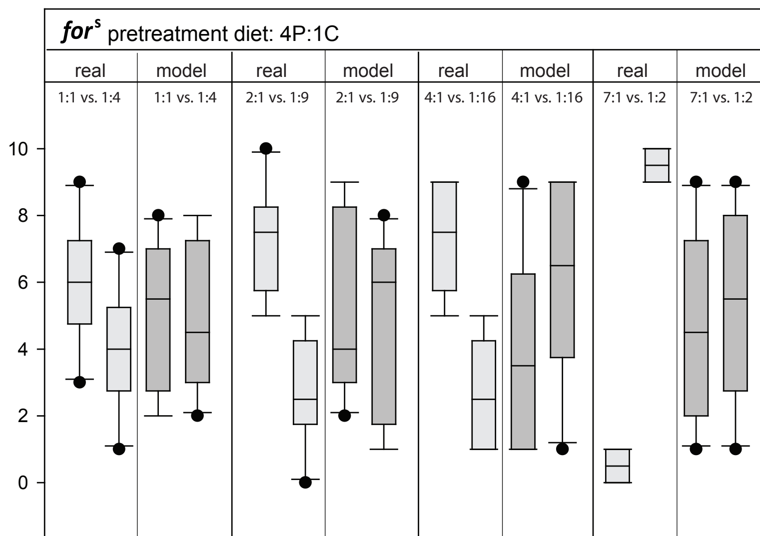
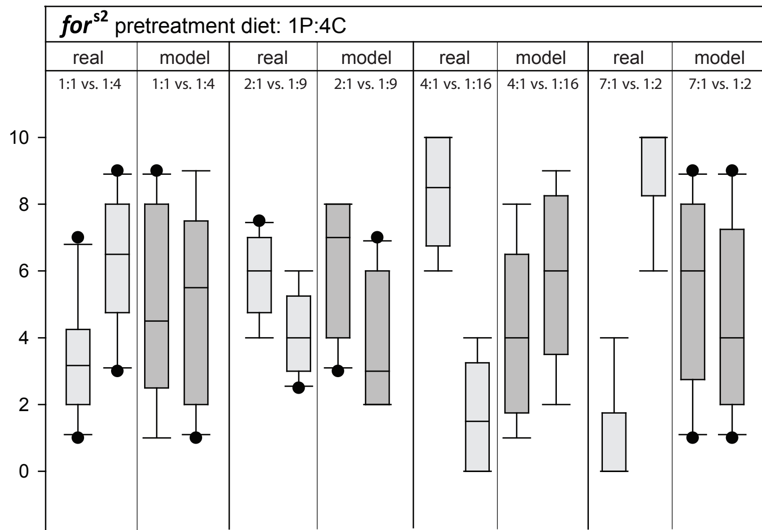
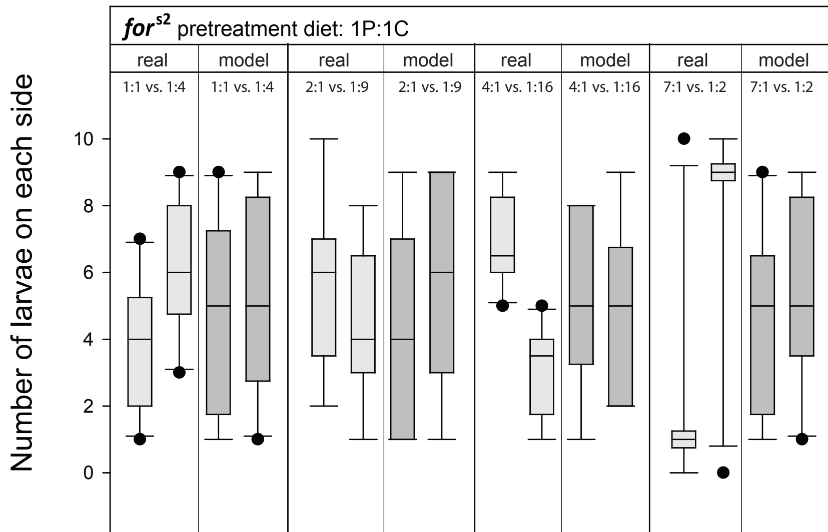




Figure 8b. Boxplots representing the number of real and null model *for*$^s$ larvae on the left (high protein) and right (high carbohydrate) sides of dishes containing pairs of high protein vs. high carbohydrate diets. *for*$^s$ panels are arranged as in the previous figure, 8a.



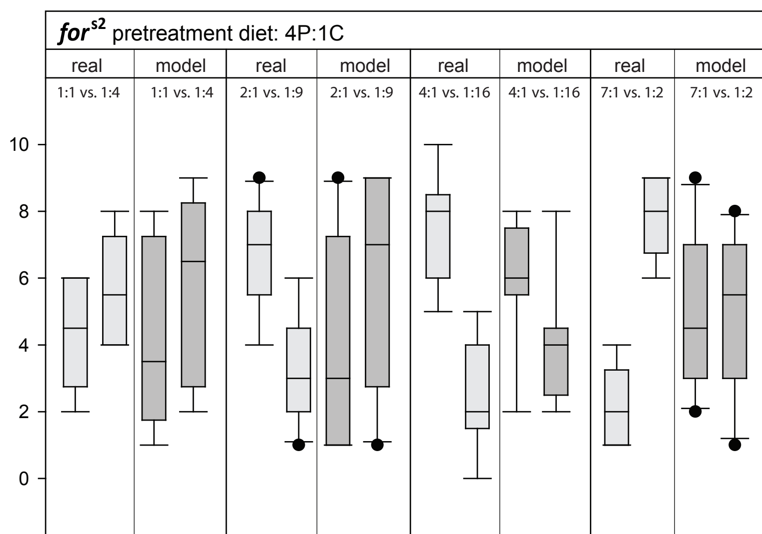
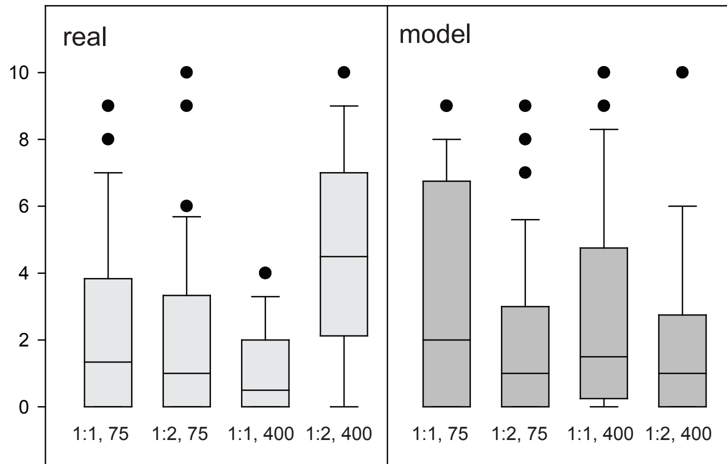
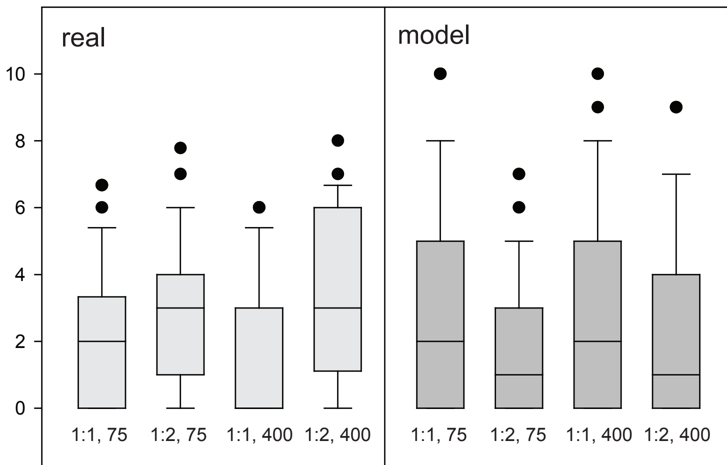




Figure 8c. Boxplots representing the number of *for*$^{s2}$ larvae, real and null model, on the left and right sides of dishes containing a protein biased diet on their left hand side, or a carbohydrate biased diet on their right hand side. *for*$^{s2}$ panels are arranged in the same order as in figures 8a & b.




Number of larvae in each quadrant



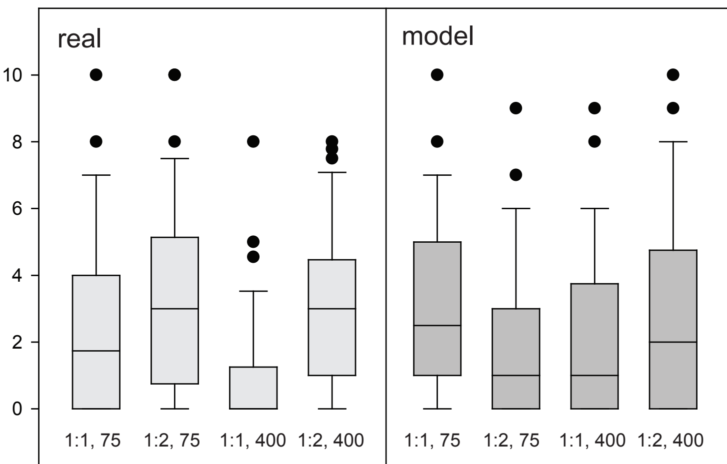




Figure 9. Panels demonstrating that experimental larvae actively regulated their protein (P) to carbohydrate (C) intake during four-way choice experiments (Please see the methods section further details.)
Each panel shows two sets of four boxplots. Pale grey plots represent the actual ('real') number of larvae counted within each experimental diet quadrant at the 22 h time point during the experiment (this time was chosen arbitrarily).
Adjacent to each set of four pale grey plots are four dark grey boxplots labeled 'model'. These plots show the number expected in each diet quadrant if larval position within the dish was due to random chance.
For both 'real' and null 'model' larvae, each boxplot shows the median, first and third quartiles. Whiskers represent the 9th and 91st percentiles. *N* for each boxplot was 36 dishes.
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**Table 2.1. Generalised linear model statistical tables for survivorship to pupation for all *foraging* genotypes.**

**Omnibus test**

| Likelihood ratio $\chi^2$ | df | Significance |
|---|---|---|
| 30.508 | 7 | $p < 0.001$ |

**Test of model effects**

| Source | Type III S.S. | | |
|---|---|---|---|
| | Wald $\chi^2$ | df | Significance |
| Intercept | 8.420 | 1 | $p = 0.004$ |
| *for* genotype | 0.134 | 2 | $p = 0.935$ |
| Protein | 3.686 | 1 | $p = 0.055$ |
| Carbohydrate | 7.358 | 1 | $p = 0.007$ |
| Protein$^2$ | 3.291 | 1 | $p = 0.070$ |
| Carbohydrate$^2$ | 14.563 | 1 | $p < 0.001$ |
| Protein*carbohydrate | 0.553 | 1 | $p = 0.457$ |

**Parameter estimates**

| Parameter | β coefficient | Standard error | 95% Wald C.I. | | Hypothesis test | | |
|---|---|---|---|---|---|---|---|
| | | | Lower | Upper | Wald $\chi^2$ | df | Significance |
| Intercept | 42.891 | 15.0294 | 13.434 | 72.348 | 8.144 | 1 | $p = 0.004$ |
| $for^R$ genotype | 0.254 | 6.1642 | -11.827 | 12.336 | 0.002 | 1 | $p = 0.967$ |
| $for^s$ genotype | -1.813 | 6.1642 | -13.894 | 10.269 | 0.086 | 1 | $p = 0.769$ |
| $for^{s2}$ genotype | . | . | . | . | . | . | . |
| Protein | 0.401 | 0.2087 | -0.008 | 0.810 | 3.686 | 1 | $p = 0.055$ |
| Carbohydrate | 0.546 | 0.2013 | 0.152 | 0.941 | 7.358 | 1 | $p = 0.007$ |
| Protein$^2$ | -0.001 | 0.0006 | -0.002 | 8.846E-005 | 3.291 | 1 | $p = 0.070$ |
| Carbohydrate$^2$ | -0.002 | 0.0006 | -0.003 | -0.001 | 14.563 | 1 | $p < 0.001$ |
| Protein*carbohydrate | -0.001 | 0.0011 | -0.003 | 0.001 | 0.553 | 1 | $p = 0.457$ |

**Table 2.2a. Generalised linear model statistical tables for developmental rate for all *foraging* genotypes.**

**Omnibus test**

| Likelihood ratio $\chi^2$ | df | Significance |
|---|---|---|

| 575.955 | 7 | $p < 0.001$ |
|---|---|---|

**Test of model effects**

| Source | Type III S.S. | | |
|---|---|---|---|
| | Wald $\chi^2$ | df | Significance |
| Intercept | 1107.439 | 1 | $p < 0.001$ |
| *for* genotype | 15.884 | 2 | $p < 0.001$ |
| Protein | 36.731 | 1 | $p < 0.001$ |
| $\text{Protein}^2$ | 38.531 | 1 | $p < 0.001$ |
| Carbohydrate | 0.178 | 1 | $p = 0.673$ |
| $\text{Carbohydrate}^2$ | 29.208 | 1 | $p < 0.001$ |
| Protein*carbohydrate | 0.853 | 1 | $p = 0.356$ |

**Parameter estimates**

| Parameter | β coefficient | Standard error | 95% Wald C.I. | | Hypothesis test | | |
|---|---|---|---|---|---|---|---|
| | | | Lower | Upper | Wald $\chi^2$ | df | Significance |
| Intercept | 0.162 | 0.0049 | 0.153 | 0.172 | 1106.953 | 1 | $p < 0.001$ |
| $for^R$ | -0.004 | 0.0020 | -0.008 | -0.001 | 4.911 | 1 | $p = 0.027$ |
| $for^s$ | -0.008 | 0.0020 | -0.012 | -0.004 | 15.811 | 1 | $p < 0.001$ |
| $for^{s2}$ | . | . | . | . | . | . | . |
| Protein | 0.0 | 6.4030E-005 | 0.0 | 0.001 | 36.731 | 1 | $p < 0.001$ |
| $\text{Protein}^2$ | -1.151E-006 | 1.8539E-007 | -1.514E-006 | -7.874E-007 | 38.531 | 1 | $p < 0.001$ |
| Carbohydrate | 2.845E-005 | 6.7487E-005 | 0.0 | 0.0 | 0.178 | 1 | $p = 0.673$ |
| $\text{Carbohydrate}^2$ | -1.060E-006 | 1.9619E-007 | -1.445E-006 | -6.758E-007 | 29.208 | 1 | $p < 0.001$ |
| Protein*carbohydrate | 3.250E-007 | 3.5178E-007 | -3.645E-007 | 1.014E-006 | 0.853 | 1 | $p = 0.356$ |

**Table 2.2b. Generalised linear model statistical tables for *for*$^R$ vs. *for*$^s$ developmental rate.**

**Omnibus test**

| Likelihood ratio $\chi^2$ | df | Significance |
|---|---|---|
| 389.987 | 6 | $p < 0.001$ |

**Test of model effects**

| Source | Type III S.S. | | |
|---|---|---|---|
| | Wald $\chi^2$ | df | Significance |

| Intercept | 700.227 | 1 | $p < 0.001$ |
|---|---|---|---|
| *for* genotype | 3.017 | 1 | $p = 0.082$ |
| Protein | 24.105 | 1 | $p < 0.001$ |
| Protein$^2$ | 25.723 | 1 | $p < 0.001$ |
| Carbohydrate$^2$ | 14.942 | 1 | $p < 0.001$ |
| Carbohydrate | 0.044 | 1 | $p = 0.833$ |
| Protein*carbohydrate | 1.106 | 1 | $p = 0.293$ |

**Parameter estimates**

| **Parameter** | **β coefficient** | **Standard error** | **95% Wald C.I.** | | **Hypothesis test** | | |
|---|---|---|---|---|---|---|---|
| | | | **Lower** | **Upper** | **Wald $\chi^2$** | **df** | **Significance** |
| Intercept | 0.156 | 0.0061 | 0.144 | 0.168 | 663.317 | 1 | $p < 0.001$ |
| *for*$^R$ | 0.004 | 0.0020 | 0.0 | 0.008 | 3.017 | 1 | $p = 0.082$ |
| *for*$^s$ | . | . | . | . | . | . | . |
| Protein | 0.0 | 8.0322E-005 | 0.0 | 0.001 | 24.105 | 1 | $p < 0.001$ |
| Protein$^2$ | -1.183E-006 | 2.3317E-007 | -1.640E-006 | -7.256E-007 | 25.723 | 1 | $p < 0.001$ |
| Carbohydrate$^2$ | -9.470E-007 | 2.4500E-007 | -1.427E-006 | -4.668E-007 | 14.942 | 1 | $p < 0.001$ |
| Carbohydrate | -1.778E-005 | 8.4400E-005 | 0.0 | 0.0 | 0.044 | 1 | $p = 0.833$ |
| Protein*carbohydrate | 4.634E-007 | 4.4074E-007 | -4.004E-007 | 1.327E-006 | 1.106 | 1 | $p = 0.293$ |

**Table 2.2c. Generalised linear model statistical tables for *for*$^R$ vs. *for*$^{s2}$ developmental rate.**

**Omnibus test**

| **Likelihood ratio $\chi^2$** | **df** | **Significance** |
|---|---|---|
| 413.514 | 4 | $p < 0.001$ |

**Test of model effects**

| **Source** | **Type III S.S.** | | |
|---|---|---|---|
| | **Wald $\chi^2$** | **df** | **Significance** |
| Intercept | 5679.032 | 1 | $p < 0.001$ |
| *for* genotype | 5.662 | 1 | $p = 0.017$ |
| Protein | 79.640 | 1 | $p < 0.001$ |
| Protein$^2$ | 43.670 | 1 | $p < 0.001$ |

| Carbohydrate$^2$ | 305.534 | 1 | $p < 0.001$ |
|---|---|---|---|

**Parameter estimates**

| Parameter | β coefficient | Standard error | 95% Wald C.I. | | Hypothesis test | | |
|---|---|---|---|---|---|---|---|
| | | | Lower | Upper | Wald $\chi^2$ | df | Significance |
| Intercept | 0.164 | 0.0024 | 0.159 | 0.159 | 4811.769 | 1 | $p < 0.001$ |
| *for*$^R$ | -0.004 | 0.0018 | -0.008 | -0.008 | 5.662 | 1 | $p = 0.017$ |
| *for*$^{s2}$ | . | . | . | . | . | . | . |
| Protein | 0.0 | 4.4882E-005 | 0.0 | 0.0 | 79.640 | 1 | $p < 0.001$ |
| Protein$^2$ | -1.146E-006 | 1.7347E-007 | -1.486E-006 | -8.064E-007 | 43.670 | 1 | $p < 0.001$ |
| Carbohydrate$^2$ | -9.028E-007 | 5.1648E-008 | -1.004E-006 | -8.016E-007 | 305.534 | 1 | $p < 0.001$ |

**Table 2.2d. Generalised linear model statistical tables for *for*$^s$ vs. *for*$^{s2}$ developmental rate.**

**Omnibus test**

| Likelihood ratio $\chi^2$ | df | Significance |
|---|---|---|
| 342.029 | 4 | $p < 0.001$ |

**Test of model effects**

| Source | Type III S.S. | | |
|---|---|---|---|
| | Wald $\chi^2$ | df | Significance |
| Intercept | 4226.391 | 1 | $p < 0.001$ |
| *for* genotype | 14.459 | 1 | $p < 0.001$ |
| Protein | 68.504 | 1 | $p < 0.001$ |
| Protein$^2$ | 37.250 | 1 | $p < 0.001$ |
| Carbohydrate$^2$ | 223.883 | 1 | $p < 0.001$ |

**Parameter estimates**

| Parameter | β coefficient | Standard error | 95% Wald C.I. | | Hypothesis test | | |
|---|---|---|---|---|---|---|---|
| | | | Lower | Upper | Wald $\chi^2$ | df | Significance |
| Intercept | 0.162 | 0.0027 | 0.157 | 0.167 | 3706.971 | 1 | $p < 0.001$ |
| *for*$^s$ | -0.008 | 0.0021 | -0.12 | -0.004 | 14.459 | 1 | $p < 0.001$ |
| *for*$^{s2}$ | . | . | . | . | . | . | . |
| Protein | 0.0 | 5.0773E-005 | 0.0 | 0.001 | 68.504 | 1 | $p < 0.001$ |
| Protein$^2$ | -1.193E-006 | 1.9555E-007 | -1.577E-006 | -8.102E-007 | 37.250 | 1 | $p < 0.001$ |

| Carbohydrate$^2$ | -8.657E-007 | 5.7861E-008 | -9.792E-007 | -7.523E-007 | 223.883 | 1 | $p < 0.001$ |
|---|---|---|---|---|---|---|---|

**Table 2.3a. Generalised linear model statistical tables for model comparing body triglycerides across all *foraging* genotypes.**

**Omnibus test**

| Likelihood ratio $\chi^2$ | df | Significance |
|---|---|---|
| 190.116 | 4 | $p < 0.001$ |

**Test of model effects**

| Source | Type III S.S. | | |
|---|---|---|---|
| | Wald $\chi^2$ | df | Significance |
| Intercept | 364.257 | 1 | $p < 0.001$ |
| *for* genotype | 61.746 | 2 | $p = 0.049$ |
| Protein | 13.477 | 1 | $p = 0.003$ |
| Carbohydrate$^2$ | 130.727 | 1 | $p < 0.001$ |

**Parameter estimates**

| Parameter | β coefficient | Standard error | 95% Wald C.I. | | Hypothesis test | | |
|---|---|---|---|---|---|---|---|
| | | | Lower | Upper | Wald $\chi^2$ | df | Significance |
| Intercept | 0.424 | 0.0445 | 0.336 | 0.511 | 90.538 | 1 | $p < 0.001$ |
| $for^R$ | 0.360 | 0.0480 | 0.266 | 0.454 | 56.204 | 1 | $p < 0.001$ |
| $for^s$ | 0.280 | 0.0471 | 0.187 | 0.372 | 35.247 | 1 | $p < 0.001$ |
| $for^{s2}$ | . | . | . | . | . | . | . |
| Protein | -0.001 | 0.0003 | -0.002 | 0.0 | 13.477 | 1 | $p < 0.001$ |
| Carbohydrate$^2$ | 1.085E-005 | 9.4925E-007 | 8.993E-006 | 1.271E-005 | 130.727 | 1 | $p < 0.001$ |

**Table 2.3b. Generalised linear model statistical tables for $for^R$ vs. $for^s$ body triglycerides.**

**Omnibus test**

| Likelihood ratio $\chi^2$ | df | Significance |
|---|---|---|
| 99.068 | 3 | $p < 0.001$ |

**Test of model effects**

| Source | Type III S.S. | | |
|---|---|---|---|
| | Wald $\chi^2$ | df | Significance |
| Intercept | 280.469 | 1 | $p < 0.001$ |
| *for* genotype | 2.474 | 1 | $p = 0.116$ |
| Protein | 11.466 | 1 | $p = 0.001$ |
| Carbohydrate$^2$ | 83.729 | 1 | $p < 0.001$ |

**Parameter estimates**

| Parameter | β coefficient | Standard error | 95% Wald C.I. | | Hypothesis test | | |
|---|---|---|---|---|---|---|---|
| | | | Lower | Upper | Wald $\chi^2$ | df | Significance |
| Intercept | 0.707 | 0.0514 | 0.607 | 0.808 | 189.169 | 1 | $p < 0.001$ |
| *for*$^R$ | 0.081 | 0.0517 | -0.020 | 0.183 | 2.474 | 1 | $p = 0.116$ |
| *for*$^s$ | 0.0 | . | . | . | . | . | . |
| Protein | -0.001 | 0.004 | -0.002 | -0.001 | 11.466 | 1 | $p = 0.001$ |
| Carbohydrate$^2$ | 1.195E-005 | 1.3062E-006 | 9.392E-006 | 1.451E-005 | 83.726 | 1 | $p < 0.001$ |

**Table 2.3c. Generalised linear model statistical tables for *for*$^R$ vs. *for*$^{s2}$ body triglycerides.**

**Omnibus test**

| Likelihood ratio $\chi^2$ | df | Significance |
|---|---|---|
| 136.204 | 3 | $p < 0.001$ |

**Test of model effects**

| Source | Type III S.S. | | |
|---|---|---|---|
| | Wald $\chi^2$ | df | Significance |
| Intercept | 205.141 | 1 | $p < 0.001$ |
| *for* genotype | 55.561 | 1 | $p < 0.001$ |
| Protein | 8.881 | 1 | $p < 0.05$ |
| Carbohydrate$^2$ | 89.234 | 1 | $p < 0.001$ |

**Parameter estimates**

| Parameter | β coefficient | Standard error | 95% Wald C.I. | | Hypothesis test | | |
|---|---|---|---|---|---|---|---|
| | | | Lower | Upper | Wald $\chi^2$ | df | Significance |

| Intercept | 0.416 | 0.0493 | 0.312 | 0.513 | 71.128 | 1 | $p < 0.001$ |
|---|---|---|---|---|---|---|---|
| *for*$^{R}$ | 0.361 | 0.0484 | 0.266 | 0.456 | 55.561 | 1 | $p < 0.001$ |
| *for*$^{s2}$ | 0.0 | . | . | . | . | . | . |
| Protein | -0.001 | 0.0003 | -0.002 | 0.000 | 8.881 | 1 | $p = 0.003$ |
| Carbohydrate$^{2}$ | 1.135E-005 | 1.2011E-006 | 8.992E-006 | 1.370E-005 | 89.234 | 1 | $p < 0.001$ |

**Table 2.3d. Generalised linear model statistical tables for *for*$^{s}$ vs. *for*$^{s2}$ body triglycerides.**

**Omnibus test**

| Likelihood ratio $\chi^2$ | df | Significance |
|---|---|---|
| 138.963 | 3 | $p < 0.001$ |

**Test of model effects**

| Source | Type III S.S. | | |
|---|---|---|---|
| | Wald $\chi^2$ | df | Significance |
| Intercept | 259.208 | 1 | $p < 0.001$ |
| *for* genotype | 48.282 | 1 | $p < 0.001$ |
| Protein | 6.895 | 1 | $p = 0.009$ |
| Carbohydrate$^{2}$ | 96.619 | 1 | $p < 0.001$ |

**Parameter estimates**

| Parameter | β coefficient | Standard error | 95% Wald C.I. | | Hypothesis test | | |
|---|---|---|---|---|---|---|---|
| | | | Lower | Upper | Wald $\chi^2$ | df | Significance |
| Intercept | 0.426 | 0.0415 | 0.344 | 0.507 | 105.171 | 1 | $p < 0.001$ |
| *For*$^{s}$ | 0.279 | 0.0401 | 0.200 | 0.357 | 48.282 | 1 | $p < 0.001$ |
| *for*$^{s2}$ | 0.0 | . | . | . | . | . | . |
| Protein | -0.001 | 0.0003 | -0.001 | 0.000 | 6.895 | 1 | $p < 0.05$ |
| Carbohydrate$^{2}$ | 9.435E-006 | 9.5991E-007 | 1.132E-005 | 1.132E-005 | 96.619 | 1 | $p < 0.001$ |

**Table 2.3e. Generalised linear model statistical tables for *for*$^{R}$ vs. *for*$^{s}$ body triglycerides in high carbohydrate regions of nutrient space.**

**Omnibus test**

| Likelihood ratio $\chi^2$ | df | Significance |
|---|---|---|

| 30.765 | 3 | $p < 0.001$ |
|---|---|---|

**Test of model effects**

| Source | Type III S.S. | | |
|---|---|---|---|
| | Wald $\chi^2$ | df | Significance |
| Intercept | 108.386 | 1 | $p < 0.001$ |
| *for* genotype | 3.868 | 2 | $p = 0.049$ |
| Protein | 8.790 | 1 | $p = 0.003$ |
| Carbohydrate$^2$ | 30.557 | 1 | $p < 0.001$ |

**Parameter estimates**

| Parameter | β coefficient | Standard error | 95% Wald C.I. | | Hypothesis test | | |
|---|---|---|---|---|---|---|---|
| | | | Lower | Upper | Wald $\chi^2$ | df | Significance |
| Intercept | 0.770 | 0.0998 | 0.574 | 0.965 | 59.500 | 1 | $p < 0.001$ |
| *for*$^R$ | 0.206 | 0.1049 | 0.001 | 0.412 | 3.868 | 1 | $p = 0.049$ |
| *for*$^s$ | 0.0 | . | . | . | . | . | . |
| Protein | -0.013 | 0.0042 | -0.021 | -0.004 | 8.790 | 1 | $p = 0.003$ |
| Carbohydrate$^2$ | 1.518E-005 | 2.7461E-006 | 9.798E-006 | 2.056E-005 | 30.557 | 1 | $p < 0.001$ |

**Table 2.4. Repeated measured MANOVA on protein and carbohydrate ratio selected by larvae of each *foraging* genotype across the 3rd larval stadium in response to pre-experimental rearing on either a 1P:1C, 1P:4C, or 4P:1C diet.**

**Multivariate test results**

| Effect | | | Value | F | Hypothesis df | Error df | Significance |
|---|---|---|---|---|---|---|---|
| **Between subjects** | **Intercept** | Wilks' λ | 0.000 | 8238802672 | 2 | 323.0 | $p < 0.001$ |
| | **Pretreatment** | Wilks' λ | 0.974 | 2.119 | 4 | 646.0 | $p = 0.077$ |
| | **Genotype** | Wilks' λ | 0.964 | 3.025 | 4 | 646.0 | $p = 0.017$ |
| | **Pretreat*genotype** | Wilks' λ | 0.940 | 2.550 | 8 | 646.0 | $p = 0.017$ |
| **Within subjects** | **Time** | Wilks' λ | 0.858 | 13.241 | 4 | 321.0 | $p < 0.001$ |
| | **Time*pretreatment** | Wilks' λ | 0.971 | 1.169 | 8 | 642.0 | $p = 0.315$ |
| | **Time*genotype** | Wilks' λ | 0.951 | 2.044 | 8 | 642.0 | $p = 0.039$ |
| | **Time*pretreatment*genotype** | Wilks' λ | 0.873 | 2.780 | 16 | 981.309 | $p < 0.001$ |

**Univariate test results**

| Source | Measure | | Type III sums of squares | df | Mean square | F | Significance |
|---|---|---|---|---|---|---|---|
| **Time** | **Protein** | Greenhouse-Geisser | 7773.608 | 1.942 | 4002.005 | 26.467 | $p < 0.001$ |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | **Carbohydrate** | Greenhouse-Geisser | 7966.038 | 1.949 | 4081.129 | 27.209 | $p < 0.001$ |
| **Time*pretreatment** | **Protein** | Greenhouse-Geisser | 569.155 | 3.885 | 146.506 | 0.969 | $p = 0.422$ |
| | **Carbohydrate** | Greenhouse-Geisser | 559.475 | 3.899 | 143.494 | 0.957 | $p = 0.429$ |
| **Time*genotype** | **Protein** | Greenhouse-Geisser | 1695.869 | 3.885 | 436.533 | 2.887 | $p = 0.023$ |
| | **Carbohydrate** | Greenhouse-Geisser | 1672.044 | 3.899 | 428.846 | 2.859 | $p = 0.024$ |
| **Time*pretreatment*genotype** | **Protein** | Greenhouse-Geisser | 5030.012 | 7.770 | 647.387 | 4.281 | $p < 0.001$ |
| | **Carbohydrate** | Greenhouse-Geisser | 4644.644 | 7.798 | 595.629 | 3.971 | $p < 0.001$ |
| **Error(time)** | **Protein** | Greenhouse-Geisser | 95161.874 | 629.347 | 151.207 | | |
| | **Carbohydrate** | Greenhouse-Geisser | 94740.085 | 631.628 | 149.993 | | |

**Post hoc Bonferroni multiple comparisons of factor 'Genotype'**

| **Measure** | **(I) Genotype** | **(J) Genotype** | **Mean difference (I-J)** | **Std. error** | **Significance** | **95% C.I. lower bound** | **95% C.I. upper bound** |
|---|---|---|---|---|---|---|---|
| **Protein** | ***for$^{R}$*** | ***for$^{s}$*** | -3.818806 | 1.3357911 | $p = 0.014$ | -7.033354 | -0.604258 |
| | | ***for$^{s2}$*** | -2.691978 | 1.3026920 | $p = 0.119$ | -5.826874 | 0.442919 |
| | ***for$^{s}$*** | ***for$^{R}$*** | 3.818806 | 1.3357911 | $p = 0.014$ | 0.604258 | 7.033354 |
| | | ***for$^{s2}$*** | 1.126828 | 1.3246003 | $p = 1.0$ | -2.060790 | 4.314446 |
| | ***for$^{s2}$*** | ***for$^{R}$*** | 2.691978 | 1.3026920 | $p = 0.119$ | -0.442919 | 5.826874 |
| | | ***for$^{s}$*** | -1.126828 | 1.3246003 | $p = 1.0$ | -4.314446 | 2.060790 |
| **Carbohydrate** | ***for$^{R}$*** | ***for$^{s}$*** | 3.820836 | 1.3359145 | $p = 0.014$ | 0.605991 | 7.035682 |
| | | ***for$^{s2}$*** | 2.695988 | 1.3028123 | $p = 0.118$ | -0.439198 | 5.831174 |
| | ***for$^{s}$*** | ***for$^{R}$*** | -3.820836 | 1.3359145 | $p = 0.014$ | -7.035682 | -0.605991 |
| | | ***for$^{s2}$*** | -1.124848 | 1.3247226 | $p = 1.0$ | -4.312761 | 2.063064 |
| | ***for$^{s2}$*** | ***for$^{R}$*** | -2.695988 | 1.3028123 | $p = 0.118$ | -5.831174 | 0.439198 |
| | | ***for$^{s}$*** | 1.124848 | 1.3247226 | $p = 1.0$ | -2.063064 | 4.312761 |

**Table 2.4a. Repeated measured MANOVA on protein and carbohydrate ratio selected by larvae of each *foraging* genotype across the 3rd larval stadium in response to pre-experimental rearing on a 1P:4C diet.**

**Multivariate test results**

| **Effect** | | | **Value** | **F** | **Hypothesis df** | **Error df** | **Significance** |
|---|---|---|---|---|---|---|---|
| **Between subjects** | **Intercept** | Wilks' λ | 0.0 | 1674890931.21 | 2 | 98.0 | $p < 0.001$ |
| | **Genotype** | Wilks' λ | 0.942 | 1.494 | 4 | 196.0 | $p = 0.205$ |
| **Within subjects** | **Time** | Wilks' λ | 0.903 | 2.592 | 4 | 96.0 | $p < 0.05$ |
| | **Time*genotype** | Wilks' λ | 0.830 | 2.337 | 8 | 192.0 | $p < 0.05$ |

**Univariate test results**

| **Source** | **Measure** | **Type III sums of squares** | **df** | **Mean square** | **F** | **Significance** |
|---|---|---|---|---|---|---|

| Time | Protein | Greenhouse-Geisser | 1385.642 | 1.987 | 697.272 | 3.733 | $p < 0.05$ |
|---|---|---|---|---|---|---|---|
| | Carbohydrate | Greenhouse-Geisser | 1540.175 | 1.989 | 774.356 | 4.198 | $p < 0.05$ |
| Time*genotype | Protein | Greenhouse-Geisser | 2909.917 | 3.974 | 732.153 | 3.920 | $p < 0.05$ |
| | Carbohydrate | Greenhouse-Geisser | 2523.155 | 3.978 | 634.285 | 3.438 | $p < 0.05$ |
| Error(time) | Protein | Greenhouse-Geisser | 36743.597 | 196.736 | 186.766 | | |
| | Carbohydrate | Greenhouse-Geisser | 36325.612 | 196.908 | 184.480 | | |

**Post hoc Bonferroni multiple comparisons of factor 'Genotype'**

| Measure | (I) Genotype | (J) Genotype | Mean difference (I-J) | Std. error | Significance | 95% C.I. lower bound | 95% C.I. upper bound |
|---|---|---|---|---|---|---|---|
| Protein | $for^{R}$ | $for^{s}$ | -3.3418 | 2.23863 | $p = 0.416$ | -8.7936 | 2.1100 |
| | | $for^{s2}$ | -0.9287 | 1.99825 | $p = 1.0$ | -5.7952 | 3.9377 |
| | $for^{s}$ | $for^{R}$ | 3.3.418 | 2.23863 | $p = 0.416$ | -2.1100 | 8.7936 |
| | | $for^{s2}$ | 2.4131 | 2.19119 | $p = 0.820$ | -2.9232 | 7.7493 |
| | $for^{s2}$ | $for^{R}$ | 0.9287 | 1.99825 | $p = 1.0$ | -3.9377 | 5.7952 |
| | | $for^{s}$ | -2.4131 | 2.19119 | $p = 0.820$ | -7.7493 | 2.9232 |
| Carbohydrate | $for^{R}$ | $for^{s}$ | 3.3428 | 2.23897 | $p = 0.416$ | -2.1098 | 8.7955 |
| | | $for^{s2}$ | 0.9358 | 1.99855 | $p = 1.0$ | -3.9314 | 5.8029 |
| | $for^{s}$ | $for^{R}$ | -3.3428 | 2.23897 | $p = 0.416$ | -8.7955 | 2.1098 |
| | | $for^{s2}$ | -2.4070 | 2.19152 | $p = 0.824$ | -7.7441 | 2.9301 |
| | $for^{s2}$ | $for^{R}$ | -0.9358 | 1.99855 | $p = 1.0$ | -5.8029 | 3.9314 |
| | | $for^{s}$ | 2.4070 | 2.19152 | $p = 0.824$ | -2.9301 | 7.7441 |

**Table 2.4b Repeated measured MANOVA on protein and carbohydrate ratio selected by larvae of each *foraging* genotype across the 3rd larval stadium in response to pre-experimental rearing on a 1P:1C diet.**

**Multivariate test results**

| Effect | | | Value | F | Hypothesis df | Error df | Significance |
|---|---|---|---|---|---|---|---|
| Between subjects | Intercept | Wilks' λ | 0.0 | 529416659408.6 | 2 | 110.0 | $p < 0.001$ |
| | Genotype | Wilks' λ | 0.870 | 3.972 | 4 | 220.0 | $p < 0.001$ |
| Within subjects | Time | Wilks' λ | 0.851 | 6.346 | 4 | 109.0 | $p < 0.001$ |
| | Time*genotype | Wilks' λ | 0.868 | 2.654 | 6 | 218.8 | $p < 0.05$ |

**Univariate test results**

| Source | Measure | | Type III sums of squares | df | Mean square | F | Significance |
|---|---|---|---|---|---|---|---|
| Time | Protein | Greenhouse-Geisser | 3153.529 | 1.827 | 1726.339 | 9.575 | $p < 0.001$ |
| | Carbohydrate | Greenhouse-Geisser | 3153.297 | 1.827 | 1726.218 | 9.574 | $p < 0.001$ |
| Time*genotype | Protein | Greenhouse-Geisser | 2473.491 | 3.653 | 677.033 | 3.755 | $p < 0.05$ |

| | Carbohydrate | Greenhouse-Geisser | 2473.697 | 3.653 | 677.091 | 3.755 | $p < 0.05$ |
|---|---|---|---|---|---|---|---|
| Error(time) | Protein | Greenhouse-Geisser | 36559.632 | 202.765 | 180.305 | | |
| | Carbohydrate | Greenhouse-Geisser | 36559.749 | 202.765 | 180.306 | | |

