Lifelong Consequences of Protein Deficiency during Development in *Drosophila*

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Declaration

I confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that his has been indicated in the thesis.

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Abstract

Development is a vulnerable time in the life of an organism. In mammals, malnutrition during early life has profound effects on the risk of developing metabolic disease later in adulthood. Despite the well-established connection between early nutrition and adult health, the underlying programming mechanisms remain unclear. This thesis develops *Drosophila* as a model for studying the long-term physiological effects of an early low protein diet. I show that *Drosophila* larvae raised on food low in yeast, the major dietary source of protein, are developmentally delayed and eclose into smaller-than-normal adult flies (henceforth called small flies). These small flies display altered organ proportions and long-term changes in metabolism. For example, I observed that lipid droplets appear specifically in the hindgut enterocytes of old control flies as a feature of normal ageing but, in small flies, they are also present in young adults. Tissue- and stage-specific genetic manipulations demonstrated that these hindgut lipid droplets are regulated by the Insulin/Target of Rapamycin signalling network and require cell-autonomous *de novo* lipogenesis. In addition, I found that a high glucose "obesogenic" adult diet interacted with low but not control [yeast] larval diets to generate flies with a two-fold increase in triglycerides and ectopic lipid droplets in podocytes. The latter metabolic phenotype is recapitulated by experimentally overexpressing Brummer lipase in adipose tissue, suggesting that the underlying mechanism corresponds to lipid overflow. I also demonstrated that larval [yeast] can programme very large extensions and reductions in adult lifespan which, surprisingly, can be exchanged between co-housed individuals. This suggests the existence of a social, infectious or transferable component to the mechanism of nutritional programming. Together, these findings reveal that nutritional programming is conserved from mammals to insects. They also establish a new genetic model for dissecting the mechanisms underlying the developmental origins of adult health and disease.

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Abbreviations

ACC = Acetyl-CoA Carboxylase

ACL = ATP citrate lyase

AEL = after egg-laying

AKH = adipokinetic hormone

AKHR = adipokinetic hormone receptor

ALH= after larval hatching

Alk = anaplastic lymphoma kinase

AMPK= AMP-activated protein kinase

Bmm= brummer lipase

CE= cholesterol ester

CNS = central nervous system

Cn:x = fatty acid containing "n" carbons and "x" double bonds

CW = critical weight

DAG= diacylglycerol

dFOXO= *Drosophila* Forkhead BoxO transcription factor

Dilp = *Drosophila* insulin-like peptide

DN = dominant-negative

DOHaD = developmental origins of health and disease

DR = dietary restriction, usually by diluting component of the food

DV = dorsal vessel

EB = ejaculatory bulb

FA = fatty acid

FAME = fatty acid methyl ester

FB= fat body

GAD = GAL4 activation domain

GC-MS= gas chromatography-mass spectrometry

GFP = green fluorescent protein

[glucose] = glucose concentration

hg= hindgut

HG= high glucose (i.e. 400 grams of glucose per litre of food, as opposed to "standard" glucose = 58.5 grams of glucose per litre)

IGF= insulin-like growth factor

IIS = insulin/ insulin-like growth factor signalling

InR= insulin receptor

IPCs = insulin-producing cells (an alternative name for MNCs)

L1-3= first to third larval instars, respectively

lexAop= LexA operator

LG = low glucose diet = 2.925% (w/v) glucose i.e. half the glucose content of standard food

Lpp = lipophorin

LpR= lipophorin receptor

Lsd2 = lipid storage droplet-2

mg= midgut

MTs= Malpighian tubules

MVW = minimum viable weight

Myr= myristoylated

NEFA= non-esterified fatty acids (i.e. free fatty acids)

NMR = nuclear magnetic resonance

NR = severe nutrient restriction on PBS/ 1% agar

oe= oenocytes

PBS = phosphate buffered saline

PDH = pyruvate dehydrogenase

PI3K = phosphoinositide 3-kinase

PG = prothoracic gland

PTEN= phosphatase and tensin homolog

ra= rectal ampulla

Rheb= Ras homolog enriched in brain

S6K = S6 kinase

SCFAs = short-chain fatty acids

SG = salivary gland

TAG = triacylglycerol

TGP = terminal period of growth

TOR = target of rapamycin

TSC= tuberous sclerosis complex

UAS = upstream activating sequence

wL3 = wandering 3rd instar larva

WPP = white pre-pupa

w^{1118}iso = white^{1118} isogenic strain used for most of the experiments in this thesis

[yeast]= yeast concentration

"n% flies" = flies that were raised on food containing n% yeast during larval development

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To the few

Chapter 1

General Introduction

1 General Introduction

Lack of food has been the main problem for the vast majority of the Earth's population for millions of years. While this problem persists in many countries, for western societies the opposite issue of too much food and too little exercise now predominates. Eating too much for one's metabolic needs is widely accepted as a sure, one-way road to obesity. Evolutionary theorists have proposed that our hunter-gatherer past has predisposed humans to a "thrifty" metabolism, which can become detrimental when food is abundant. Hence, one of the biggest economic challenges for healthcare at present is how to prevent and treat an ever-increasing proportion of the population that is medically overweight (body mass index (BMI) higher than 25). A related issue is what happens when the same individual faces a period of scarcity, followed by a time of plenty? Research suggests that it all comes down to timing. Obesity and metabolic diseases have been put down to either genetic, nutritional or lifestyle causes. During the last couple of decades, a fourth, developmental dimension has been added to this model. An overwhelming amount of evidence seems to suggest that risk for obesity and metabolic diseases can be programmed early on during the life of an organism, during development. This might explain why individuals with similar genetic constitutions and lifestyles are discordant in terms of metabolic outcomes as adults.