**Post hoc Bonferroni multiple comparisons of factor 'Genotype'**

| Measure | (I) Genotype | (J) Genotype | Mean difference (I-J) | Std. error | Significance | 95% C.I. lower bound | 95% C.I. upper bound |
|---|---|---|---|---|---|---|---|
| Protein | $for^R$ | $for^s$ | -8.2656623 | 2.31189190 | $p < 0.05$ | -13.8854160 | -2.6459086 |
| | | $for^{s2}$ | -2.1866548 | 2.32665433 | $p = 1.0$ | -7.8422931 | 3.4689834 |
| | $for^s$ | $for^R$ | 8.2656623 | 2.31189190 | $p < 0.05$ | 2.6459086 | 13.8854160 |
| | | $for^{s2}$ | 6.0790075 | 2.29622877 | $p < 0.05$ | 0.4973278 | 11.6606872 |
| | $for^{s2}$ | $for^R$ | 2.1866548 | 2.32665433 | $p = 1.0$ | -3.4689834 | 7.8422931 |
| | | $for^s$ | -6.0790075 | 2.29622877 | $p < 0.05$ | -11.6606872 | -0.4973278 |
| Carbohydrate | $for^R$ | $for^s$ | 8.2656601 | 2.31186438 | $p < 0.05$ | 2.6459733 | 13.8853469 |
| | | $for^{s2}$ | 2.1863620 | 2.32662663 | $p = 1.0$ | -3.4692089 | 7.8419330 |
| | $for^s$ | $for^R$ | -8.2656601 | 2.31186438 | $p < 0.05$ | -13.8853469 | -2.6459733 |
| | | $for^{s2}$ | -6.0792981 | 2.29620143 | $p < 0.05$ | -11.6609113 | -0.4976848 |
| | $for^{s2}$ | $for^R$ | -2.1863620 | 2.32662663 | $p = 1.0$ | -7.8419330 | 3.4692089 |
| | | $for^s$ | 6.0792981 | 2.29620143 | $p < 0.05$ | 0.4976848 | 11.6609113 |

**Table 2.4c Repeated measured MANOVA on protein and carbohydrate ratio selected by larvae of each *foraging* genotype across the 3rd larval stadium in response to pre-experimental rearing on a 4P:1C diet.**

**Multivariate test results**

| Effect | | | Value | F | Hypothesis df | Error df | Significance |
|---|---|---|---|---|---|---|---|
| Between subjects | Intercept | Wilks' λ | 0.0 | 1884188904.3 | 2 | 113.0 | $p < 0.001$ |
| | Genotype | Wilks' λ | 0.928 | 2.149 | 4 | 226.0 | $p = 0.076$ |
| Within subjects | Time | Wilks' λ | 0.749 | 12.519 | 3 | 112.0 | $p < 0.001$ |
| | Time*genotype | Wilks' λ | 0.883 | 2.397 | 6 | 224.0 | $p < 0.05$ |

**Univariate test results**

| Source | Measure | | Type III sums of squares | df | Mean square | F | Significance |
|---|---|---|---|---|---|---|---|
| Time | Protein | Greenhouse-Geisser | 4058.615 | 1.931 | 2101.797 | 21.167 | $p < 0.001$ |
| | Carbohydrate | Greenhouse-Geisser | 4059.731 | 1.931 | 2102.540 | 21.177 | $p < 0.001$ |
| Time*genotype | Protein | Greenhouse-Geisser | 1256.625 | 3.862 | 325.378 | 3.277 | $p < 0.05$ |
| | Carbohydrate | Greenhouse-Geisser | 1256.199 | 3.862 | 325.294 | 3.276 | $p < 0.05$ |
| Error(time) | Protein | Greenhouse-Geisser | 21858.644 | 220.136 | 99.296 | | |
| | Carbohydrate | Greenhouse-Geisser | 21854.724 | 220.119 | 99.286 | | |

**Post hoc Bonferroni multiple comparisons of factor 'Genotype'**

| Measure | (I) Genotype | (J) Genotype | Mean difference (I-J) | Std. error | Significance | 95% C.I. lower bound | 95% C.I. upper bound |
|---|---|---|---|---|---|---|---|
| Protein | ***for***$^{R}$ | ***for***$^{s}$ | 0.4365116 | 2.36359488 | *p* = 1.0 | -5.3065990 | 6.1796222 |
| | | ***for***$^{s2}$ | -5.2316056 | 2.39410423 | *p* = 0.093 | -11.0488485 | .5856373 |
| | ***for***$^{s}$ | ***for***$^{R}$ | -0.4365116 | 2.36359488 | *p* = 1.0 | -6.1796222 | 5.3065990 |
| | | ***for***$^{s2}$ | -5.6681172 | 2.37928957 | *p* = 0.057 | -11.4493631 | .1131287 |
| | ***for***$^{s2}$ | ***for***$^{R}$ | 5.2316056 | 2.39410423 | *p* = 0.093 | -.5856373 | 11.0488485 |
| | | ***for***$^{s}$ | 5.6681172 | 2.37928957 | *p* = 0.057 | -.1131287 | 11.4493631 |
| Carbohydrate | ***for***$^{R}$ | ***for***$^{s}$ | -0.4316272 | 2.36394942 | *p* = 1.0 | -6.1755993 | 5.3123449 |
| | | ***for***$^{s2}$ | 5.2364885 | 2.39446335 | *p* = 0.092 | -.5816269 | 11.0546040 |
| | ***for***$^{s}$ | ***for***$^{R}$ | 0.4316272 | 2.36394942 | *p* = 1.0 | -5.3123449 | 6.1755993 |
| | | ***for***$^{s2}$ | -5.6681157 | 2.37964647 | *p* = 0.057 | -.1139974 | 11.4502288 |
| | ***for***$^{s2}$ | ***for***$^{R}$ | -5.2364885 | 2.39446335 | *p* = 0.092 | -11.0546040 | .5816269 |
| | | ***for***$^{s}$ | -5.6681157 | 2.37964647 | *p* = 0.057 | -11.4502288 | .1139974 |

**Table 2.5. Repeated measured MANOVA on protein and carbohydrate ratio and caloric content selected by larvae of each *foraging* genotype over 3rd larval stadium following pre-experimental rearing on a 1P:1.5C diet.**

**5a. Multivariate test results**

| Effect | | | Value | F | Hypothesis df | Error df | Significance |
|---|---|---|---|---|---|---|---|
| Between subjects | Intercept | Wilks' λ | 0.071 | 267.961 | 2.0 | 41.0 | *p* < 0.001 |
| | Genotype | Wilks' λ | 0.854 | 1.689 | 4 | 82.0 | *p* = 0.160 |
| Within subjects | Time | Wilks' λ | 0.845 | 1.783 | 4 | 39.0 | *p* = 0.152 |
| | Time*genotype | Wilks' λ | 0.863 | 0.744 | 8 | 78 | *p* = 0.653 |

**5b. Univariate test results**

| Source | Measure | | Type III sums of squares | df | Mean square | F | Significance |
|---|---|---|---|---|---|---|---|
| Time | Protein | Greenhouse-Geisser | 5201.544 | 1.897 | 2742.549 | 3.158 | *p* = 0.05 |
| | Carbohydrate | Greenhouse-Geisser | 9043.685 | 1.980 | 4566.901 | 2.385 | *p* < 0.01 |
| Time*genotype | Protein | Greenhouse-Geisser | 5078.441 | 3.793 | 1338.821 | 1.542 | *p* = 0.201 |
| | Carbohydrate | Greenhouse-Geisser | 8979.166 | 3.961 | 2267.160 | 1.184 | *p* = 0.324 |
| Error(time) | Protein | Greenhouse-Geisser | 69182.821 | 79.658 | 868.503 | | |
| | Carbohydrate | Greenhouse-Geisser | 159281.698 | 83.171 | 1915.106 | | |

**Table 2.6a. Univariate ANOVA comparing body triglycerides of larvae of each *foraging* genotype allowed either to self-regulate protein to carbohydrate intake for either the entire larval period vs. only the 3$^{rd}$ larval stadium.**

| Source | Type III sums of squares | df | Mean square | F | Significance |
|---|---|---|---|---|---|
| Corrected model | 0.340 | 5 | 0.068 | 3.182 | $p = 0.014$ |
| Intercept | 35.409 | 1 | 35.409 | 1655.832 | $p < 0.001$ |
| Genotype | 0.095 | 2 | 0.048 | 2.230 | $p = 0.117$ |
| Genotype*feedingregime | 0.245 | 3 | 0.082 | 3.818 | $p = 0.015$ |
| Error | 1.155 | 54 | 0.021 | | |
| Total | 36.904 | 60 | | | |
| Corrected total | 1.495 | 59 | | | |

**Table 2.6b. Post-hoc multiple ANOVA comparisons between mean body triglycerides from each treatment and *foraging* genotype from the above (Table 6i) analysis. '3$^{rd}$ to pup.' denotes larvae permitted to self-regulate their protein and carbohydrate intake for only the 3$^{rd}$ and final larval stadium, 'entire' denotes larvae allowed to self-regulate their protein and carbohydrate intake for the entire larval period.**

| Comparison | Sums of squares | df | Mean square | F | Significance |
|---|---|---|---|---|---|
| ***for*$^{R}$ 3$^{rd}$ to pup. vs. *for*$^{s}$ 3$^{rd}$ to pup.** | | | | | |
| Between groups | 0.050 | 1 | 0.050 | 1.686 | $p = 0.211$ |
| Within groups | 0.534 | 18 | 0.030 | | |
| **Total** | 0.583 | 19 | | | |
| ***for*$^{R}$ 3$^{rd}$ to pup. vs. *for*$^{s2}$ 3$^{rd}$ to pup.** | | | | | |
| Between groups | 0.230 | 1 | 0.230 | 9.301 | $p < 0.01$ |
| Within groups | 0.445 | 18 | 0.025 | | |
| **Total** | 0.675 | 19 | | | |
| ***for*$^{R}$ 3$^{rd}$ to pup. vs. *for*$^{R}$ entire** | | | | | |
| Between groups | 0.194 | 1 | 0.194 | 9.031 | $p < 0.01$ |
| Within groups | 0.388 | 18 | 0.022 | | |
| **Total** | 0.582 | 19 | | | |
| ***for*$^{R}$ 3$^{rd}$ to pup. vs. *for*$^{s}$ entire** | | | | | |
| Between groups | 0.014 | 1 | 0.014 | 0.453 | $p = 0.509$ |
| Within groups | 0.565 | 18 | 0.031 | | |
| **Total** | 0.580 | 19 | | | |
| ***for*$^{R}$ 3$^{rd}$ to pup. vs. *for*$^{s2}$ entire** | | | | | |
| Between groups | 0.079 | 1 | 0.079 | 3.554 | $p = 0.076$ |
| Within groups | 0.399 | 18 | 0.022 | | |
| **Total** | 0.478 | 19 | | | |
| ***for*$^{s}$ 3$^{rd}$ to pup. vs. *for*$^{s2}$ 3$^{rd}$ to pup.** | | | | | |

| | | | | | |
|---|---|---|---|---|---|
| Between groups | 0.066 | 1 | 0.066 | 3.024 | $p < 0.01$ |
| Within groups | 0.391 | 18 | 0.022 | | |
| **Total** | 0.456 | 19 | | | |
| ***for*$^{s}$ 3$^{rd}$ to pup. vs. *for*$^{R}$ entire** | | | | | |
| Between groups | 0.047 | 1 | 0.047 | 2.557 | $p = 0.127$ |
| Within groups | 0.333 | 18 | 0.018 | | |
| **Total** | 0.380 | 19 | | | |
| ***for*$^{s}$ 3$^{rd}$ to pup. vs. *for*$^{s}$ entire** | | | | | |
| Between groups | 0.011 | 1 | 0.011 | 0.383 | $p = 0.544$ |
| Within groups | 0.511 | 18 | 0.028 | | |
| **Total** | 0.522 | 19 | | | |
| ***for*$^{s}$ 3$^{rd}$ to pup. vs. *for*$^{s2}$ entire** | | | | | |
| Between groups | 0.003 | 1 | 0.003 | 0.171 | $p = 0.684$ |
| Within groups | 0.345 | 18 | 0.019 | | |
| **Total** | 0.348 | 19 | | | |
| ***for*$^{s2}$ 3$^{rd}$ to pup. vs. *for*$^{R}$ entire** | | | | | |
| Between groups | 0.001 | 1 | 0.001 | 0.110 | $p = 0.744$ |
| Within groups | 0.245 | 18 | 0.014 | | |
| **Total** | 0.246 | 19 | | | |
| ***for*$^{s2}$ 3$^{rd}$ to pup. vs. *for*$^{s}$ entire** | | | | | |
| Between groups | 0.130 | 1 | 0.130 | 5.532 | $p < 0.05$ |
| Within groups | 0.423 | 18 | 0.023 | | |
| **Total** | 0.552 | 19 | | | |
| ***for*$^{s2}$ 3$^{rd}$ to pup. vs. *for*$^{s2}$ entire** | | | | | |
| Between groups | 0.040 | 1 | 0.040 | 2.778 | $p = 0.113$ |
| Within groups | 0.256 | 18 | 0.014 | | |
| **Total** | 0.296 | 19 | | | |
| ***for*$^{R}$ entire vs. *for*$^{s}$ entire** | | | | | |
| Between groups | 0.103 | 1 | 0.103 | 5.104 | $p < 0.05$ |
| Within groups | 0.365 | 18 | 0.020 | | |
| **Total** | 0.468 | 19 | | | |
| ***for*$^{R}$ entire vs. *for*$^{s2}$ entire** | | | | | |
| Between groups | 0.026 | 1 | 0.026 | 2.326 | 0.145 |
| Within groups | 0.199 | 18 | 0.011 | | |
| **Total** | 0.224 | 19 | | | |
| ***for*$^{s}$ entire vs. *for*$^{s2}$ entire** | | | | | |
| Between groups | 0.026 | 1 | 0.026 | 1.246 | 0.279 |
| Within groups | 0.377 | 18 | 0.021 | | |

| Total | 0.403 | 19 | | | |
|---|---|---|---|---|---|

**Table 2.7. P:C selection targets displayed by larvae of each *foraging* genotype across each choice experiment. We display the mean P:C selection target displayed by each genetic strain across the 44 h period of each choice experiment. Note that both mean and SD for *for*$^R$ larvae P:C selections are more similar across the choice experiments than *for*$^s$ and *for*$^{s2}$ selections**

| | *for*$^R$ rovers | | *for*$^s$ sitters | | *for*$^{s2}$ sitters | |
|---|---|---|---|---|---|---|
| **Choice experiment** | **Mean P:C** | **s.d. (from C selection)** | **Mean P:C** | **s.d. (from C selection)** | **Mean P:C** | **s.e. (from C selection)** |
| **1P:4P pre-treatment** | 1:1.2 | 0.17 | 1:1.13 | 0.05 | 1:1.32 | 0.34 |
| **1P:1C pre-treatment** | 1:1.2 | 0.017 | 1: 0.9 | 0.25 | 1:1.23 | 0.06 |
| **4P:1C pre-treatment** | 1:1.2 | 0.12 | 1:1.22 | 0.25 | 1:1.1 | 0.4 |
| **1P:1.5C pre-treatment** | 1:1.6 | 0 | 1:1.5 | 0.1 | 1:1.46 | 0.05 |

# Chapter 3.

## The interaction of *foraging* gene polymorphism with nutritional environments unleashes two modes of continuous phenotypic plasticity in *Drosophila melanogaster*

## Abstract

This study shows that naturally occurring allelic variation in a single gene, the *foraging* gene, generates alternate patterns of phenotypic plasticity between *Drosophila melanogaster* individuals raised in common nutritional environments. Our results indicate that the *foraging* gene can function as a 'plasticity gene' in *D. melanogaster* populations, allowing populations comprised of different allelic variants to express variable phenotypes, which may, 1) facilitate greater performance for populations in both realised environments and, 2) generate exaptations that can enhance population 'evolvability' under nutritional environment shifts. We used the Geometric Framework to both design our experimental rearing diets, and to analyse inter-genotypic trait expression. Phenotypes we compared between the *foraging* allelic variants, $for^R$, $for^s$ and $for^{s2}$ were: adult fly wing size and shape, which we quantified using geometric morphometrics, and developmental time and survivorship to eclosion. We found that for all genotypes analysed, trait performance typically increased with increase in dietary protein, but even in 'best' and 'worst' food environments, inter-genotypic performance of a given trait was rarely equivalent. So while trait expression was sensitive to the environment, showing graded responses, the absolute magnitude of performance achievable was genotype-dependent.

## Introduction

Holometabolous insect larvae face a dual nutritional challenge. Sufficient calories must be consumed throughout larval development to fuel growth and the metabolically-demanding process of metamorphosis (Sewell *et al.*, 1975; Merkey *et al.*, 2011). Larvae are also challenged with the acquisition of an appropriate complement and quantity of nutrients to produce an optimal adult body (Robertson, 1936; Bodenstein, 1950; Bainbridge & Bownes, 1981; Aguila *et al.*, 2013), the morphology of which is virtually fixed upon eclosion. The lack of opportunity for post-eclosion modifications to the adult form means that larval feeding and post-ingestive processing of nutrients have direct consequences for many adult-fitness relevant larval life history traits and subsequent adult phenotypes (House, 1967; Stockhoff, 1991; Gotthard, *et al.*, 1994; Leclaire & Brandl, 1994; Emlen, 1994; Foley & Luckinbill, 2001; Burns *et al.*, 2012; Aguila *et al.*, 2013; Boggs, 2009). Significant selection pressure is therefore exerted on the larval stage of holometabolous insects,

with evolution working to promote and integrate feeding behaviour and metabolic physiology for enhanced adult fitness (Stearns & Koella, 1986; Nylin & Gotthard, 1998).

The capacity of individual larvae to develop an optimal suite of adult phenotypes will be determined by the interaction of the genes (and epigenetic markers) controlling feeding and metabolism with the nutritional environments encountered by larvae during development (Nylin & Gotthard, 1998; Pigliucci, 2001; Sgrò & Hoffmann, 2004; Burns *et al*., 2012). Across a species distribution, there may exist considerable spatial and temporal variation in the quality and character of nutritional environments encountered by individuals and populations. It follows that there must be sufficient capacity within and between populations to tolerate and respond plastically to such environmental variability (Via & Lande, 1985; Via & Lande, 1987; Vieira *et al*., 2000). Given the importance of optimal larval nutrition to subsequent adult performance, one might predict holometabolous insect species have evolved specialised adaptations for mitigating potential costs of environmental variability encountered between and within larval nutritional environments (Jones & Probert, 1980; Hedrick, 1986; Nylin & Gotthard, 1998; Pigliucci, 2001; Burns *et al*., 2012).

One such adaptation is hypothesised to exist at the genetic level in the model species *Drosophila melanogaster* (Sokolowski, 1985; Rodriguez *et al*., 1992; Sokolowski *et al*., 1997; Fitzpatrick *et al*., 2007; Kaun *et al*., 2007a; Kaun & Sokolowski, 2009). Natural populations of *D. melanogaster* are comprised of individuals that possess either of two allelic variants of the *foraging* gene, $for^{R}$ 'rovers' and $for^{s}$ 'sitters' (de Belle & Sokolwoski, 1989; de Belle *et al*., 1989; Sokolowski, 2001). These genetic strains differ in the behavioural and physiological phenotypes they express in response to characteristics of the prevailing nutritional environment.

During feeding on yeast paste, $for^{R}$ larvae consistently move more and display greater levels of between food-patch foraging than do their $for^{s}$ counterparts (rovers *rove*). When the food environment is changed in the extreme to an absence of food, sitter larvae begin to rove, presumably 'searching' for food (Sokolowski, 1980; Sokolowski, 2001). In adult flies, rovers have a higher tendency to leave patches of

food than do sitters, which show low levels of this 'exploratory' behaviour (Pererira & Sokolowski, 1993). However, when exposed to chronic food deprivation as larvae, adult sitters shift to display rover-like between-patch exploration behaviour (Burns *et al*., 2012), improving their food source encounter rates (Morris & Kareiva, 1990). Larval rovers and sitters also differ in their use of odour cues to help locate food sources. Under classical conditioning, rover larvae more readily learn and remember olfactory cues associated with food sources than do larval sitters (Kaun, *et al*., 2007b).

Inter-genotypic differences are not confined to movement and olfactory responses to the food environment. Larval *for*$^{R}$ rovers have higher nutrient absorption efficiencies than sitters, and are able to adjust the quantity of food they ingest according to its energy content (i.e. demonstrate compensatory feeding) (Kaun, *et al*., 2007a). In response to the developmental threat imposed by a drop in the energy density of food, *for*$^{R}$ larvae react by increasing total food intake – thus maintaining developmental rate. Concurrent with this adjustment is a drop in rover *foraging* gene expression. Sitter larvae do not alter feeding rate or *foraging* expression when available food energy density decreases. Together with their lower nutrient absorption rates, sitters take longer than rovers to reach pupation and experience lower survivorship in these energy-reduced food environments as a consequence (Kaun *et al*, 2007a).

Mediation of rover *vs*. sitter differences is by a cyclic guanosine monosphosphate (cGMP)-dependent protein kinase (PKG), encoded by the *foraging* gene (Osborne *et al*., 1997). *for*$^{R}$ larvae have higher PKG levels circulating in their nervous system than *for*$^{s}$ larvae (Osborne *et al*., 1997; Kaun *et al*., 2007a) and adult *for*$^{R}$ have higher brain PKG levels than *for*$^{s}$ flies (Osborne *et al*., 1997; Belay *et al*., 2007). Rover behavioural phenotypes can be induced in natural sitter larvae through experimental manipulation of PKG levels (Osborne *et al*., 1997; Belay *et al*., 2007; Kaun *et al*., 2007a). These patterns of intrinsically-different responses to nutritional environments due to *foraging* genetic strain, controlled by rover/sitter expression level differences in PKG, provide compelling evidence that *foraging* is a 'gene for plasticity' (Schlichting & Pigliucci, 1993; Pigliucci, 2001).

In this study, we ask, 'is *foraging* a plasticity gene?'. Plasticity genes are theorised to provide alternative adaptive phenotypic plasticity in response to

environmental stressors through population-level allelic variation (Schlichting & Pigliucci, 1993; Pigliucci, 2001; Scheiner, 1993; Via & Lande, 1985; Via *et al*., 1995; Kent *et al*., 2009). Each genotype of a plasticity gene is thought to contribute to higher net population performance through increasing niche breadth (Via & Lande, 1985; Lynch & Gabriel, 1987; Lande & Shannon, 1996) by providing alternative phenotypic and/or trait specialisations in response to the stressor. Although plasticity genes are much theorised over (Schlichting & Pigliucci, 1993; Pigliucci, 2001; Scheiner, 1993, 1998; Via *et al*., 1995; Zhivotovsky *et al*., 1996; Wu, 1998), there are few empirical demonstrations of their existence (Meier & Leuschner, 2008; Shimizu *et al*., 2010), especially in animals. Here, through comprehensive gene-by-environment interaction analyses (GEI) (Via & Lande, 1985; West-Eberhard, 1989) we provide a cogent assessment of the role natural allelic variation in *foraging* plays in maximising adult *D. melanogaster* performance under a range of larval nutritional environments via alternate genotype-dependent phenotypic plasticity.

Using the Geometric Framework, a state-space modeling framework for studies of nutrition (Simpson & Raubenheimer, 2012), we systematically explore the phenotypic reaction norms to three key nutritional dimensions: protein, carbohydrate and caloric density. Unlike using typical 1D reaction norms, this provides a multidimensional assessment, allowing the main and interactive effects of protein, carbohydrates and calories to be quantified. Integrated with our assessment of adaptive phenotypic plasticity across each *foraging* genotype are concurrent comparisons of inter-genotypic larval life history trait performance and developmental instability. We consider multiple means through which *foraging* may act as a plasticity gene. For example, as hypothesised by Via *et al*. (1995) one genotype may deliver the mean phenotype under frequently-encountered nutritional environments (over evolutionary time), while another delivers maximal phenotype performance under extremes (Via *et al*, 1995) (see Figure 1 for further hypothetical scenarios considered). Through this multivariate approach we hope to characterise any differences in performance, and therefore potential adaptive differences, between *foraging* genotypes in response to heterogeneity of larval nutrition.

*Response variables*

Across *foraging* genotypes we measure and compare survivorship to eclosion and developmental rate. Beyond life history, we use geometric morphometric techniques to quantify and compare inter-genotypic differences in adult fly wing size. Wings are particularly useful for comparing adult performance. Wing quality influences flight to food sources, to prospective mates, from predators, and, in females flies, to oviposition sites (Brodskiĭ, 1994). For males, wing size influences the quality of courtship song delivered to females (Partridge & Farquhar, 1983) and male body size, which scales allometrically with wing size (Shingleton, *et al*., 2007), is positively correlated with male mating success (Partridge & Farquhar, 1983; Partridge *et al*., 1987). Indeed, taken with developmental rate data, this allometric scaling relationship allows wing size comparisons to be indicative of how efficiently each *foraging* genotype converts dietary nutrients to growth.

We also compare wing shape variability across *foraging* genotypes using geometric morphometrics. Measuring the fidelity with which a genotype can generate a fitness-relevant phenotype within and between environments enables inter-genotype performance analysis. Stabilising selection maintains that fitness relevant traits will display canalised reaction norms across environmental gradients (Schmalhausen, 1949; Falconer, 1990; Stearns & Kawecki, 1994). However, less 'memory' or inherited instruction for how phenotype development should proceed persists in a species' genome (or epigenome) for infrequently encountered, and therefore stressful, environments (Danchin, 2013). This 'genomic naivety' diminishes the capacity for stable, optimal phenotype development in novel environments. The reliability with which individuals of a shared genotype can produce the same phenotype following development in a common environment can be quantified as the phenotypic variance (phenodeviance) and is termed developmental instability (Campbell *et al*., 1998). Here, in response to varying larval nutritional environments, we measure developmental instability of wing shape between *foraging* genotypes. We demonstrate whether *foraging* genotypes show differential susceptibility or resistance to developmental stress across multidimensional nutritional gradients. We further discuss concepts in phenotypic plasticity relevant to the response variables used in this study in Box 1.

*Experimental nutritional environment heterogeneity*

We systematically sampled larval dietary macronutrient composition using the Geometric Framework for nutrition (GF). The GF employs the concept of nutrient space, an *n*-dimensional Cartesian geometric space in which foods are represented as vectors (rails) radiating from the origin (Simpson and Raubenheimer, 1995, 2012; Simpson *et al*., 2004), where the angle of individual food rails to the axes is determined by the relative concentrations of nutrients the food contains. Here we formulate a nutrient space delimited by increasing dietary concentrations of protein and carbohydrate, the two energy-yielding macronutrients most relevant to growing herbivorous insect larvae (Behmer, 2009). Response variables are then plotted as topological surfaces, mapped onto diet composition-based nutrient space, enabling identification of performance maxima and minima (Simpson *et al*., 2004). Nutritional geometry response surfaces are analogous to fitness landscapes (Wright, 1988; Orr, 2005), but with mapping of fitness or performance being in response to graded environmental variation, rather than inherent genetic variation. Diet regions of nutrient space wherein performance maxima co-localise are interpreted as 'optimal' diets, – as 'calibrated' by natural selection (Simpson *et al*., 2004).

## Materials and Methods

*Fly stocks*

We used three separate *foraging* genotypic strains in this study: natural rover (*for*$^{R}$), natural sitter (*for*$^{s}$) and a sitter mutant (*for*$^{s2}$). All strains share a common isogenic third chromosome from *for*$^{R}$ and a common X chromosome. Genetic variation on the small fourth and Y chromosomes was not controlled (Fitzpatrick *et al*., 2007); de Belle & Sokolowski, 1987; de Belle *et al.,* 1989; Pereira & Sokolowski, 1993). The sitter mutant *for*$^{s2}$ strain was generated on a *for*$^{R}$ rover genetic background using gamma radiation (de Belle *et al*., 1993; Pereira & Sokolowski, 1993). *for*$^{s2}$ larval and adult flies display behaviour and PKG expression levels that do not differ from those of natural *for*$^{s}$ sitter larvae and adults (Osborne *et al*., 1997; Belay *et al*., 2007; Fitzpatrick *et al*, 2007; Kaun *et al*., 2007a; Kaun *et al*., 2007b; Kaun *et al*., 2008). Historically, given its rover genetic background yet sitter phenotypes, *for*$^{s2}$ has been used as a control strain for the involvement of *foraging*-dependent PKG in

phenotype mediation. It is now known that the DNA sequences of *for*$^{s}$ and *for*$^{s2}$ differ at a number of sites (A. Allen & M. B. Sokolowski, unpublished data). Notwithstanding this, we have included *for*$^{s2}$ in our experiments to facilitate inter-study comparison, and to model the contribution to species-level phenotypic plasticity made by naturally arising mutant allelic variants in the wild.

All strains were maintained prior to experiments for hundreds of generations on a standard laboratory diet comprising 50 g of dry yeast, 100 g sucrose, and 16 g agar per L of distilled water. The culture diet and subsequent experimental diets also contained 8 g of $KNaC_4H_4O_6$, 1 g of $KH_2PO_4$, 0.5 g of $CaCl_2$, 0.5 g of $MgCl_2$, 0.5 g of $Fe_2(SO_4)_3$ and 5 mL of propionic acid per L of distilled water. All culture rearing and experiments were conducted at 25°C, under a 12L:12D light cycle, with lights on at 08:00 h.

*Experimental diets and rearing protocol*

Larvae from each genotype were sourced from three replicate parent vials containing approximately 100 male and 100 female adult flies that were ~ five days post-eclosion. Parent flies were left to mate and females to oviposit in fresh vials covered with a Petri-dish containing oviposition media topped with ~ 0.5 g fresh yeast paste. Oviposition medium was 1.8 g agar, 50 mL distilled $H_2O$ and 45 mL dilute grape juice. The media mix was boiled then allowed to cool and set. Oviposition media and yeast were replaced in each vial after 24 h. After another 12 h all visible hatchlings were removed from each Petri dish. After an additional 3 h, larvae were introduced to the experiment. This process ensured all experimental larvae were within 6 h post-hatch.

Larvae were reared from egg hatch to pupation on one of 24 diets varying in protein to carbohydrate ratio (P:C) and total energy density, providing a comprehensive sampling across P-C nutrient space. Diet P:C ratios were 1:1, 1:2, 1:4, 1:9, 1:16, 2:1, 1:16, 2:1, 4:1 and 7:1, and each ratio was formulated at three energy densities: 75, 150 and 300 $g{\cdot}Kg^{-1}$ of P+C. Diet macronutrient sources were: thermolysed baker's yeast (containing 47.4% P, 24% digestible C, Lowan Whole Foods), sucrose, and casein (95.7% P, 0.8% C, Sigma-Aldrich). Agar (93.9% C) was used to gel diets (comprising < 2 $g{\cdot}kg^{-1}$ of any experimental diet) and, following Lee

*et al.* (2008), distilled water was used to dilute P:C mixes to the required energy density.

Next, 1 mL of each diet was allocated to a separate well in a sterile, 24-well tissue culture plate (Falcon # 08-772-1). Once set, the surface of each diet was scored with a flame-sterilised probe to facilitate larval feeding, after which a single larva was introduced. Larvae were enclosed in individual wells by 1.5 x 2.5 cm, snuggly-fitting, cylindrical foam caps. We raised larvae individually as they can cannibalise one another (Vijendravarma, *et al.,* 2013). Diets were maintained at close to 100% relative humidity by placing each well plate into a 15 x 20 cm plastic container fitted with a perforated lid. A total of 12 plates was set up per genotype, resulting in 288 larvae of each genotype entering the experiment.

Development was checked daily for all 864 larvae from day 3. Survivorship and total days to eclosion were recorded. For ease of visual representation, we plotted the inverse number of days to eclosion, calculated as 1 over the number of days.

*Wing morphology: designation of landmarks and generalised orthogonal Procrustes superimposition*

We used geometric morphometric techniques to compare wing size and the variability of wing shape for female and male flies of each genotype across nutrient space. Briefly, the technique relies on designation of ontogenetically-homologous morphological landmarks (Patterson, 1982) on digital photographs of specimens. Landmarks chosen must allow for one to one morphological landmark correspondence between all specimens subject to analysis (Klingenberg, 2011). Generalised orthogonal Procrustes superimposition (GPA) is then performed on the set of landmarks for all specimens. This procedure removes variation in size, position and orientation from the landmark data arising from specimens, preserving only shape information (Dryden & Mardia, 1998; Klingenberg, 2011). GPA results in the calculation of $x$,$y$ Procrustes tangent space coordinates (Procrustes coordinates) in shape space (Drydan & Mardia, 1998). Procrustes coordinates and/or specimen-specific variables derived from them can then be subjected to multi- or univariate analyses, enabling cogent inter-specimen comparisons (see Drydan & Mardia, 1998 for detailed explanation of the methodology).

In preparation for geometric morphometrics, all 558 flies that successfully eclosed had their left and right wings removed and wet-mounted under a coverslip on a microscope slide. Digital photographs of each wing were taken using an Olympus DP70 digital camera mounted on a Leica MZ8 stereo light microscope under 5.0 x magnification. Following Klingenberg & Zaklan (2000) we designated 12 landmarks located at the intersections of *D. melanogaster* wing veins (Figure 2). Landmarks were digitised using tpsDig2 software (ver. 2.16) (Rohlf, 2010).

We used MorphoJ (ver. 1.05) (Klingenberg, 2011) to conduct GPA on landmark data. Prior to further intra- and inter-genotypic comparisons via GPA-derived variables, we used tpsSmall software (ver. 1.20) (Rohlf, 2003) to certify that shape variation between our specimens (as calculated by GPA) was small enough to accept a linear tangent space approximation of non-linear Kendall's shape space (Drydan & Mardia, 1998; Rohlf *et al*., 1996). MorphoJ was then used to extract specimen partial warp scores and centroid size from the GPA. The Geomorph package (ver. 1.1-3) (Adams & Otarola-Castillo, 2013) in R (ver. 3.0.2) was next used to extract the Reinmanian distance, *Rho* for each wing specimen. We used these variables in our morphological analyses in the several ways as detailed below.

*Centroid size: computing wing size*

Centroid size for each specimen in a data set is the square root of the sum of squared Euclidean distances from each landmark to the specimen's landmark centroid, or centre of gravity (Drydan & Mardia, 1998; Slice *et al*., 1996). We used the centroid size of both left and right wings from each individual fly in our experiment to compare wing size, and therefore body size, between flies of each genotype across nutrient space. Due to *D. melanogaster* sexual size dimorphism, we analysed female and male wings separately.

*Partial warp scores: computation of relative warps to explore contribution of genotype and sex to wing shape variation*

The partial warp scores of a specimen represent the shape of that individual specimen relative to the mean shape calculated from all specimens in the data set (Bookstein, 1991; Rohlf *et al*., 1996; Drydan & Mardia, 1998). They are calculated from Procrustes tangent coordinates during GPA and are defined as the rotation of the

Procrustes residuals around the Procrustes mean configuration; *i.e.* they are values that represent the location of each specimen in the space of the entire data set's partial warps (Bookstein, 1991; Rohlf *et al*., 1996). Using MorphoJ, we conducted a principal components analysis (PCA) on all specimen partial warp scores to compute relative warp scores. Relative warp scores describe the main modes of variation in shape (Dryden & Mardia, 1998) and are synonymous with principal components (PCs) of shape (Bookstein, 1991; Dryden & Mardia, 1998), thus we shall refer to them as PCs. Using PCA we explored the contribution of the factors '*foraging* genotype' and 'fly sex' to wing shape variation. We formally characterised the contribution these factors made to shape variation by conducting MANOVA in SPSS (ver. 21) on specimen Procrustes coordinates using Type II sums of squares, testing first for a main effect of genotype and then of sex.

*Reinmannian shape distance, Rho: comparing within and between genotype developmental instability (wing shape variability)*

We calculated *Rho*, the Reinmannian shape distance for each right-hand-side fly wing in our data set. *Rho* is the shape distance of each wing specimen's Procrustes tangent coordinate configuration to the mean shape configuration (Kendall, 1984). We used *Rho* scores to measure of how variable fly wing shape was across our data set in a nested fashion according to the specimen's genetic strain, sex and rearing diet. This was done by calculating the standard deviation (SD) of the mean *Rho* score for all fly wings of the same sex and genotype raised on a common diet. For example, all the male rover flies from the 1:4 P:C, 150 g P+C.$Kg^{-1}$ diet treatment group had *Rho* scores for their right wings calculated. As a measure of how variable wing shape for rovers in this food environment was, the SD of *Rho* scores for this treatment group were calculated.

*Trait and phenotype response surface visualisation: thin plate splines*

Trait and phenotypic performance response surfaces (or phenotypic reaction norms –Box 1) were plotted for survivorship to eclosion, developmental rate and wing size. Trait-based surfaces were fitted onto P-C nutrient space using the fields package (ver. 6.8) in R (ver. 3.0.2). Fields facilitates data visualisation by fitting 2D *x*, *y*, *z* (protein, carbohydrate, response variable) response surfaces as thin plate splines

(TPSs). Fields TPSs display points of equal *z* value as the same colour, and connect regions of equivalent value with isolines. Smoothness of the surfaces, *i.e.* the fidelity with which the experimenter allows the TPS to show data variation across nutrient space, can be determined by varying λ (lambda), the TPS tuning parameter. For consistency of interpretation, when creating TPS for each phenotype, we maintained the same λ. For example, all TPS response surfaces for development rate had $\lambda = 0.01$. While statistical comparisons between males and females were not made, to allow for visual comparisons we plotted TPS surfaces for males and females on the same scale for each phenotype.

*Trait and phenotype response surface statistical analysis: generalised linear modeling*

Phenotype response surfaces were statistically analysed using a Lande & Arnold generalised linear modeling (GLM) approach (following Lee *et al.,* 2008 and Dussutour *et al.,* 2010). This approach allows for positive and negative influences of the linear terms, linear term cross products, and *n*th order polynomial terms of a model to be statistically analysed. Terms were protein (*x*) and carbohydrate (*y*). We built our models using SPSS (ver. 21) and used a Taylor series function to predict the shape of our trait response surfaces. We used a backwards entry model building approach whereby all terms were forcibly entered into the first iteration of the model. Non-significant terms were removed from successive iterations of the model in sequence of their non-significant contribution to fit. Model iterations were ceased once an optimal fit was achieved. Inter-genotype differences in response surface shape were tested by incorporating genotype as a factor. The probability of our terms significantly contributing to the shape of phenotype response surface was calculated using maximum likelihood. If a model showed that response variable surfaces differed due to genotype, we built three comparative pairwise models to determine which genotypes differed. These were $for^{R}$ *vs.* $for^{s}$, $for^{R}$ *vs.* $for^{s2}$, and $for^{s}$ *vs.* $for^{s2}$. The relative positive or negative contribution of each model term to the surface was evaluated through the term's β coefficient and through *p*. We established α of 0.05. Here we report the best-fitting version of each model.