Depending on the environmental conditions to which the organism was exposed during development, the same genotype can give rise to a variety of phenotypes, a concept known as 'developmental plasticity' (Bateson et al., 2004). It has been proposed that there is a critical, formative period in the early life of any living creature when the system is 'plastic' and sensitive to the environment. Outside this early time window, the developing organism is believed to be much less affected by environmental conditions. Developmental life is, therefore, a vulnerable period of the lifespan when adverse environmental factors may induce irreversible changes in tissue structure, gene expression patterns and physiological function. In mammals, it is now clear that impaired foetal growth is an important factor in determining the long-term health and well-being of the developing foetus. This concept is part of the more general idea that health and disease at all stages of life are the product of cumulative experiences across the lifespan.

1.1 Evidence from mammalian studies linking early-life nutrition with adult health and physiology

1.1.1 Epidemiological studies

Studying the relationship between maternal diet and disease in later life is not a realistic possibility for most researchers, in terms of cost, manpower and time. Proper exploration of this relationship would require detailed records of maternal diet and lifestyle during pregnancy and long-term follow-up of children into late adulthood. Moreover, experiments modifying the mother's diet to study the outcome on the offspring are not only impractical, but also unethical.

Therefore, epidemiological studies in this field have mainly relied on retrospective cohort studies i.e. locating adults for whom some indicator of prenatal exposure to putative risk factors is available, and relate these exposure indicators to disease patterns. Often, information on conditions and foetal growth during pregnancy is not available. Instead, the data that are recorded about pregnancy outcomes are infant measurements, such as birth weight, length at birth, and head circumference. Even though these measurements give only a crude indicator to nutritional influences, birth weight has often been used as a proxy for foetal growth.

A few exceptional historical situations have given epidemiologists the chance to study the offspring of mothers subjected to brief periods of food restriction. One such situation was the Dutch hunger winter. During the Second World War, in the winter of 1944-1945, an area of western Holland was subject to a famine that lasted for approximately 6 months as the occupying Nazi forces blocked delivery of rations. As this was a relatively short period, some women were exposed to famine during the last trimester of pregnancy, while others in the early stages. Comparisons of the adult offspring of these women with individuals whose mothers gave birth before or fell pregnant after the famine have suggested that exposure to *in utero* dietary restriction during late gestation significantly decreases weight at birth but is associated with increased adult obesity and glucose intolerance. In contrast, it was found that exposure to famine during the first trimester increased birth weight, in comparison to the Dutch average of those times, and was associated with adult hypertension (Ravelli et al., 1998; 1976).

The first to formulate a clear hypothesis linking early life nutrition with later disease was the population epidemiologist David Barker. In the 1980s, Barker and his colleagues at the university of Southampton sought to understand the North-South health divide noted in

England and Wales. They found that birthplace was a strong predictor of death from coronary heart disease, with greatest risk associated with birth in the industrial North (Barker and Osmond, 1986). Importantly, the risk was independent of subsequent migration. To study further the relationships between birth anthropometry and disease in later life they used a unique set of records from the county of Hertfordshire, England. A follow up of those individuals still living in the county revealed that both weight at birth and at 1 year of age were strong predictors for death from cardiovascular disease (Barker et al., 1990). Shortly after, studies of the same cohort also revealed a strong association between birth weight and decreased glucose tolerance and risk for non-insulin-dependent type 2 diabetes in adult life (Hales and Barker, 1992; Hales et al., 1991). Strikingly, infants who weighed less than 2.5 kg at birth were twice as likely to die from coronary heart disease, six and a half times more likely to develop type 2 diabetes and eighteen times more likely to develop the metabolic syndrome, as compared to individuals who weighed more than 4.3 kg. Based on such epidemiological findings linking poor early growth and an increased risk for cardiovascular and metabolic disease 60-70 years later, Barker proposed the "thrifty phenotype" theory. According to this theory, if *in utero* nutrition is poor, the developing foetus reacts by making predictive adaptive responses to maximize nutrient uptake and conservation. If postnatal diet is similar to the conditions it has faced *in utero*, then this "thrifty phenotype" confers an advantage as the individual will be well-equipped biologically to withstand a poor diet. Problems arise, however, when the postnatal diet is adequate or plentiful and, thus, different from the one that the developing organism has been programmed for. It has been, therefore, proposed that disease arises only when the postnatal environment is considerably different from that predicted by the *in utero* experience (Gluckman et al., 2005; Hanson and Gluckman, 2008). The "thrifty phenotype" theory was based on and complemented the "thrifty genotype" theory proposed by Neel in 1962, which attempted to explain how genetically-determined negative phenotypes, such as diabetes, have been selected for in the process of evolution (Neel, 1962). Neel proposed that genes that predispose to diabetes (referred to as "thrifty genes") have been advantageous in the history of humankind, but have become detrimental in the modern world where food is plentiful. Such "thrifty" genes have enabled individuals carrying them to process and store fat reserves efficiently during periods of food abundance, which represented an important energy conserving mechanism when food intake was irregular. The two theories, therefore, distinguish between the effects of nature (genotype) versus nurture (environment).

In humans, it is difficult to distinguish between "thrifty genotype" and "thrifty phenotype". However, studies of monozygotic twins have shown that the effects of poor early growth can be independent of genetic effects. A study of 64yr old monozygotic twins who were discordant for diabetes found that the affected monozygotic twin had a significantly lower birth weight (Poulsen et al., 1997).

The initial observations made by Barker *et al.* were then reproduced in a variety of studies and populations worldwide (reviewed by Demmelmair et al., 2006), and the link between birth weight and later outcome was extended to include insulin resistance, hypertension, raised plasma triglycerol concentrations, abdominal adiposity and unfavourable high- to low-density lipoprotein (HDL/LDL) cholesterol ratios, all major components of the metabolic syndrome. In consequence, the "thrifty phenotype" theory has been reformulated as the Developmental Origins of Health and Disease theory (DOHaD; (Barker, 1998; Langley-Evans and McMullen, 2010), which states that environmental factors acting at sensitive periods during development can result in long-term or permanent changes in the structure and function of an organism (Lucas, 1991).

Using birth weight as a proxy for maternal nutritional status, however, is the topic of ongoing debate. The link between maternal nutrition and foetal growth in well-nourished populations is controversial. Godfrey and colleagues have suggested that high carbohydrate intake in early pregnancy and low protein intake in the last trimester correlate with lower birth weight (Godfrey et al., 1996). Recently, caffeine intake during pregnancy has also been associated with impaired foetal length growth (Bakker et al., 2010), while another study found significant relationships between maternal vitamin A and calcium intake, and birth weight (Bawadi et al., 2010). However, other studies have failed to establish an association. For example, a study among pregnant women living in Portsmouth, UK, found no significant associations between babies' weights at birth and maternal intake of any nutrient (Mathews et al., 1999). It may be that maternal nutrition has a greater impact in women of lower socioeconomic status. In support of this idea, Wynn and colleagues found that, among impoverished women in Hackney, London, vitamin B intake was correlated with birth weight (Wynn et al., 1991).

The fact that the link between maternal nutrition and babies' weights at birth is not clear is unsurprising, as foetal growth depends on more than just maternal intake of nutrients and well-nourished women probably have adequate stores that can be mobilised in order to provide most substrates to foetal tissues. In developed countries, one of the most important determinants of intrauterine growth restriction is cigarette smoking

(Nordentoft et al., 1996; Wang et al., 2002)– a factor that is not always taken into account in studies that evaluate maternal nutrition. Furthermore, it must not be forgotten that foetal nutrition also depends on other factors, such as placental function and size, which in turn will depend on maternal-derived cues such as hormones, nutrients or adaptations to certain conditions. Impaired foetal growth can, therefore, have more causes than just maternal malnutrition even though, premature, low birthweight of <2.5kg, small for gestational age (SGA, <10th percentile), or intrauterine growth-restricted (IUGR) newborns may have similar weights.

Despite the original controversy and the inevitable caveats of human epidemiological studies, there now is a substantial body of evidence that suggests that a poor *in utero* environment, elicited by an inappropriate maternal diet or placental insufficiency, for example, may 'programme' susceptibility of the foetus to later development of cardiovascular and metabolic disease. Remarkably, high birth weight, maternal obesity and high fat intake during pregnancy, which increase birth weight, have also been associated with higher risk of cardiovascular disease, obesity and metabolic disease in the adult (Akyol et al., 2009; Bruce et al., 2009; Freeman, 2010; Han et al., 2005; Heerwagen et al., 2010). This might suggest a common underlying mechanism and highlights the importance of a balanced maternal nutrition.

1.1.2 Animal studies

Animal models have been instrumental in demonstrating the biological plausibility of the associations observed in human populations, providing proof of principle to the theory of the developmental origins of health and disease (DOHaD). The use of appropriate animal models for a specific hypothesis is essential as it can overcome many of the limitations of epidemiological studies i.e. it allows control over confounding factors, measurement of invasive end points and follow-up of the effects across the full lifespan and into subsequent generations.

Already in the 1960s, McCance and Widdowson showed that alteration of early growth by manipulation of feeding conditions during sensitive pre- and postnatal periods predetermined the weight of rats in adulthood (Widdowson and McCance, 1963). To explore the link between foetal growth and outcome in later life, animal models of maternal global calorie restriction, protein undernutrition, intrauterine artery ligation, iron deficiency, hypoxia and high fat-feeding have been developed. Despite the widely different approaches, all of these show the same outcome and offer strong evidence that

alterations of the foetal environment can lead to disturbed lipid metabolism and increased risk of metabolic diseases in later life.

By far, the most commonly described models of nutritional programming have involved pregnant rats, whose diet has been manipulated. The single largest limitation of rodent models lies in the fundamental differences between rat and human development. The rat is born with a poorly developed CNS and endocrine system, both of which mature significantly during the weaning period. One alternative would be the guinea pig, which is born with well-developed CNS, endocrine and vascular systems, but has been used in very few studies to date. However, as rodents have multiple pregnancies, concerns have been raised that nutrient supply to individual offspring might vary within the same litter. This problem can be overcome using sheep models, which usually have single or twin pregnancies. Each organism will have its advantages and disadvantages, and the best model in each case will depend on the hypothesis being tested. While one perfect model does not exist, different approaches and model organisms can be used to model different aspects of nutritional programming. Integrating evidence from a variety of models will then hopefully lead to the advancement of knowledge in the field and might reveal evolutionarily conserved mechanisms of developmental programming.

Taken together, evidence from mammalian model organisms suggests that nutrition during early life mediates outcomes in the adult largely through: (1) permanent structural changes (mainly affecting brain structure, kidney nephron number and pancreatic β-cell mass); (2) epigenetic programming of gene expression by DNA methylation and/or histone modifications at the enhancer and/or promoter regions of specific genes (primarily, those encoding for transcription factors such as PPARα, PDX-1 and HNF4α); and (3) altered rate of cellular senescence, due to effects on telomere length in key metabolic organs such as the endocrine pancreas (reviewed by: Bruce and Hanson, 2010; Langley-Evans, 2009; Martin-Gronert and Ozanne, 2012).