*Developmental instability (wing shape variability) response surface visualisation and interpretation*

Significant generalised linear models could not be fitted for SD of *Rho* scores, our measure of developmental instability. Following the method described above for phenotype response surface visualisation, we fitted wing shape variability TPS response surfaces across nutrient space for each genotype as above. To make intra- and inter-genotype comparisons, we interpreted raw results as shown on TPS response surfaces.

## Results

*Survivorship to eclosion*

Generalised linear modeling (GLM) showed there was no significance difference between *foraging* genotypes in the percentage of individuals that survived to eclosion across diet composition space (*genotype*, Wald $\chi^2 = 1.272$, d.f. = 2, $p = 0.529$, Table 1), with thin plate spline (TPS) response surfaces showing all *foraging* genotypes displayed a survivorship maximum in the mid-energy 1P:1C region of nutrient space. These maxima extend outwards as broad performance plateaus across nutrient space for each genotype as dietary protein increases (Figure 3). Visual comparison between each TPS showed that while larvae of all *foraging* strains displayed reduced survivorship on high energy, high carbohydrate diets, natural sitter $for^{s}$ individuals suffered up to 10% lower survival than $for^{R}$ or $for^{s2}$ flies (Figure 3). GLM showed that the quadratic term of carbohydrate significantly contributed to response surface shape ($carbohydrate^2$, Wald $\chi^2 = 2.295$, d.f. = 1, $p = 0.038$, Table 1), with a negative β coefficient sign for $carbohydrate^2$, demonstrating that high dietary carbohydrate was associated with lower survivorship for all *foraging* genotypes (Table 1).

*Developmental rate*

*Females*

Females of $for^{R}$, $for^{s}$ and $for^{s2}$ did not differ significantly in their nutrition-dependent developmental rate (Figure 4, A-C). GLM analysis of female developmental rate across *foraging* genotypes showed genotype did not significantly

contribute to the shape of the response surfaces (*genotype* $\chi^2 = 2.063$, d.f. = 2, $p = 0.356$, Table 2a). Diets offering approximately 1P:1C to 4P:1C led to a broad, shared performance maximum of 9 days to reach eclosion for all genotypes (Figure 4, A-C). GLM showed the cross product of dietary protein and carbohydrate (*protein∗carbohydrate* $\chi^2 = 29.586$, d.f. = 1, $p < 0.001$, Table 2a) and the squared term of dietary carbohydrate (*carbohydrate*$^2$, Wald $\chi^2 = 149.881$, d.f. = 1, $p = 0.001$, Table 2a) significantly contributed to the shape of response surfaces for female developmental rate. The positive sign of the β coefficient for *protein∗carbohydrate* (Table 2a) indicates that as dietary protein and carbohydrate simultaneously increased, development rate increased, while the negative β coefficient sign for *carbohydrate*$^2$ (Table 2a) indicates high dietary carbohydrate decreased developmental rate. Indeed females of all *foraging* genotypes raised in very low P:C, high calorie margins of nutrient space took up to 15 days to reach eclosion, with *for*$^s$ showing the slowest development (Figure 4, A-C).

*Males*

Despite *foraging* genotypes sharing a broad performance maximum over the mid-energy 1P:1C region of nutrient space, within which eclosion was reached in ≤ 10 days, *foraging* genotypes significantly differed in the shape of their response surfaces for male developmental rate (*genotype*, Wald $\chi^2 = 10.293$, d.f. = 2, $p = 0.006$, Table 2b, Figure 4, D-F). Subsequent inter-genotype comparative GLM showed that *for*$^s$ males differed significantly in developmental rate compared to *for*$^R$ and *for*$^{s2}$ males (*for*$^R$ *vs. for*$^s$ *genotype*, Wald $\chi^2 = 6.224$, d.f. = 1, $p = 0.013$, Table 2c; *for*$^s$ *vs. for*$^{s2}$ *genotype*, Wald $\chi^2 = 6.766$, d.f. = 1, $p = 0.009$, Table 2e) while *for*$^R$ and *for*$^{s2}$ males took an equivalent number of days to achieve eclosion (*for*$^R$ *vs. for*$^{s2}$ *genotype,* Wald $\chi^2 = 0.02$, d.f. = 1, $p = 0.879$, Table 2d). TPS response surfaces (Figure 4, D-F) showed *for*$^s$ males developed more slowly than *for*$^R$ and *for*$^{s2}$ males across 50% of nutrient space from the 1P:1C to the 7P:1C diet rail. However, for nutrient space from the 1P:1C rail to the high carbohydrate 1P:16C rail, *for*$^s$ males maintained faster development than shown by *for*$^R$ and *for*$^{s2}$ males (Figure 4, D-F). These patterns cause the *for*$^s$ response surface to be lower and flatter.

The *carbohydrate*$^2$ term significantly contributed to surface shape for all *foraging* genotypes (*carbohydrate*$^2$, Wald $\chi^2 = 47.082$, d.f. = 1, $p < 0.001$, Table 2b)

with a negative β coefficient for *carbohydrate*$^2$ indicating high carbohydrate was associated with an decrease in developmental rate for males. Comparing the β coefficient value of *carbohydrate*$^2$ across *for*$^R$ *vs. for*$^s$, *for*$^R$ *vs. for*$^{s2}$ and *for*$^s$ *vs. for*$^{s2}$ GLMs showed models featuring *for*$^s$ have relatively less negative c*arbohydrate*$^2$ β coefficient values (Tables 2c-e), supporting the TPS surface trend of *for*$^s$ males taking relatively less time to reach eclosion on very high carbohydrate food (Figure 4, D-F).

Linear increases in dietary protein concentration were associated with faster development for males of all genotypes (*protein*, Wald $\chi^2$ = 20.661, d.f. = 1, $p <$ 0.001, positive β coefficient, Table 2b), while very high dietary protein was significantly associated with slower male development (*protein*$^2$*,* Wald $\chi^2$ = 9.267, d.f. = 1, $p <$ 0.001, negative β coefficient, Table 2b) (Figure 4, D-F). The TPS surfaces also show this pattern. Developmental rate for all males increased as dietary protein increased to 140 g.kg$^{-1}$ along the 1P:2C, 1P:1C, 2P:1C and 4P:1C diet rail regions of nutrient space; however, concentrations of dietary protein above 4P:1C and between 140 g.kg$^{-1}$ and 200 g.kg$^{-1}$ were associated with decreases in development to eclosion. Higher energy diets along the 2P:1C, 4P:1C and 7P:1C food rails, which supply more than 200 g.kg$^{-1}$, are associated with a return to a maximal developmental rate for males of all genotypes (Figure 4, D-F). A TPS surface trend whereby *for*$^s$ genotype males showed relatively slower development in response to high dietary protein was validated by inter-genotype GLMs, which featured *for*$^s$ showing more negative *protein*$^2$ β coefficient values (Tables 2c-e).

*Wing size*

*Females*

Generalised linear modeling showed response surfaces for female wing size differed significantly according to *foraging* genotype (*genotype*, Wald $\chi^2$ = 60.615, d.f. = 2, $p <$ 0.001, Table 3a). Between genotype GLM showed all female wing size response surfaces (Figure 5, A-C) significantly differed in their relief, with relative β coefficient values for *genotype* across models showing *for*$^s$ females had the largest wings across all nutrient environments, followed by *for*$^{s2}$ and then *for*$^R$ (*for*$^R$ *vs. for*$^s$ *genotype*, Wald $\chi^2$ = 59.516, d.f. = 1, $p <$ 0.001, Table 3b; *for*$^R$ *vs. for*$^{s2}$ *genotype*, Wald $\chi^2$ = 12.373, d.f. = 1, $p <$ 0.001, Table 3c; *for*$^s$ *vs. for*$^{s2}$ *genotype*, $\chi^2$ = 19.507, d.f. = 1, $p <$ 0.001, Table 3d). The linear term for carbohydrate was significantly

associated with a decrease in female wing size for all genotypes (*carbohydrate*, Wald $\chi^2 = 7.682$, d.f. = 1, $p = 0.006$, negative β coefficient, Table 3a). TPS surfaces show that for all genotypes, female wing size decreased as dietary carbohydrate increased (Figure 5, A-C). TPS surfaces clearly demonstrate the size differences between females of each genotype. The $for^{s}$ surface grades from orange in high energy, high carbohydrate areas of nutrient space progressively toward deep red (larger wings) as relative concentration of dietary protein increases and carbohydrate decreases (Figure 5, B). The $for^{s2}$ surface is a similar shape to the $for^{s}$, showing a common trend where the very highest energy, highest P:C diets support the growth of the largest wings. However, the $for^{s2}$ surface height is approximately 0.1 units lower than the $for^{s}$ surface (Figure 5, B *vs.* C). The $for^{R}$ surface (Figure 5, A), which is another 0.1 units lower than the $for^{s2}$ surface, shows the same decrease in elevation associated with increase in dietary carbohydrate. Unlike the other *foraging* genotypes, $for^{R}$ females develop the largest wings when flies are raised in lower energy, balanced P:C diets, not on the highest protein diets. $for^{s2}$ females also show a small peak in wing size in the same low-energy-balanced P:C region of nutrient space (*cf.* Figure 5, A & C).

*Males*

Wing size response surfaces of males from each *foraging* genotype significantly differed in shape and relief (*genotype*, Wald $\chi^2 = 36.671$, d.f. = 2, $p < 0.001$, Table 3e). As shown for females, $for^{s}$ males had the largest wings overall followed by $for^{s2}$ and then $for^{R}$, as indicated by *genotype* β coefficient signs of comparative GLMs (Tables 3f-3h). The shape of the male wing size response surfaces for the two natural *foraging* genotypes $for^{R}$ and $for^{s}$ were similar to those of females. Wing size in these males also decreased with dietary carbohydrate increase. This pattern is evident on the $for^{R}$ and $for^{s}$ TPS surfaces as areas of deep blue (small wings) overlaying high carbohydrate regions of nutrient space (Figure 5, D & E). $for^{R}$ *vs.* $for^{s}$ GLM supported this, showing the *carbohydrate*$^2$ term significantly associated with smaller wings (*carbohydrate*$^2$, Wald $\chi^2 = 9.963$, d.f. = 1, $p = 0.002$, negative β coefficient, Table 3f). $for^{R}$ and $for^{s}$ GLM also showed male wing size was statistically associated with a combined increase in protein and carbohydrate (*protein∗carbohydrate*, Wald $\chi^2 = 20.340$, d.f. = 1, $p < 0.001$, positive β coefficient, Table 3f). The combination of the low positive value ($8.664 \times 10^{-6}$) of the

*protein*∗*carbohydrate* β coefficient, with the distinct flatness of both the *for*$^R$ and *for*$^s$ wing size TPS surfaces indicates that the simultaneous increase in dietary protein and carbohydrate has only a weak positive effect on wing size – with *for*$^s$ sitters affected more. Wing size only varied between 1.72-1.86 units of centroid size for *for*$^R$ males across all nutrient space (Figure 5, D) while in sitter males, wing size rose from 1.9-2.1 units of centroid size from the 1P:2C to the 7P:1C diet rail (Figure 5, E).

In contrast to the natural *foraging* genotypes, *for*$^{s2}$ males developed larger wings as dietary carbohydrate concentration increased. Higher protein diets were associated with progressively smaller *for*$^{s2}$ wings (Figure 5, F). *for*$^{s2}$ GLM showed linear increase in carbohydrate was significantly associated with larger wing size (Wald $\chi^2 = 5.789$, d.f. = 1, $p < 0.016$, positive β coefficient, Table 3i). As shown for *for*$^R$ and *for*$^s$ males, the *for*$^{s2}$ response surface is distinctly flat (units of centroid size ranging from 1.84–2.06) and the cross product of protein and carbohydrate significantly contributed to wing size response surface shape (*protein*∗*carbohydrate*, Wald $\chi^2 = 9.106$, d.f. = 1, $p < 0.003$, positive β coefficient, Table 3i).

*Wing shape variation*

*Linearisation of shape space - TpsSmall results*

TpsSmall (ver. 1.03) (Rohlf, 2003) verified that the tangent plane projection did not significantly distort distances among the digitised *Drosophila* wing specimens (Pearson product–moment correlation for both *x* and *y* axes = 1.0; slope of relationship between tangent space *vs.* Procrustes distances > 0.999). The mean and maximum Procrustes shape distances to the consensus configuration were 0.0266 and 0.1549 units of Procrustes shape distance respectively, suggesting there was not excessive (i.e. > 0.2) shape variation between wing specimens as recommended by Dryden & Mardia (1998).

*Relative warps principal components analysis and MANOVA on Procrustes coordinates*

The first principal component of shape variation explained 36.1% of total shape variation among wings (Eigenvalue, 0.00029136), and the second explained 21.3% of total shape variation (Eigenvalue, 0.0001784). Colour coding principal

component 1 and then 2 showed that the shape variation associated with principal component was due to *foraging* genotype (Figure 6, A) and the variation associated with principal component 2 was due to fly sex (Figure 6, B). These patterns were statistically investigated using MANOVA on the set of each specimen's Procrustes coordinates, with each set analysed simultaneously. Multivariate test results showed that Procrustes coordinates differed between wing specimens due to *genotype* ($F_{(40, 2230)} = 153.03$, $p < 0.001$, Wilks' $\lambda = 0.071$, Table 4) and *sex* ($F_{(20, 1115.0)} = 105.18$, $p < 0.001$, Wilks' $\lambda = 0.346$, Table 4).

*Developmental instability, Riemannian shape distance, Rho – comparing within and between genotype diet-dependent shape variability*

Our question was whether different *foraging* genetic strains show differential sensitivity to developmental instability (DI) across larval nutritional environments. This was measured as within diet treatment wing shape variability, the SD of *Rho*. As MANOVA revealed significant wing shape variation between *foraging* genotypes and between the sexes, we analysed response surfaces for SD of *Rho* in response to nutrition separately for each sex and *foraging* genetic strain.

*Females*

Female *D. melanogaster* of the two natural *foraging* genotypes show greatest DI when reared on diets offering less than 100 g of P+C per kilogram (Figure 7, A-B). *for*$^{R}$ rover females displayed DI maxima of 0.009 when reared on low energy, 1P:1C and 1P:2C diets (Figure 7, A). *for*$^{s}$ females displayed much higher DI when reared on low energy foods than *for*$^{R}$ females, with a DI maxima of 0.015 located over the high carbohydrate, low energy 1P:2C-1P:16C nutrient space region (Figure 7, B). *for*$^{R}$ and *for*$^{s}$ females displayed very low DI (SD of *Rho* < 0.007) across the remainder of nutrient space (Figure 7, A-B). Notably, *for*$^{s}$ females achieved their lowest DI on very high energy, high protein rearing diets (Figure 7, B).

Mutant sitter *for*$^{s2}$ females showed higher overall DI than females of natural *foraging* genotypes (Figure 7, C). The distribution pattern of female *for*$^{s2}$ SD of *Rho* scores across nutrient space was the inverse of *for*$^{R}$ and *for*$^{s}$ scores (Figure 7, A-C). Aside from the small minima in SD of *Rho* scores of 0.0055-0.007 located over the lowest energy, 1P:1C – 7P:1C region, the remainder of nutrient space yielded SD of

*Rho* scores of 0.007-0.012. In direct contrast to the patterns of DI displayed by females of the natural *foraging* genotypes, the highest DI for $for^{s2}$ females was for those reared on high energy diets, with a maximum located over 1P:1C-1P:4C diet rails offering greater than 220 $g.kg^{-1}$ of P (Figure 7, C).

*Males*

Thin plate spline response surfaces for male SD of *Rho* scores show that of the natural *foraging* genotypes, $for^{R}$ males display slightly higher levels DI across a broader area of nutrient space than $for^{s}$ males (Figure 7, D-E). SD of *Rho* scores of $for^{R}$ males form a broad plateau, ranging from only 0.0085-0.0075 across the majority of nutrient space (Figure 7, D). The $for^{R}$ DI maximum of 0.009-0.010, located over the 1P:9C diet rail at 150 $g.kg^{-1}$ C, and two concentrated minima of 0.0065 (located over low energy 1P:2C diets) and 0.0065-0.0045 (located over diets offering > 200 $kg^{-1}$ P) form the only deviations in surface relief (Figure 7, D).

$for^{s}$ males showed a larger range of SD of *Rho* scores across nutrient space with a higher DI maxima (0.014) and lower DI minima (0.003) (Figure 7, E). As shown for $for^{R}$ males, the $for^{s}$ DI maximum is located over mid-energy, high carbohydrate diets, although the $for^{s}$ maximum is higher (0.0004 SD of *Rho* points higher) and broader, extending across the mid-energy 1P:2C-1P:16C diet rail area of nutrient space (Figure 7, E). The lowest male $for^{s}$ DI occurred on low- to mid-energy high-protein diets with SD of *Rho* score minima of 0.003 (Figure 7).

In contrast to the pattern in females, mutant $for^{s2}$ males showed lower diet-dependent wing shape variability than the natural *foraging* genotypes. Male $for^{s2}$ shape variability was below 0.0065 SD of *Rho* score units across almost all of nutrient space (Figure 7, F). Contrasting with natural *foraging* males, $for^{s2}$ wing shape variability was highest on high-energy, high-protein diets offering greater than 220 $g.Kg^{-1}$ P (SD of *Rho* maxima of 0.012) and lowest (minima of 0.035) on diets of the highest carbohydrate concentration. High-energy diets ranging from the 1P:4C-1P:9C rails supported the low wing shape variability for $for^{s2}$ males (Figure 7, F).

## Discussion

We have identified multiple gene by environment interactions (GEI), indicating *foraging* functions as a plasticity gene in *D. melanogaster* populations, though not as anticipated. Each natural genotype did not deliver higher overall performance in alternate, discrete regions of nutrient space (*e.g.* Figure 1, B-D). Nor did one genotype function as a typical specialist and the other a generalist (Figure 1, A & B). Instead, the data suggest that natural *foraging* genotypes act as trait specialists.

Across all larval nutritional environments analysed, natural *foraging* genetic strains, rover and sitter, apparently specialise in the delivery of maximal performance of alternative traits. No matter the nutritional environment, in each trait measured here, one natural genetic strain outperformed the other. Environmental characteristics influenced the magnitude of trait expression; for example, trait performance typically increased with increase in dietary protein, but even in 'best' and 'worst' food environments, inter-genotypic performance of a given trait was rarely equivalent. So while trait expression was sensitive to the environment, showing graded responses, the absolute magnitude of performance achievable was typically genotype-dependent. This outcome is most similar to the scenario we considered in Figure 1i.

These patterns of differential trait level expression in common environments indicate that developing larvae of each *foraging* strain differ in their approach to nutrient allocation, metabolism and investment to traits. Each genotype appears to respond to a given nutritional environment by making its own unique, within-genotype, life history trait and phenotype performance tradeoffs (Collins, 1980; Pigliucci, 2001; Boggs, 2009). For a population of flies that possess, for example, 70% $for^{R}$ to 30% $for^{s}$ individuals (Sokolowski, 1980), these genotype-specific nutrient allocation and subsequent performance differences would see adults eclosing at different times, in different number and at different sizes. *Drosophila melanogaster* therefore possesses an adaptation – *foraging* gene polymorphism – that in response to the environment can coordinate two modes of continuous and potentially-adaptive phenotype variation. In this sense, *foraging* could be considered a polymorphism for polyphenism (Ellegren & Sheldon, 2008).

Here we discuss the trait trade-offs faced by larvae of each of the natural *foraging* genotypes that result in adult variation. We discuss potential physiological drivers of measured trait differences and speculate as to how these patterns of alternative trait maximisation may benefit a *D. melanogaster* population across heterogeneous larval nutritional environments. We also discuss potential molecular mechanisms through which these genotype-dependent patterns of alternative sensitivity to the environment might be achieved.

foraging *gene strain trait and phenotype differences and trade-offs*

Rovers showed slightly higher overall survivorship to eclosion than sitters. While individuals of both *for*$^{R}$ and *for*$^{s}$ showed their highest survivorship on mid- to high-energy 1P:1C and 4P:1C diets, possessing the *for*$^{R}$ genotype boosted survivorship to adulthood 10% above that achievable by *for*$^{s}$ on the same high-protein foods. Rovers were also better able to withstand the apparent mortality threat posed by high dietary carbohydrate. The *for*$^{R}$ survivorship advantage exceeded 20% on the highest-energy, high-carbohydrate foods. While not statistically significant, this level of inter-genotypic difference in survivorship could be relevant to populations subject to generations of natural selection.

The patterns of natural *foraging* genotype differences in developmental timing are broadly similar to those of survivorship. The 1P:1C and 2P:1C diet region of nutrient space supported both maximal survival for rovers and sitters, and the fastest time to eclosion for males and females, with females of both natural genetic strains developing marginally faster than males. Diets that supplied a carbohydrate bias were associated with lower performance for both genotypes. Diets providing carbohydrate in excess of 1P:1C led to graded increase in the number of days to reach adulthood for all males and females. Intriguingly, male sitters displayed some resilience to the development delaying effect of high dietary carbohydrate. Their development rate in response to very high carbohydrate diets was faster than that of female sitters, which appeared especially sensitive to high dietary carbohydrate, and rovers of either sex. Across the entire diet composition space analysed, sitter males took longer to eclose than rovers.

Marginally higher *for*$^{R}$ survival and faster development came at a cost: size. Female and male *for*$^{s}$ achieved much larger sizes than their rover counterparts. Sitter

size increased strongly in both sexes with the concentration of dietary protein. The largest sitter wings – and likely also sitter bodies – were generated on the highest-energy, high-protein diet (7P:1C, at 300 g P+C.$Kg^{-1}$). High dietary carbohydrate led to smaller wings for females and males of both natural *foraging* genotypes, but even in these most nutritionally-challenging environments, sitters grew bigger. Rovers also differed from sitters, in their wing size variability. The rover male and female wing size response surfaces (reaction norms) were completely different in shape to those of sitters. Regardless of the nutritional environment, our results showed *for*$^{R}$ flies to be invariably small, showing almost no phenotypic plasticity for wing size across nutrient space. Unlike female sitters, female rovers did not increase in size as dietary protein increased. Instead, the largest female rovers developed on relatively low-energy, balanced P:C diets.

Why might growing larger be associated with lower survivorship and being small with enhanced survival? Growth rate, while not explicitly quantified here, differed dramatically between natural *foraging* genotypes. While rovers reached eclosion earlier than sitters, the considerable size differences between the genotypes is indicative of sitters having invested far more larval-acquired nutrition into becoming large, quickly. In addition to the marginally-higher pre-reproductive mortality of sitters, a good indicator that rapid *for*$^{s}$ growth bears costs is the higher level of developmental instability displayed by sitters. Absolute levels of diet-dependent developmental stress or developmental instability (DI), measured here as wing-shape phenodeviance, was higher for adult *for*$^{s}$ flies than *for*$^{R}$. In both females and male *for*$^{s}$ flies, DI was highest in regions of nutrient space associated with low survival to eclosion. In sitter males, DI was extremely high when flies were raised on heavily carbohydrate-biased diets. These were the very same rearing diets that supported the most rapid male sitter development, suggesting that rapid growth on high-carbohydrate food was especially costly for male sitters.

*Rover vs. sitter physiological differences*

Known differences in the metabolic physiology of *Drosophila* may be proximate drivers of the rover *vs.* sitter trait differences measured here. Work by Kaun *et al*. (2007a) has indicated that larval rovers have a higher nutrient absorption capacity than larval sitters on a standard dietary regime. Rovers can also increase their

total intake when food energy density drops (Kaun *et al.,* 2007a). If the *for*$^{R}$ rover ability to increase intake in response to low caloric density food extends to situations of specific macronutrient imbalance, combined with their *for*$^{R}$ higher nutrient absorption affinity, this could account for our measurement of higher rover survivorship and faster development. The rover's greater sensitivity, and capacity to respond to the prevailing nutritional environment, is likely due to *for*$^{R}$ *vs. for*$^{s}$ allelic variation in PKG expression. Rover larvae and adults express higher PKG than sitters (Osborne *et al*., 1997; Belay *et al*., 2007; Kaun *et al*., 2007a). Larval rovers, when starved, display dramatic reduction in PKG expression, while larval sitter PKG levels remain consistently low regardless of changes to the food environment (Kaun *et al*., 2007a). Together these observations show lower PKG levels are associated with higher food intake and that *for*$^{R}$ individuals have flexible PKG expression. Studies on mice and *Caenorhabditis elegans* worms have demonstrated PKG has a conserved influence on food intake across animals. Working with mice, Valentino *et al*. (2011) found reducing PKG expression, by blocking cGMP production, led to mice losing appetite control and subsequently becoming obese. *Caenorhabditis elegans* worms lacking the *pkg-1* gene, the *C. elegans* homologue of *D. melanogaster foraging* gene, displayed no 'satiety quiescence' and fed continuously, while gain of function *pkg-1* worms showed 'excessive' quiescence, despite food deprivation (You *et al*., 2008).

Indiscriminate feeding in *Drosophila* is driven by the activity of neuronal neuropeptide F (NPF), a homolog of mammalian neuropeptide Y (NPY) (Wu *et al*., 2003; Lingo *et al*., 2007). NPF receptor 1 overexpression is necessary and sufficient to drive feeding in sated flies (Lingo *et al*., 2007). PKG expression may influence NPF levels in *D. melanogaster*. If so, lower PKG levels would be associated with higher NPF and thus higher food intake, with *for*$^{R}$ rover flies showing flexible NPF expression in response to the prevailing macronutrient imbalance. For this to occur *for*$^{R}$ individuals would have to be more sensitive than *for*$^{s}$ individuals to specific macronutrient deficits in order for a PKG-> NPF mediated increase in feeding to occur at an appropriate juncture, for example, under a carbohydrate deficiency induced by feeding on a high P:C diet, or under a protein deficiency induced by a high C:P diet.

Work by Ikeya *et al.* (2002) and Colombani *et al*. (2003) showed that in adult *D. melanogaster*, low dietary carbohydrate – but not low amino acids – reduced the

expression of *Drosophila* insulin-like peptides (dilp3 and 5), homologues of mammalian insulin (Rajan & Perrimon, 2013). In mice post-prandial insulin release inhibits NPY (Porte *et al*., 2005), while loss of function insulin receptor mice show increased food intake (Garofola, 2002). In *D. melanogaster*, over expression of dilps suppresses feeding in starved larvae (Wu *et al*., 2005). Together, these findings suggest that when feeding on low dietary carbohydrate, *Drosophila* insulin-like peptide production is reduced, which, through release of *Drosophila* NPF inhibition, drives increased food intake. Adult *D. melanogaster* can behaviourally redress an experimentally-induced dietary protein deficiency by selectively feeding on high yeast foods (Ribeiro & Jackson, 2010; Vargas *et al*., 2010). As in higher animals, in *Drosophila*, low circulating amino acid levels inhibit TOR/S6K signaling (Arsham & Neufeld, 2006; Ribeiro & Jackson, 2010). S6K is a downstream effector of TOR (Wullschleger *et al*., 2006; Arsham & Neufeld, 2006). Neuronal down-regulation of S6K is associated with increase in bulk food intake in larval *D. melanogaster* (Wu *et al*., 2005), while in mice, up-regulation of hypothalamus S6K, replicating high circulating amino acids and high TOR expression, has been shown to suppress appetite (Blouet *et al*., 2008). Suppression of TOR→S6K signaling in adult *Drosophila* by over-expression of upstream regulators Tsc1 and 2, replicating low circulating amino acids, can stimulate preferential yeast feeding (Riberio & Jackson, 2010). High-protein diets are associated with higher TOR signaling and reduced food intake in mice (Solon-Biet *et al*., 2014) and up-regulation of S6K in rats is associated with lower food intake and lower NPY expression (Blouet *et al*., 2008). These results indicate that in larval *Drosophila* low protein diets may suppress TOR signaling, which in turn reduces S6K activity and promotes high NPF expression.

We propose that *for*$^{R}$ allelic variants may have higher sensitivity to circulating nutrients and thus respond more strongly, in terms of PKG expression and subsequent NPF activity, to insulin and TOR pathway signaling than *for*$^{s}$ individuals. Evidence for enhanced sensitivity of the *for*$^{R}$ allele to signaling from the nutrient-sensing pathways comes from work on adult *D. melanogaster.* Adult rovers show greater insulin signaling (IIS) when food deprived than sitters (Kent *et al*., (2009). Also, when comparing change in gene expression in adult flies that have been fed *vs.* food-deprived, *for*$^{R}$ adults display significant change in more genes of known affinity to the TOR signaling pathway than *for*$^{s2}$ sitter adults (Kent *et al*., 2009).

Larval sitter *vs.* rover behavioural differences could also contribute to trait differences we have observed. Sitters eat more (Kaun *et al*., 2007a) and move less (Sokolowski, 2001) than rovers. Could the marked differences in rover/sitter size be partially attributed to sitters acquiring more food per unit time plus allocating those nutrients to body tissues (thus size) rather than 'roving'? Another potential sitter/rover resource allocation difference that may account for the size and growth rate differences measured could exist between growth and storage – a classic life history tradeoff (Boggs, 2009). While sitters allocated nutritional resources to growth rate, rovers may have invested nutritional resources obtained as larvae to lipid storage. Measuring relative lipid stores could help resolve this (see Chapter 2).

*Ecological relevance of trait differences*

How might the environmentally induced differences in *foraging* genotype traits we have measured translate into adaptive ecological strategies that promote survival of adult *D. melanogaster*? While the benefits of higher survival are obvious, having a population of individuals that eclose at different times could help stagger predation risk, or allow better utilisation of finite food resources. Theory and experimental evidence suggests that increased predation risk in the larval stage is linked to earlier metamorphosis and smaller size at metamorphosis (Vonesh & Warkentin, 2006). As *Drosophila* larvae and pupae are susceptible to attack by parasitic wasps (Kraaijeveld & van der Wel, 1994; Sokolowski & Turlings, 1987), genetically-determined variation in developmental rate may allow entire localised populations of closely-related larvae (Shan & Langley, 1979) to avoid being simultaneously attacked. The earlier development in *for*$^{R}$ rovers could partially be reinforced by *for*$^{R}$ genotype-specific predation pressure. Prey movement can facilitate predation (Werner & Anholt, 1993) and higher levels of larval *D. melanogaster* movement increases risk of attack by parasitic wasps (Sokolowski & Turlings, 1987). Rovers may have evolving faster development to escape parasitic wasp attack at the cost of reduced body size.

Another situation where maintaining population-level variation in developmental rate is advantageous is under developmental time constraints (Johansson *et al.,* 2001). Work on the damselfly *Lestes viridis* has shown larvae developing under time constraints, for example toward the end of a breeding season,

have increased foraging and developmental rates yet eclose with lower mass (Joahnsson, *et al*., 2001; De Block & Stoks, 2005). *Drosophila* larvae typically live in discrete patches of food, *i.e.* pieces of rotting fruit. The rover allele may allow for maximisation of survival through rapid development within these finite resource patches when roving off is not possible (Wiegmann *et al*., 1997).

The more pronounced difference in development time between male rovers and sitters compared to females may be reflective of intra-sexual competition processes. Male *D. melanogaster* engage in aggressive encounters with one another (Hoyer *et al*., 2008), with size being positively correlated with mating success (Partridge & Farquhar, 1983; Partridge *et al*., 1987). The more rapid male rover development may be a mitigation strategy for enhancing rover competitiveness against the much larger male sitters. Our results indicate females of both genotypes eclose a full 24 h prior to males. Rover males eclosing sooner than sitters may allow them to garner more mating opportunities before the larger sitters 'arrive'.

In other insect species, intra-specific adult size differences often link to migration *vs.* settlement ecological strategies. Populations of the Glanville fritillary butterfly, *Melitaea cinxia*, consist of two natural allelic variants of the *PGI* gene. Variants consistently differ in body size, with largeness being positively correlated with higher flight metabolic rate and larger clutch size in females (Haag *et al*., 2005). The cricket *Gryllus firmus* is a famous model species known for its migration *vs.* reproduction life history tradeoff. The species shows a marked phenotypic plasticity for either long or short wings. Longer-winged individuals are associated with dispersal while those with short wings are obligatory non-migratory (Clark *et al*., 2013; Zera & Larsen, 2001). The trait specialisation we have identified here may be reflective of similar adult-stage alternate life history trade-offs occurring in *Drosophila* as adaptation to environmental heterogeneity. The bigger sitters may be dispersers, while smaller rovers, with their higher localised exploration behaviour (Burns *et al*., 2012) may specialise in efficient exploitation of the local environment. It would be interesting to build on the work of Burns *et al*. (2012) and examine the food search and flight behaviour of adult *foraging* flies that were reared in different nutritional environments.

*Significant wing shape variation exists between foraging genetic strains*

We quantified each specimen's wing shape to compare the level of diet-dependent phenodeviance or DI between *foraging* genotypes. Through doing this, we also identified subtle, but significant genotype-dependent and sex-dependent, differences in *D. melanogaster* wing shape. The two natural *foraging* genotypes were more similar in their shape profile than the mutant *for*$^{s2}$. Between genotypes, shape differences seem to be driven by the position of wing vein intersections demarked here as landmarks 5, 7, 8 and 11. How these shape differences translate into differential ecological advantage, if at all, is difficult to speculate upon. It may be easier to speculate on ecological significance of female *vs.* male shape difference. The 'most male' wing shape configuration (Figure 6, Ciii) is for a relatively rounder wing, while the 'most female' (Figure 6, Civ) is elongate and narrow. The rounder male wing could be associated with maximising courtship song quality, or perhaps enhancing flight manoeuverability while pursuing females. The tapered 'female' wing profile may be optimised for longer distance flying to and from oviposition sites. Sex differences also exist between the level of wing shape phenodeviance measured across our experimental nutritional environments. Males of both natural *foraging* genotypes generated higher levels of wing shape variability than females. Wing shape being more variable in males than females may be due to wing shape phenotype being under sexual selection, rather than stabilising natural selection. It may be somehow developmentally 'costly' (Zahavi, 1975) for males to produce an optimal wing shape configuration across all larval nutritional environments. Our results indicate that high carbohydrate diets are the most challenging for males of naturally-occurring *foraging* genotypes to produce wings of consistent shape.

*Mutant sitter foraging genotype for*$^{s2}$

Given its rover genetic background, the *for*$^{s2}$ genotype has been used as a control for the role of the *foraging* gene in mediating variation in phenotypic response to environmental stimuli, typically the nutritional environment. Previous work had shown that *for*$^{s2}$ larvae respond as sitter *for*$^{s}$ equivalents in their larval foraging behaviour and metabolism (Pereira & Sokolowski, 1998; Fitzpatrick *et al*., 2007; Kaun *et al*., 2007a, 2007b; Kent *et al.*, 2009). For traits measured here, there was no consistent correspondence in expression between *for*$^{s}$ and *for*$^{s2}$. This could be because

we have investigated new traits, measured in response to hitherto-unexplored experimental nutritional environments. The *for*$^{s2}$ mutant's *foraging* allele may be natural sitter equivalent in the gene region driving larval behaviour under previously-assayed food environments, but gene regions responsible for the development of other traits and phenotypes may differ in sequence between *for*$^{s}$ and *for*$^{s2}$. There are two ways sequence differences at the *foraging* allele could lead to trait differences between natural and mutant sitter. The novel *for*$^{s2}$ nucleotide sequences could themselves drive trait expression, or mutant sequence could break down currently unknown signaling interactions between *foraging* and other genes. Whichever the case, the transcriptional relationships between natural sitter's *foraging* allele and other genes – honed by natural selection – have been 'released' in *for*$^{s2}$, generating completely novel traits. For example, *for*$^{s2}$ patterns of diet-dependent developmental instability are completely opposite to the natural *foraging* genotypes, showing increase with dietary protein rather than decrease. Patterns of male *for*$^{s2}$ wing size are similarly opposite to those of natural *foraging* males. Mutant sitter male wings develop to be their largest on high-carbohydrate diets rather than high-protein. These results provide an example of difficulties in working with mutants organisms when trying to understand GEIs shaped by natural selection.

*An optimal diet*

Though using the Geometric Framework, we have identified a common optimal larval diet for the traits measured populations of *D. melanogaster*. The region of nutrient space where the performance maxima for each trait from males and females of each *foraging* genotype most over-lapped were diets offering approximately 90 – 150 g.kg$^{-1}$ of P+C at 1P:1C - 1.5P:1C. Interestingly, this balance of P:C offers higher protein per g ingested than most standard laboratory *D. melanogaster* culture diets. It would be interesting to see if larval diets of this P:C ratio are associated with high performance in other traits not measured here. We have also demonstrated that high dietary carbohydrate is consistently associated with lower trait performance in developing *D. melanogaster*.

*Conclusions*

Using nutritional geometry we have demonstrated that regardless of the developmental nutritional environment, natural allelic variation in *foraging* leads to population level trait and phenotype variation. The nature of larval food environments influences the magnitude of trait or phenotype expression, but neither natural *foraging* genotype facilitates adaptation to environmental heterogeneity through alternate environmental specialisation. Determining the characteristics of the initial selective environments that drove the evolution of *foraging* allelic variation is challenging, although it could be modeled (Simpson *et al*., 2009). Speculating on how *foraging* may contribute to population evolvability is, however, more straight-forward. The allelic variation allows for two separate, and genetically-discrete, modes of continuous trait and/or phenotypic variation. This variation could well be relevant to microevolution across changing environments: *foraging* may be a means of maintaining the evolutionary potential of *D. melanogaster* populations.

While the specific rover/sitter sequence differences are not yet characterised, it is fascinating to consider how sequence differences, and differences in the regulation of those sequences in response to the developmental environment, can generate such marked trait variation in a single species (Danchin, 2013). Sokolowski and colleagues are currently examining the role of a euchromatin histone methy-transferase (Kramer *et al*., 2011) in regulating rover/sitter phenotypic differences in response to food environments. Work like this will improve our understanding of the origins of developmental plasticity and, specifically, the regulation of within-species GEI at the finest of molecular scales. Natural genetic variation of *foraging* and close homologues is present in other animals, including humans. While GEI studies of the human *foraging* homologue PRKG1 are in their infancy (Zakharkin *et al*., 2005), the homologues in ants, *ppfor* (Lucas *et al*., 2009) and bees, *Amfor* (Ben-Shahar *et al*., 2003), are demonstrated to drive developmental trait plasticity in response to nutrition in these species. It would be immensely interesting to study further the role of *foraging* and its homologues in mediating trait performance variation and generating phenotypic plasticity in other animal species.

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**Box 1. Concepts in phenotypic plasticity as applied in this study**

*Phenotypic plasticity versus developmental instability*

We consider the phenotypic plasticity and developmental instability (DI) expressed by a genotype across an environmental gradient as being distinct yet related concepts. We extend Bradshaw's (1965) concept of DI to be a measure of how variably a genotype yields its phenotype in a discrete environment. Phenotypic plasticity instead refers to the capacity for a genotype to yield different iterations of a phenotype across an entire environmental gradient (West-Eberhard, 1989). Phenotypic plasticity does not refer to the specific, *within environment*, phenotypic variability produced by a genotype. We use DI to describe this variability. Additionally, DI can also encompass any within-individual variation in the expression in a pair of bilaterally symmetrical traits (*e.g.* wings) whose ontogeny is dependent upon a single set of genetic processes.