1.2 *Drosophila* as a model organism for integrative physiology

A holometabolous insect, *Drosophila* develops through distinct embryonic, larval, pupal and adult stages. Flies develop through a series of three larval stages (L_1, L_2 and L_3) called instars (Figure 1.1.A). Progression from one larval instar to the next involves shedding of the old exoskeleton and producing a new, larger one, a process known as moulting. The bulk of the larval tissues are polyploid and grow by endoreplication. Also developing in the larva are a smaller number of diploid cells organised into imaginal tissues. These are

the undifferentiated progenitors of adult (imaginal) structures that grow and proliferate in the larva. The best-characterised imaginal cells are those of the imaginal discs i.e. sac-like structures composed of cells destined to form adult external cuticular structures (Figure 1.1.A, inset). These are named after the adult structure that they will make, e.g. wing, haltere, leg, eye, genital (Morata, 2001). Following the last larval instar, the insect enters a pupal stage during which it undergoes complete metamorphosis to its adult, reproductive form. Metamorphosis is a complex process during which nearly all of the larval tissues are destroyed and adult organs are made by differentiation of the imaginal tissues. Of special interest to this project is the fact that the larval adipocyte-like cells of the fat body undergo apoptosis during metamorphosis and are completely eliminated during the first week of adult life (Aguila et al., 2007; Nelliot et al., 2006). The adult fat body forms from a different set of cells and is, therefore, a completely different tissue from its larval counterpart, even though little is known about the origins of this tissue. Given this, the life cycle of holometabolous insects, in general, and *Drosophila*, in particular, actually involves two different organisms: the larva and the adult. Seen from this perspective, in the context of developmental programming, one could say that the larva acts like the "mother" of the adult, with the imaginal tissues from which adult structures will form, developing inside the larva until they are ready to form adult organs. In a crude parallel to mammalian development, the larval stages where most growth occurs can, therefore, be considered to cover both foetal and post-natal development up to adulthood. Adult flies, like all arthropods, are encased in a stiff exoskeleton, which limits their adult body size. Since the pupa is a closed system, the size of the adult and its internal organs at eclosion are largely dependent upon the size that the larva has attained at the end of development. Also dependent on growth during the premetamorphic, larval stages is the size of adult cuticular structures (e.g. wings, legs etc), which are believed to be entirely defined by the growth of the imaginal discs. In turn, the final size attained by the larva and its internal organs depends on both growth rate and length of the growth period (Edgar, 2006; Shingleton, 2010). In *Drosophila*, commitment to metamorphosis depends on the larva reaching a size checkpoint referred to as "minimum viable weight" (MVW), operationally defined as the weight threshold after which larvae will progress to pupariation even if food is completely withdrawn. If starved at this point, progress to pupariation will be delayed, but the larva will, nonetheless, give rise to a small but viable adult. This checkpoint was first described in *Drosophila melanogaster* in the 1930s (Beadle et al., 1938), and it wasn't until twenty years later that it was distinguished from "critical weight" (CW; Bakker, 1959), which is attained a few hours later and is the weight

Figure 1.1: *Drosophila* as a model organism. (A) Cartoon shows the lifecycle of *Drosophila*. Fruit flies develop through distinct embryonic, larval, pupal and adult stages. Adult organs develop within the larva as imaginal discs. Inset shows the approximate positions of the imaginal discs in a 3rd instar larva (colour coded according to the organs they will give rise to; from Shingleton, 2010). Permission to reproduce this figure was granted by the Copyright Clearance Center on behalf of Landes Bioscience.

(B) Schematic drawing of a *Drosophila* larva showing the major organs involved in metabolic homeostasis: gut (grey), fat body (yellow), oenocytes (red) and the neurosecretory insulin-producing cells (IPCs; pink). Adapted from Gutierrez *et al.* (2007).

threshold above which nutrition does not influence the time course to pupariation i.e. even if completely starved, larvae will proceed to pupariation without delay. Critical weight is believed to be determined genetically and differs between species (Edgar, 2006). The time it takes for an insect larva's physiology to sense critical weight and initiate the behaviours related to metamorphosis, including cessation of feeding and wandering out of the food, is termed as the interval to cessation of growth (ICG) or terminal growth period (TGP). Throughout this document I refer to this period using the latter term. *Drosophila* larvae grow so fast that they can more than triple their mass during TGP, depending upon the food they eat during this period (reviewed by Edgar, 2006; Shingleton, 2010).

At 25^0 C, *Drosophila* embryonic development lasts only 24 hours. The newly hatched L_1 larvae can then progress to pupariation, i.e. formation of the white pre-pupa (WPP), in only 4-5 days under standard laboratory conditions. Following another 5-day pupal period, during which metamorphosis occurs, the adult fly emerges. Adult flies can become reproductively active as soon as 8 hours after eclosion and reach their full reproductive potential 2-3 days later. Apart from the short life cycle, small size and ease to culture in large numbers, there are other reasons why the humble fruit fly is one of the most exploited model organisms. *Drosophila* has been used by geneticists for over a century, but only in recent years has it emerged as an attractive model for studies of integrative physiology. With only four pairs of chromosomes and little gene redundancy, *Drosophila* still shares most of the basic metabolic functions and interactions between organs found in vertebrates (reviewed by Baker and Thummel, 2007). Therefore, it is likely that discoveries made in *Drosophila* will be informative for uncovering mechanisms in more complex organisms.

1.2.1 The organs involved in metabolic homeostasis

Animals have to constantly assess, integrate information from hundreds of feedback mechanisms and adapt to ever-changing environmental conditions, including nutritional status, in order to ensure survival. Metabolic regulation and physiological feedback systems are central in maintaining metabolic homeostasis in all multicellular organisms. The past few years have seen a resurgence in the number of genetic studies of physiological responses in *Drosophila*. The fruit fly has emerged as a simpler, well-balanced integrated system that shares most of the same metabolic functions and interactions between organs as seen in vertebrates (Fig 1.1.B; reviewed by Andersen et al., 2013; Baker and Thummel, 2007; Edgar, 2006; Léopold and Perrimon, 2007; Liu and Huang, 2013).

As with vertebrates, ingested food is digested and absorbed in the midgut, the functional equivalent of the stomach and small intestine. Excess energy is stored in the form of glycogen granules and lipid droplets in the fat body, the fly equivalent of the vertebrate white adipose tissue and, partially, the liver. The fat body is an amorphous organ made up by sheets of adipose tissue distributed throughout the body. As with vertebrates, glycogen can also be deposited in the muscles for more immediate usage. Sugar levels in the haemolymph, the fly circulating fluid, are maintained by neurosecretory cells located in the brain and ring gland that together form a bipartite "endocrine pancreas". Two clusters of medial neurosecretory cells (MNCs) located in the *pars intercerbralis* secrete *Drosophila* insulin-like peptides (dilps), whose actions are mediated by the only insulin receptor homolog (InR) found in *Drosophila*. In the literature, these are often referred to as insulin-producing cells (IPCs), which is also the term I adopt throughout this document. Upon nutrient shortage, corpora cardiaca cells, a region of the neuroendocrine ring gland, release adipokinetic hormone (AKH; a neuropeptide similar in function to mammalian glucagon) into the circulation. AKH mobilises glycogen stored in the fat body, by activating glycogen phosphorylase and, thus, reducing fat body glycogen and increasing circulating sugars. Lipids are also mobilised by AKH via increased activity of the triacylglycerol lipase Brummer (ortholog of the vertebrate ATGL) under conditions of starvation (Gronke et al., 2005; 2007). Lipids are carried through the haemolymph as either high density or low-density lipophorin particles (Brankatschk and Eaton, 2010; Palm et al., 2012). Previous research in our lab has shown that, upon starvation, lipids accumulate in specialised clusters of cells called oenocytes, reminiscent of mammalian hepatocytes (Gutierrez et al., 2007). In this way, mammalian liver functions appear to be shared between the oenocytes and the fat body in *Drosophila*. Recently, Unpaired 2 (Upd2), a protein synthesised and secreted by the fat body, was shown to control growth and metabolism and was proposed to be the *Drosophila* functional homolog of mammalian leptin (Rajan and Perrimon, 2012). One important difference between mammalian and insect metabolism, however, is the fact that insects are unable to synthesise cholesterol, which renders them cholesterol auxotrophs (Karlson, 1970).

1.2.2 Challenges of using *Drosophila* for studies of metabolism

While fruit flies are the rising stars of metabolism research, *Drosophila* studies of metabolism face a number of challenges inherent to any young field, including the fact that not enough time has passed for methods to be fully established. Protocols that are standard practice in mammalian model organisms, from quantifying adiposity to implementing dietary restriction or measuring food intake, are still being disputed,

tweaked and reinvented (Al-Anzi and Zinn, 2010; Bass et al., 2007; Carvalho et al., 2005; Grandison et al., 2009b; Hildebrandt et al., 2011; Ja et al., 2007; Wong et al., 2009). The side effect of this is a lack of standardisation among the protocols employed by the different laboratories. For example, a seemingly trivial but consequential matter is the lack of a standardised fly diet. In Table 10.1 (see Appendix), I have compiled fly food recipes employed by different laboratories in published studies of *Drosophila* metabolism and/or dietary manipulations. The information in Table 10.1 highlights the extent of the problem and can be used as a reference when comparing the work presented in this thesis with published literature. Moreover – also stemming from the relatively short time that has elapsed since the fly has started to be used for studies of metabolism – many of the basic questions of how fly physiology and metabolism work have not yet been elucidated. In practical terms this means that studies investigating the mechanistic bases of a metabolic phenotype have first to characterise the biological process of interest under wildtype conditions. Finally, physiological parameters of most metabolic readouts, including adiposity and concentrations of circulating metabolites, have not yet been defined in *Drosophila*. For this reason, small differences should not be over-interpreted as they might simply fall within physiological limits.

Additional challenges are posed by the minute size of our model organism of choice. For example, measuring the concentrations of circulating metabolites, such as sugars, is common practice in mammals. Indeed, commercial glucose quantification kits exist that require only 9 µL of blood and do not rely on specialist training. Considering that an adult fly contains less than 100 nL of haemolymph (Albers and Bradley, 2004), obtaining 9 uL of fly haemolymph would require extracting from at least 1000 adult flies. Because of this and similar considerations, obtaining basic readouts of metabolic status in flies can be time consuming and often requires highly specialised equipment, such as mass spectrometry or nuclear magnetic resonance (Cheng et al., 2011; Piyankarage et al., 2012). Equally, the small size of the internal organs means that studying the metabolic profile of individual organs is not trivial. Ideally, samples would comprise of homogenous cell types or one would be able to study metabolism at single cell level. Towards the former, attempts have been made in the direction of separating out pure populations of cells from heterogenous tissues, but these usually require large amounts of starting material (Berger et al., 2012; Neufeld et al., 1998; Shigenobu et al., 2006). To overcome the problem of either studying bulk tissues or having to start with large amounts of material, Andrew P Bailey and Alex P Gould in our lab have recently collaborated with researchers at Harvard Medical School to provide proof-of-principle that multi-isotope imaging mass

spectrometry (MIMS) can be used to study metabolism at single-cell level in *Drosophila* tissues (Steinhauser et al., 2012). MIMS enables one to visualise and measure the incorporation of stable isotope-labelled metabolites with sub-micrometre resolution. However, while highly informative, this technique is costly and low throughput, and, therefore, reserved for only crucial experiments. However, while all of the above is true, the benefits of using *Drosophila* as a model organism – with its simplicity and virtually limitless possibilities for genetic manipulations – are numerous and far outweigh the cons. With the technology available nowadays, a little more time is all that the *Drosophila* physiology and metabolism research community needs.