*Canalisation, phenotypic plasticity, developmental instability*

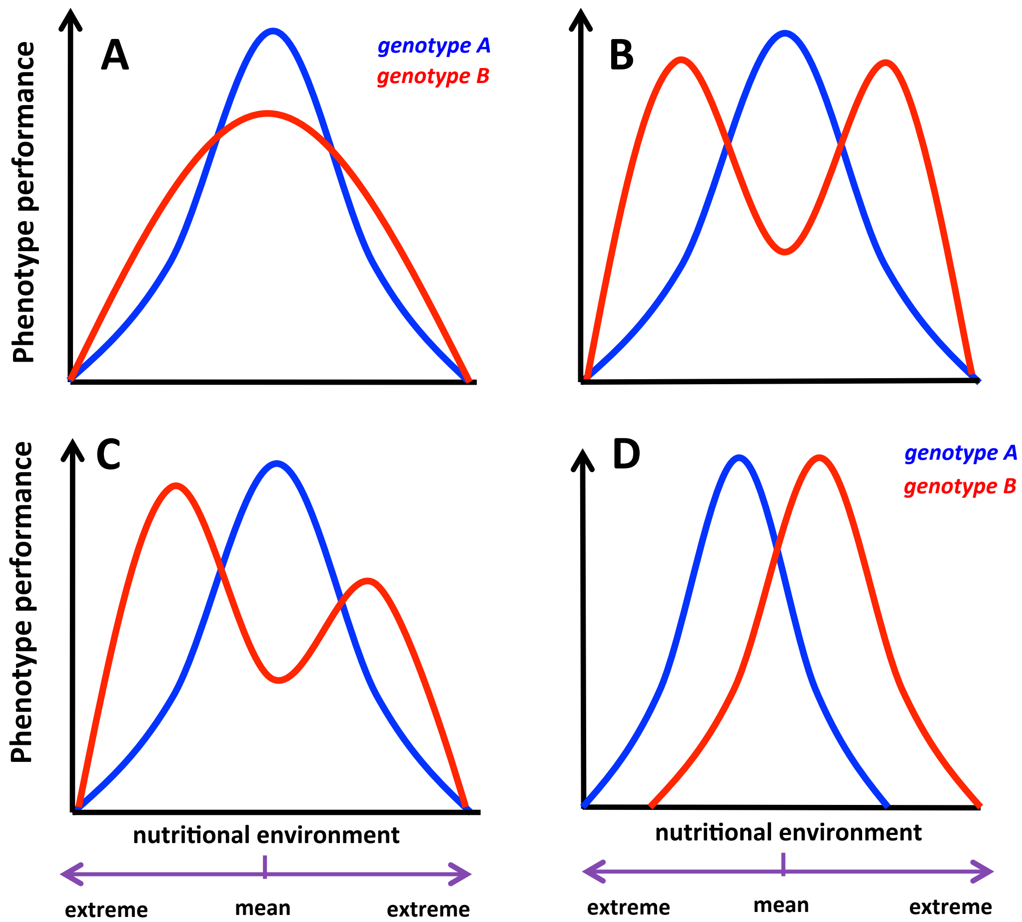
Genotypes that produce the same or a very similar phenotype across all environments are described as having high phenotypic canalisation and low phenotypic plasticity (Waddington, 1956; Bradshaw, 1965). Genotypes that produce low plasticity across all environments also display low developmental instability. Genotypes which produce a phenotype invariantly are likely to have been exposed to a high stabilising selection (Schmalhausen, 1949; Falconer, 1990; Stearns & Kawecki, 1994).

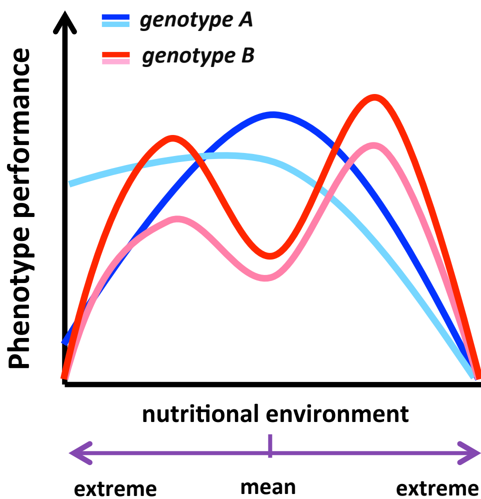
*Reaction norms versus phenotype performance surfaces*

A reaction norm is also a property of a genotype. It is a plot of the measured range of phenotypic variants a genotype yields in response to an environmental gradient (Schmalhausen, 1949; Stearns *et al*., 1986; Falconer, 1990). The reaction norm itself may be described as being canalised if the phenotype plotted upon it is near invariant. Though not the default purpose of reactions norms, in our study we impute the concept of 'performance' onto measured reaction norms. We have expressly measured phenotypes whose relative expression is relevant to the performance (and fitness) of individuals of a given genotype. Therefore the reaction norms we have measured simultaneously serve as phenotype or trait performance surfaces.

*Performance surface of genotype-specific developmental instability*

Here we have measured the DI of genotypes. We have not measured within-individual genotypic DI, for example, fluctuating asymmetry (Palmer & Strobeck, 1986). We have plotted our DI index as a response surface. This response surface is not however a reaction norm. While our measure of DI is a property of a genotype, it is not a phenotype. While we use DI as a marker of developmental stress or as a negative marker of performance, we do not discount that there may exist some evolutionary scenarios in which the widely ranging phenotypic variants generated by a comparatively unstable genotype in a novel environment may be of potential adaptive benefit to a species. For example act as an exaptation (Gould & Vrba, 1982).



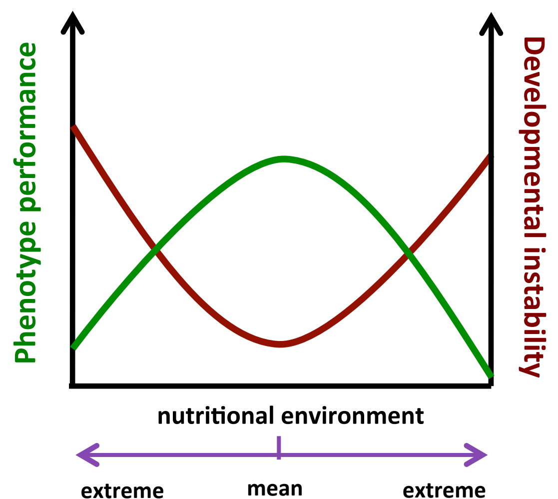




Figure 1. Differential phenotype performance curves (also 'reaction norms,' see box 1.) for two hypothetical genotypes exposed to a one dimension nutritional environment. Here 'phenotype' encompasses larval life history traits. A) – D) represent alternate hypotheses predicting how genetic variation for phenotype performance may enable *Drosophila melanogaster* to maintain performance across variable nutritional environments. In scenario a) genotype A outperforms B in the mean environment, however genotype B provides higher performance in less frequently encountered extremes. These alternate strategies are referred to as specialist (genotype A) and generalist (genotype B) strategies. Scenario b) and c) show genotype A specialising in the mean environment, while genotype B serves as an extremes specialist. Scenario b) shows genotype B specialising in any extreme environment, while scenario c) shows genotype B providing relatively higher performance at one extreme. Scenario d) shows both genotypes performing comparably in the mean environment, with genotype A providing higher performance toward one end of the nutritional spectrum. Genotype B, the other.

Figure 1i. Multiple phenotype performance curves for two hypothetical *D. melanogaster* phenotypes. Curves demonstrate that a single genotype may yield multiple disparately performing phenotypes across the same nutritional environmental spectrum. We hypothesise that genotypes differ in their response to tradeoffs between the performance of different phenotypes across nutrient space. Here genotype A faces the potential costs of co-maximisation of phenotype performance better in the mean environment, whereas genotype B manages performance tradeoffs better at the extremes.

Figure 1ii. Phenotype performance curve and developmental instability curve for a single genotype across a nutritional environment spectrum. Figure 1ii demonstrates our prediction that within a genotype, low phenotype performance and high developmental instability will co-localise in nutrient space.

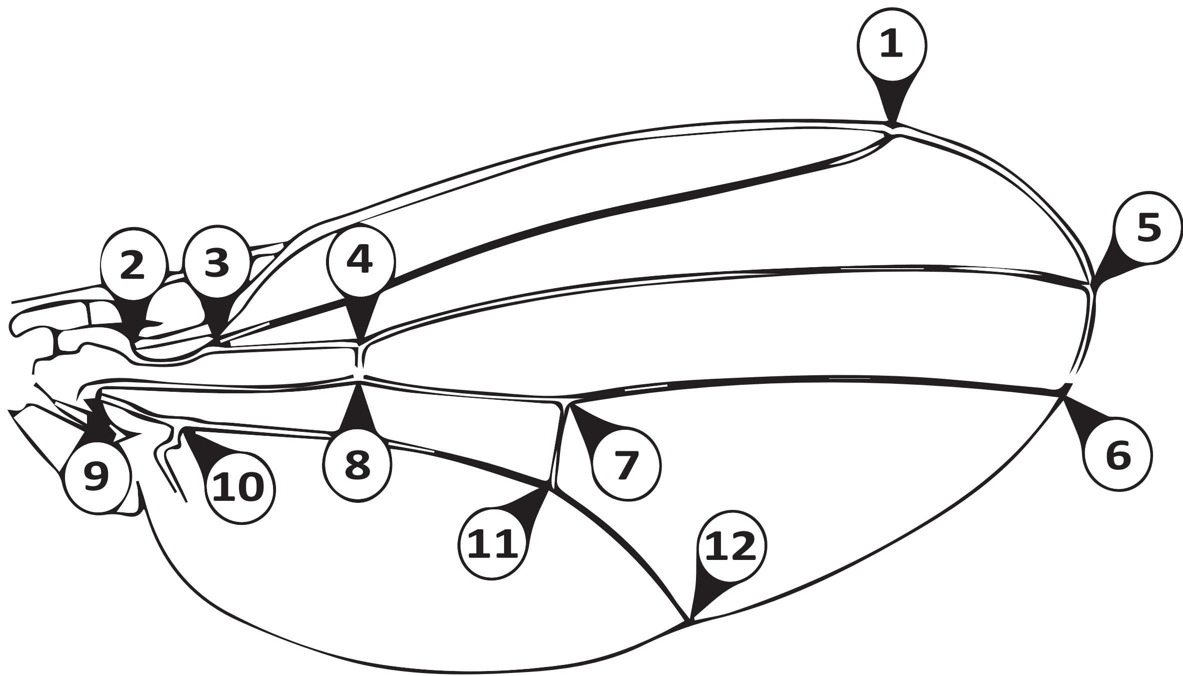



Figure 2. *Drosophila melanogaster* wing showing the 12 wing-vein intersection landmarks used in geometric-morphometric analyses (after Klingenberg & Zaklan, 2000).

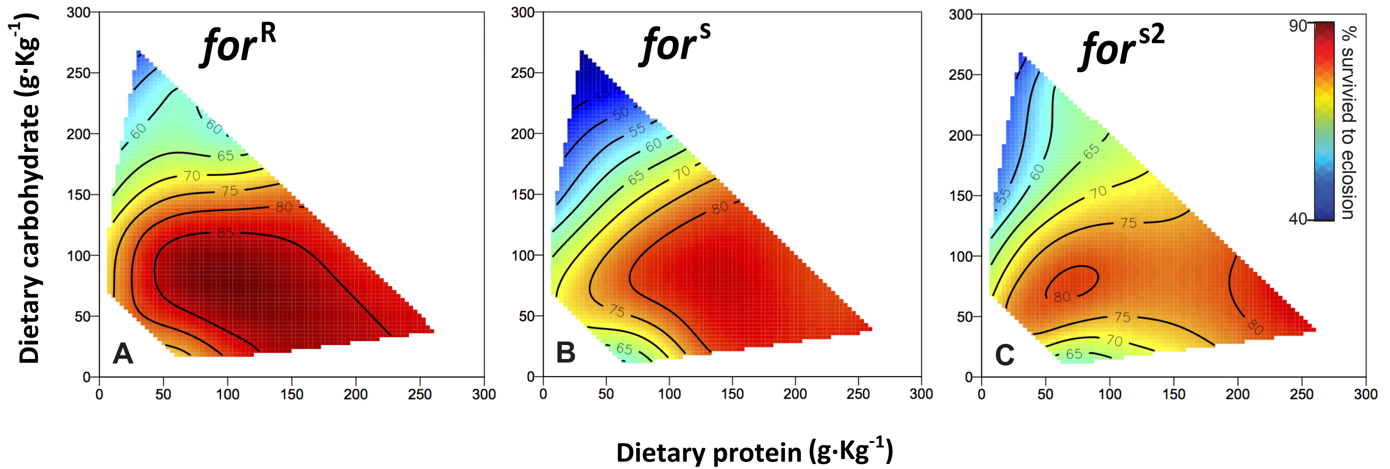



Figure 3. A) – C) Thin plate spline (TPS) response surfaces showing diet-dependent survivorship of *Drosophila melanogaster* larvae from each *foraging* genotype. Response surfaces grade across protein-carbohydrate (P-C) nutrient space from deep blue where ≤ 40% of larvae reached eclosion, to deep red where ≥ 90% reached eclosion. Mutant sitter genotype *for*$^{s2}$ (panel C) showed lowest survivorship across nutrient space, *for*$^{s}$ sitter (panel B) intermediate survivorship, and *for*$^{R}$ rovers (panel A) highest survivorship. A) *for*$^{R}$ rover TPS shows a maximum of ≥ 85% survived extending from 50 g.Kg$^{-1}$ P:110 g.Kg$^{-1}$ C to 220 g.Kg$^{-1}$ P: 50 g.Kg$^{-1}$ C. B) *for*$^{s}$ TPS maximum of 80% survived extending from 100 g.Kg$^{-1}$ P: 100 g.Kg$^{-1}$ C, to 250 g.Kg$^{-1}$ P: 140 g.Kg$^{-1}$ C. C) *for*$^{s2}$ mutant sitter TPS shows two survivorship maxima of >80% survived, one at 80 g.Kg$^{-1}$ P: 80 g.Kg$^{-1}$ C and another extending from 200 g.Kg$^{-1}$ P: ≥ 50 g.Kg$^{-1}$ C. A) – C) Co-localisation of each *foraging* genotype's maxima occured under the mid-energy density 1P:1C diet rail. Across all *foraging* genotypes C rich diets, offering greater than 150 g.Kg$^{-1}$ of C were significantly associated with lower survivorship for all *foraging* genetic strains. Generalised linear modeling (GLM) showed the quadratic term of dietary carbohydrate was significantly associated with all genotype's surface shape (*carbohydrate*$^2$, Wald $\chi^2$ = 2.295, d.f. = 1, $p$ = 0.038). GLM showed, despite TPS trends, there was no significant difference in diet-dependent survivorship to eclosion due to *foraging* genotype (Wald $\chi^2$ = 1.272, d.f. = 2, $p$ = 0.529).

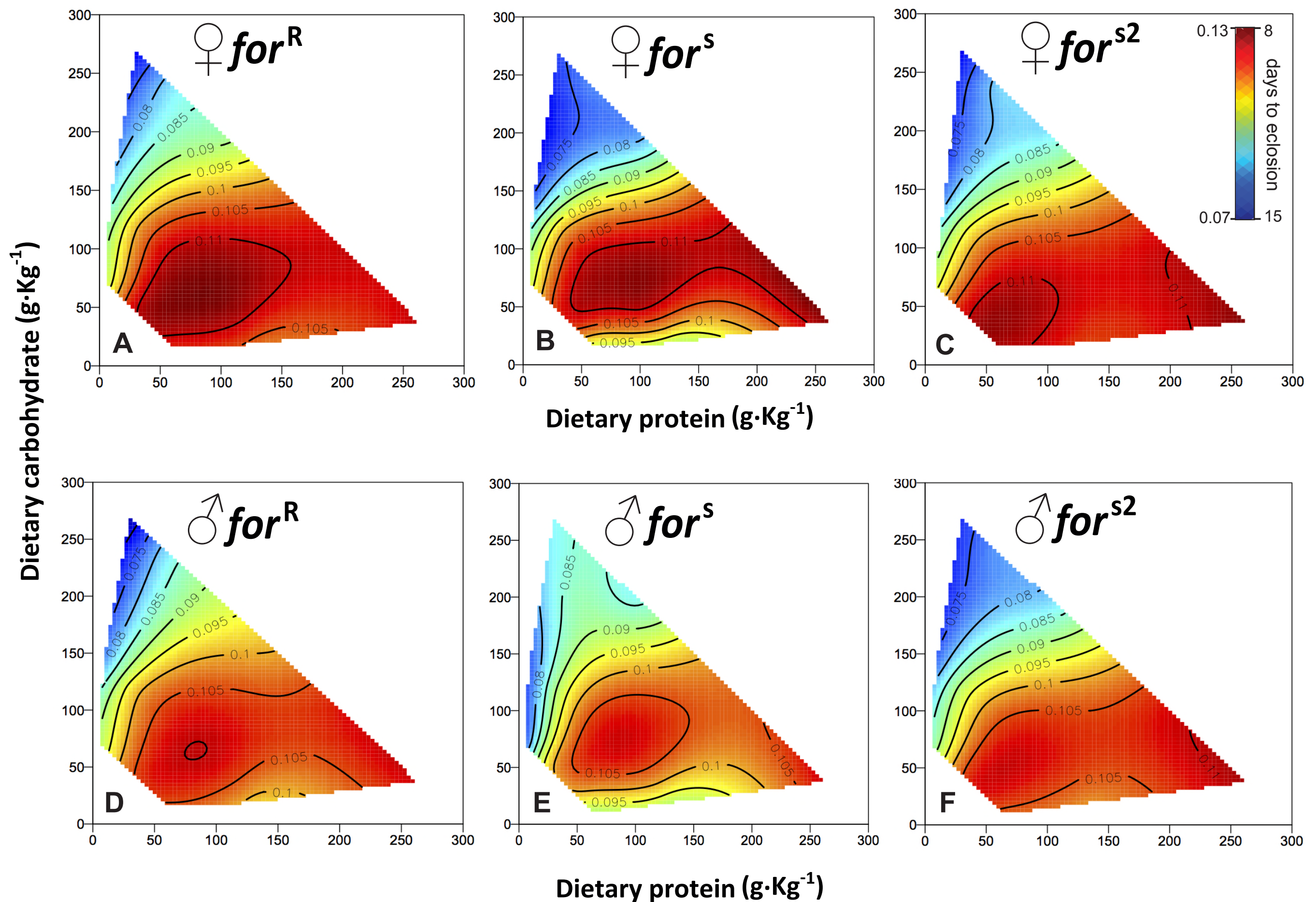



Figure 4. A) – F) Thin plate spline (TPS) response surfaces showing nutrition-dependent developmental rate of *Drosophila melanogaster* females and males from each *foraging* genotype. Surfaces range across nutrient space from red, high elevation areas where larvae took a minimum of 8 days to eclose, to low elevation deep blue areas where larvae took 15 days eclose.

A) – C) Generalised linear modeling (GLM) showed simultaneous increase in dietary protein (P) and carbohydrate (C) was associated with faster development for all females (*protein*carbohydrate*, Wald $\chi^2 = 29.586$, d.f. = 1, $p < 0.001$). High and low dietary C was significantly associated with slower development (*carbohydrate*$^2$, Wald $\chi^2 = 149.881$, d.f. = 1, $p = 0.001$). This trend is visible on TPS surfaces as blue, low elevation regions where larvae were reared on > 150 g.Kg$^{-1}$ of C. Developmental rate did not differ due to *foraging* genotype (Wald $\chi^2 = 2.063$, d.f. = 2, $p = 0.356$), with all females sharing a developmental rate maximum of eclosion in ≤ 9 days over regions of nutrient space offering 1P:1C to 4P:1C.

D) – F) GLM showing high and low dietary C was significantly associated with slower development in all males (*carbohydrate*$^2$, Wald $\chi^2 = 47.082$, d.f. = 1, $p < 0.001$). Linear increase in P was associated with faster development for all genotypes (*protein*, Wald $\chi^2 = 20.661$, d.f. = 1, $p < 0.001$), while the quadratic term of P was significantly associated with slower development at low P levels and faster development when dietary P was very high (*protein*$^2$, Wald $\chi^2 = 9.267$, d.f. = 1, $p < 0.001$). Male developmental rate surfaces differed significantly with *foraging* genotype (Wald $\chi^2 = 10.293$, d.f. = 2, $p = 0.006$). Between genotype GLM showed the $for^s$ surface was significantly different to both the $for^R$ and $for^{s2}$ surfaces ($for^R$ vs. $for^s$, Wald $\chi^2 = 6.224$, d.f. = 1, $p = 0.013$; $for^s$ vs. $for^{s2}$, Wald $\chi^2 = 6.766$, d.f. = 1, $p = 0.009$) which were equivalent ($for^R$ vs. $for^{s2}$, Wald $\chi^2 = 0.02$, d.f. = 1, $p = 0.879$). Surface shape differed as male sitter developmental rate was relatively slower than $for^R$ and $for^{s2}$ in response to high dietary P, and yet faster than $for^R$ and $for^{s2}$ on high C diets.

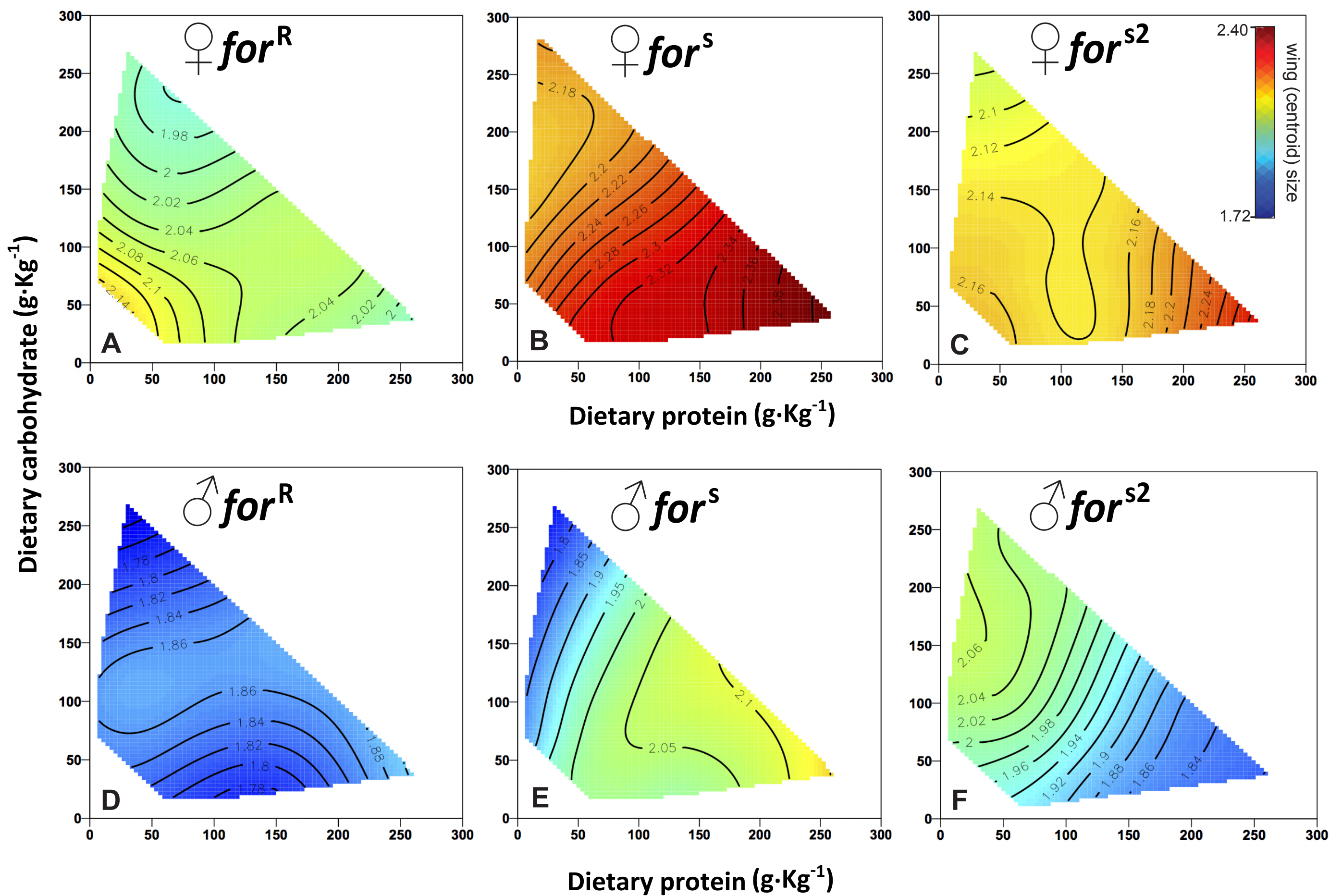



Figure 5. A) – F) Thin plate spline (TPS) response surfaces of female and male *Drosophila melanogaster* nutrition-dependent wing size (expressed in units of centroid size). Areas of deep red represent large wings, while deep blue depicts small wings.

A) – C) GLM showed that for females, linear increase in dietary carbohydrate (C) was significantly associated with smaller wings (*carbohydrate*, Wald $\chi^2 = 7.682$, d.f. = 1, $p = 0.006$). GLM showed female wing size differed significantly with genotype (Wald $\chi^2 = 60.615$, d.f. = 2, $p < 0.001$) and that wing size differed significantly between each genotype ($for^R$ vs. $for^s$, Wald $\chi^2 = 59.516$, d.f. = 1, $p < 0.001$; $for^R$ vs. $for^{s2}$, Wald $\chi^2 = 12.373$, d.f. = 1, $p < 0.001$; $for^s$ vs. $for^{s2}$, Wald $\chi^2 = 19.507$, d.f. = 1, $p < 0.001$). $for^s$ females developed significantly larger wings than $for^{s2}$ females. Female $for^R$ developed the smallest wings. In natural ($for^s$) and mutant ($for^{s2}$) sitters, wing size was maximal when flies were reared on high-energy, high-protein (P) diets. $for^R$ females developed larger wings on lower energy, balanced-P:C diets.

D) – F) GLM showed male wing size surface shape differed significantly with *foraging* genotype (Wald $\chi^2 = 36.671$, d.f. = 2, $p < 0.001$). Comparative GLM showed $for^s$ males developed the largest wings followed by $for^{s2}$ and $for^R$. D) – E) $for^R$ vs. $for^s$ GLM showed dietary C was significantly associated with the development of smaller wings in $for^R$ and $for^s$ males (*carbohydrate*$^2$, Wald $\chi^2 = 9.963$, d.f. = 1, $p = 0.002$). The P and C cross product was significantly associated with larger male wings ($for^R$ vs. $for^s$ *protein*carbohydrate*, Wald $\chi^2 = 20.340$, d.f. = 1, $p < 0.001$). $for^s$ males attained larger wings than $for^R$ males within the 1P:1C to 7P:1C region of nutrient space. F) Opposite to natural genotypes, $for^{s2}$ males developed smaller wings as dietary as P increased, and larger wings as C increased. $for^{s2}$ GLM showed linear increase in C was significantly associated with larger wing size (*carbohydrate*, Wald $\chi^2 = 5.789$, d.f. = 1, $p < 0.016$). As for $for^R$ and $for^s$ males, the cross product of P and C significantly contributed to the $for^{s2}$ wing size response surface shape (Wald $\chi^2 = 9.106$, d.f. = 1, $p < 0.003$).

.

A

B

Principal component 2

0.06
0.03
0.00
-0.03
-0.06
-0.09
-0.12
-0.15

-0.09 -0.06 -0.03 0.00 0.03 0.06

*for*^R^
*for*^s^
*for*^s2^

♂
♀

Principal component 1

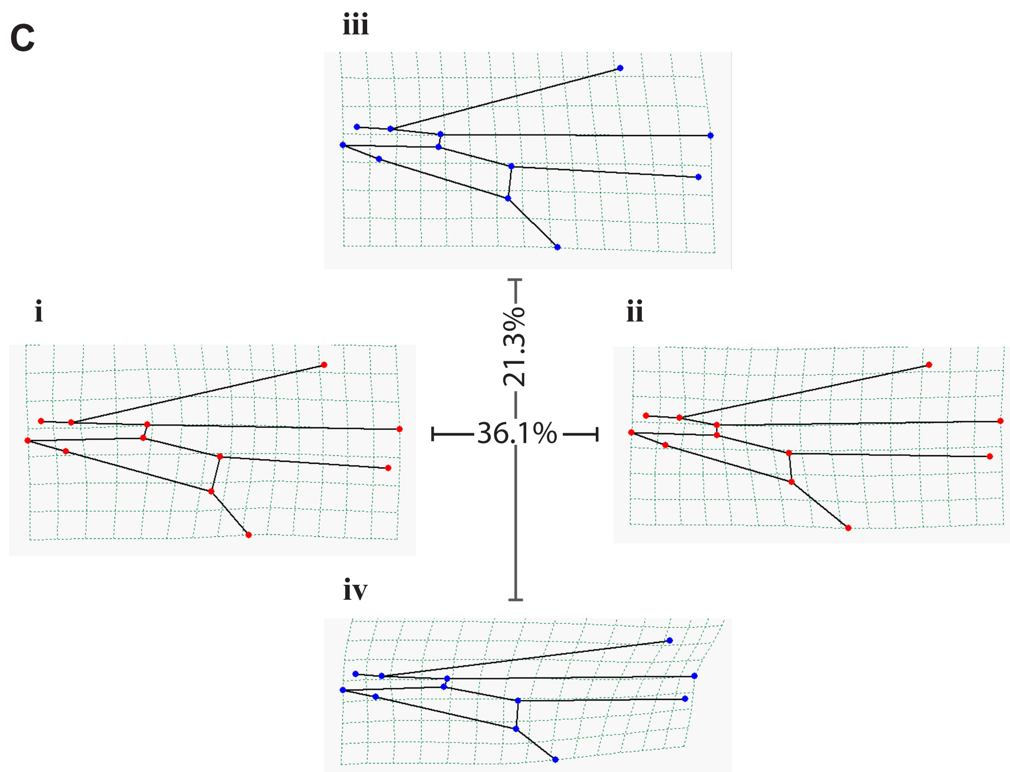



Figure 6. Caption on following page.

Figure 6 (On previous page). A) - B) Principal components analysis plots showing two major axes of *Drosophila melanogaster* wing shape variation, principal component 1 (*x* axis) and principal component 2 (*y* axis). Principal component 1 explained 36.1% of total wing shape variation. Principal component 2 explained 21.3%. A) Colour coding specimens according to *foraging* genotype demonstrated that the shape variation described by principal component 1 was associated with *foraging* genotype. B) Colour coding specimens by sex showed that shape variation described by principal component 2 was associated with fly sex. MANOVA on wing Procrustes coordinates supported these trends. Coordinate sets differed significantly between specimens due to genotype ($F_{(40, 2230)} = 153.03$, $p < 0.001$, Wilks' $\lambda = 0.071$) and sex ($F_{(20, 1115.0)} = 105.18$, $p < 0.001$, Wilks' $\lambda = 0.346$). C) i) – ii) Shows extremes in wing shape configurations due to genotype, iii) – iv) shows extreme configurations due to fly sex.

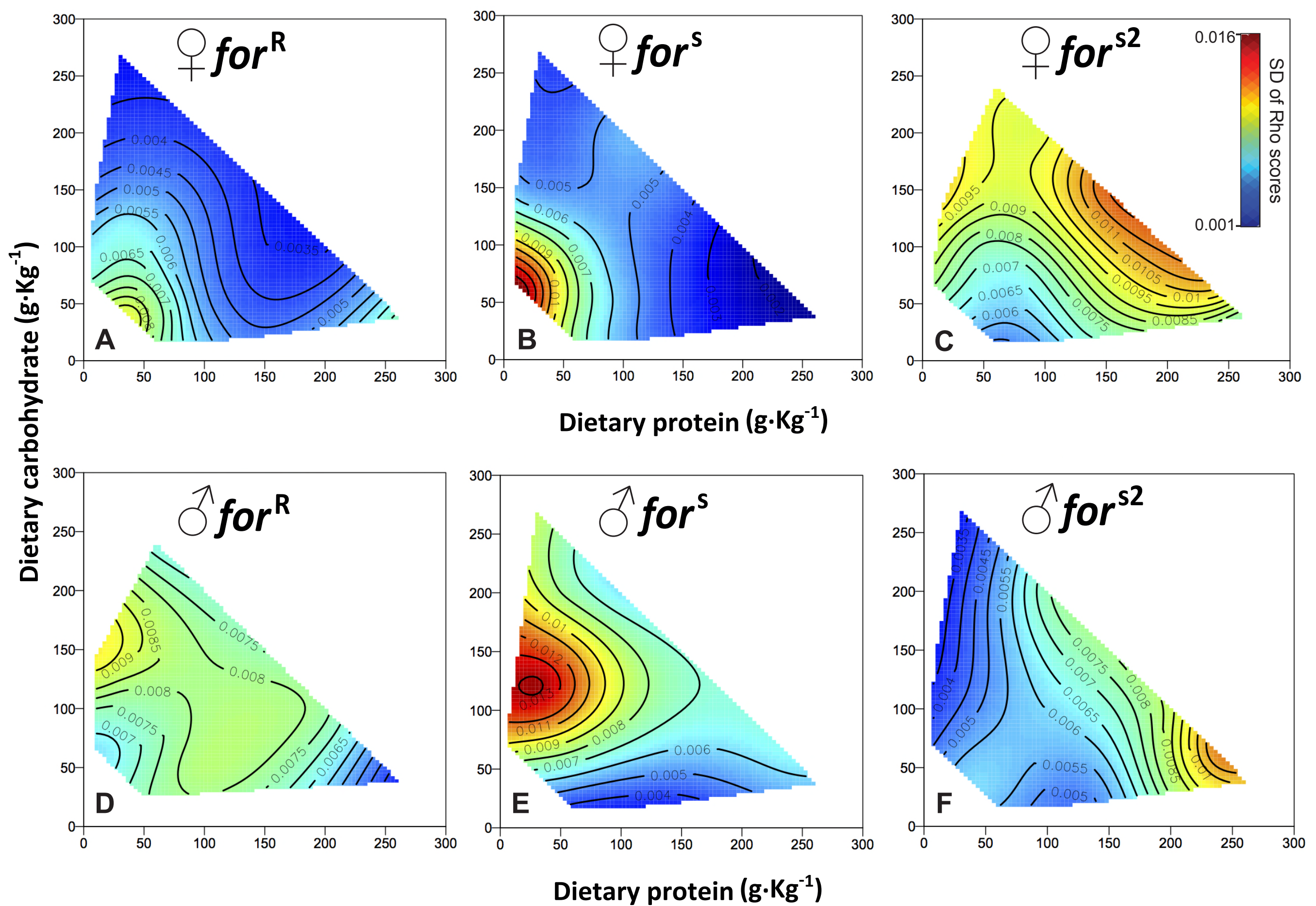



Figure 7. A) – F) Thin plate spline response surfaces for development instability (DI), represented by standard deviation of *Rho* scores, for female and male flies of each *foraging* genotype. Standard deviation (SD) of *Rho* scores describe how variable wing shapes are among flies raised on common larval food environments. Areas of deep red show high within-diet wing shape variability (high DI). Deep blue depicts the opposite, and thus low DI. *for*$^{R}$ flies showed the smallest range of DI across food environments.
A) & B) Females of natural *foraging* genotypes showed highest DI when reared on diets of less than 100 g.Kg$^{-1}$ of P and C. *for*$^{s}$ females displayed a higher DI maximum in this region of nutrient space than *for*$^{R}$ females. The *for*$^{s}$ maximum was located over more carbohydrate (C) biased, low-energy diets than the *for*$^{R}$ maximum. *for*$^{R}$ and *for*$^{s}$ females showed low within-diet wing shape variability across the remainder of nutrient space, achieving lowest overall DI on high-protein (P) diets. C) Mutant *for*$^{s2}$ females showed higher DI than natural genotypes. All rearing diets of > 100 g.Kg$^{-1}$ of P or C led to DI scores above 0.0085. *for*$^{s2}$ females reached highest wing shape variability on high energy diets, with the DI maximum located over the high-energy 1P:1C to 1P:2C region of nutrient space.
D) & E) Males of the natural *foraging* genotypes show greater overall DI than *for*$^{R}$ and *for*$^{s}$ females. *for*$^{R}$ males display higher DI across the breadth of nutrient space, while *for*$^{s}$ males displayed a larger DI range across rearing diets. Maximal DI for *for*$^{R}$ and *for*$^{s}$ males occurred over mid-energy density, high-C diets. The *for*$^{R}$ maximum is located in nutrient space over mid-energy 1P:9C diet rail, while the broader and higher *for*$^{s}$ maximum is located over diets which offered up to 50 g P.Kg$^{-1}$, and 75 g to 150 g.Kg$^{-1}$ C.
F) Mutant *for*$^{s2}$ males showed lower variability in DI across diets than *for*$^{R}$ and *for*$^{s}$ males. *for*$^{s2}$ wing shape variability was below 0.008 SD of *Rho* score units across the majority of nutrient space. Male *for*$^{s2}$ DI was highest on high-energy, P-biased diets, offering greater than 220 g.Kg$^{-1}$ P.