1.3 Systemic regulation of growth in *Drosophila*

1.3.1 IIS and TOR signalling match nutrient supply with growth rate

For a long time, the problem was simple: organisms eat, organisms grow. However, it is now clear that nutrients do not directly promote cell growth and are much more than just the raw materials for making cell components. Macronutrients, such as carbohydrates and amino acids, have been shown to stimulate signalling pathways, which, in turn, regulate the rate of growth. The molecular pathways controlling the rate of growth have been revealed to be remarkably well conserved across species. The number of pathways that have been described to be implicated in the control of growth is extensive, but the two principal ones that match nutrient supply with growth rate are the highly inter-connected Insulin/ Insulin-like Growth Factor signalling (IIS) and TOR (Target of Rapamycin) signalling pathways (Figure 1.2) (reviewed by Hietakangas and Cohen, 2009; Laplante and Sabatini, 2012; Polak and Hall, 2009).

The role of insulin in carbohydrate metabolism has long been acknowledged and investigated. However, over the past decade or so it has become clear that altered insulin signalling can also lead to metabolic disorders, such as obesity and diabetes (O'Rahilly, 2007). Since then, great efforts have been made to elucidate the molecular mechanisms underlying insulin signalling and its effects on growth, size, longevity and reproduction. Insulin-like molecules have been identified in both vertebrates and invertebrates and include the mammalian insulins and insulin-like growth factors (IGFs), the *Drosophila* insulin-like peptides (Dilps), the *Caenorhabditis* insulins and the lepidopteran bombyxins.

In *Drosophila*, seven different Dilp molecules were originally identified and described (Brogiolo et al., 2001). These are mainly produced by a set of insulin-producing cells (IPCs) in the brain, but the gut, enteric neurons, glia, imaginal discs, principal cells of the

Malpighian tubules and, recently, the fat body and perivisceral muscles have also been reported to synthesise some of them (Ikeya et al., 2002; Miguel-Aliaga et al., 2008; O'Brien et al., 2011; Slaidina et al., 2009; Sousa-Nunes et al., 2011; Söderberg et al., 2011). Recently, an eighth one, Dilp8, has been described as a signal secreted by the developing imaginal discs to communicate abnormal growth and postpone pupariation (Colombani et al., 2012; Garelli et al., 2012). Increased Dilp expression increases adult body size in *D. melanogaster*, whereas ablation of the IPCs results in reduced body size and diabetic phenotypes (Ikeya et al., 2002; Rulifson et al., 2002). The activity of the eight Dilps is mediated by a single insulin receptor (dInR) and its downstream effectors, which are

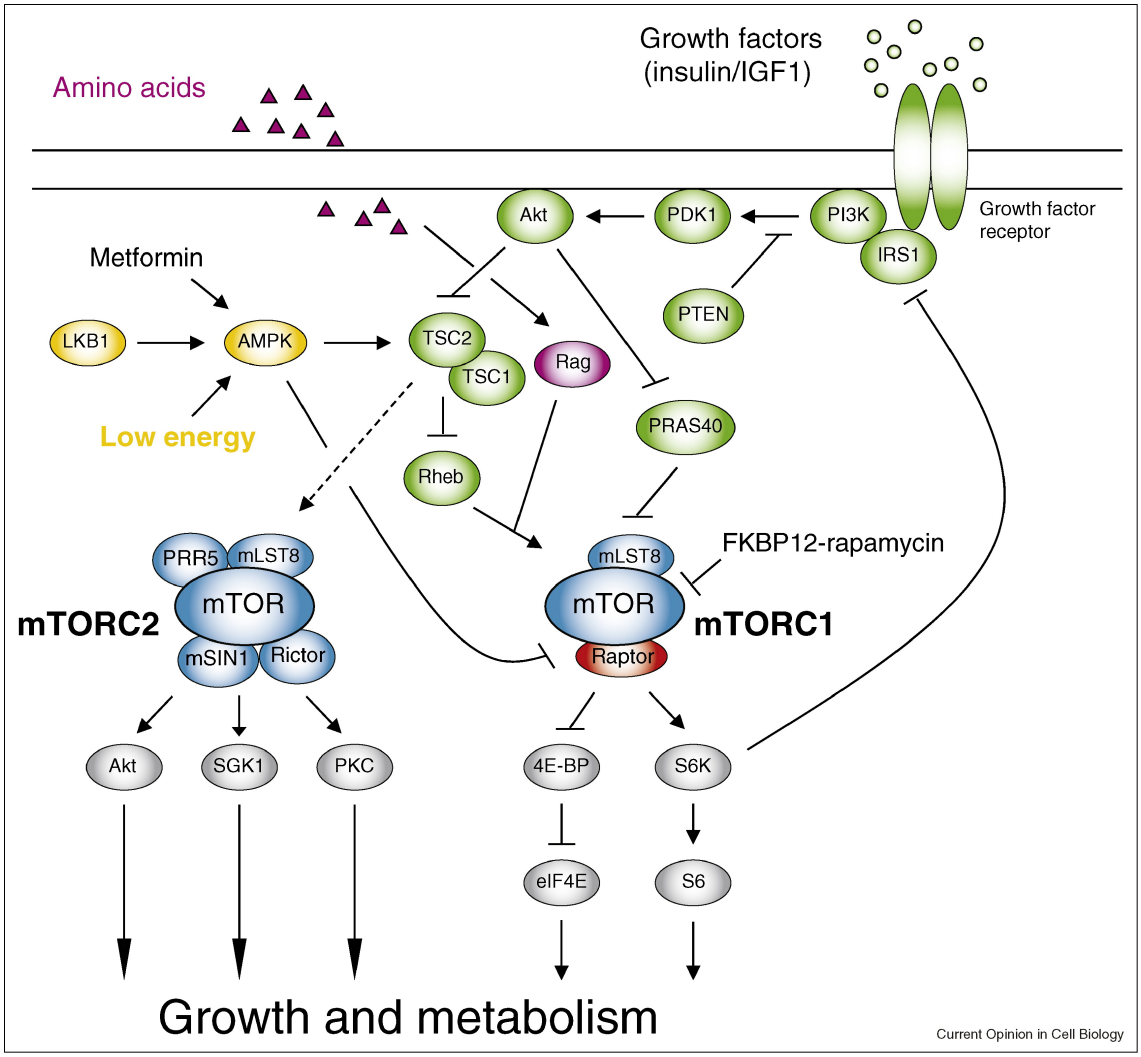

Figure 1.2: Schematic diagram of the mammalian TOR and IIS signalling networks. The main components and functions of these signalling pathways are conserved from yeast to humans. Amino acids (purple) enter the cell and act via Rag GTPases to positively regulate the rapamycin-sensitive mTORC1. Growth factors (green), such as insulin and IGF1, bind to growth factor receptors to activate the Akt-PI3K signalling cascade, which in turn positively regulates mTORC1. Energy status (yellow) is sensed via AMPK, which in turn feeds into and regulates the mTORC1 branch of the pathway. Substrates of the mTORCs are depicted in grey (from Polak and Hall, 2009). Permission to reproduce this figure was granted by the Copyright Clearance Center on behalf of Elsevier Limited.

expressed ubiquitously (Brogiolo et al., 2001; Chen et al., 1996; Fernandez et al., 1995). The insulin receptor becomes active upon binding of an insulin-like peptide, which triggers a downstream phosphorylation cascade that involves CHICO (*Drosophila* Insulin Receptor Substrate) and phosphatidylinositide 3-kinase (PI3K) (Böhni et al., 1999; Leevers et al., 1996; Yenush et al., 1996). PI3K converts phosphatidylinositol 4,5-bisphosphate (PIP_2) to phosphatidylinositol 3,4,5-trisphosphate (PIP_3), which stimulates membrane recruitment of phosphoinositide-dependent protein kinase 1 (PDK1) and Akt (also known as protein kinase B, PKB). Akt promotes cell growth by at least three mechanisms: (1) It increases cells' energy supplies by promoting glucose uptake through a phosphorylation cascade which promotes the insertion of glucose transporters into the membrane and by activating glycogen synthase kinase, which leads to the synthesis of glycogen and, thus, increased glucose stores; (2) Akt phosphorylates, and thus sequesters in the cytoplasm, the transcriptional repressor Forkhead box, class O (FOXO) which is a negative regulator of cell growth (Jünger et al., 2003; Kramer et al., 2003; Puig et al., 2003); and (3) Akt inhibits the Tuberous Sclerosis Complex dimer (TSC1/TSC2; Kwiatkowski and Manning, 2005; Potter et al., 2001; Tapon et al., 2001), thus relieving inhibition of the small GTPase RHEB (Ras Homolog Enhanced in Brain; Patel et al., 2003; Stocker et al., 2003), which increases TOR pathway activity (reviewed by Andersen et al., 2013; Edgar, 2006; Hietakangas and Cohen, 2009; Mirth and Shingleton, 2012).

In addition to sensing systemic nutrient levels indirectly through the insulin-signalling pathway via Akt, TOR senses amino acid levels in a way that is not yet entirely understood, but recent work in mammalian cell lines suggests that amino acids induce GTP loading of the Rag proteins, which mediate the correct binding of mTOR to RAPTOR, its partner in the mTORC1 complex (reviewed by Goberdhan and Ögmundsdóttir, 2009; Hietakangas and Cohen, 2009; Kim and Guan, 2011; Polak and Hall, 2009). The complex is then transported to an endomembrane where mTORC1 interacts with its activator RHEB. RHEB is independently activated by growth factors, which makes this a very attractive model as it explains why growth factors fail to stimulate mTORC1 activity in the absence of amino acids. Activated TORC1 promotes growth by phosphorylating both, the translation suppressor 4E-binding protein (4EBP), which suppresses its activity, and the ribosomal protein S6 kinase (S6K). This results in enhanced translation and ribosome biogenesis, respectively. TOR activity also suppresses autophagy, promotes bulk endocytosis and prevents the targeted endocytosis of the amino acid transporter Slimfast, all effects that are believed to support the positive cell growth effects of the TOR signalling pathway.