**Table 3.1. Generalised linear model statistical table for percentage survived to eclosion for all *foraging* genotypes.**

**Omnibus test**

| Likelihood ratio $\chi^2$ | df | Significance |
|---|---|---|
| 12.658 | 6 | $p = 0.049$ |

**Test of model effects**

| Source | Type III S.S. | | |
|---|---|---|---|
| | Wald $\chi^2$ | df | Significance |
| Intercept | 32.693 | 1 | $p < 0.001$ |
| *for* genotype | 1.272 | 2 | $p = 0.529$ |
| Protein | 1.598 | 1 | $p = 0.206$ |
| Carbohydrate | 2.058 | 1 | $p = 0.151$ |
| Protein$^2$ | 0.723 | 1 | $p = 0.395$ |
| Carbohydrate$^2$ | 2.295 | 1 | $p = 0.038$ |

**Parameter estimates**

| Parameter | β coefficient | Standard error | 95% Wald C.I. | | Hypothesis test | | |
|---|---|---|---|---|---|---|---|
| | | | Lower | Upper | Wald $\chi^2$ | df | Significance |
| Intercept | 56.332 | 10.8598 | 35.047 | 77.617 | 26.907 | 1 | $p < 0.001$ |
| *for*$^R$ genotype | 6.122 | 7.1914 | -7.973 | 20.217 | 0.725 | 1 | $p = 0.395$ |
| *for*$^s$ genotype | -1.582 | 7.1093 | -15.516 | 12.352 | 0.050 | 1 | $p = 0.824$ |
| *for*$^{s2}$ genotype | . | . | . | . | . | . | |
| Protein | 0.179 | 0.1417 | -0.099 | 0.457 | 1.598 | 1 | $p = 0.206$ |
| Carbohydrate | 0.221 | 0.1543 | -0.081 | 0.524 | 2.058 | 1 | $p = 0.151$ |
| Protein$^2$ | 0.000 | 0.0006 | -0.002 | 0.001 | 0.723 | 1 | $p = 0.395$ |
| Carbohydrate$^2$ | -0.001 | 0.0006 | -0.002 | -6.462E-005 | 4.295 | 1 | $p = 0.038$ |

**Table 3.2a. Generalised linear model statistical tables for female development rate .**

**Omnibus test**

| Likelihood ratio $\chi^2$ | df | Significance |
|---|---|---|
| 123.584 | 4 | $p < 0.001$ |

**Test of model effects**

| Source | Type III S.S. | | |
|---|---|---|---|
| | Wald $\chi^2$ | df | Significance |
| Intercept | 8924.665 | 1 | $p < 0.001$ |
| *for* genotype | 2.063 | 2 | $p = 0.356$ |
| Carbohydrate$^2$ | 149.881 | 1 | $p < 0.001$ |
| Protein*carbohydrate | 29.586 | 1 | $p < 0.001$ |

**Parameter estimates**

| Parameter | β coefficient | Standard error | 95% Wald C.I. | | Hypothesis test | | |
|---|---|---|---|---|---|---|---|
| | | | Lower | Upper | Wald $\chi^2$ | df | Significance |
| Intercept | 0.103 | 0.0016 | 0.100 | 0.106 | 4019.940 | 1 | $p < 0.001$ |
| $for^R$ | 0.001 | 0.0019 | -0.002 | 0.005 | 0.504 | 1 | $p = 0.478$ |
| $for^s$ | -0.001 | 0.0020 | -0.005 | 0.002 | 0.493 | 1 | $p = 0.482$ |
| $for^{s2}$ | . | . | . | . | . | . | . |
| Carbohydrate$^2$ | -6.172E-007 | 5.0413E-008 | -7.160E-007 | -5.184E-007 | 149.881 | 1 | $p < 0.001$ |
| Protein*carbohydrate | 7.225E-007 | 1.3283E-007 | 4.621E-007 | 9.828E-007 | 29.586 | 1 | $p < 0.001$ |

**Table 3.2b. Generalised linear model statistical tables for male developmental rate.**

**Omnibus test**

| Likelihood ratio $\chi^2$ | df | Significance |
|---|---|---|
| 92.551 | 5 | $p < 0.001$ |

**Test of model effects**

| Source | Type III S.S. | | |
|---|---|---|---|
| | Wald $\chi^2$ | df | Significance |
| Intercept | 2116.666 | 1 | $p < 0.001$ |
| *for* genotype | 10.293 | 2 | $p = 0.006$ |
| Protein | 20.661 | 1 | $p < 0.001$ |
| Protein$^2$ | 9.267 | 1 | $p < 0.001$ |
| Carbohydrate$^2$ | 47.082 | 1 | $p < 0.001$ |

**Parameter estimates**

| Parameter | β coefficient | Standard error | 95% Wald C.I. | | Hypothesis test | | |
|---|---|---|---|---|---|---|---|
| | | | Lower | Upper | Wald $\chi^2$ | df | Significance |
| Intercept | 0.103 | 0.0016 | 0.100 | 0.106 | 4019.940 | 1 | $p < 0.001$ |
| *for*$^R$ | 0.001 | 0.0019 | -0.002 | 0.005 | 0.504 | 1 | $p = 0.478$ |
| *for*$^s$ | -0.001 | 0.0020 | -0.005 | 0.002 | 0.493 | 1 | $p = 0.482$ |
| *for*$^{s2}$ | . | . | . | . | . | . | . |
| Protein | 0.000 | 4.1358E-005 | 0.000 | 0.000 | 20.661 | 1 | $p < 0.001$ |
| Protein$^2$ | -4.821E-007 | 1.5836E-007 | -7.925E-007 | -1.717E-007 | 47.082 | 1 | $p = 0.002$ |
| Carbohydrate$^2$ | -3.902E-007 | 5.6868E-008 | -5.017E-007 | -2.787E-007 | 47.082 | 1 | $p < 0.001$ |

**Table 3.2c. Generalised linear model statistical tables for time to eclosion for males of *for*$^R$ and *for*$^s$.**

**Omnibus test**

| Likelihood ratio $\chi^2$ | df | Significance |
|---|---|---|
| 50.972 | 4 | $p < 0.001$ |

**Test of model effects**

| Source | Type III S.S. | | |
|---|---|---|---|
| | Wald $\chi^2$ | df | Significance |
| Intercept | 1145.247 | 1 | $p < 0.001$ |
| *for* genotype | 6.224 | 1 | $p = 0.013$ |
| Protein | 13.249 | 1 | $p < 0.001$ |
| Protein$^2$ | 5.652 | 1 | $p < 0.017$ |
| Carbohydrate$^2$ | 17.641 | 1 | $p < 0.001$ |

**Parameter estimates**

| Parameter | β coefficient | Standard error | 95% Wald C.I. | | Hypothesis test | | |
|---|---|---|---|---|---|---|---|
| | | | Lower | Upper | Wald $\chi^2$ | df | Significance |
| Intercept | 0.087 | 0.0028 | 0.082 | 0.093 | 952.833 | 1 | $p < 0.001$ |
| *for*$^R$ | 0.006 | 0.0023 | 0.001 | 0.010 | 6.224 | 1 | $p = 0.013$ |
| *for*$^s$ | . | . | . | . | . | . | . |
| Protein | 0.000 | 5.3527E-005 | 8.992E-005 | 0.000 | 13.249 | 1 | $p < 0.001$ |
| Protein$^2$ | -4.889E-007 | 2.0566E-007 | -8.920E-007 | -8.586E-008 | 5.652 | 1 | $p = 0.017$ |
| Carbohydrate$^2$ | -3.261E-007 | 7.7639E-008 | -4.783E-007 | -1.739E-007 | 17.641 | 1 | $p < 0.001$ |

**Table 3.2d. Generalised linear model statistical tables time to eclosion for males of *for*$^R$ and *for*$^{s2}$.**

**Omnibus test**

| Likelihood ratio $\chi^2$ | df | Significance |
|---|---|---|
| 89.722 | 4 | $p < 0.001$ |

**Test of model effects**

| Source | Type III S.S. | | |
|---|---|---|---|
| | Wald $\chi^2$ | df | Significance |
| Intercept | 2311.916 | 1 | $p < 0.001$ |
| *for* genotype | 0.023 | 1 | $p = 0.879$ |
| Protein | 15.595 | 1 | $p < 0.001$ |
| Protein$^2$ | 7.923 | 1 | $p = 0.005$ |
| Carbohydrate$^2$ | 77.597 | 1 | $p < 0.001$ |

**Parameter estimates**

| Parameter | β coefficient | Standard error | 95% Wald C.I. | | Hypothesis test | | |
|---|---|---|---|---|---|---|---|
| | | | Lower | Upper | Wald $\chi^2$ | df | Significance |
| Intercept | 0.097 | 0.0021 | 0.092 | 0.101 | 2102.582 | 1 | $p < 0.001$ |
| *for*$^R$ | 0.000 | 0.0016 | -0.003 | 0.003 | 0.023 | 1 | $p = 0.879$ |
| *for*$^{s2}$ | . | . | . | . | . | . | . |
| Protein | 0.000 | 4.1182E-005 | 8.191E-005 | 0.000 | 15.595 | 1 | $p < 0.001$ |
| Protein$^2$ | -4.395E-007 | 1.5614E-007 | -7.455E-007 | -1.335E-007 | 7.923 | 1 | $p = 0.005$ |
| Carbohydrate$^2$ | -4.665E-007 | 5.2957E-008 | -5.703E-007 | -3.627E-007 | 77.597 | 1 | $p < 0.001$ |

**Table 3.2e. Generalised linear model statistical tables for time to eclosion for males of *for*$^{s}$ and *for*$^{s2}$.**

**Omnibus test**

| Likelihood ratio $\chi^2$ | df | Significance |
|---|---|---|
| 56.304 | 4 | $p < 0.001$ |

**Test of model effects**

| Source | Type III S.S. | | |
|---|---|---|---|
| | Wald $\chi^2$ | df | Significance |
| Intercept | 1227.862 | 1 | $p < 0.001$ |
| *for* genotype | 6.766 | 1 | $p = 0.009$ |
| Protein | 13.395 | 1 | $p < 0.001$ |
| Protein$^2$ | 5.595 | 1 | $p = 0.018$ |
| Carbohydrate$^2$ | 24.006 | 1 | $p < 0.001$ |

**Parameter estimates**

| Parameter | β coefficient | Standard error | 95% Wald C.I. | | Hypothesis test | | |
|---|---|---|---|---|---|---|---|
| | | | Lower | Upper | Wald $\chi^2$ | df | Significance |
| Intercept | 0.093 | 0.0028 | 0.088 | 0.099 | 1101.676 | 1 | $p < 0.001$ |
| *for*$^{s}$ | -0.006 | 0.0023 | -0.010 | -0.001 | 6.766 | 1 | $p = 0.009$ |
| *for*$^{s2}$ | . | . | . | . | . | . | . |
| Protein | 0.000 | 5.4758E-005 | 9.309E-005 | 0.000 | 13.395 | 1 | $p < 0.001$ |
| Protein$^2$ | -4.987E-007 | 2.1085E-007 | -9.120E-007 | -8.550E-007 | 5.595 | 1 | $p = 0.018$ |
| Carbohydrate$^2$ | -3.731E-007 | 7.6152E-008 | -5.224E-007 | -2.239E-007 | 24.006 | 1 | $p < 0.001$ |

**Table 3.3a. Generalised linear model statistical tables for female wing size.**

**Omnibus test**

| Likelihood ratio $\chi^2$ | df | Significance |
|---|---|---|
| 69.690 | 5 | $p < 0.001$ |

**Test of model effects**

| Source | Type III S.S. | | |
|---|---|---|---|
| | Wald $\chi^2$ | df | Significance |
| Intercept | 6236.294 | 1 | $p < 0.001$ |
| *for* genotype | 60.615 | 2 | $p < 0.001$ |
| Protein | 1.939 | 1 | $p = 0.164$ |
| Carbohydrate | 7.682 | 1 | $p = 0.006$ |
| Protein$^2$ | 2.966 | 1 | $p = 0.085$ |

**Parameter estimates**

| Parameter | β coefficient | Standard error | 95% Wald C.I. | | Hypothesis test | | |
|---|---|---|---|---|---|---|---|
| | | | Lower | Upper | Wald $\chi^2$ | df | Significance |
| Intercept | 2.224 | 0.0331 | 2.159 | 2.289 | 4516.927 | 1 | $p < 0.001$ |
| *for*$^R$ | -0.094 | 0.0267 | -0.146 | -0.041 | 12.322 | 1 | $p < 0.001$ |
| *for*$^s$ | 0.115 | 0.0276 | -0.061 | 0.169 | 17.491 | 1 | $p < 0.001$ |
| *for*$^{s2}$ | . | . | . | . | . | . | . |
| Protein | -0.001 | 0.0005 | -0.002 | 0.000 | 1.939 | 1 | $p = 0.164$ |
| Carbohydrate | 0.000 | 0.0002 | -0.001 | 0.000 | 7.682 | 1 | $p = 0.006$ |
| Protein$^2$ | -3.490E-006 | 2.0265E-006 | -4.817E-007 | 7.462E-006 | 2.966 | 1 | $p = 0.085$ |

**Table 3.3b. Generalised linear model statistical tables for wing size for *for*$^R$ and *for*$^s$ females.**

**Omnibus test**

| Likelihood ratio $\chi^2$ | df | Significance |
|---|---|---|
| 63.385 | 4 | $p < 0.001$ |

**Test of model effects**

| Source | Type III S.S. | | |
|---|---|---|---|
| | Wald $\chi^2$ | df | Significance |
| Intercept | 4202.970 | 1 | $p < 0.001$ |
| *for* genotype | 59.516 | 1 | $p < 0.001$ |
| Protein | 0.179 | 1 | $p = 0.672$ |
| Carbohydrate | 7.468 | 1 | $p = 0.006$ |
| Protein$^2$ | 0.154 | 1 | $p = 0.695$ |

**Parameter estimates**

| Parameter | β coefficient | Standard error | 95% Wald C.I. | | Hypothesis test | | |
|---|---|---|---|---|---|---|---|
| | | | Lower | Upper | Wald $\chi^2$ | df | Significance |
| Intercept | 2.339 | 0.0378 | 2.265 | 2.413 | 3832.208 | 1 | $p < 0.001$ |
| *for*$^R$ | -0.207 | 0.0268 | -0.260 | -0.154 | 59.516 | 1 | $p < 0.001$ |
| *for*$^s$ | . | . | . | . | . | . | . |
| Protein | 0.000 | 0.0006 | -0.002 | 0.001 | 0.179 | 1 | $p = 0.672$ |
| Carbohydrate | -0.001 | 0.0002 | -0.001 | 0.000 | 7.468 | 1 | $p = 0.006$ |
| Protein$^2$ | 9.638E-007 | 2.4572E-006 | -3.852E-006 | 5.780E-006 | 0.154 | 1 | $p = 0.695$ |

**Table 3.3c. Generalised linear model statistical tables for wing size for *for*$^R$ and *for*$^{s2}$ females.**

**Omnibus test**

| Likelihood ratio $\chi^2$ | df | Significance |
|---|---|---|
| 21.016 | 4 | $p < 0.001$ |

**Test of model effects**

| Source | Type III S.S. | | |
|---|---|---|---|
| | Wald $\chi^2$ | df | Significance |
| Intercept | 4166.875 | 1 | $p < 0.001$ |
| *for* genotype | 12.373 | 1 | $p < 0.001$ |
| Protein | 4.929 | 1 | $p = 0.026$ |
| Carbohydrate | 4.381 | 1 | $p = 0.036$ |
| Protein$^2$ | 4.867 | 1 | $p = 0.027$ |

**Parameter estimates**

| Parameter | β coefficient | Standard error | 95% Wald C.I. | | Hypothesis test | | |
|---|---|---|---|---|---|---|---|
| | | | Lower | Upper | Wald $\chi^2$ | df | Significance |
| Intercept | 2.255 | 0.0379 | 2.181 | 2.330 | 3535.790 | 1 | $p < 0.001$ |
| *for*$^R$ | -0.096 | 0.0272 | -0.149 | -0.042 | 12.373 | 1 | $p < 0.001$ |
| *for*$^{s2}$ | . | . | . | . | . | . | . |
| Protein | -0.001 | 0.0007 | -0.003 | 0.000 | 4.929 | 1 | $p = 0.026$ |
| Carbohydrate | 0.000 | 0.0002 | -0.001 | -2.846E-005 | 4.381 | 1 | $p = 0.036$ |
| Protein$^2$ | 5.592E-006 | 2.5345E-006 | 6.240E-007 | 1.056E-005 | 4.867 | 1 | $p = 0.027$ |

**Table 3.3d. Generalised linear model statistical tables for wing size for *for*$^{s}$ and *for*$^{s2}$ females.**

**Omnibus test**

| Likelihood ratio $\chi^2$ | df | Significance |
|---|---|---|
| 34.982 | 4 | $p < 0.001$ |

**Test of model effects**

| Source | Type III S.S. | | |
|---|---|---|---|
| | Wald $\chi^2$ | df | Significance |
| Intercept | 4168.108 | 1 | $p < 0.001$ |
| *for* genotype | 19.507 | 1 | $p < 0.001$ |
| Protein | 0.492 | 1 | $p = 0.483$ |
| Carbohydrate | 3.429 | 1 | $p = 0.064$ |
| Protein$^2$ | 2.576 | 1 | $p = 0.108$ |

**Parameter estimates**

| Parameter | β coefficient | Standard error | 95% Wald C.I. | | Hypothesis test | | |
|---|---|---|---|---|---|---|---|
| | | | Lower | Upper | Wald $\chi^2$ | df | Significance |
| Intercept | 2.185 | 0.0376 | 2.111 | 2.259 | 3535.790 | 1 | $p < 0.001$ |
| *for*$^{s}$ | 0.119 | 0.0268 | 0.066 | 0.171 | 12.373 | 1 | $p < 0.001$ |
| *for*$^{s2}$ | . | . | . | . | . | . | . |
| Protein | 0.000 | 0.0006 | -0.002 | 0.001 | 4.929 | 1 | $p = 0.483$ |
| Carbohydrate | 0.000 | 0.0002 | -0.001 | 2.232E-005 | 4.381 | 1 | $p = 0.064$ |
| Protein$^2$ | 3.905E-006 | 2.4329E-006 | -8.635E-007 | 8.673E-006 | 4.867 | 1 | $p = 0.108$ |

**Table 3.3e. Generalised linear model statistical tables for male wing size.**

**Omnibus test**

| Likelihood ratio $\chi^2$ | df | Significance |
|---|---|---|
| 48.394 | 5 | $p < 0.001$ |

**Test of model effects**

| Source | Type III S.S. | | |
|---|---|---|---|
| | Wald $\chi^2$ | df | Significance |
| Intercept | 3781.417 | 1 | $p < 0.001$ |
| *for* genotype | 36.671 | 2 | $p < 0.001$ |
| Carbohydrate | 6.171 | 1 | $p = 0.013$ |
| Carbohydrate$^2$ | 9.220 | 1 | $p = 0.002$ |
| Protein*carbohydrate | 3.262 | 1 | $p = 0.071$ |

**Parameter estimates**

| Parameter | β coefficient | Standard error | 95% Wald C.I. | | Hypothesis test | | |
|---|---|---|---|---|---|---|---|
| | | | Lower | Upper | Wald $\chi^2$ | df | Significance |
| Intercept | 1.885 | 0.0321 | 1.822 | 1.948 | 3452.029 | 1 | $p < 0.001$ |
| *for*$^R$ | -0.126 | 0.0260 | -0.177 | -0.075 | 23.482 | 1 | $p < 0.001$ |
| *for*$^s$ | 0.021 | 0.0260 | -0.030 | 0.072 | 0.666 | 1 | $p = 0.414$ |
| *for*$^{s2}$ | . | . | . | . | . | . | . |
| Carbohydrate | 0.002 | 0.0007 | 0.000 | 0.003 | 6.171 | 1 | $p = 0.013$ |
| Carbohydrate$^2$ | -7.369E-006 | 2.4268E-006 | -1.213E-005 | -2.612E-006 | 9.220 | 1 | $p = 0.002$ |
| Protein*carbohydrate | 3.246E-006 | 1.7971E-006 | -2.767E-007 | 6.768E-006 | 3.262 | 1 | $p = 0.071$ |

**Table 3.3f. Generalised linear model statistical tables for wing size for *for*$^R$ and *for*$^s$ males.**

**Omnibus test**

| Likelihood ratio $\chi^2$ | df | Significance |
|---|---|---|
| 70.152 | 4 | $p < 0.001$ |

**Test of model effects**

| Source | Type III S.S. | | |
|---|---|---|---|
| | Wald $\chi^2$ | df | Significance |
| Intercept | 2842.362 | 1 | $p < 0.001$ |
| *for* genotype | 36.716 | 1 | $p < 0.001$ |
| Carbohydrate | 2.980 | 1 | $p = 0.084$ |
| Carbohydrate$^2$ | 9.963 | 1 | $p = 0.002$ |
| Protein*carbohydrate | 20.340 | 1 | $p < 0.001$ |

**Parameter estimates**

| Parameter | β coefficient | Standard error | 95% Wald C.I. | | Hypothesis test | | |
|---|---|---|---|---|---|---|---|
| | | | Lower | Upper | Wald $\chi^2$ | df | Significance |
| Intercept | 1.911 | 0.0365 | 1.839 | 1.982 | 2746.684 | 1 | $p < 0.001$ |
| *for*$^R$ | -0.145 | 0.0240 | -0.192 | -0.098 | 36.716 | 1 | $p < 0.001$ |
| *for*$^s$ | . | . | . | . | . | . | . |
| Carbohydrate | 0.001 | 0.0007 | 0.000 | 0.003 | 2.980 | 1 | $p = 0.084$ |
| Carbohydrate$^2$ | -8.658E-006 | 2.7432E-006 | -1.404E-005 | -3.282E-006 | 9.963 | 1 | $p = 0.002$ |
| Protein*carbohydrate | 8.664E-006 | 1.9210E-006 | 4.899E-005 | 1.243E-006 | 20.340 | 1 | $p < 0.001$ |

**Table 3.3g. Generalised linear model statistical tables for wing size for *for*$^R$ and *for*$^{s2}$ males.**

**Omnibus test**

| Likelihood ratio $\chi^2$ | df | Significance |
|---|---|---|
| 33.390 | 4 | $p < 0.001$ |

**Test of model effects**

| Source | Type III S.S. | | |
|---|---|---|---|
| | Wald $\chi^2$ | df | Significance |
| Intercept | 2316.430 | 1 | $p < 0.001$ |
| *for* genotype | 22.261 | 1 | $p < 0.001$ |
| Carbohydrate | 15.053 | 1 | $p < 0.001$ |
| Carbohydrate$^2$ | 12.525 | 1 | $p < 0.001$ |
| Protein*carbohydrate | 2.624 | 1 | $p = 0.105$ |

**Parameter estimates**

| Parameter | β coefficient | Standard error | 95% Wald C.I. | | Hypothesis test | | |
|---|---|---|---|---|---|---|---|
| | | | Lower | Upper | Wald $\chi^2$ | df | Significance |
| Intercept | 1.842 | 0.0378 | 1.768 | 1.916 | 2375.345 | 1 | $p < 0.001$ |
| *for*$^R$ | -0.126 | 0.0268 | -0.179 | -0.074 | 22.261 | 1 | $p < 0.001$ |
| *for*$^{s2}$ | . | . | . | . | . | . | . |
| Carbohydrate | 0.003 | 0.0008 | 0.002 | 0.005 | 15.053 | 1 | $p < 0.001$ |
| Carbohydrate$^2$ | -1.058E-005 | 2.9894E-006 | -1.644E-005 | -4.721E-006 | 12.525 | 1 | $p < 0.001$ |
| Protein*carbohydrate | -3.819E-006 | 2.3578E-006 | -8.441E-005 | 8.019E-007 | 2.624 | 1 | $p = 0.105$ |

**Table 3.3h. Generalised linear model statistical tables wing size for *for*$^{s}$ and *for*$^{s2}$ males.**

**Omnibus test**

| Likelihood ratio $\chi^2$ | df | Significance |
|---|---|---|
| 6.318 | 4 | $p = 0.177$ |

**Test of model effects**

| Source | Type III S.S. | | |
|---|---|---|---|
| | Wald $\chi^2$ | df | Significance |
| Intercept | 2670.720 | 1 | $p < 0.001$ |
| *for* genotype | 0.817 | 1 | $p = 0.366$ |
| Carbohydrate | 0.370 | 1 | $p = 0.543$ |
| Carbohydrate$^2$ | 0.925 | 1 | $p = 0.336$ |
| Protein*carbohydrate | 3.161 | 1 | $p = 0.075$ |

**Parameter estimates**

| Parameter | β coefficient | Standard error | 95% Wald C.I. | | Hypothesis test | | |
|---|---|---|---|---|---|---|---|
| | | | Lower | Upper | Wald $\chi^2$ | df | Significance |
| Intercept | 1.924 | 0.0383 | 1.849 | 2.000 | 2521.890 | 1 | $p < 0.001$ |
| *for*$^{s}$ | 0.024 | 0.0267 | -0.028 | 0.076 | 0.817 | 1 | $p = 0.366$ |
| *for*$^{s2}$ | . | . | . | . | . | . | . |
| Carbohydrate | 0.001 | 0.0008 | 0.001 | 0.002 | 0.370 | 1 | $p = 0.543$ |
| Carbohydrate$^2$ | -2.942E-006 | 3.0591E-006 | -8.937E-006 | 3.054E-006 | 0.925 | 1 | $p = 0.336$ |
| Protein*carbohydrate | 4.009E-006 | 2.2550E-006 | -4.105E-007 | 8.429E-006 | 3.161 | 1 | $p = 0.075$ |

**Table 3.3i. Generalised linear model statistical tables for wing size for *for*$^{s2}$ males.**

**Omnibus test**

| Likelihood ratio $\chi^2$ | df | Significance |
|---|---|---|
| 12.935 | 3 | $p = 0.005$ |

**Test of model effects**

| Source | Type III S.S. | | |
|---|---|---|---|
| | Wald $\chi^2$ | df | Significance |
| Intercept | 1264.851 | 1 | $p < 0.001$ |
| Carbohydrate | 5.789 | 1 | $p = 0.016$ |
| Protein$^2$ | 2.264 | 1 | $p = 0.132$ |
| Protein*carbohydrate | 9.106 | 1 | $p = 0.003$ |

**Parameter estimates**

| Parameter | β coefficient | Standard error | 95% Wald C.I. | | Hypothesis test | | |
|---|---|---|---|---|---|---|---|
| | | | Lower | Upper | Wald $\chi^2$ | df | Significance |
| Intercept | 1.865 | 0.0524 | 1.762 | 1.968 | 1264.851 | 1 | $p < 0.001$ |
| Carbohydrate | 0.003 | 0.0012 | 0.001 | 0.005 | 5.789 | 1 | $p = 0.016$ |
| Protein$^2$ | -6.576E-006 | 4.3710E-006 | -1.514E-005 | 1.991E-006 | 2.264 | 1 | $p = 0.132$ |
| Protein*carbohydrate | -1.100E-005 | 3.6446E-006 | -1.814E-005 | -3.855E-006 | 9.106 | 1 | $p = 0.003$ |

**Table 3.4. Two–way MANOVA on Procrustes coordinates derived from all fly wing specimens with *foraging* genotype and fly sex as factors.**

**Multivariate Test Results**

| Effect | Wilks' λ value | *F* | Hypothesis d.f. | Error df | Significance |
|---|---|---|---|---|---|
| Intercept | 0.0 | 351355560.9 | 20.0 | 1115.0 | $p < 0.001$ |
| Genotype | 0.071 | 153.003 | 40.0 | 2230.0 | $p < 0.001$ |
| Sex | 0.346 | 105.18 | 20.0 | 1115.0 | $p < 0.001$ |

# Chapter 4.

## Nutritional environment dictates secondary sexual trait quality: how to eat your way bigger and more beautiful

## Abstract

This study demonstrates the central role nutritional environments play in shaping secondary-sexual trait variation – variation requisite for evolution via sexual selection to proceed. We experimentally controlled male *Drosophila melanogaster* 'condition' using artificial diets, and measured the condition-dependent expression of male traits, including the size and symmetry of a secondary sexual trait, the sex comb. Using the Geometric Framework (GF) for nutrition we show that different diets, and thus condition, support maximal expression of alternate male traits. High-protein diets support rapid development and high survivorship, while modestly carbohydrate-biased diets support the development of larger bodied males and larger sex combs. High-calorie formulations of these same carbohydrate-biased diets drive the development of the largest sex combs per unit body size, and the most symmetrical sex combs. Intriguingly, when mated female *D. melanogaster* were given the option of which diets to oviposit upon, thus influencing the 'condition' of their male offspring, they do not choose the sex comb quality optimising carbohydrate-biased diet. Unambiguously, they chose to lay eggs on the survivorship and developmental rate maximising high-protein diet – suggesting *D. melanogaster* may be subject to an intergenerational, sexual-conflict.


## Introduction

The interaction between an animal's genetic potential and the resources it obtains throughout life dictate its 'condition'. Theory claims an animal's condition determines the level of resources it can invest into competing life history traits and phenotypes, with animals in better condition having a larger resource pool from which to allocate simultaneously to all traits (Rowe & Houle, 1996). Animals whose mating systems are subject to inter- or intrasexual selection possess secondary sexual characters whose expression is markedly condition-dependent – more so than non-sexual traits (Zahavi, 1977; Cotton *et al*., 2004; Andersson & Simmons, 2006). The 'handicap' theory of sexual selection posits characters under direct sexual selection exhibit heightened condition dependence, as they act as intraspecific honesty signals of the bearer's quality (Zahavi, 1975, 1977; Rowe & Howle, 1996). Relative mating success of individuals within a population is then determined by the quality of the sexual trait (Darwin, 1871; Zahavi, 1977; Andersson, 1982; Cotton *et al*., 2004).

Under inter-sexual selection this is via enhanced attractiveness. The sexual trait acts as an advertisement. Under intra-sexual selection, a superior trait usually functions as a superior weapon, affording its owner a competitive edge (Darwin, 1871; Eberhard, 1985; Polak *et al*., 2004).

Traditionally, secondary sexual characters are considered costly for their bearer to produce ontogenetically and/or to sustain display of throughout life – or both (Zahavi, 1977; Pomiankowski, 1987), hence 'handicap'. Male peacock tail feathers provide a famous example (Zahavi, 1977; Maynard Smith & Harper, 1995). Considerable resources are utilised in producing the feathers and their possession is thought to increase vulnerability to predation or disease (Folstad & Karter, 1992). It also follows, however, that individuals simply in better overall condition can produce and maintain higher quality secondary sexual traits (Maynard Smith & Harper, 1995). Rather than the sexual trait appearing as a measure of how well the individual copes with the putative handicap of trait possession, it is possible the trait simply marks the individual as being of the highest overall quality. Sexual selection on the trait would still proceed under this scenario (Cornwallis & Uller, 2009).

Whether an animal reaches the reproductive stage in prime condition is driven largely by juvenile and sub-adult experiences, with access to appropriate nutrition a key determinant of adult health and performance (Delisle & Hardy, 1997; Burns *et al*., 2012). Secondary sexual characters are typically exaggerations of existing morphological traits exhibited by individuals of a given lineage, whose expression has been elaborated by many generations of sexual selection. (Eberhard, 1985; Andersson, 1994; Panhuis *et al*., 2001). Traits wherein elaboration has been driven by intra-sexual selection include horns in deer (Ditchkoff *et al*., 2001), sheep (Robinson *et al*., 2008), rhinoceros (Jarman, 1983) and, indeed, rhinoceros beetles (Emlen *et al*., 2005). Traits thought to be driven by inter-sexual selection include peacock tail feathers (Zahavi, 1977), bird of paradise display feathers (Diamond, 1986) and elongated eye stalks in Diopsidae flies (Burkhardt *et al*., 1994; David *et al*., 2000). Production of these morphological structures requires a considerable volume of keratin or chitin to be synthesised endogenously by the animal. Developing secondary sexual traits is additional to standard somatic ontological demands, presenting a significant nutritional resource acquisition and allocation challenge.

It is not known whether nutritional resources required to maximise traits shaped largely by natural selection, versus secondary sexual traits shaped exclusively sexual selection, are equivalent. What of somatic traits whose expression is informed partially through natural *and* sexual selection? Does achieving optimal condition – thus supporting optimal sexual trait expression - require 'more of the same', or does maximal expression of sexual traits require specialised nutrition? Here we address this question by examining male trait condition dependence in the important model organism *Drosophila melanogaster*. We use the Geometric Framework for nutrition (GF) (Simpson & Raubenheimer, 2012) to control the specific condition of males experimentally, rearing larval *Drosophila* from hatch to adult on nutritionally-defined diets. This method avoids the statistical and conceptual problems of condition indices (Jakob *et al*., 1996; Cotton *et al*., 2004; Tomkins *et al*., 2004) and allows for accurate comparison of relative trait expression between individuals in the same condition (*i.e.* raised on the same diet). We measure condition dependence of the non-sexual traits, survivorship to eclosion, developmental rate and body size, and compare their responses to that of the sex comb, a *D. melanogaster* secondary sexual trait. (Refer to previous chapters for a detailed explanation of the GF.)

Males of many *Drosophila* species possess sex combs: hard, chitinous, bristle-like structures of the foreleg tibia. Among closely-related *Drosophila* species, sex comb morphology is strikingly diverse (Kopp & True, 2002) –

typical of secondary sexual characters under active sexual selection (Eberhard, 1985; Andersson, 1994; Panhuis *et al*., 2001). Relative to the considerable knowledge of *D. melanogaster* developmental genetics, the species' behavioural ecology is poorly understood. While the exact role of sex combs is still being characterised, they are likely to be involved in numerous behaviours important to primary and secondary sexual activities. Males use their sex combs to grasp female genitalia during copulation (Hurtado-Gonzales *et al*., in prep), during 'lunging' in intra-sexual fights (Chen *et al*., 2002; Hoyer *et al*, 2008) and are potentially displayed to females during courtship 'tapping' (Sokolowski, 2001). Here we consider the males that produce relatively bigger, more symmetrical sex combs (Markow, 1987; Polak & Tomkins, 2012) to be expressing the trait optimally.

Quantification of secondary sexual trait fluctuating asymmetry (FA), the deviation from perfect symmetry expressed by bilateral morphological traits (Polak, 2003), is a tractable means of assessing trait quality. Measuring an individual's trait FA, for example that of a male fly's sex comb pair, provides quantification of the environmental stress incurred by the individual across development (Bradshaw, 1965; Polak & Tomkins, 2012). As common molecular processes drive the physical development of each trait within a pair, FA reveals how well an individual, given its genetic background and broader developmental setting, has withstood environmental perturbations during trait synthesis (Palmer & Strobeck, 1986; Møller, 2006). In this study, we control the environmental setting of larvae, such that only nutrition varies. Evidence that larger, more symmetrical combs are of relatively higher quality than smaller, asymmetrical sex combs in *Drosophila* comes from a species closely related to *D. melanogaster*: *D. bipectinata*. Both symmetry and size of male sex combs have been demonstrated to be under sexual selection in *D. bipectinata* (Polak *et al*., 2004). Male *D. bipectinata* with larger sex combs also sire more offspring than males with smaller combs (Polak & Simmons, 2009).

We complement our direct study of condition-dependence of male traits with a maternal oviposition substrate choice experiment. A strong determinant of a *Drosophila* larva's early nutrition is the food substrate it is laid upon as an egg (Refsnider & Janzen, 2010; Thompson, 1988). We test for correspondence between rearing diets that support optimal maximal trait expression and the diets upon which mother flies choose to lay their eggs. Do mothers, through their selection of oviposition substrate, bias their offspring toward optimal expression of specific traits and thus entrain offspring toward particular 'sexual phenotypes' (Cornwallis & Uller, 2009)?

## Materials and Methods

### *Fly stocks*

We used a long-term laboratory reared culture of Canton-S *D. melanogaster* for all experiments. Prior to experimentation, flies had been raised under standard outbreeding conditions for hundreds of generations, on a medium comprising 16 g agar, 80 g brewer's yeast (thermolysed), 125 g semolina, 250 g golden syrup, and 0.5

g of anti-fungal methylparaben ('Nipagin' Sigma-Aldrich) per L distilled water. All culture rearing and experiments were conducted at 25°C, under 12L:12D light cycle, with lights on at 0700 h.

*Controlling condition: 'no-choice' experimental rearing diets*

We raised larvae from egg hatch to adulthood on one of 24 diets that ranged over eight protein (P) to carbohydrate (C) ratios (1:1, 1:2, 1:4, 1:9, 1:16, 2:1, 4:1 and 7:1) and three energy densities (75, 200, and 400 $g.kg^{-1}$ P+C). Dietary macronutrient sources were thermolysed baker's yeast (containing 47.4% P, 24% digestible C, Lowan whole foods), sucrose (98% C), and casein (95.7% P, 0.8% C, Sigma-Aldrich). Agar (93.9% C) was used to gel diets (comprising < 2 $g.kg^{-1}$ of any experimental diet) and following Lee *et al.* (2008) distilled water was used to dilute P:C mixes to the appropriate energy density. 10 mL of each experimental diet was poured into separate, sterilised 60 mL, 3 cm diameter plastic vials. The experiment was conducted in two rounds. Within each round, each diet was replicated five times, resulting in ten replicate diet-vials in total. Within each round, one vial of each of the 24 diets were maintained within 25 x 25 x 7 cm plastic containers to maintain ~100 % relative humidity.

For each experimental run, larvae were sourced from three replicate parent-vials containing approximately 100 male and 100 female adult flies that were ~5 days post-eclosion. Parent flies were left to mate and females to oviposit in fresh vials capped with a Petri dish containing oviposition medium topped with ~0.5 g fresh yeast paste. Oviposition medium was 1.8 g agar, 50 mL deionised water and 1 g golden syrup. The media mix was boiled then allowed to cool and set. Oviposition media and yeast were replaced in each vial after 24. After another 12 h hatchlings were removed from each Petri dish. After an additional 3 h, in each round, 12 larvae were introduced into each experimental diet-vial. Vials were then capped with snuggly-fitting foam caps and a perforated lid was fitted to each of the larger plastic containers. Twelve larvae per vial, with 24 diets replicated 10 times, gave a total of 2880 larvae entering the experiment.

In the first round, all male larvae that successfully eclosed were collected under $CO_2$ anaesthesia 12 to 24 h post eclosion. While remaining anaesthetized, flies were transferred to 1.5 mL centrifuge tubes containing 70% ethanol. Subsequently,

thorax length was measured to quantify body size using an ocular micrometer. Thorax length was measured as the distance from the anterior edge of the thorax to the distal end of the scutellum. Under a dissecting light microscope, each male's left and right hand sex comb tooth number was counted. Following Polak *et al*. (2004), sex comb size was quantified as total sex comb teeth (number on left + number of right) and sex comb symmetry was calculated by subtracting the number of sex comb teeth on the side of the body with fewer teeth, from the side of the body with more – thus calculating absolute symmetry. From the second round we measured two response variables: the percentage of male larvae that survived to eclosion and the number of days it took to reach eclosion. We subsequently plotted and analysed developmental rate as the inverse of the number of days to eclosion.

High measurement error is a common criticism of the use of FA as a measure of individual and/or sexual trait quality (Merilä & Björklund, 1995; Palmer & Strobeck, 2003). The critique states that FA is typically subtle, and that the level of inter-individual measurement error incurred when quantifying trait size can be as high, or higher than, the within-individual differences in trait size, which are the targets of measurement (Merilä & Björklund, 1995; Palmer & Strobeck, 2003). This is a substantial challenge when measuring continuous morphological characters (Palmer & Strobeck, 2003); however, for discrete characters, like sex comb teeth, this methodological shortcoming, given accurate counting, is not applicable (see Polak *et al*., 2004).

*Visualisation of condition-dependent trait response surfaces using thin plate splines*

Trait performance response surfaces were plotted for survivorship, developmental rate, body size, sex comb size (total tooth number) and sex comb absolute asymmetry. Sex comb size, corrected for body size, was also calculated and fitted as a response surface. Correction was achieved by obtaining the residual values from a linear regression analysis (performed in SPSS ver. 21) where each male's sex comb total tooth number was regressed over thorax length. Ten units were then added to each value such that only positive values would appear on the response surface, with higher values representing relatively bigger combs 'per unit' body size. Traits were mapped onto P-C nutrient space as thin plate splines (TPSs) using the fields

package (ver. 6.8) in R (ver. 3.0.2). (Refer to Materials and Methods Chapters 2 & 3 for the approach we used when fitting TPS.)

*Trait response surface statistical analysis: generalised linear modeling*

Response surfaces were statistically analysed using a Lande & Arnold generalised linear modeling (GLM) approach, following Lee *et al*. (2008) and Dussutour *et al*. (2010). (Refer to the Materials and Methods sections of previous chapters for a detailed explanation of the modeling methodology we used.)

Unfortunately, round one samples from the 4P:1C and 7P:1C 400 g P+C.kg$^{-1}$ diets were lost due to a fungal contaminant. Response surfaces for body size, sex comb size and sex comb symmetry lack measures for this region of nutrient space.

*Maternal oviposition substrate choice experiment*

We allowed mother flies to choose between five experimental diets upon which to oviposit, set up in 20 replicate choice arenas. Diets were: 1P:1C at 200 g P+C.kg$^{-1}$, 1P:2C at 400 g P+C.kg$^{-1}$, 2P:1C at 200 and 400 g P+C.kg$^{-1}$, and 4P:1C at 400 g P+C.kg$^{-1}$. Diets were formulated using the ingredients described above. Three mL of each diet was then poured into 20 separate 1 cm-deep, 2 cm-diameter replicate dishes, totaling 100 dishes across the five diets. One dish of each diet was then placed inside one of 20 large, 30 x 25 x 8 cm plastic containers fitted with a non-air-tight lid. Diets were arranged in a ring, with the sequence of diets in each container designated haphazardly. Ten adult female *D. melanogaster*, 1.5 days post- eclosion, were then introduced to each of the 20 arenas. Females were left to oviposit for 12 hours. The total number of eggs on each diet dish was then counted under a dissecting microscope. The percentage of the total number of eggs laid in the experiment on each individual dish was then calculated. Using SPSS (ver. 21) we conducted a non-parametric Kruskal-Wallis test with *post-hoc*, Bonferroni α corrected, multiple comparisons to determine on which of the diets females laid more eggs.

Mother flies were sourced using the technique described above for obtaining age-staged larvae. However, instead of placing hatchlings into experimental diets, 100 hatchlings from each parent-vial were placed into each of five fresh culture vials, containing standard culture diet. Newly-eclosed females remained within these vials,

along with any newly-eclosed male mates, for 36 h, after which they were collected under light $CO_2$ anaesthesia and allocated haphazardly to oviposition choice arenas.

## Results

*Survivorship*

The thin plate spline (TPS) response surface shows highest male survivorship to eclosion of 90-95% on high-P diets offering > 200 g $P^{\cdot}.kg^{-1}$ and between 100 and 200 g $C^{\cdot}.kg^{-1}$ (Figure 1a). This location on the nutritional plane corresponds to high calorie 2P:1C and 4P:1C rearing diets. Survivorship on any diet offering between 100-200 g $C^{\cdot}.kg^{-1}$ was associated with > 75% survivorship. Lower survivorship occurred on diets containing > 200 g $C^{\cdot}.kg^{-1}$, and > 300 g $P^{\cdot}.kg^{-1}$, and on all high-P diets offering < 200 g P and 100 g $C^{\cdot}.kg^{-1}$. Generalised linear modeling (GLM) corroborated these TPS patterns. The linear term of C was significantly positively associated with survivorship (Wald $\chi^2 = 40.548$, d.f. = 1, $p < 0.001$, Table 1), while the quadratic term was significantly associated with decrease (Wald $\chi^2 = 54.129$, d.f. = 1, $p < 0.001$, negative β coefficient, Table 1). High levels of dietary protein were also significantly associated with an increase in survivorship .(*protein*$^2$, Wald $\chi^2$ 19.526, d.f. = 1, $p < 0.001$, positive β coefficient, Table 1).

*Development rate*

The TPS response surfaces for developmental rate shows that the fastest time to eclosion was achieved by males raised on high-protein, high-energy diets. Rearing diets offering between 275-300 g $P^{\cdot}.kg^{-1}$ and 75-100 g $C^{\cdot}.kg^{-1}$ led to males eclosing within 7 d (Figure 1b). All males raised on diets offering ≥ 2P:1C eclosed within 9 d. Development rate decreased gradually for males raised on any diet containing ≥ 175 g $C^{\cdot}.kg^{-1}$. GLM showed both linear and quadratic terms of protein and carbohydrate predicted the developmental rate response surface shape. The linear protein term was associated with decrease in developmental rate (*protein*, Wald $\chi^2$ 9.412, d.f. = 1, $p = 0.002$, negative β coefficient, Table 2), while high levels of dietary protein were associated with increase (*protein*$^2$, Wald $\chi^2 = 14.744$, d.f. = 1, $p < 0.001$, positive β coefficient, Table 2). The linear and quadratic terms of dietary carbohydrate were significantly associated with the shape of the developmental rate surface. The linear term was significantly associated with increase in elevation (*carbohydrate*, Wald $\chi^2 =$

40.237, d.f. = 1, $p < 0.001$, positive β coefficient, Table 2) and the quadratic term with decrease at high value of C (*carbohydrate*$^2$,Wald $\chi^2 = 160.562$, d.f. = 1, $p < 0.001$, negative β coefficient, Table 2).

*Body size*

Balanced protein to carbohydrate rearing diets, offering up to 200 g P+C·kg$^{-1}$ supported development of the largest males. Body size decreased for males on rearing diets comprising > 175 g P·.kg$^{-1}$ and > 200 g C·.kg$^{-1}$ (Figure 2a). Supporting this, GLM showed both the linear terms of protein and carbohydrate significantly contributed to increase in body size (Wald $\chi^2 = 6.659$, d.f. = 1, $p < 0.001$ and Wald $\chi^2 = 4.727$, d.f. = 1, $p < 0.05$ respectively, positive β coefficients, Table 3). Both the quadratic terms of dietary protein and carbohydrate were associated with decrease in surface elevation at high nutrient concentrations (*protein*$^2$, Wald $\chi^2 = 8.404$, d.f. = 1, $p = 0.004$, negative β coefficient; *carbohydrate*$^2$, Wald $\chi^2 = 8.184$, d.f. = 1, $p = 0.004$, negative β coefficient).

*Sex comb size: total sex comb tooth number*

Larval rearing diets associated with the region of nutrient space between the 1P:1C and 1P:2C food rails, offering greater than > 100 g P+C·.kg$^{-1}$, supported the development of the largest male sex combs. This is visible on the sex comb size TPS surface as a thick ridge of deep red, denoting males with ~22 comb teeth (Figure 2b). This ridge dropped sharply in elevation as the proportion of carbohydrate in the rearing diet increased above 4C:1P. Low- to mid-energy high-P, rearing diets of P:C ratio of > 1.5P:C were associated with smaller sex combs (Figure 2b). GLM showed the cross-product of P and C was significantly associated with increase in sex comb size (Wald $\chi^2 = 6.561$ d.f. = 1, $p < 0.05$, positive β coefficient, Table 4), while the quadratic term of dietary protein was associated with smaller combs at high values for dietary P (Wald $\chi^2 = 7.138$ d.f. = 1, $p < 0.05$, negative β coefficient, Table 4, Figure 2b).

*Sex comb size per unit body size*

There was a distinct hot spot in high-energy, high-C nutrient space wherein the largest sex combs, corrected for body size, developed. Males with the largest sex

combs relative to their body size were those reared on diets offering between 100-200 g P.kg$^{-1}$ and 200-300 g C.kg$^{-1}$ (Figure 2c). Sex comb size decreased gradually as the percentage of total dietary P increased away from the comb-size maximum, and decreased steeply with increase in dietary C above a 1P:3C ratio. GLM showed combined simultaneous increase in dietary protein and carbohydrate (Wald $\chi^2 = 7.831$, d.f. = 1, $p = 0.005$, positive β coefficient, Table 5), which, combined with linear increases in dietary carbohydrate (Wald $\chi^2 = 7.712$, d.f. = 1, $p = 0.005$, positive β coefficient, Table 5), significantly predicted increase in sex comb size response surface relief. Decrease in response surface relief at high value for dietary P, was reflected by the significant quadratic term for dietary protein (Wald $\chi^2 = 19.794$, d.f. = 1, $p < 0.001$, negative β coefficient, Table 5) and carbohydrate (Wald $\chi^2 = 21.181$, d.f. = 1, $p < 0.001$, negative β coefficient, Table 5).

*Sex comb absolute fluctuating asymmetry*

The response surface of male sex comb absolute FA is markedly divided by a deep valley of near-zero values, denoting males with highly symmetrical sex combs. Males raised on diets containing between 1P:1C and 1P:2C developed more symmetrical combs, with diets offering > 200 g C.kg$^{-1}$ and between 75-150 g P.kg$^{-1}$ supporting the most symmetrical combs. Gradual increase in dietary carbohydrate within this protein range led to increase in sex comb symmetry (Figure 2d). GLM showed the quadratic term of carbohydrate was significantly associated with decrease in surface relief (Wald $\chi^2 = 15.282$, d.f. = 1, $p < 0.001$, negative β coefficient, Table 6), as was the quadratic term of protein (Wald $\chi^2 = 16.829$, d.f. = 1, $p < 0.001$, negative β coefficient, Table 6). Linear increase in dietary carbohydrate was significantly associated with increase in surface relief (Wald $\chi^2 = 13.610$, d.f. = 1, $p < 0.001$, positive β coefficient, Table 6).

*Oviposition choice between five trait-optimising diets*

We compared maternal oviposition substrate choice among the five rearing diets that supported maximal performance of the following traits: survivorship to eclosion (2P:1C at 200 and 400 g P+C .kg$^{-1}$), developmental rate (4P:1C, 400 g P+C .kg$^{-1}$), body size (1P:1C, 200 g P+C .kg$^{-1}$), sex comb size per unit body size (1P:2C, 400 g P+C .kg$^{-1}$), and sex comb symmetry (also 1P:2C, 400 g P+C .kg$^{-1}$). Kruskal-

Wallis analysis showed that the nutritional content of the laying substrate significantly influenced oviposition choice ($\chi^2 = 32.812$, d.f. = 4, $p < 0.001$, Table 7a) with *post-hoc* Bonferroni multiple comparisons showing mother flies laid over 60% of all experimental eggs on the highest protein, highest energy diet; 4P:1C at 400 g P+C $.kg^{-1}$ (Figure 3, Table 7b).

## Discussion

We have identified divergence between the nutritional conditions that support maximal survival and developmental rate in male *D. melanogaster* and those that support the (putatively) highest-quality secondary sexual traits. Surprisingly, no single diet composition, or sub-set of similar diets, simultaneously drove maximal expression of traits subject to both sexual and natural selection. There was no single 'best condition' or attendant single optimal sexual phenotype for male *D. melanogaster.* Traversing nutrient space delimited by the 1P:1C – 1P:2C food rails saw alternate adult male traits optimally expressed. Lower energy diets in this region optimised body size and mid- to high-energy diets optimised sex comb size. This demonstrates that *D. melanogaster* males possess the capacity to develop alternate, sexual phenotypes (Conwallis & Uller, 2009) according to their larval nutritional environments. Sex comb quality, quantified here as body-size-corrected comb size and comb symmetry, was maximal on the high-calorie region of 1P:1C to 1P:2C nutrient space. With remarkable adherence to the predictions of the handicap hypothesis (Zahavi, 1975, 1977), achieving this condition presented developing male *D. melanogaster* with severe fitness costs: a one in four probability of dying during the larval stage and 75% longer time to eclose than high-protein reared conspecifics. The most rapid male development and highest survivorship to eclosion was, contrastingly, maximised by high-energy, heavily protein-biased foods.

Intriguingly, when presented the option of five different performance enhancing foods to lay eggs on, mother flies laid the overwhelming majority on a single diet: high-calorie 4P:1C food. This diet does not support maximal sex comb quality or body size but maximal survival and rapid development to adulthood. While we concede that larval nutrition is not wholly restricted to the oviposition medium, our findings suggest *D. melanogaster* may be subject to parent-offspring conflict. Mothers appear to be biased toward promoting an alternative, less-developmentally

costly, sexual phenotype. Rather than producing fewer, yet 'sexier' sons (Pomiankowski & Iwasa, 1998), our experiment reveals mated females are biased toward producing offspring that reach adulthood more quickly and that, in 95% of cases, survive.

*Do mother flies spread fitness outcomes evenly between female and male offspring, or do they 'ignore' their son's nutritional needs?*

There are competing explanations for maternal choice of oviposition substrate. Raising offspring primarily on high-energy, high-protein diets might return the greatest overall fitness benefits for mothers by co-maximising lifetime reproductive success of their male and female offspring (Uller, 2008). Evidence supporting this comes from Hunt *et al.* (2004), who showed male and female *Teleogryllus commodus* crickets raised on a 45% protein diet survived better and grew faster and bigger than lower-protein-reared conspecifics. Females reared on high protein also lived longer and males delivered higher lifetime courtship song than individuals reared on lower protein foods. However, more recent work by Maklakov *et al* (2008) indicates that to achieve optimal fitness, males and females require different dietary compositions. Again working with *T. commondus*, these authors found male lifetime calling effort was actually maximal on lower P:C diets (of a similar ratio to our sex comb optimising 1P:2C diet) while female maximal life time egg production was supported by equal protein to carbohydrate food. Lee (2010) has demonstrated similar sex-specific nutritional needs in *Spodoptera litura* caterpillars. When males and females are given the option of either protein- or carbohydrate-biased meals, females consistently chose protein-biased food and males, carbohydrate.

The proposition that mothers are choosing high-protein foods to average fitness outcomes across offspring of both sexes is also at odds with our finding of striking condition dependence of male sex comb quality. We found that a highly-restricted area of nutrient space, high-energy 1P:2C-1P:1C diets, supported the condition linked to optimal comb expression, *i.e.* large, symmetrical combs. Achieving this condition even exacted a 25% chance of pre-reproductive mortality. These findings - extreme condition-dependence and an associated viability handicap - are obvious hallmarks of sexual selection shaping secondary sexual trait expression (Zahavi, 1977; Cotton *et al.*, 2004; Andersson & Simmons, 2006). Following theory,

males with optimal combs should have higher fitness. Some evidence of this already exists for *D. bipectinata* fruit flies. Male *D. bipectinata* possessing larger sex combs sire more offspring than males with smaller combs (Polak & Simmons, 2009), and symmetrical combs are under positive sexual selection in wild populations (Polak *et al*., 2004).

Body size is also relevant for male *Drosophila* fitness. Larger bodies are correlated with enhanced mating success in numerous *Drosophila* species (Partridge & Farquhar, 1983; Partridge *et al*., 1987a, b) and large male *D. melanogaster* have longer sperm, longer sperm receptacles (Amitin & Pitnick, 2007) and win more fights than smaller conspecifics (Partridge & Farquhar, 1983). Maximal body size was supported by similar (yet lower calorie) 1P:1C-1P:2C diets to those supporting optimal sex combs and not the maternally-chosen high protein diets. Although we lack body size measures for males raised on the 4P:1C diet, extrapolation of our body size response surface indicates body size associated with this maternally-selected region of nutrient space would be very small indeed.

Resolving whether female *D. melanogaster* oviposition substrate choice reflects an attempt to co-maximise male and female offspring fitness requires measuring male and female condition-dependent lifetime reproductive success. Complete measurement of lifetime reproductive success is challenging. To overcome confounding influences of mates, proxy measures of reproductive 'potential' are typically made from virgin animals. These include the number of eggs laid by females (Lee *et al*., 2008) and calling or courtship effort by males (Hunt *et al*., 2004; Partridge *et al*., 1987a). As our question pertains to sexual selection, we would need to append such measures with actual comparisons of mating success. Combining protocols of Partridge *et al.* (1987a) and Polak & Simmons (2009), wherein male competition and female selection would be accounted for, would identify which condition supported optimal male fitness. To tackle the potential confounding influence of female mate nutritional background, female mates should be raised on a common diet and *vice versa*.

If large-bodied males with large symmetrical sex combs were identified as the top sexual performers, then parent-offspring sexual conflict (Trivers, 1972; Parker, 1979; Crespi & Semeniuk, 2004: Pizzari & Snook, 2003) presents as an explanation

for female oviposition substrate choice. The conflict could take two forms. Mothers may avoid the production of males of this sexual phenotype as it somehow threatens female fitness (Parker, 1979). However, this is unlikely as males court females (von Schilcher, 1976) rather than engage in coercive mating (Rowe *et al*., 1994; Pizzari & Snook, 2003) and females can also readily 'decamp' males (Partridge *et al*., 1987a), thus terminating copulation. Instead, females may be preferentially investing in the quality of their female offspring. Across time and varying nutritional environments, investing in females, the sex with the higher reproductive 'guarantee' (Robertson, 2006; Whittingham & Dunn, 2000), may be a better bet-hedging evolutionary strategy (Crean & Marshall, 2009; Bateman, 1948; Uller, 2008). Some support for this idea comes from experiments on *D. melanogaster* by Bateman (1948). In trans-generational rearing experiments, he found the likelihood of a given male making a genetic contribution to the subsequent generation was lower than for his female equivalents. If mothers are preferencing female offspring quality, selective pressure would then be exerted on developing males to self-select the viability-reducing, yet sex comb-optimising high energy 1P:2C-1P:1P diet. Known larval *Drosophila* behaviour certainly suggests capacity for nutritional decision-making (Sokolowski *et al*., 1983; Wallin, 1988) and adult *D. melanogaster* can behaviourally regulate their protein to carbohydrate intake (Lee *et al*., 2008; Riberio & Jackson, 2010: Vargas *et al*., 2010; Itskov & Ribeiro, 2013). Adult female *Drosophila*, for example, select a 1P:4C diet that promotes maximal lifetime reproductive success (Lee *et al*., 2008). Males of the cockroach species *Nauphoeta cinerea* self-select a diet that promotes their pheromonal attractiveness to females (South *et al*., 2011). Whether male larval *Drosophila* do select diets that promote their future 'sexual attractiveness' could be easily measured (see Chapter 2, this thesis).

*Does a sex comb-optimising condition handicap male immunity?*

What is it about high-energy, 1P:1C-1P:2C diets that promotes the largest, most symmetrical sex combs for some males, yet imposes a serious viability cost in others? Sexual selection theory suggests that the survivors have overcome a handicap associated with the diet, and now bear their high performance combs as status 'badges' (Hansen & Rohwer, 1986). Indeed, individuals with highly-symmetrical bilateral morphological features, like sex combs, are those understood to have

experienced less, or better overcome, environmental developmental stress (Polak & Tomkins, 2012). It appears that, when raised on our 1P:1C-1P:2C diets, flies either survive with high quality combs, or perish. The high-carbohydrate content of the diet may be of benefit to the 'survivors', as it would support the synthesis of chitin, a complex polysaccharide (Chapman, 2013) from which the combs are made. In adult insects (Maklakov, *et al*., 2008; Lee *et al*., 2008; Skorupa *et al*., 2008; Grandison *et al*., 2009; Fanson *et al*., 2009; Fanson & Taylor, 2011; Pirk *et al*., 2010) and mammals (Solon-Biet *et al*., 2014) maintenance of high-carbohydrate feeding is associated with increased longevity. Perhaps there is an association between high-carbohydrate larval diets and increased adult longevity (regardless of subsequent adult feeding) and that this attribute is 'displayed' to prospective females in the form of large symmetrical combs.

Why these diets are harmful and associated with mortality during the larval period is even less clear. The diet must impose a significant physiological challenge for developing larvae. Larval locusts raised on high-carbohydrate diets experience higher mortality and a prolonged developmental period relative to conspecifics raised on higher-protein food (Simpson & Raubenheimer, 1993; Simpson *et al*., 2002). The developmental delay is attributable to the locust nymphs on fixed, high-C:P rearing diets maintaining protein-dependent growth targets (Simpson & Raubenheimer, 1993). Possibly larval *Drosophila* maintain similar growth targets and the increased mortality, observed here in *D. melanogaster* males and previously in locusts, is incurred due to metabolic costs associated with excess intake of dietary carbohydrate (Raubenheimer & Simpson, 1993; Simpson *et al*., 2002). In *Spodoptera exempta* caterpillars, feeding on carbohydrate-rich yet protein-poor diets causes increased lipid deposition relative to higher-P:C foods (Lee *et al*., 2004). High-carbohydrate diets, especially for males (Maklakov *et al*., 2008), can cause 'obesity' in larval and adult insects (Warbrick-Smith *et al*., 2006; Skorupa *et al*., 2008), a state associated with inflammation-like responses of the insect innate immune system (Schilder & Marden, 2006). Secondary sexual trait quality is traded off with immune function in a plethora of animals (Roberts *et al*., 2004) in what Folstad & Karter (1992) call the immunocompetence handicap hypothesis (ICHH). In future studies we could measure both the obesogenic potential of our experimental diets and related measures of male immune state (Siva-Jothy *et al*., 2005; Ponton *et al*., 2013).

Genetic variation, while likely minimal in our flies, may account for why some males failed to survive (Kirkpatrick & Ryan, 1991). While our experimental population of flies was drawn from a long-term laboratory-reared culture, genetic differences driving differential response is plausible, and could be quantified in future work by comparing survivorship across different genetic strains (*e.g.* the strain by environment interaction approach taken in Chapters 2 and 3 of this thesis).

*Condition is not as simple as 'genes' and 'resources': environmental variation as well as genetic contributes to secondary sexual trait phenotypic plasticity*

While mothers may not be promoting sons with optimal sex combs, through laying eggs on high-energy, high-protein foods they are generating high viability, rapidly-maturing male offspring, a reasonable alternative sexual phenotype. The power of the environment in shaping phenotypic variation in secondary sexual characters has been downplayed historically (Andersson & Simmons, 2006; Cornwallis & Uller, 2009). Most studies examining secondary sexual traits regard 'condition' as a univariate response (South *et al*., 2011), with increase or decrease in condition extending along a single axis of expression, increasing or decreasing relative to two extremes, 'good' and 'poor' (Ahuja *et al*., 2011). Typically, only two inputs are considered to influence where an animal sits along the spectrum, 'genes' and 'resources', with condition toward 'good' equaling better secondary sexual traits. Standard thinking is that good genes interacting with a large resource pool equals good condition (Rowe and Houle, 1996).

Cornwallis and Uller (2009) remark that this reductionist theoretical approach has contributed to confusion when trying to understand the origins of phenotypic plasticity for secondary sexual traits, citing the lek paradox as an example (Kirkpatrick & Ryan, 1991; Rowe & Houle, 1996). Rowe and Houle (1996) made substantial improvements to secondary sexual trait variation theory by introducing the concept of 'genic capture'. Shifting away from modern synthesis thinking (Mayr, 1963; Huxley, 1952), these authors acknowledge multiple genes and loci working together are likely responsible for genetic contributions to secondary sexual trait phenotypic plasticity. However, Rowe & Houle still conceive the ultimate source of sexual trait phenotypic variation being underlying genetic variation. Here, we have demonstrated that nutrition, which is inherently multidimensional and poorly

characterised by a single measure such as energy content or 'quality', also has a substantial influence on the generation of trait variation. Our population of flies had been laboratory reared, without novel environmental stimuli, for hundreds of generations. They are likely quite genetically homogenous. Yet within a generation of rearing across a diversity of nutritional environments, we unlocked substantial phenotypic plasticity relevant to *D. melanogaster* sexually-selective processes. The *capacity* for plasticity is likely epi/genetic in origin, but the phenotypic variation yielded is a product of gene by environment interactions (Via *et al*., 1995; Danchin, 2013).

Our demonstration of the nutritional environment driving alternate sexual phenotypes in male *D. melanogaster* shows that 'condition' does not increase and decrease along a single dimension, with increase leading to more optimal secondary sexual trait expression. Optimal condition, driven by the interaction of two multivariate inputs, genes and environment, is evolutionarily and environmentally context dependent. While it will be hugely challenging, it is time to permanently incorporate the action of environmental variability into our conceptual models of all microevolutionary processes, including those of sexual selection.

*Conclusions*

Our results demonstrate that environmental variation, in this case of the nutritional environment (whether maternally dictated or otherwise), profoundly affects secondary sexual trait phenotypic expression. We are yet to understand exactly how past evolution, due to the combined influence of natural and sexual selection, determines the way present generations respond to environmental variation. We simply do not understand how the capacity for phenotypic plasticity to arise in response to the environment in a living generation is maintained in the epi/genome of a species over evolutionary time (Gross, 1996; Andersson & Simmons, 2006; Robinson *et al*., 2012; Danchin, 2013). Here we have demonstrated that variation of the developmental nutritional environment can unlock phenotypic variation, which is of relevance to future microevolution.

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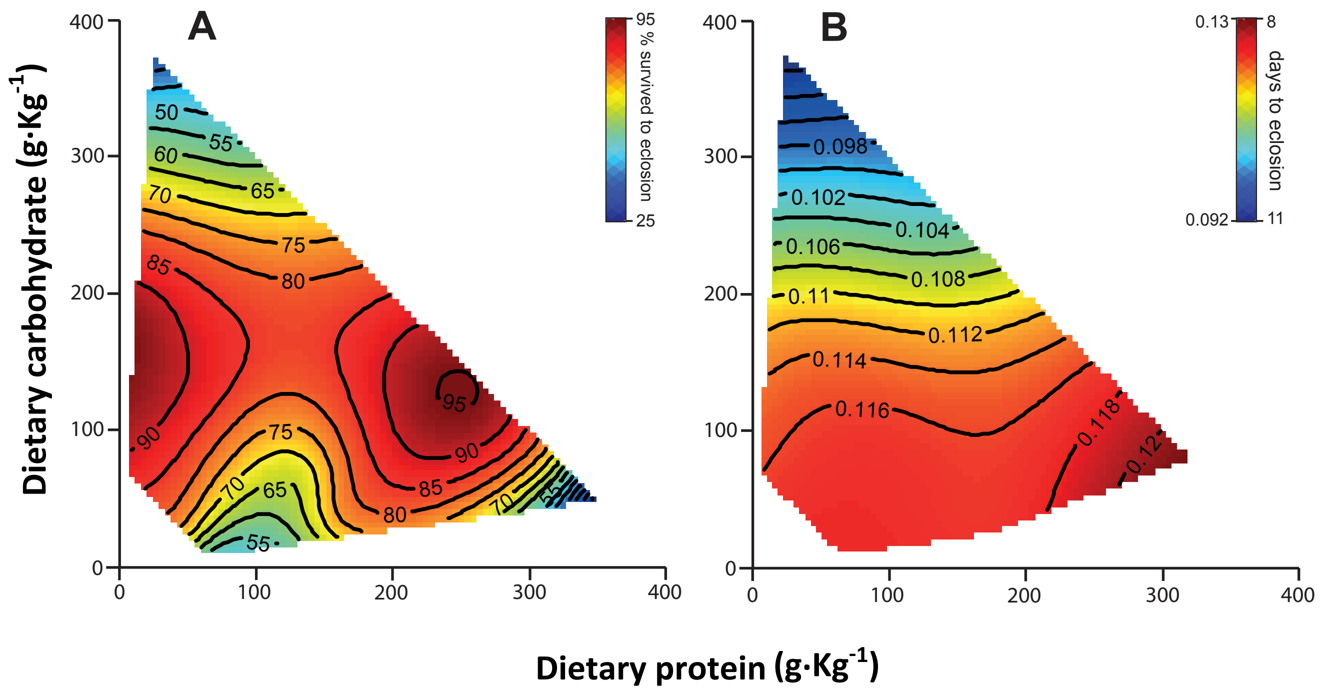


Figure 1. A) Thin plate spline (TPS) response surfaces of nutrition-dependent survivorship to eclosion by male *Drosophila melanogaster*. Highest survivorship, wherein > 95% males reached eclosion, was supported by high-energy, high-protein (P) food. Generalised linear modeling (GLM) showed quadratic increase in dietary P significantly contributed to higher survivorship (Wald $\chi^2$ 19.526, d.f. = 1, $p < 0.001$). Any rearing diet that offered between 100 and 200 g C.Kg$^{-1}$ was associated with > 75% survivorship. The significant contribution of dietary carbohydrate (C) to response surface shape was verified by GLM showing the linear C term supported higher survivorship (Wald $\chi^2 = 40.548$, d.f. = 1, $p < 0.001$). Increasing the total proportion of C to ≥ 1P:4C or increasing the total proportion of dietary P ≥ 2P:1C - 4P:1C, while maintaining 100-200 g C.Kg$^{-1}$, was associated with higher survivorship. Any diet offering > 200 g C.Kg$^{-1}$ was associated with a sharp decline in male survivorship. GLM showed the quadratic term of dietary C was significantly associated with lower survivorship (Wald $\chi^2 = 54.129$, d.f. = 1, $p < 0.001$).
B) TPS showing nutrition-dependent developmental rate of male *D. melanogaster*. High-energy, high-P diets drove the most rapid development. Males raised on diets offering ≥ 2P:1C eclosed within 9 days. GLM showed the quadratic term of P was significantly associated with increase in developmental rate (Wald $\chi^2 = 14.744$, d.f. = 1, $p < 0.001$), as was the linear term of C (Wald $\chi^2 = 40.237$, d.f. = 1, $p < 0.001$). The quadratic term of dietary C was also significantly associated with male development rate (Wald $\chi^2$ 9.412, d.f. = 1, $p = 0.002$).

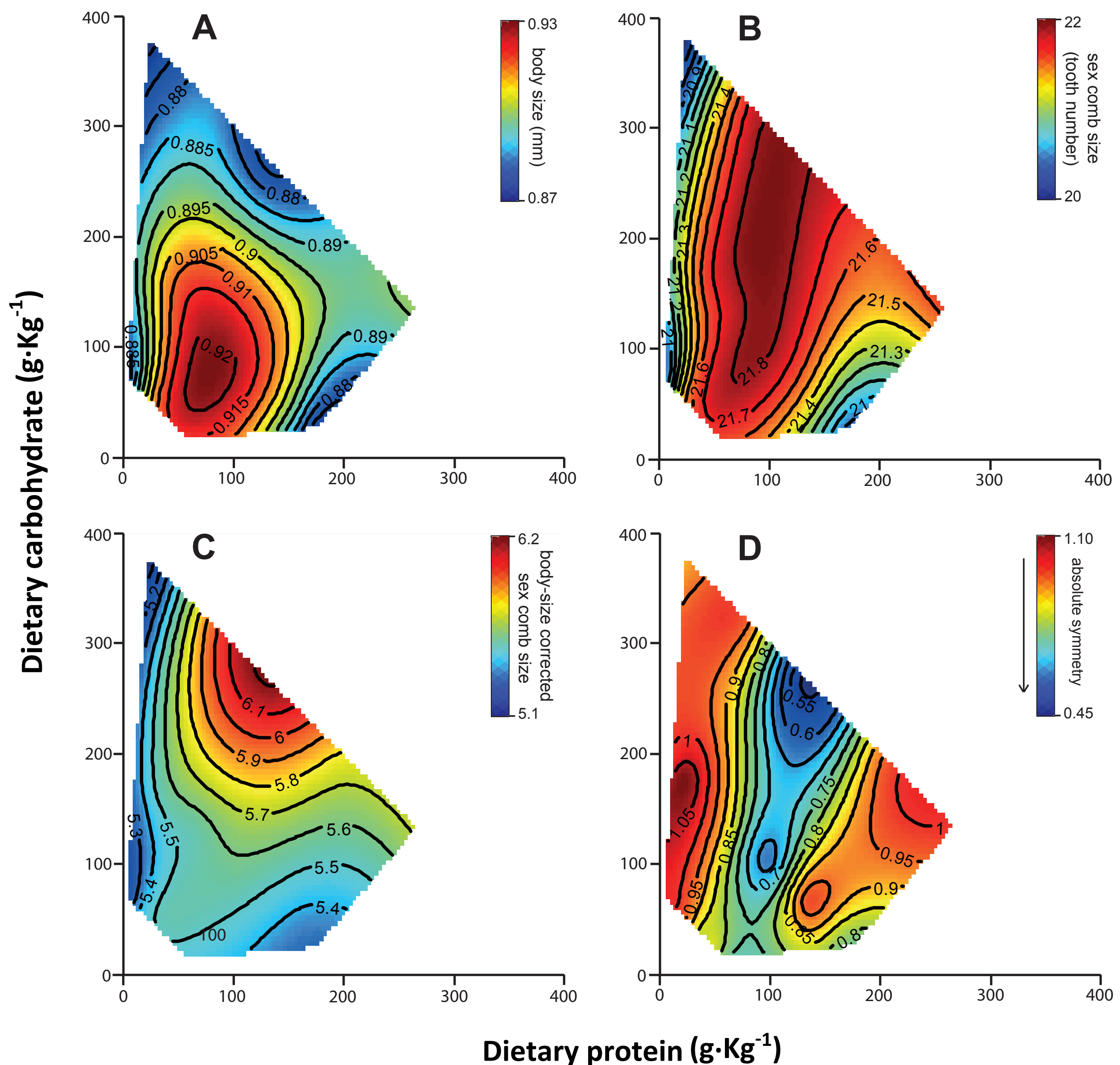


Figure 2. A) – D) Thin plate spline (TPS) response surfaces showing condition-dependent expression of male *Drosophila melanogaster* traits. A) Low- to medium-energy, balanced-protein to carbohydrae (P:C) diets supported maximal body size. Generalised linear modeling (GLM) showed that linear increases in protein (P) and carbohydrate (C) were significantly associated with increased male body size (*protein,* Wald $\chi^2 = 6.659$, d.f. = 1, $p < 0.001$; *carbohydrate*, Wald $\chi^2 = 4.727$, d.f. = 1, $p < 0.05$). Rearing diets offering > 200 g P+C.Kg$^{-1}$ led to the development of smaller male flies, with GLM showing high dietary P ($\chi^2 = 8.404$, d.f. = 1, $p = 0.004$) and C ($\chi^2 = 8.184$, d.f. = 1, $p = 0.004$) were significantly associated with smaller body size.

B) Diets lying between the 1P:1C and 1P:2C food rails, offering greater than > 100 g P+C.Kg$^{-1}$ supported the development of the largest male sex combs. Comb size decreased sharply as the proportion of C in the rearing diet increased above 4C:1P. Mid- to low-energy, high-P diets of P:C ratio of > 1.5P:C were also associated with smaller sex combs. GLM showed the cross-product of P and C was significantly associated with larger sex combs (Wald $\chi^2 = 6.561$ d.f. = 1, $p < 0.05$), while quadratic term of dietary protein was associated with smaller combs (Wald $\chi^2 = 7.138$ d.f. = 1, $p < 0.05$). Caption continues on the following page.

Figure 2. (Continued from previous page.) C) High-energy 1P:2C to 1P:1C diets supported maximal sex combs size (per unit body size). GLM showed the cross-product of dietary P and C (*protein*carbohydrate*, Wald $\chi^2 = 7.831$, d.f. = 1, $p = 0.005$) and the linear term of dietary C (Wald $\chi^2 = 7.712$, d.f. = 1, $p = 0.005$) were significantly associated with increase in response surface relief. The quadratic terms of both dietary P (Wald $\chi^2 = 19.794$, d.f. = 1, $p < 0.001$) and C (Wald $\chi^2 = 21.181$, d.f. = 1, $p < 0.001$) were also significantly associated with the body-size corrected ,sex comb size surface.
D) Sex comb absolute fluctuating asymmetry (FA) was lowest on the same high-energy, 1P:2C to 1P:1C diets that supported maximal sex comb size. Gradual increase in dietary C, within the range of 75 - 50 g $P.Kg^{-1}$ supported development of the most symmetrical sex combs. Both the quadratic terms of dietary P and C were significantly associated with sex comb symmetry ($protein^2$, Wald $\chi^2 = 16.829$, d.f. = 1, $p < 0.001$; $carbohydrate^2$, Wald $\chi^2 = 15.282$, d.f. = 1, $p < 0.001$. The linear term of dietary C was significantly associated with greater sex comb asymmetry (Wald $\chi^2 = 13.610$, d.f. = 1, $p < 0.001$).

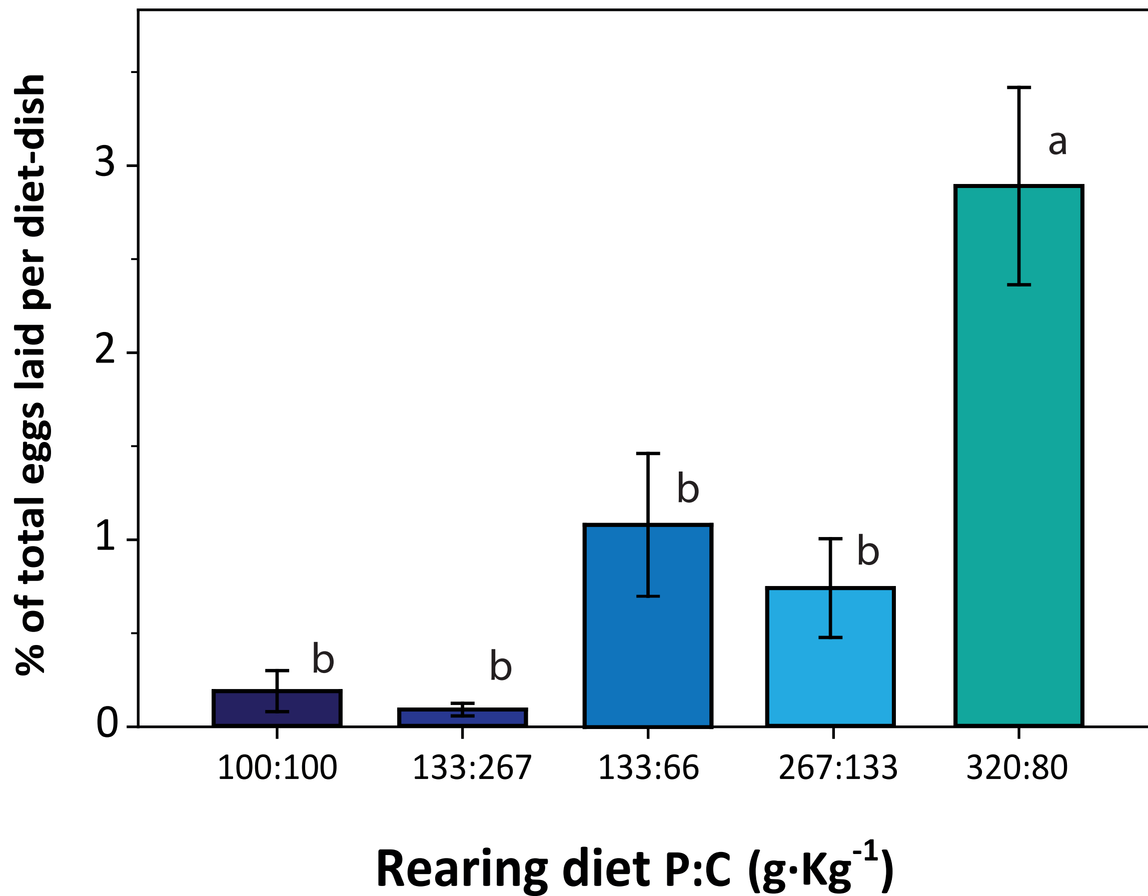


Figure 3. Bar-chart showing oviposition substrate selection by mated, adult female *Drosophila melanogaster*. Bars display the mean percentage (± sem) of total eggs laid on each dish containing one of five experimental diets varying in P:C ratio. Female *D. melanogaster* laid up to 60% of all eggs on dishes that contained the 4P:1C, 400 g P+C.Kg$^{-1}$ (320P:80C) diet. This diet supported maximal developmental rate and survivorship in adult male *D. melanogaster*. 'a' denotes significant statistical difference, at $p < 0.05$, from 'b'.

**Table 4.1. Generalised linear model for male survivorship.**

**Omnibus test**

| Likelihood ratio $\chi^2$ | df | Significance |
|---|---|---|
| 57.066 | 3 | $p < 0.001$ |

**Test of model effects**

| Source | Type III S.S. | | |
|---|---|---|---|
| | Wald $\chi^2$ | df | Significance |
| Intercept | 123.219 | 1 | $p < 0.001$ |
| Carbohydrate | 40.548 | 1 | $p < 0.001$ |
| Protein$^2$ | 19.526 | 1 | $p < 0.001$ |
| Carbohydrate$^2$ | 54.129 | 1 | $p < 0.001$ |

**Parameter estimates**

| Parameter | β coefficient | Standard error | 95% Wald C.I. | | Hypothesis test | | |
|---|---|---|---|---|---|---|---|
| | | | Lower | Upper | Wald $\chi^2$ | df | Significance |
| Intercept | 57.472 | 5.1774 | 47.324 | 67.619 | 123.219 | 1 | $p < 0.001$ |
| Carbohydrate | 0.491 | 0.771 | 0.340 | 0.642 | 40.548 | 1 | $p < 0.001$ |
| Protein$^2$ | <0.001 | 6.9122E-005 | 0.000 | <0.001 | 19.526 | 1 | $p < 0.001$ |
| Carbohydrate$^2$ | -0.002 | 0.0002 | -0.002 | -0.001 | 54.129 | 1 | $p < 0.001$ |

**Table 4.2. Generalised linear model for male developmental rate.**

**Omnibus test**

| Likelihood ratio $\chi^2$ | df | Significance |
|---|---|---|
| 510.059 | 4 | $p < 0.001$ |

**Test of model effects**

| Source | Type III S.S. | | |
|---|---|---|---|
| | Wald $\chi^2$ | df | Significance |
| Intercept | 16651.713 | 1 | $p < 0.001$ |
| Protein$^2$ | 14.744 | 1 | $p < 0.001$ |
| Carbohydrate$^2$ | 160.562 | 1 | $p < 0.001$ |
| Protein | 9.412 | 1 | $p = 0.002$ |
| Carbohydrate | 40.237 | 1 | $p < 0.001$ |

**Parameter estimates**

| Parameter | β coefficient | Standard error | 95% Wald C.I. | | Hypothesis test | | |
|---|---|---|---|---|---|---|---|
| | | | Lower | Upper | Wald $\chi^2$ | df | Significance |
| Intercept | 0.115 | 0.0009 | 0.113 | 0.117 | 16651.713 | 1 | $p < 0.001$ |
| Protein$^2$ | 1.493E-007 | 3.8877E-008 | 7.308E-008 | 2.255E-007 | 14.744 | 1 | $p < 0.001$ |
| Carbohydrate$^2$ | -4.026E-007 | 3.1772E-008 | -4.649E-007 | -3.403E-007 | 160.562 | 1 | $p < 0.001$ |
| Protein | -3.558E-005 | 1.1598E-005 | -5.831E-005 | -1.285E-005 | 9.412 | 1 | $p = 0.002$ |
| Carbohydrate | 7.107E-005 | 1.1203E-005 | 4.911E-005 | 9.303E-005 | 40.237 | 1 | $p < 0.001$ |

**Table 4.3. Generalised linear model for male body size (thorax length).**

**Omnibus test**

| Likelihood ratio $\chi^2$ | df | Significance |
|---|---|---|
| 16.401 | 4 | $p = 0.003$ |

**Test of model effects**

| Source | Type III S.S. | | |
|---|---|---|---|
| | Wald $\chi^2$ | df | Significance |
| Intercept | 11224.390 | 1 | $p < 0.001$ |
| Protein | 6.659 | 1 | $p = 0.010$ |
| Carbohydrate | 4.727 | 1 | $p = 0.030$ |
| $\text{Protein}^2$ | 8.404 | 1 | $p = 0.004$ |
| $\text{Carbohydrate}^2$ | 8.184 | 1 | $p = 0.004$ |

**Parameter estimates**

| Parameter | β coefficient | Standard error | 95% Wald C.I. | | Hypothesis test | | |
|---|---|---|---|---|---|---|---|
| | | | Lower | Upper | Wald $\chi^2$ | df | Significance |
| Intercept | 0.881 | 0.0083 | 0.864 | 0.897 | 11224.390 | 1 | $p < 0.001$ |
| Protein | > 0.001 | 0.0001 | 7.597E-005 | 0.001 | 6.659 | 1 | $p = 0.010$ |
| Carbohydrate | > 0.001 | 0.001 | 2.168E-005 | >0.001 | 4.727 | 1 | $p = 0.030$ |
| $\text{Protein}^2$ | -1.448E-006 | 4.9935E-007 | -2.426E-006 | -4.689E-007 | 8.404 | 1 | $p = 0.004$ |
| $\text{Carbohydrate}^2$ | -8.521E-007 | 2.9785E-007 | -1.436E-006 | -2.683E-007 | 8.184 | 1 | $p = 0.004$ |

**Table 4.4. Generalised linear model for sex comb size.**

**Omnibus test**

| Likelihood ratio $\chi^2$ | df | Significance |
|---|---|---|
| 14.520 | 5 | $p < 0.05$ |

**Test of model effects**

| Source | Type III S.S. | | |
|---|---|---|---|
| | Wald $\chi^2$ | df | Significance |
| Intercept | 4375.139 | 1 | $p < 0.001$ |
| Protein | 1.753 | 1 | $p = 0.185$ |
| Carbohydrate | 0.154 | 1 | $p = 0.695$ |
| Protein$^2$ | 7.138 | 1 | $p < 0.05$ |
| Carbohydrate$^2$ | 2.017 | 1 | $p = 0.159$ |
| Protein*carbohydrate | 6.561 | 1 | $p < 0.05$ |

**Parameter estimates**

| Parameter | β coefficient | Standard error | 95% Wald C.I. | | Hypothesis test | | |
|---|---|---|---|---|---|---|---|
| | | | Lower | Upper | Wald $\chi^2$ | df | Significance |
| Intercept | 21.164 | 0.3200 | 20.791 | 21.791 | 4375.139 | 1 | $p < 0.001$ |
| Protein | 0.006 | 0.0042 | -0.003 | 0.014 | 1.753 | 1 | $p = 0.185$ |
| Carbohydrate | 0.002 | 0.0039 | -0.006 | 0.009 | 0.154 | 1 | $p = 0.695$ |
| Protein$^2$ | -4.336E-5 | 1.6228E-5 | -7.517E-5 | -1.155E-5 | 7.138 | 1 | $p < 0.05$ |
| Carbohydrate$^2$ | -1.403E-5 | 9.8822E-6 | -3.340E-5 | 5.335E-6 | 2.017 | 1 | $p = 0.159$ |
| Protein*carbohydrate | 4.566E-5 | 1.7826E-5 | 1.072E-5 | 8.605E-5 | 6.561 | 1 | $p < 0.05$ |

**Table 4.5. Generalised linear model for sex comb size controlled for body size.**

**Omnibus test**

| Likelihood ratio $\chi^2$ | df | Significance |
|---|---|---|
| 70.038 | 5 | $p < 0.001$ |

**Test of model effects**

| Source | Type III S.S. | | |
|---|---|---|---|
| | Wald $\chi^2$ | df | Significance |
| Intercept | 31.945 | 1 | $p < 0.001$ |
| Protein | 1.288 | 1 | $p = 0.256$ |
| Carbohydrate | 7.712 | 1 | $p = 0.005$ |
| Protein$^2$ | 19.794 | 1 | $p < 0.001$ |
| Carbohydrate$^2$ | 21.181 | 1 | $p < 0.001$ |
| Protein*carbohydrate | 7.831 | 1 | $p = 0.005$ |

**Parameter estimates**

| Parameter | β coefficient | Standard error | 95% Wald C.I. | | Hypothesis test | | |
|---|---|---|---|---|---|---|---|
| | | | Lower | Upper | Wald $\chi^2$ | df | Significance |
| Intercept | 1.713 | 0.3030 | 1.119 | 2.307 | 31.945 | 1 | $p < 0.001$ |
| Protein | 0.004 | 0.0036 | -0.003 | 0.011 | 1.288 | 1 | $p = 0.256$ |
| Carbohydrate | 0.010 | 0.0037 | 0.003 | 0.017 | 7.712 | 1 | $p = 0.005$ |
| Protein$^2$ | -4.477E-005 | 1.0064E-005 | -6.450E-005 | -2.505E-005 | 19.794 | 1 | $p < 0.001$ |
| Carbohydrate$^2$ | -4.104E-005 | 8.9178E-006 | -5.852E-005 | -2.356E-005 | 21.181 | 1 | $p < 0.001$ |
| Protein*carbohydrate | 4.472E-005 | 1.5982E-005 | 1.340E-005 | 7.605E-005 | 7.831 | 1 | $p = 0.005$ |

**Table 4.6. Generalised linear model for sex comb absolute fluctuating asymmetry**

**Omnibus test**

| Likelihood ratio $\chi^2$ | df | Significance |
|---|---|---|
| 25.846 | 3 | $p < 0.001$ |

**Test of model effects**

| Source | Type III S.S. | | |
|---|---|---|---|
| | Wald $\chi^2$ | df | Significance |
| Intercept | 54.768 | 1 | $p < 0.001$ |
| Carbohydrate | 13.610 | 1 | $p < 0.001$ |
| Protein$^2$ | 16.829 | 1 | $p < 0.001$ |
| Carbohydrate$^2$ | 15.282 | 1 | $p < 0.001$ |

**Parameter estimates**

| Parameter | β coefficient | Standard error | 95% Wald C.I. | | Hypothesis test | | |
|---|---|---|---|---|---|---|---|
| | | | Lower | Upper | Wald $\chi^2$ | df | Significance |
| Intercept | 0.326 | 0.0441 | 0.240 | 4.12 | 54.768 | 1 | $p < 0.001$ |
| Carbohydrate | 0.003 | 0.0007 | 0.001 | 0.004 | 13.610 | 1 | $p < 0.001$ |
| Protein$^2$ | -2.7840E-006 | 6.9232E-007 | -4.197E-006 | -1.483E-006 | 16.829 | 1 | $p < 0.001$ |
| Carbohydrate$^2$ | -7.876E-006 | 2.0148E-006 | -1.183E-005 | -3927E-006 | 15.282 | 1 | $p < 0.001$ |

**Table 4.7. Kruskal-Wallis test on percentage of total eggs laid on each diet dish of each diet**

**Table 7a. Ranks**

| Diet | *N* | Mean rank (for % total eggs laid on each dish of each diet) |
|---|---|---|
| 1P:1C, 200 g P+C | 20 | 34.13 |
| 1P:2C, 400 g P+C | 20 | 38.80 |
| 2P:1C, 200 g P+C | 20 | 51.45 |
| 2P:1C, 400 g P+C | 20 | 49.33 |
| 4P:1C, 400 g P+C | 20 | 78.80 |
| **Total** | 100 | |

**Test statistics**

| | % total eggs laid on each dish of each diet |
|---|---|
| $\chi^2$ | 32.812 |
| **df** | 4 |
| **Significance** | $p < 0.001$ |

**Table 7b. Post-hoc Bonferroni multiple-comparisons for % total eggs laid on each dish of each diet**

| (I) Diet | (J) Diet | Mean difference (I-J) | Std. error | Significance (α adjusted) | 95% C.I. | |
|---|---|---|---|---|---|---|
| | | | | | Lower | Upper |
| 1:1, 200 | 1:2, 400 | 0.0980 | 0.45006 | 1.00 | -1.1955 | 1.3915 |
| | 2:1, 200 | -0.8955 | 0.45006 | 0.495 | -2.1890 | 0.3980 |
| | 2:1, 400 | -0.5510 | 0.45006 | 1.00 | -1.8445 | 0.7425 |
| | 4:1, 400 | -2.70 | 0.45006 | 0.000 | -3.9935 | -1.4065 |
| 1:2, 400 | 1:1, 200 | 0.0980 | 0.45006 | 1.00 | -1.3915 | 1.1955 |
| | 2:1, 200 | -0.9935 | 0.45006 | 0.297 | -2.2870 | 0.30 |
| | 2:1, 400 | -0.6490 | 0.45006 | 1.00 | -1.9425 | 0.6445 |
| | 4:1, 400 | -2.7980 | 0.45006 | 0.000 | -4.0915 | -1.5045 |
| 2:1, 200 | 1:1, 200 | 0.8955 | 0.45006 | 0.495 | -0.3980 | 2.1890 |
| | 1:2, 400 | 0.9935 | 0.45006 | 0.297 | -0.30 | 2.2870 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | 2:1, 400 | 0.3445 | 0.45006 | 1.00 | -0.9490 | 1.6380 |
| | 4:1, 400 | -1.8045 | 0.45006 | 0.000 | -3.0980 | -0.5110 |
| 2:1, 400 | 1:1, 200 | 0.5510 | 0.45006 | 1.00 | -0.7425 | 1.8445 |
| | 1:2, 400 | 0.6490 | 0.45006 | 1.00 | -0.6445 | `.9425 |
| | 2:1, 200 | -0.3445 | 0.45006 | 1.00 | -1.6380 | 0.9490 |
| | 4:1, 400 | -0.21490 | 0.45006 | 0.000 | -3.4425 | -0.8555 |
| 4:1, 400 | 1:1, 200 | 2.700 | 0.45006 | 0.000 | 1.4065 | 3.9935 |
| | 1:2, 400 | 2.7980 | 0.45006 | 0.000 | 1.5045 | 4.0915 |
| | 2:1, 200 | 1.8045 | 0.45006 | 0.001 | 0.5110 | 3.0980 |
| | 2:1, 400 | 2.1 | 0.45006 | 0.000 | 0.8555 | 3.4425 |

# Chapter 5.

## Gene expression changes associated with acquisition of lifespan protecting tolerance to a high-protein diet in *Drosophila melanogaster*

## Abstract

Prolonged feeding on high protein diets is associated with decreased life span in many animals, but not in carnivorous animals. Here, using *Drosophila melanogaster* and Illumina Hi-seq mRNA sequencing, we investigate the changes in gene expression associated with populations of these fruit-flies becoming adapted to feeding on high-protein, low-carbohydrate food. High-protein feeding usually leads to death within ten days post-ecolosion in *Drosophila.* We show that over five generations of laboratory selection on high-protein diets, *D. melanogaster* populations can evolve high-protein tolerance, and display 'natural' *Drosophila* longevity. Our findings indicate that in order to evolve a carnivore-like tolerance to utilising high-protein food as a principle source of calories, major changes in the expression of proteolysis and immune and stress response genes are required – supporting the 'multiplex stress response' hypothesis of ageing. Notably, very few genes of known membership of candidate 'longevity' pathways, TOR, AMPK, or insulin signaling pathways significantly changed in expression. Vitellogenin and hexamerin encoding genes did though, providing further evidence that expression of these proteins may play an important role in determining insect life span.

## Introduction

A high-protein diet is associated with decreased lifespan (LS) in many animals. Ants, crickets, flies, honeybees, mice (Dussutour & Simpson, 2009; Cook *et al.*, 2010; Hunt *et al.*, 2004; Maklakov, *et al.*, 2008; Mair *et al.*, 2005; Lee *et al.*, 2008; Skorupa *et al.*, 2008; Grandison *et al.*, 2009; Carey *et al.*, 2008; Fanson *et al.*, 2009; Fanson & Taylor, 2011; Pirk *et al.*, 2010; Solon-Biet *et al.*, 2014) and even humans (Lagiou *et al.*, 2007) are susceptible to early death following prolonged high-protein:carbohydrate (P:C) feeding. How high-P:C diets shorten lifespan is unresolved, although premature ageing *via* increased protein metabolism under high-P:C feeding is a likely candidate. Increase in dietary protein positively correlates with mitochondrial malfunction *via* increased production of radical oxygen species (ROS) (Sanz *et al.*, 2004; Ayala *et al.*, 2007), cellular stress responses (Lithgow & Miller, 2008), pathologies caused by toxic nitrogenous waste (Raubenheimer & Simpson, 2009; Walker *et al.*, 1989; St. Jeor *et al.*, 2001), and major changes in immune state (Ponton *et al.*, 2011; Dzhumagaziev, 1980). Maintaining energy balance through

gluconeogenesis can also represent a substantial metabolic challenge. When humans are restricted in high-P:C diets, only 67% of total glucose generated *via* gluconeogenesis is released for somatic use (Veldhorst *et al*., 2009), driving demand for further intake of potentially-damaging protein.

The reduced LS experienced by many animals when maintained on high-protein diets might appear to present a barrier to the evolution of carnivory. Yet entire animal lineages have evolved to survive by feeding on animal tissue, wherein carbohydrate is rare and protein and fat are the most abundant forms of dietary energy (Mayntz *et al*., 2009; Jensen *et al*., 2011, 2012; Hewson-Hughes *et al*., 2011, 2013). The evolution of carnivory presents animals with new and diverse foraging opportunities. Prey animals, which are sometimes conspecifics (Via, 1999; Vijendravarama *et al*., 2013, Simpson *et al*., 2009), are concentrated packages of nutrition (Mayntz & Toft, 2006). Feeding on other animals relieves carnivores of time-intensive herbivorous and omnivorous foraging for, feeding on, and processing of, lower-protein foods (White, 1978). Wilder *et al*. (2013) found that carnivorious mammals (possibly due to their consumption of and tolerance to high-protein diets) can better withstand longevity costs associated with sexual reproduction, typically incurred by herbivores and omnivores: this is a pattern conceptualised as the disposable soma theory of ageing (Kirkwood, 1977). How have carnivores achieved this?

Research on the anti-ageing effects of caloric restriction (CR) provides some indication of how carnivores resist the longevity costs of a high-protein diet. Recent studies have shown that rather than calories, restriction of protein intake in CR dietary protocols is responsible for the widely-reported life-extension effect (Mair *et al*., 2005; Lee *et al*., 2008, Solon-Biet *et al*., 2014). Of all amino acids in dietary protein, the branched-chain amino acids, leucine, isoleucine and valine (D'Antona *et al*., 2010; Avruch *et al*., 2009), and the essential amino acid methionine (Miller *et al*., 2005; Grandison *et al*., 2009; Lee *et al*., 2013) have emerged as the probable LS-altering protein constituents. Complementary studies of gene expression change in animals subject to LS-extending CR have identified especially the target of rapamycin (TOR) but also the AMPK, insulin signaling (IIS) and insulin-like growth factor (IGF) pathways as the possible regulators of animal longevity. Indeed, studies using targeted expression manipulations of genes in these regulatory pathways have

demonstrated that even under 'full feeding' LS can be altered (Lithgow *et al*., 1994; Guarente & Kenyon, 2000; Clancy *et al*., 2001; Vellai *et al*., 2003; Kapahi *et al*., 2004; Cox & Mattison, 2009; Harrison *et al*., 2009; Stanfel *et al*., 2009; Stenesen *et al*., 2013; Zhang, *et al*., 2013; Solon-Biet *et al*., 2014; Epel & Lithgow, 2014). These findings demonstrate that the pathway's longevity-affecting action can proceed independently of dietary composition, opening the possibility that permanent regulatory changes in these pathways might be protective of lifespan under sustained high-protein feeding.

*Discovery of high-P:C tolerant* Drosophila melanogaster

We discovered, through maintaining adult $w^{1118}$ *Drosophila melanogaster* flies on four diets of varying sugar (S) to yeast (Y) ratios (0.25S:5Y, 5S:5Y, 1S:5Y, 10S:0.25Y), that a minority of flies kept on the highest-P:C diet, 0.25S:5Y, displayed longevity near equivalent to that of flies maintained on the lifespan optimising 5S:5Y diet (Figure 1a). We then demonstrated that this resistance to the LS reducing effects of high-P:C feeding was also expressed in a similar minority of flies from two separate genetic strains of *D. melanogaster*, first in a Canton-S and next in an Oregon R population (Figure 1b & c). The $w^{1118}$ *D. melanogaster* population was then artificially selected for protein resistance, simulating positive selection for a 'protein-resistant' metabolic phenotype that might occur under a nutritional environment shift in a wild population (Figure 2).

*Current aim: use mRNA sequencing to describe gene expression changes as a high-P:C tolerant phenotype evolves*

Here, we examine how the evolution of a carnivorous or at least high-P:C tolerant metabolic phenotype changes gene expression in three separate outbreeding animal populations. Using the $w^{1118}$ *Drosophila melanogaster* population described above, we show that over just five generations of laboratory selection, individual resistance to the LS shortening effects of a high protein diet can increase from being expressed in 10% of flies to greater than 60%. *Drosophila* provides an excellent model for understanding the mechanistic evolution of carnivory. Several species of drosophilid 'fruit flies' have entirely carnivorous larvae: *D. cogani*, *D. simulivora* (Tsacas & Disney, 1974) and *Lissocephala powelli* (Carson & Wheeler, 1973). Both

*D. hydae* (Gregg *et al*., 1990) and *D. melanogaster* larvae also routinely commit necrophagy on deceased conspecifics (pers. obs) and recently *D. melanogaster* larvae have been shown to evolve outright predatory cannibalism (Vijendravarama *et al*., 2013). While the metabolic capability and concomitant predatory behaviours requisite for carnivory are obviously present and perhaps prevalent across the Drosophilidae, no prior researchers have studied – in real time – the evolution of resistance to high-P:C induced early-death in a population. Here we take advantage of this drosophilid capacity to express carnivory, and the species group's genetic model status, to begin investigating the physiological mechanisms underlying the evolution of a carnivore-like metabolism.

We used Illumina Hi-Seq mRNA-sequencing (mRNA-seq) to compare gene expression differences between control flies and those that had evolved high-P:C tolerance, *i.e*. resistance to the LS-curtailing effects of high dietary protein. Illumina mRNA-seq offers substantial advantages over older transcriptomic techniques. Unlike microarray and qRT-PCR methods, candidate gene discovery and description is not constrained by existing knowledge. Any gene, even those with unknown affinities to candidate pathways (or even unknown function), whose expression changes with experimental manipulation can have their relative expression level and direction quantified using mRNA-seq (Werner, 2010). Through mRNA-seq we ask: are existing candidate longevity pathways responsible for the observed protein-dependent LS changes or are novel physiological processes driving high-P:C tolerance?

This chapter presents the preliminary results of our mRNA-seq analysis. It also provides comment on further analytical techniques we are applying to the large gene expression dataset derived from this ongoing, collaborative project. Fly longevity data presented here in Figures 1 and 2 were collected by our co-authors Ihor S. Yurkevych and Oleh V. Lushchak.

## Material and Methods

### *Fly specimens*

All fly specimens analysed were obtained from a laboratory selection regime conducted by Ihor S. Yurkevych and Oleh V. Lushchak at the Prykarpatian National University, Ukraine. Three separate populations of $w^{1118}$ *D. melanogaster* were

established and raised for hundreds of generations on a 5 sugar(S):5 yeast(Y) diet under standard laboratory conditions. Before experimental treatments were imposed, an 'initial' sample of 50 adult male flies from each population was collected under light $CO_2$ anaesthesia, snap frozen, and then stored, in liquid nitrogen. Freshly-eclosed adults, removed from a 5S:5Y 'larval rearing' diet were then transfered to vials containing either the 0.25S:5Y 'selection' diet, or the 5S:5Y 'control' diet. After 24 days, all surviving adults were removed and left to mate in fresh vials containing the 5S:5Y larval diet. Larvae were again allowed to develop to adulthood and this process was repeated for a total of five consecutive generations. After five generations, 50 24-day old adult males from both the selected and control lines were collected and snap frozen in liquid nitrogen for mRNA sequencing (see Figure 3 for a schema of this selection regime).

*mRNA extraction*

*Drosophila* RNA was extracted using TRIzol reagent (Life Technologies, ref. 15596-026). From each $w^{1118}$ population, and each diet-treatment, 20 whole male flies (haphazardly chosen from the sample of 50) were placed in separate sterile 2 mL microfuge tubes (nine in total). 1 mL of TRIzol and a sterile 7 mm stainless steel ball were added to each tube. Samples were homogenised for 40 s using a tissue grinder at 25 Hz. Following incubation at room temperature for 15 min, samples were centrifuged at 4°C at 12,000 *g* for 10 min. 800 uL of the supernatant was then removed to a new sterile 1.5 mL centrifuge tube. Following addition of 0.2 mL of chloroform, the tube was shaken vigorously for 15 s, incubated at room temperature for 3 min and then centrifuged at 4°C at 12,000 *g* for 20 min. To purify the RNA, 350 uL of each sample's upper aqueous phase was transferred to a gDNA eliminator column from an RNeasy Plus Mini Kit (Qiagen, ref. 74134). Samples were then centrifuged at room temperature for 1 min at 8000 *g*, until all liquid had passed through the gDNA collection column membrane. 350 uL of 70% ethanol was then added to the flow-through, and mixed by pipetting. Next, samples were transferred to an RNeasy MinElute spin column placed within a sterile 2 mL collection tube, and centrifuged for 1 min at 10,000 *g*. To purify RNA, we followed the exact purification procedure described in the RNeasy Plus Mirco Handbook (Qiagen, www.qiagen.com/ingenuity). Samples were then air dried for 10 min to allow any

ethanol used in the purification step to evaporate. The $A_{260}/A_{280}$ ratio of the purified RNA was then analysed by nanodrop spectrophotometry. The required $A_{260}/A_{280}$ ratio of > 1.8 for reliable mRNA-sequencing was successfully achieved for all nine samples.

*Illumina HiSeq 2500 mRNA sequencing*

All samples were submitted to the Australian Genomics Research Facility (AGRF) in Melbourne Australia for Illumina Hiseq 2500 mRNA sequencing (www.illumina.com/systems/hiseq_2500_1500.ilmn). Following cDNA library preparation, 100 base pair, single end read sequencing was conducted on each sample. To avoid confounding effects, all nine mRNA samples were allocated to a single flow-cell lane of the sequencer (following Werner, 2010). Sample reads ranged between 16,000,000 and 21,000,000 (Table 1).

*Statistical analysis – differential expression*

Gene expression in selected flies was compared to that of initial and control flies within each fly population. To control for the potential influence of genetic drift over five generations, only genes that were significantly up- or down-regulated in the selected flies relative to both the initial and control samples were considered. Differential expression analysis was performed with DESeq (Anders & Huber, 2006) in R (ver. 3.0.2, R Project, 2013). Genes with an adjusted $p$ value of $< 0.05$ were taken as differentially expressed between comparisons. We constructed a gene expression heat-map, displaying log2-fold change in expression. Significantly differentially expressed genes for each population are highlighted on the expression heat map (Figure 4) by red (populations A & C) and pink borders (population B). To facilitate inter-population comparisons of differential gene expression, on the heat map we included the expression level of any gene that was significantly differentially expressed in any population. For example, even though the immune response gene, TotC, was only significantly unregulated in population B, we have also included the expression level achieved by this gene in populations A and C in the heatmap.

*Functional annotation analysis*

We used functional annotation analysis to infer the function of significantly up- and down-regulated genes in selected flies of each fly population. Official gene symbols for each gene were uploaded to the DAVID Bioinformatics Functional Annotation Tool (ver. 6.7, david.abcc.ncifcrf.gov) and over-represented keywords from the Swiss-Prot Protein Information Resource (SP PIR Keywords) were identified by comparing to the *Drosophila melanogaster* background (Huang *et al*., 2009a; 2009b). For the genes for which DAVID did not identify a function, we obtained biological process details by searching the FlyBase database (www.flybase.org).

*Crosscheck of significantly up- & down-regulated genes against candidate pathways*

To complement our functional annotation analysis, using the gene lists available within the Inter*active* Fly database (www.sdbonline.org) we crosschecked for presence of any known genes from within the TOR, IIS and IGF signaling pathways that significantly changed in their expression due to our selection regime. For the AMPK signaling pathway, we used the gene list published in Mihaylova & Shaw (2011), although as yet there are few known *Drosophila* homologues of mammalian AMPK pathway genes.

## Results and Discussion

We designed our selection experiment to facilitate tight correlation between the expression of high-P:C tolerance and associated gene expression. We artificially selected for 'protein-resistance' until a majority of individuals in each $w^{1118}$ population expressed the high-P:C tolerant phenotype. By examining the gene expression differences that were shared only between the selected and control, and selected and initial samples, the genes most closely associated with the phenotype change were brought into sharp focus. In contrast to expectations, the analysis has identified very few genes with existing, characterised relationships with candidate longevity pathways – TOR, AMPK, IIS or IGF. Instead, we have identified a largely novel set of ~100 genes, the majority of which have no known relationship with LS extension (Figure 4 & Table 2). Two explanations exist for our finding of unique gene sets. The first is that molecular processes putatively responsible for LS extension within a high-P:C environment are separate to those responsible for extended

longevity under low-P:C. Perhaps LS extension can be achieved via different environment-dependent biochemical processes (Ardent & Reznick, 2007). The alternative is that the genes we have identified have previously uncharacterised, or poorly-characterised, relationships with existing candidate longevity pathways. Our initial, somewhat coarse-grained analyses may have failed to reveal these important relationships. We explore these possibilities below.

*Do changes in proteasome function mediate longevity in carnivores?*

If the major nutrient sensing and signaling pathways previously associated with longevity are not responsible for coordinating extended longevity in the face of high-protein feeding in our flies, and potentially facilitating the evolution of carnivory, what pathways might be involved? Our functional annotation analysis reveals that of the 93 genes that significantly changed in their expression, the major subsets for which function is known were hydrolase-proteolysis/proteolysis genes, and genes associated with environmental stress and/or immune responsiveness (Figure 4).

A characteristic of ageing is the loss of proteasome-mediated control of proteolysis and a subsequent increase in circulating oxidised proteins and protein subunits (Shringarpure & Davies, 2002). These 'rogue' proteins can then compound ageing by interfering with vital physiological processes, including neurological function (Floyd, 1999; Keller *et al*., 2004) and DNA replication (Nash *et al*., 2001). Demands on proteasome processing of damaged and damaging proteins would be predicted to be intensified on a high P:C diet. Our findings, of altered expression of a large cluster of genes associated with proteolysis, may indicate altered activity of the proteasome or similar proteolytic cellular mechanisms (Rock *et al*., 1994; Grune *et al*., 2004; Epel & Lithgow, 2014) could be associated with LS protection in high-P:C tolerant *Drosophila.* Many genes associated with insect innate immunity and stress response were significantly up-regulated in our high-P:C tolerant *vs.* control *Drosophila* (Figure 4). Extensive work using *C. elegans* worms has demonstrated that enhanced longevity in response to environmental stress is promoted through the simultaneous response of cellular protein homeostasis pathways and stress-resistance pathways (Epel & Lithgow, 2014) in what Lithgow and Miller (2008) term 'multiplex stress resistance'.

Epel & Lithgow (2014) hypothesise that coordinated and sustained signalling between stress response pathways to cellular protein systems such as the proteasome is protective of lifespan. Our results are supportive of this idea and could provide an informed basis from which to test this hypothesis using *Drosophila* by combining high-protein feeding experiments with the UAS-Gal4 system (Brand & Perrimon, 1993) and RNAi techniques (Elbashir *et al*., 2001). First, using RNAi we could test for a signaling relationship between immune/stress response genes and proteolysis genes by down regulating immune/stress response gene activity in our high-P:C tolerant line of $w^{1118}$ flies, and by then measuring fly longevity and proteolysis gene expression. Next, using high-protein-fed, non-high-P:C tolerant selected flies, we could up and down regulate key proteolysis genes (informed by the gene list generated by the current study, Figure 4), via UAS-GAL4 and monitor for attendant LS extension.

Manipulative experiments like these can verify the role of proteolysis and immune/stress response genes in conferring longevity under high-P:C feeding but they will not demonstrate a structural, mechanistic basis for the evolution of the protective phenotype. Our existing mRNA-seq gene expression dataset can act as a launch pad for exactly this kind of search. As an example of one approach, take that we discover some proteolysis genes from our list (Figure 4) are associated with LS extension under high-P:C feeding, we can return to our mRNA-seq data and look for control *vs.* selected RNA sequence variation between those genes, *e.g.* using DNASTAR's SeqMan programme (following Koepke *et al*., 2012). Next we would establish whether the expression differences at the loci are due to either new genotypes having evolved (*i.e.* structural change in nucleotide sequence has occurred), or whether regulation of expression has been modified epigenetically, for example via histone-methyl-transferases (Kramer *et al*., 2011). This kind of locus-level enquiry would then be complemented by examining structural and regulatory differences in these new candidate longevity genes in existing carnivorous and non-carnivorous *Drosophila* species.

*Some genes with known links to life-span extension, vitellogenin and hexamerin genes, changed expression with acquisition of the protein-resistant phenotype*

Proteolysis and immune/stress response genes were not the only functional groups of genes that changed their expression significantly across our populations selected for 'protein-resistance'. Some genes with known links to LS extension and the TOR pathway from other model organism systems appeared in our list of 93 genes. *Drosophila* vitellogenin genes, Yp1, Yp2, Yp3, expression of which controls production of the glycolipoprotein vitellogenin, were intensely up-regulated in population A flies (Figure 4). In ticks, suppression of TOR->S6K signaling reduces production of vitellogenin protein (Umemiya-Shirafuji *et al*., 2012). In mosquitos, increase in circulating amino acids stimulates expression of vitellogenin genes via requisite TOR signaling (Hansen *et al*., 2004). Vitellogenin titres are also high in the long-living queen caste of honeybees (Amdam *et al*., 2004). This is not surprising given the protein's role as a yolk precursor, although even non-reproductive, longer-living worker bees possess higher vitellogenin titres than shorter LS workers (Amdam *et al*., 2004). RNAi knockdown of a vitellogenin gene in honeybee workers also substantially reduces their longevity (Nelson *et al*., 2007). Vitellogenin is thought to affect longevity by acting as a potent antioxidant (Seehuus *et al*., 2006). Our finding of three vitellogenin genes significantly up-regulated in flies selected for protein resistance supports the hypothesis that carnivores have evolved physiology to combat pro-ageing effects of protein metabolism.

Hexamerin, an amino acid storage protein (Martins *et al*., 2010), is associated with LS extension in herbivorous grasshoppers (Badisco *et al*., 2013; Linquist, 2013). Hexamerin genes, known as larval serum protein (Lsp) genes in *D. melanogaster*, showed significant up-regulation in two of our three protein-resistance selected $w^{1118}$ populations. Population B displayed up-regulation of Lsp1beta and Lsp2, and population C of Lsp1gamma (Figure 3). While hexamerin proteins do not have a putative antioxidant function like vitellogenin, more hexamerin-encoding genes are expressed in the longer-living solitarious *vs*. shorter-living gregarious phase of the polyphenic desert locust *Schistocera gregaria* (Badisco *et al*., 2013), which at least suggests an anti-ageing role. Interestingly, RNAi inhibition of vitellogenin gene expression in the grasshopper *Romalea micropter* leads to both a 13-21% increase in LS and a concomitant 50% increase in haemolymph hexamerin concentrations

(Linquist, 2013). Perhaps these proteins play compensatory roles in LS regulation. Our results tentatively support this idea. Our population A *D. melanogaster* displayed up-regulation of vitellogenin genes but not hexamerin. The opposite pattern was observed in populations B and C (Figure 4).

A relationship between hexamerin levels and TOR pathway signaling is partially characterised. In termites hexamerin binds to, and likely actively sequesters, juvenile hormone (JH) (Gilbert *et al*., 2000), prolonging larval development and conferring worker caste status (Zhou *et al*., 2007). Mutti *et al*. (2011) provide evidence that TOR signalling is an upstream mediator of JH levels; when TOR signalling is suppressed, JH levels remain low, retarding development of the long-living queen caste. High-protein diets are also known to increase JH titers in insects (Schal *et al*., 1993; Clifton & Noriega, 2011). Taken together this evidence is suggestive of hexamerin being an intermediate of diet->TOR->hexaerin->JH signaling, wherein LS is somehow affected.

The function of vitellogenin and hexamerin genes in promoting longer LS under a high P:C diet could be studied using the RNAi, UAS-Gal4 approach discussed above. Even the importance of potential epistasis between the vitellogenin and Lsp genes, and proteasome and immunity/stress response gene expression, in conferring longevity could be characterised using these molecular tools. As an example, experimenting with our protein-resistant $w^{1118}$ *Drosophila* under high-protein feeding, we can manipulate expression of either vitellogenin or hexamerin genes and monitor for attendant change in proteolysis and immunity/stress gene expression, and *vice versa*. Longevity would also be traced.

*Higher resolution bioinformatics could reconcile relationships between existing candidate longevity pathways and genes associated with longevity here*

Despite the exciting role UAS-Gal4 and RNAi techniques can play in identifying causal physiological roles, and/or important epistatic relationships among proteasome, immunity, vitellogenin and hexamerin genes in conferring longevity under high-P:C feeding, we believe our existing mRNA-seq dataset will yield further insights in the near-term. Given marked expression change of vitellogenin and hexamerin genes and their relationships to the TOR pathway, the failure of functional annotation analysis to highlight them has motivated a re-analysis of our entire mRNA-

seq dataset. While only in the most preliminary phase, we are using a new pathway analysis bioinformatics tool, *Pathview* (see Lou & Brouwer, 2013 for details) to evaluate our data, *Pathview* will map our entire gene expression data, candidate pathway by candidate pathway, to pre-articulated signaling cascades, allowing appraisal of the probability of each pathway's involvement and contribution to the high-P:C tolerant *Drosophila* phenotype. *Pathview* will also help disentangle the complexity of potential epistatic relationships between gene types. *Pathview* allows for this, as the gene network maps it generates indicate directionality and mode of inter-molecule signal transduction.

We further hope that applying programmes like *Pathview* to our data will better capture relationships between the major signaling pathways and their upstream stimulators and downstream targets – the primary gene types we suspect our existing differential expression analysis has identified as being up- and down-regulated in high-P:C tolerant flies. Better characterisation of the major conserved metazoan signaling cascades (including those of the proteasome and immune systems) implicated in the evolution of a carnivore-style metabolism will allow for the insect-specific findings we have elucidated thus far to be extended to other animals. Being able to translate our findings beyond understanding insect carnivore evolution, while inherently interesting, is an important goal. If the bases of ageing are, as widely hypothesised, due to metabolic damage, discovering the physiology carnivores use to overcome the pro-ageing influence of high-protein diets could stimulate major advances in our own species' healthcare. While ageing is a natural component of human life history, ageing-related or dependent diseases can be emotionally and economically devastating to the elderly and their caregivers. Dementia caused by neurodegenerative Alzheimer's Disease, thought to be caused by impaired neuronal proteolysis (Selkoe, 2001; Keller *et al*., 2004; Lushsinger *et al*., 2007; Epel & Lithgow, 2014), provides a prime example. As populations age worldwide, developing therapies to improve quality of life for older people is imperative.

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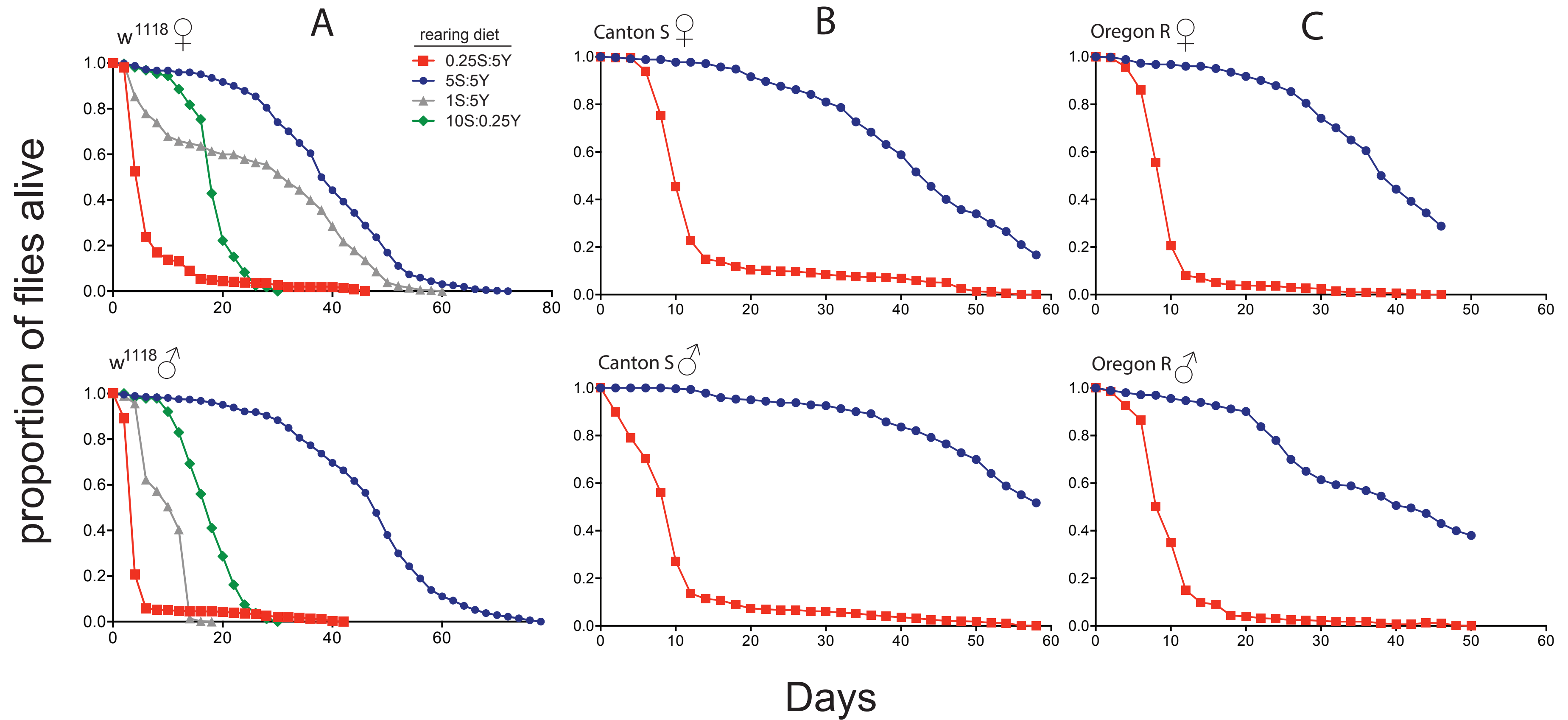


Figure 1. A) Survival curves of adult female (top) and male w1118 *Drosophila melanogaster* kept from eclosion to death on one of four diets varying in sugar (S) to yeast (Y) ratio. The ancestral 5S:5Y diet supported lifespan of over 60 days for males and females (blue circles). The highest protein diet, 0.25S:5Y, led to early death, with 80% of females and 90% of males dying within the first 10 days post-eclosion. Despite the lethal effect of the 0.25S:5Y diet for most flies, a minority of less than 10% of both males and female displayed high-P:C tolerance – and remained alive for over 40 days (red squares). Intermediate longevity occurred in male and female flies raised on 1S:5Y (grey triangles) and 10S:0.25Y (green diamonds) diets.
B & C) Survival curves for Canton S (middle panel) and Oregon R (right-hand panel) *D. melanogaster* adult females (top) and males kept from eclosion to death on either the lifespan supporting 5Y:5S (blue circles) or life span shortening 0.25S:5Y (red squares) diets. As for w1118, flies over 80% of Canton S and Oregon R flies kept on the 0.25Y:5S diet died within 10 days post eclosion, with a minority expressing the high-P:C tolerant phenotype.

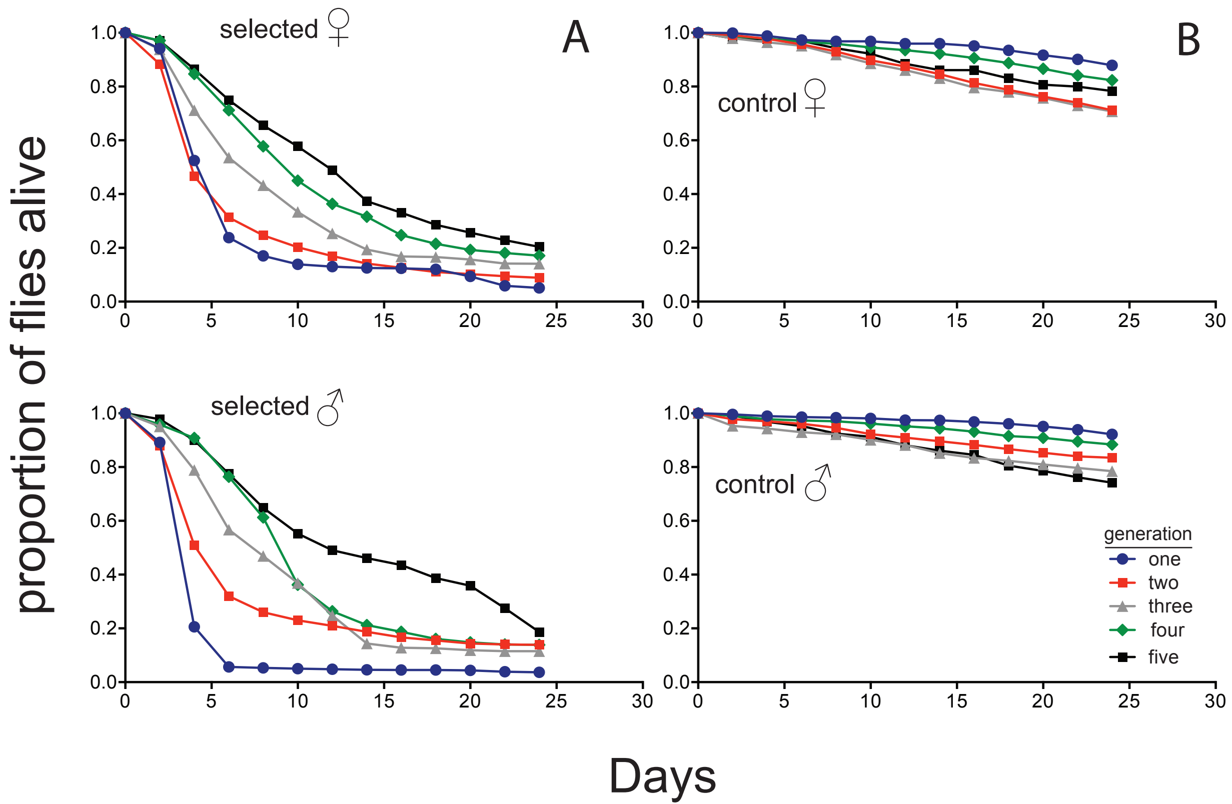


Figure 2. Survival curves comparing w[1118] *Drosophila melanogaster* females (top) and males selected for high-P:C tolerance over five generations of feeding on a high-protein, 0.25S:5Y diet (left hand panels, labelled A) and those maintained for five generations on the ancestral 5S:5Y diet (right hand side panels labelled B). Artificial selection substantially increased the proportion of females and especially males expressing the high-P:C tolerant phenotype. The control population showed no change in their lifespan over the same five-generation period.

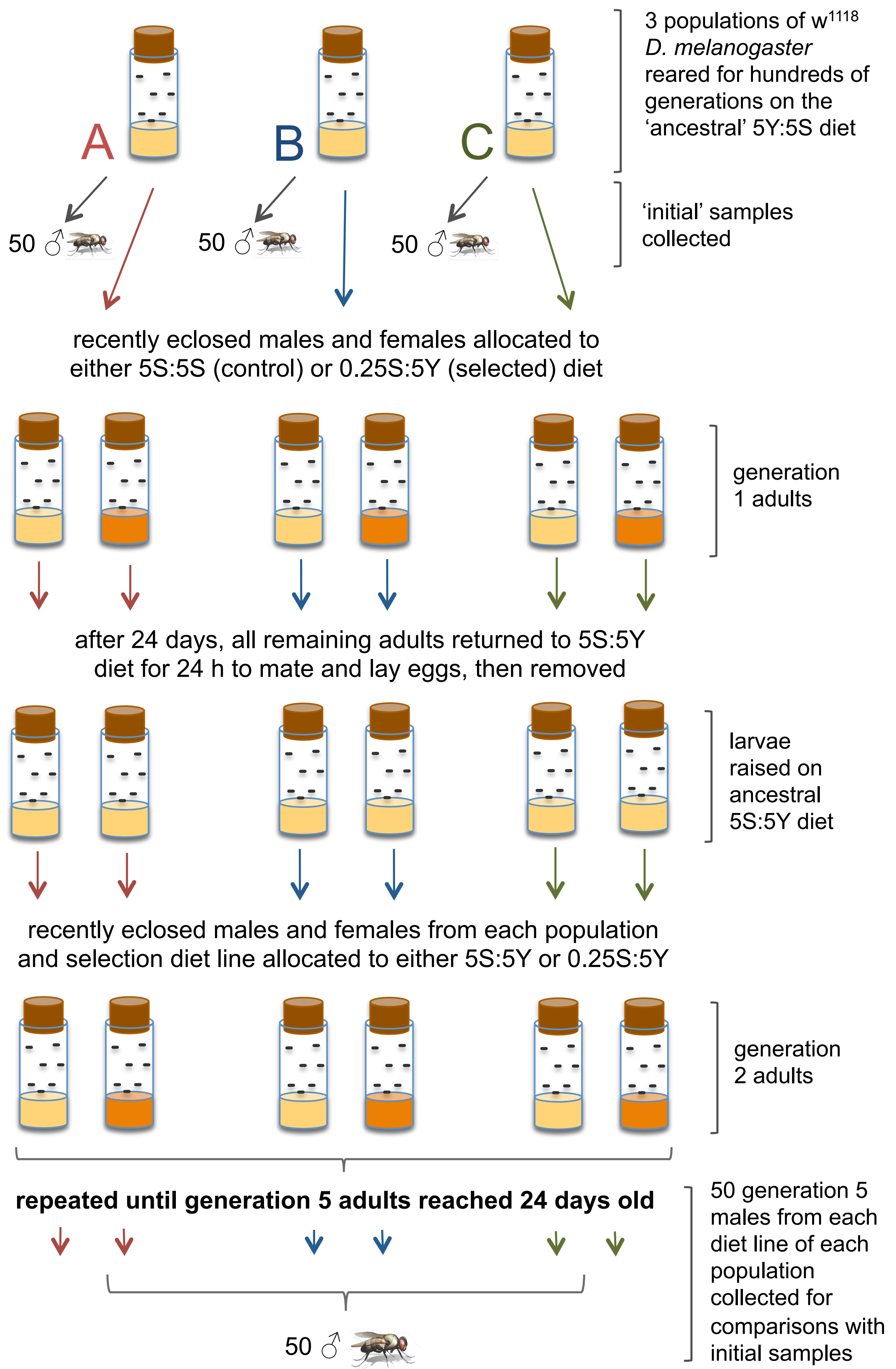


**Figure 3. Schema of laboratory selection regime used to obtain 'high-P:C tolerant' control and initial samples used in mRNA-seq gene expression comparisons.**

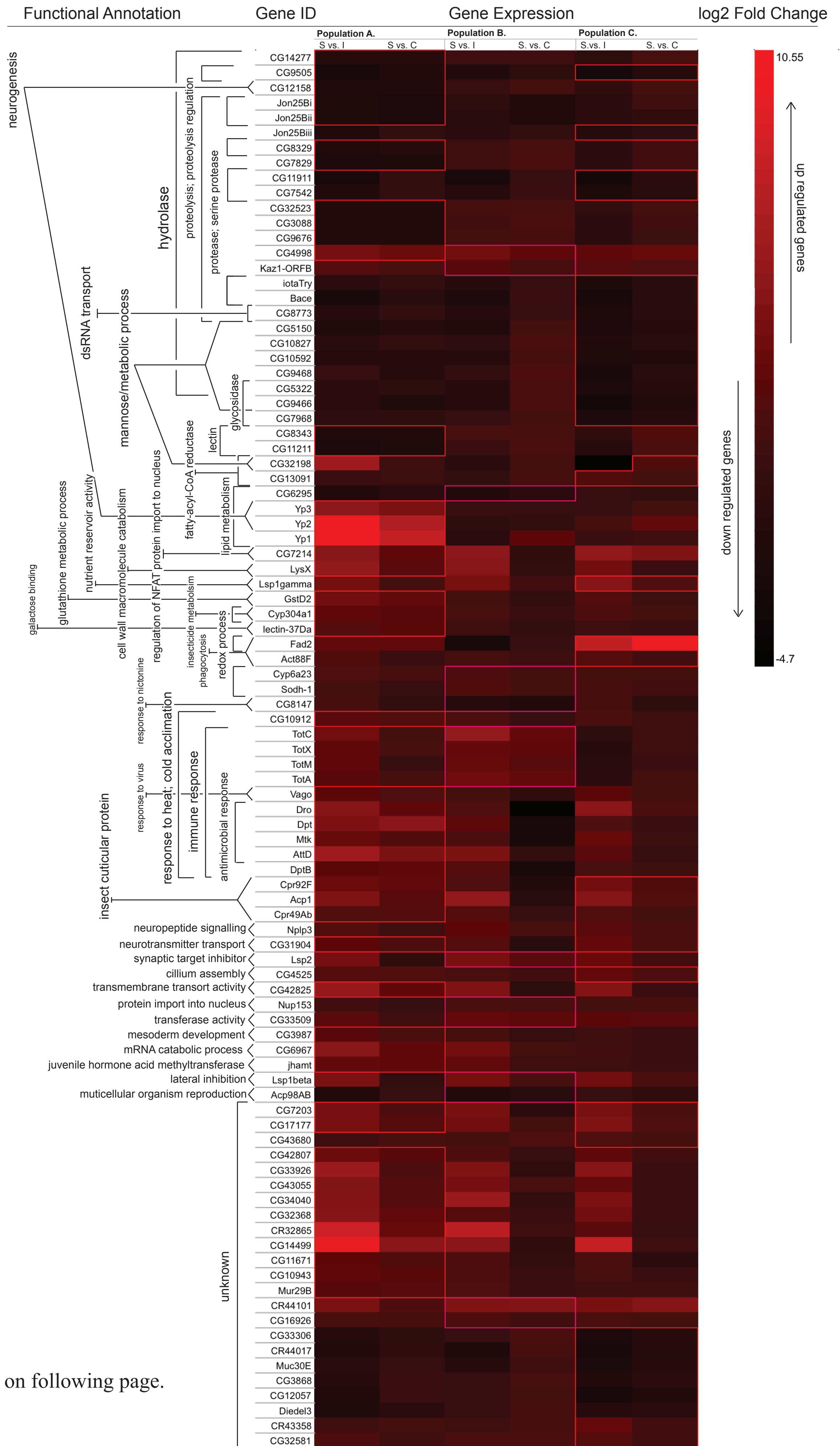


Figure 4. Caption on following page.

Figure 4. Heat-map showing *Drosophila melanogaster* genes differentially expressed between male flies from selected, control and initial treatment groups replicated across three $w^{1118}$ populations (populations A, B & C). Red and pink highlighted borders denote genes that significantly differentially expressed between selected and initial (S vs. I) and selected and control (S vs. C) samples, at $p$ of $< 0.05$. Functional annotation of genes derived from DAVID analysis and FlyBase searches are shown. Major gene subsets whose expression levels differed significantly between flies selected for high-P:C tolerance and control and initial individuals were proteolysis genes and immune/stress response genes. Notably, vitellogenin and hexamerin genes, gene types with known relationships with lifespan extension in other organisms, also significantly differed in their expression in selected flies. Vitellogenin encoding genes (here listed as Yp1, Yp2 and Yp3) were markedly unregulated in population A selected flies. Hexamerin encoding genes, listed as larval serim protein (Lsp) genes, were significantly up regulated in population B (Lsp2) and C flies (Lsp1gamma and Lsp1beta).

**Table 5.1. Illumina Hiseq 2500 mRNA sequencing single end read numbers from 9 *Drosophila melanogaster* samples. Individual samples comprised 20 newly eclosed male flies drawn from three separate populations of $w^{1118}$ flies raised on sugar (S) and yeast (Y) diets. 'Control' and 'selected' individuals were raised for 5 generations on a 5S:5Y and a 0.25S:5Y diet, respectively. 'Initial' flies were raised or a single generation on a 5S:5Y diet.**

| RNA sample name | Read number | Data yield (bp) |
|---|---|---|
| Population A | | |
| Initial | 21,028,980 | 2.103 Gb |
| Control | 19,165,955 | 1.917 Gb |
| Selected | 15,709,346 | 1.571 Gb |
| Population B | | |
| Initial | 18,102,756 | 1.81 Gb |
| Control | 20,933,556 | 2.093 Gb |
| Selected | 19,461,985 | 1.946 Gb |
| Population C | | |
| Initial | 16,639,181 | 1.664 Gb |
| Control | 17,519,189 | 1.752 Gb |
| Selected | 16,027,228 | 1.603 Gb |

**Table 5.2. List of *Drosophila melanogaster* genes with known membership of TOR, IIS, IGF or AMPK pathways as listed on the Inter*active* Fly database and Mihaylova & Shaw (2011) for AMPK genes. These genes were crosschecked against the list of significantly up or down regulated genes derived from our three populations of protein resistant *D. melanogaster.* No genes with known membership to these candidate longevity pathways were significantly up or down regulated in the protein-resistant, carnivory capable versus control or initial individuals.**

| Gene name | Annotation symbol | Significantly up/down regulated in population A, B or C? |
|---|---|---|
| S6k | CG10539 | no |
| TOR | CG5092 | no |
| rictor | CG8002 | no |
| Pi3K21B | CG2699 | no |
| Pi3K59F | CG5373 | no |
| Pi3K68D | CG11621 | no |
| InR | CG18402 | no |
| foxo | CG3143 | no |
| ampkaalpha | CG3051 | no |
| toll6 | CG7250 | no |
| sir2 | CG5216 | no |
| NAAT1 | CG3252 | no |
| Maf1 | CG40196 | no |
| TSC1 | CG6147 | no |
| TSC2 | CG6975 | no |
| Thor/4E-Bp1 | CG8846 | no |

| RHEB | CG1081 | no |
|---|---|---|
| FK506-bp2 | CG11001 | no |
| dlip1 | CG14173 | no |
| dlip2 | CG8167 | no |
| dlip3 | CG14167 | no |
| dlip4 | CG6736 | no |
| dlip5 | CG33273 | no |
| dlip6 | CG14049 | no |
| dlip7 | CG13317 | no |
| chico/IRS | CG5686 | no |
| slimfast | CG11128 | no |
| TIF1A | CG3278 | no |
| convoluted | CG8561 | no |
| Ecdysone-inducible gene L2 | CG15009 | no |
| dlip8 | CG14059 | no |
| Secreted decoy of InR | CG3837 | no |
| short neuropeptide F precursor | CG13968 | no |
| Tachykinin-like receptor at 99D | CG7887 | no |
| Akt1 | CG4006 | no |
| Histone deacetylase 3 | CG2128 | no |
| Focal adhesion kinase | CG10023 | no |
| Lnk | CG17367 | no |
| Neural Lazarillo | CG33126 | no |
| melted | CG8624 | no |
| mir-8 stem loop | CR42988 | no |
| Nucleostemin3 | CG3983 | no |
| Pdk1 | CG1210 | no |
| Pten | CG5671 | no |
| scylla | CG7590 | no |
| chrb | CG7533 | no |
| small wing | CG4200 | no |
| steppke | CG11628 | no |
| twins | CG6235 | no |
| PP2A-B | CG7913 | no |
| Diminutive | CG10798 | no |
| S6kII | CG17596 | no |
| RpS6 | CG10944 | no |
| mRpS6 | CG15016 | no |
| spargel | CG9809 | no |
| happyhour | CG7097 | no |
| lobe | CG10109 | no |
| sestrin | CG11299 | no |

| | | |
|---|---|---|
| tctp | CG4800 | no |
| Lkb1 | CG9374 | no |
| raptor | CG4320 | no |
| mir-14 stem loop | CR43013 | no |
| mlc2 | CG2184 | no |
| spaghetti squash | CG3595 | no |

# Chapter 6.

## General Discussion

## General Discussion

This thesis demonstrates the decisive influence nutritional environments, especially developmental nutritional environments, have in shaping plasticity of larval life-history traits and adult phenotypes. The thesis also demonstrates the fundamental importance of taking a multivariate approach to studying individual and species level phenotypic plasticity, both at the level of describing the multivariate nature of the environment and of the phenotype. The results highlight the inadequacy of attempting to understand how organisms have become adapted to their nutritional environments, or how nutritional environments may influence their future evolvability, through using either one-dimensional reaction norm approaches (Falconer, 1990; Chevin *et al*., 2010), or through measuring phenotypic response to serial dilutions of a single food composition (*e.g.* Ajuha *et al*., 2011; Kaun *et al*., 2007). Findings from laboratory studies utilising 'univariate' nutritional environment manipulations, claiming to demonstrate genotypic or species level phenotypic performance, or even fitness, are difficult to extrapolate meaningfully to what are the inherently multivariate natural environments wherein selection takes place.

Consider the following example to further emphasise the capacity 'univariate' studies have for generating confounded results. If the study reported in Chapter 4 of condition-dependent trait expression in *Drosophila melanogaster* males had not systematically sampled P-C nutrient space, we would have failed to identify what may be a highly important inter-generational sexual conflict for the species. For example, if larval flies had been reared only on diets offering a range of low- to high-energy formulations of the 1P:2C diet (a similar approached to that used by Ajuha *et al*., 2013 and Pavković-Lučić *et al*., 2013) we would have identified that low- to mid-energy preparations of this diet support maximal body size, while high energy preparations support the development of the largest, most symmetrical sex combs. However, we would not have noted the survivorship and performance maxima associated with the very high-energy, protein rich, 4P:1C food. Thus, when posing the question, 'which larval diet do females choose to oviposit on to sustain high levels of larval performance?', the high-protein food, which mothers unambiguously chose to lay eggs upon, would never have been offered as an option. Only a range of 1P:2C

diets would have been offered for oviposition, and the potentially important discovery about the species' ecology would have been missed.

This is not to suggest experiments presented in this thesis were exhaustive in their representation of ecologically relevant *Drosophila melanogaster* nutritional environments. While the thesis encompassed analysis of interactions between two dimensional nutritional environments, genetic polymorphism, gene expression and behaviour, the work lacks an appreciation of how other nutrient dimensions, temperature, humidity, day-length, and interactions with other species and pathogens would further contribute to the *D. melanogaster* phenotype. Analysing the impact of these additional ecological dimensions on *D. melanogaster* phenotypic plasticity presents an important challenge for future work. Studies examining the interactive effects of developmental nutrition and temperature using locusts as a model has commenced. e.g. by Coggan *et al*. (2011) and Clissold *et al*. (2013), and by van Den Berg *et al*. (2013) using spiders. To my knowledge, no-one is currently researching temperature and nutritional environment interactions using *Drosophila*. Studies are ongoing detailing the interaction of pathogens and gut symbionts with nutritional environments (Ponton *et al*., 2013) using *Drosophila*, and the influence of day-length, seasonality and nutrition on shaping phenotypes in *Drosophila* is under investigation (see Bartok *et al*., 2013 for a review). Advances made in the field of 'information theory' multivariate statistical analyses could allow for the comprehensive analysis of such data. For example, using a canonical analysis of principal coordinates approach (Anderson & Willis, 2003) might allow for phenotypic expression to be modeled given *n* predictive environmental dimensions (e.g. see Pastro *et al*. 2013).

Results from Chapters 2, 3, and 4 also highlight the importance of taking a multivariate approach when attempting to understand the phenotypic trade-offs faced by animals across their developmental period. Results from these chapters indicate that measuring the plasticity of a sub-set of different phenotypes across common nutritional environments is necessary for capturing a more complete picture of animal performance. Studies routinely rely on one or two phenotypic response metrics of individual performance, with 'survived to pupation' a typical performance measure used in *Drosophila* biology (Haymer & Hartl, 1983; Fitzpatrick *et al*., 2007). My results show that a single developmental nutritional environment can drive optimal expression in alternate traits. A particular environment may optimise phenotype

performance in one fitness-relevant trait, yet lead to deficits in the performance of another. This is most clear in results for female survivorship and development rate *vs*. wing size presented in Chapter 3. These data show that across *foraging* genotypes female *D. melanogaster* survivorship and developmental rate are maximised on diets that are similar in P:C ratio and energy density. Yet wing and putative body size, are maximised on a different range of diets in a genotypic-dependent fashion. For example, had my comparison of the performance of different *foraging* gene allelic variants ended with measurement of inter-genotypic survivorship and developmental rate, I would have concluded that mid-energy 1P:1C and high-energy 2P:1C diets supported maximal performance, and that this performance was equivalent between *foraging* genotypes. In this case I would have been forced to reject my hypothesis that *foraging* was a 'gene for plasticity'.

Chapters 2 and 3 demonstrated the remarkable role polymorphism of a single gene can play in shaping individual and species level phenotypic plasticity. Chapter 2 showed that genetically determined differences in food selection and intake behaviour displayed by larvae can drive plasticity in life-history traits whose expression is relevant to adult fitness. Chapter 3 revealed the role genetic polymorphism can play in mediating alternate modes of plasticity in response to common nutritional environments. These results demonstrate the important contribution genetic polymorphisms can make in: 1) allowing inter-individual flexibility in response to environmental variability experienced from one generation to the next, and 2) providing phenotypic 'options' for species regarding their future evolvability under environmental change. Chapter 2 and 3 demonstrated that given natural populations of *D. melanogaster* always contain alternative *foraging* allelic variants, no matter the prevailing developmental nutritional environment, populations will always contain phenotypically variable individuals whose relative fitness, given a change in the prevailing environment, should differ; thus facilitating natural selection (West-Eberhard, 1989).

How genetic polymorphisms, like that of *foraging* gene arise in nature and are retained in species' genomes over time is an important, unresolved question. Like the $for^{s2}$ mutant sitter strain, did the natural genotypes, $for^{R}$ rovers, and $for^{s}$ sitters come into existence through the action of an environmental mutagen (Jablonka & Lamb, 2005)? Were the alternate modes of continuous, environment-dependent phenotypic

plasticity encoded by each genotype of sufficient selective value over time, that both allelic variants have been 'retained'? Or did genetic assimilation, due to a consistent environmental influence (Bateman, 1959a,b; Badyaev, 2005; Jablonka & Lamb, 2005), like patchy larval food sources, drive for example the evolution of the 'roving' *for*$^{R}$ allelic variant? Conducting an experiment investigating whether *Drosophila* have the capacity to evolve such a genetic polymorphim via genetic assimilation, although possible (Bateman, 1959a,b), would be complex and time consuming. We do, however, already have access to data in the form of the *for*$^{s2}$ reaction norms detailed in Chapters 2 and 3, which can help evaluate the hypothesis that an environmental mutagen may be the origin of *foraging* polymorphisms.

The mutant *for*$^{s2}$ larvae displayed similar survivorship and developmental rates to the natural *foraging* allelic variants, however *for*$^{s2}$ individuals differed to natural *foraging* larvae in the level of triglycerides they accumulated across development, and their patterns of adult male and female wing size and wing shape. If the *for*$^{s2}$ mutation had occurred in nature, and was similarly non-lethal as it is in the laboratory, these unique patterns of phenotypic plasticity would likely have been of adaptive benefit, and lead to the mutant being retained within the *D. melanogaster* genome. Two phenotypes expressed by the *for*$^{s2}$ flies were especially interesting, and could potentially place at least male *for*$^{s2}$ flies at a performance advantage in nature. Chapter 1 data showed that on high-energy, high-carbohydrate (C), low-protein (P) diets, *for*$^{s2}$ larvae were markedly 'obesity resistant', especially compared for *for*$^{R}$ rover larvae. Chapter 2 data revealed that unlike natural *foraging* males, *for*$^{s2}$ males achieved their greatest wing and putative body size these same high-C:P diets. Natural *foraging* males were consistently small when reared on these high-C:P diet. This capacity to utilise high-C:P to support growth without 'obesity' would be beneficial under confinement to high carbohydrate diets in nature, for example, fruit. These results from *for*$^{s2}$ flies provides some evidence to support the arguments of Slatkin (1978), Lenormand (2002), and more recently Dickins & Rahman (2012) that random mutation in genes, through either crossing over or environmental mutagenesis, or potential through horizontal gene transfer (Cooper, 2014) are the ultimate origins of trait diversity and phenotypic plasticity, and that natural selection acting on the expressed phenotype arising as a result of a gene by environment interaction refines and maintains the mutations position in a species' genome over time. Following these

ideas, gene by environment interactions would therefore act as only the proximate originator of phenotypic plasticity and trait diversity. This proposal is of course at odds with the evidence for genetic assimilation. Perhaps both origins are possible.

Results from Chapter 4 demonstrate the importance of nutritional environments in shaping plasticity in traits relevant to sexual selection. Findings from this Chapter help to resolve how species retain the capacity for variation in secondary-sexual traits under persistent sexual selection. Chapter 4 showed a central role for larval developmental nutrition, or 'condition', in shaping the quality of male *D. melanogaster* sexual traits, seemingly independently of genetic variation. Recent work by Robinson *et al*. (2008, 2009) working on a wild population of the Soay sheep *Ovis aries* has similarly demonstrated a critical relationship between environmental variability and the maintenance of variability in the expression of secondary sexual traits. In Soay sheep, horn size is under sexual selection. Robinson *et al* (2008) showed that under 'poor' environmental conditions, males which allocate nutritional resources to growing large horns suffer lower survivorship, thus the more abundant smaller-horned males experience greater overall reproductive success. Under 'good' environmental conditions, however, males with larger horns survive better. As large-horned males are the better intra-sexual competitors, they experience higher total reproduction in 'good' environment breeding seasons. Presumably the nutrition available to males in 'good' seasons facilitates their synthesis of both large horns and their improved survivorship. It would be interesting to separate the environmental variables, nutrition and other factors that comprise Robinson *et al*.'s definitions of 'good' and 'poor' environments (Robinson *et al*., 2008).

Considering results from Chapter 2 and 4 together indicates that more than just nutritional environmental variability *per se* may drive variation in male 'condition' and thus variation in secondary sexual traits. Results suggest that the way animals interact behaviourally with their nutritional environments can also contribute to variation in sexual trait quality. The experiment detailed in Chapter 4, which demonstrated that male *D. melanogaster* sexual traits are condition-dependent, involved raising hundreds of larvae from egg to hatch on individual, defined diets. In nature, larval nutritional environments are more temporally and spatially heterogeneous. Two *D. melanogaster* behaviours, observed in Chapters 2 and 4 of this thesis, would contribute to larvae in nature experiencing heterogeneous food

environments – thus introducing yet further potential for variability in secondary sexual trait expression: maternal oviposition choice behaviour, and larval P-C intake regulation behaviour. Chapter 2 identified that larval *D. melanogaster*, of both sexes, regulate their P-C intake to fall between 1P:1C and 1P:2C. Larvae regulate towards this P-C intake regardless of prior rearing in either high-C:P or high-P:C food environments. Remarkably, this P-C target diet composition supported the development of highest quality male sexual traits, suggesting male larvae at least may feed in a way that maximises their 'sexual attractiveness' as adults. However, larvae in nature would have to locate foods that would allow the intake target to be reached. Chapter 4 demonstrated that mother flies lay almost all of their eggs on very high-protein foods. If this behaviour is consistently displayed by wild female *D. melanogaster*, then larval flies would have leave their oviposition site and forage for appropriate foods to allow for this putative 'attractiveness' target to be met. Given the shortness of the *Drosophila* larval period, and the highly localised nature of larval *D. melanogaster* food sites, reaching this P:C target may be challenging. Larvae can travel over reasonable distances, however, and the 1P:1C-1P:2C target may be achieved at some point during the larval period due to active foraging. Thus, both maternal and larval behaviour may contribute to realised nutritional environments being yet more variable, and thus contribute to further variability of male secondary sexual traits.

These findings pose another interesting hypothesis that could be pursed in future studies. Chapter 2 showed that *for*$^{R}$ larvae more tightly regulate their ~1P:2C intake target than *for*$^{s}$ sitters. *for*$^{R}$ larvae are also known to show greater food 'exploration' behaviour than sitters (Sokolowski, 2001; Hughson, *et al*., 2014). Could larval, male rovers feed in a way that supports their sexual attractiveness as adults? Analysis of the adult sex comb morphology from *for*$^{R}$ and *for*$^{s}$ specimens (results from which are presented in Chapter 3), in combination with the male mating success experiments proposed in Chapter 4, could resolve this question.

Chapter 5 detailed the complexity of gene expression changes associated with, and likely required for, population-level acquisition of an adaptive metabolic phenotype: high-P:C tolerance. Each of the *D. melanogaster* genetic strains investigated, w$^{1118}$, Canton S, and Oregon R, possessed the capacity, in low numbers, to express the high-P:C tolerant phenotype upon initial exposure to high-P nutritional

environments. This mode of advantageous phenotype expression fits well with the definition of an exaptation (Gould & Vrba, 1982). After just five generations of artificial selection on high-protein diets, up to 60% of the experimental population of $w^{1118}$ *Drosophila* flies expressed the lifespan protecting high-P:C tolerant phenotype. The artificial selection thus 'fast tracked' the selection for this 'exaptation', which would likely have occurred through natural selection under a sustained nutritional environmental shift in nature. A number of disparate molecular pathways were significantly changed in their expression in association with the population level acquisition and expression of the high-P:C tolerant phenotype. While results from Chapters 2 and 3 demonstrate that allelic variants are at least one origin of adventitious phenotypic plasticity, Chapter 5 results demonstrate that networks of genes are changed in their expression conferring the expression of phenotypes that provide targeted environmental adaptations.

An important finding from Chapter 5, relevant to our understanding of how genes mediate trait expression, was not just the large number of genes involved, but also the diversity of expression displayed by the same genes and gene groups across the experimental $w^{1118}$ *Drosophila* populations. The experimental populations were all of the same sex, the same genetic strain and the same species. They all stemmed from the same laboratory culture. They were all exposed to the same experimental diet and selection regime. They all expressed the same high-P:C tolerant phenotype to the same degree. Yet a gene or gene group significantly up-regulated in population A could be significantly down-regulated in population B, and again significantly up-regulated in population C flies. These findings strongly suggesting that gene expression, in conferring and maintaining the expression of complex metabolic phenotypes, is a far more dynamic and instantaneous process than evolutionary biology has previously held. This makes intuitive sense given that the other physiological processes responsible for the maintenance of homeostasis across variable environments are far from static or unidirectional, *e.g.* hormone and neuromodulator and neurotransmitter levels remain in flux (Bak *et al.*, 2006; Murphy & Bloom, 2006; Marshal, 2006; Chapman, 2013). Our experimental protocol was as close to measuring 'real time' changes in gene expression that is currently technologically possible and financially viable. No doubt future generations of evolutionary biologists will develop means to measure and analyses the real-time

expression of tissue-specific epi/genomes as an organism is exposed to environmental stimuli. Possibly only then will we be able to fully appreciate the molecular underpinnings of phenotypic plasticity.

This returns to the central question of this discussion, which will no doubt sustain decades of future work. Where did the capacity for the ‘exaptation’ phenotype, the plasticity of metabolism in the face of a nutritional environment shift, come from in the first place? Was it due to a ‘gene for plasticity’ as we hypothesise the *foraging* gene may be? Did a plasticity gene, due to its capacity to yield a broad reaction norm happened to yield a protective phenotype at the reaction norm periphery, which was subsequently selected for? Or was it a specific adaptive phenotype retained in the *Drosophila* genome, which had evolved through genetic assimilation via previous experience of high protein diets?

A final potential mechanism, which this thesis did not explore, was the contribution to phenotypic plasticity made by the epigenome. Epigenetic markers can alter the gene expression at specific loci from one generation to the next, affording rapid adaptation in the wake of environmental change (Jablonka & Lamb, 2005; Suter *et al*., 2013). Exposure to environmental stressors by a future parent-animal in a single generation like a marked change in diet can lead to epigenetic modifications of the parent’s germline DNA, which can influence gene expression in both the animal’s direct offspring and ‘grand-offspring’ (Cropley *et al*., 2012). Across animal species, these modifications include DNA methylation, histone tail protein-sequence modification and other changes in the physical configuration of chromatin, such that the manner with which transcriptional machinery interacts with DNA at the locus alters gene expression. These environmentally induced modifications are ‘Lamarckian’ in nature and represent a new frontier in our understanding of adaptive phenotypic plasticity (Ellers & Stuefer, 2010). The manner with which epigenetic markers influence gene expression and thus phenotype expression and therefore micro-evolutionary processes are unresolved, though epigenetic *vs*. genetic inheritance are likely complementary (Jablonka & Lamb, 2005; Suter *et al*., 2013; Danchin, 2013). It would be interesting to examine the role epigenetic markers may have played in altering gene expression in the high-P:C tolerant flies discussed in Chapter 5, and in other experimental studies demonstrating rapid evolution of population level protective metabolic phenotypes (*e.g.* Warbrick-Smith *et al*., 2006).

A final more prosaic finding of this thesis, though a no less important one, is identification of what may be an optimal larval *D. melanogaster* diet. Mid-energy, 1P:1C-1P:1.5C diets appear to be those that across all the phenotypes measured and genetic strains analysed in this thesis support, on average, optimal larval performance. These diets also correspond closely to the P:C intake ratio larvae self-select. Consistent lower survivorship and decreased developmental rates of *D. melanogaster* were associated with flies raised on very high-carbohydrate diets. Indeed, Chapter 4 results indicate that the capacity to survive on high-energy formulations of the 1P:1C 'optimal' P:C ratio, is possibly what adult male *D. melanogaster* 'advertise' via their large, symmetrical sex combs.

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