As described above, individual cells can also respond directly to nutrient levels. Growth of the entire organism in relation to nutrient status, however, needs to be coordinated. In *Drosophila*, it has been suggested repeatedly that the fat body may modulate the growth of other tissues according to nutrient levels (Britton and Edgar, 1998; Colombani et al., 2003; Davis and Shearn, 1977). The mechanism via which the fat body might sense nutrient levels and influence the growth of other tissue accordingly has been partially uncovered by Colombani and colleagues (2003). They showed that downregulating *slimfast* (a gene encoding for an amino acid transporter) specifically in the fat body, and thus lowering amino acid levels in the fat body cells alone, results in a global growth defect that phenocopies the effect of poor nutrition in the *Drosophila* larva. The effects of downregulating *slimfast* are mediated via decreased TOR signaling in the fat body, which, in turn, decreases the production of a putative growth-promoting humoral signal produced by the fat body. Work from the same laboratory by Geminard et al. then showed that the fat body couples amino acid levels with organismal growth by remotely controlling the release of Dilps into the haemolymph from the IPCs (Géminard et al., 2009). Furthermore, recent work in our lab and by others shows that an unidentified fat body-derived signal is also required for neuroblast exit from quiescence and reactivation at the end of the first larval instar stage (Chell and Brand, 2010; Sousa-Nunes et al., 2011). In this case, the aforementioned humoral signal indirectly promotes neuroblast exit from quiescence by promoting dilp production by nutritionally responsive glia, whereas IPC-derived dilps have no effect on neuroblast reactivation.

1.3.2 Ecdysteroids and juvenile hormone are negative regulators of growth

Along with the rate of growth, the other factor determining the final body size of an organism is the duration of growth. In insects, the latter is determined by fluctuating levels of hormones: attainment of critical weight is associated with a rise in ecdysone levels that will eventually trigger the cessation of larval feeding and subsequent metamorphosis (reviewed by Edgar, 2006; Shingleton, 2010). Caldwell et al., Colombani et al. and Mirth et al. have found that ecdysone activity is positively regulated by IIS activity in the prothoracic gland, the site of production of this steroid hormone (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005). Studies have revealed that suppressing insulin signalling in the prothoracic gland reduces ecdysone activity and increases the final body size of the insect. Conversely, up-regulating insulin signalling in the prothoracic gland accelerates its growth, which results in increased ecdysone activity, ultimately producing smaller-than-normal adults. In extreme cases, the changes in the final body size of the insect were due to changes in the timing of the onset of metamorphosis and, thus,

altered duration of the TGP. What is more surprising, however, is the observation that even relatively subtle changes in IIS in the prothoracic gland have a clear impact on the growth rates from early larval development, without altering developmental timing. The underlying molecular mechanism seems to be that low levels of ecdysone antagonise the ability of insulin signalling to activate PI3K and Akt and sequester the transcription factor FOXO in the cytoplasm (Colombani et al., 2005). The cell-autonomous effects of ecdysone activity on FOXO, suggest that insulin signalling and ecdysone have opposing effects on cell growth and metabolism.

1.4 The relative growth of body parts

It has been erroneously assumed by many in the scientific community that, among comparable individuals of the same species, *"the relative size of body parts [...] is more or less constant"* (Robertson, 1962). Even recently, an annual review article on the growth of the wing started by saying that *"external factors such as nutrient availability and crowding can alter overall animal size, but individual body parts scale reproducibly to match the body even with challenges from a changing environment."* (Neto-Silva et al., 2009).

However, it has long been known that the form of organisms is the result of differential growth, be it general growth that differs in the three planes of space or because different parts grow at different rates (Gayon, 2000; Huxley, 1950; Thompson, 1917). The problems of relative growth were discussed seven decades ago in a seminal work by Julian Huxley who provided extensive evidence that changes in form are accompanied by differential growth of the component parts (Huxley, 1932). In order to avoid confusion in the field of relative growth, Julian Huxley and Georges Teissier coined the term "allometry" in 1936. In a joint paper published simultaneously in French and English (Huxley and Teissier, 1936), they agreed to use this term, instead of the previous one "heterogony", to designate the changes in the dimensions of body parts relative to overall changes in size. Huxley and Teissier were also the first ones to propose the algebraic formula of allometric growth: $y = bx^k$, which describes the relative growth of one part compared to another or to the whole. In this formula, y and x are measurements of morphological traits, b is a constant, while k is the scaling exponent, also referred to as the allometric coefficient, and gives information about the growth ratio. If $k<1$, the relationship between y and x is referred to as negative allometry. Conversely, if $k>1$, organ y shows positive allometry relative to x. Finally, when $k=1$, the size of y is isometric, i.e. proportionate, to the size of x. In the context of insect development, static allometry can be defined as the coordination of final adult organ sizes with body size at the end of larval feeding (Stern and Emlen, 1999).

Up until now, few have considered the importance of body proportions in mediating the effects of developmental programming. However, it has long been known that undernutrition during development results in offspring with altered proportions. In humans, Gruenwald showed almost four decades ago (Gruenwald, 1963), that intra-uterine growth restriction (IUGR) late in human gestation can result in newborns that are small for gestational age (SGA) but have relatively large heads. Even back then this idea was not new. One decade earlier, Isabella Leitch had used examples from McMeekan's studies on the growth of pigs (Leitch, 2001; Victora and Barros, 2001) to raise awareness of what the wrong nurture can do to animal nature. She pointed that if a bacon pig is underfed for half of its life and then given a full ration, this "low-high" pig will certainly put on weight. However, she emphasised the fact that, while under these conditions the pig might reach the same ultimate weight, the skeleton and muscle will not grow as they would have done if they had had the opportunity at the right time and the extra food will be used mainly to lay on fat. She concluded that if the growth potential of an animal is not used all the way along, one does not get full development. Leitch used the "low-high" pig example as a parallel to human growth. Finally, she explained that, in man, if the rate of growth is sufficiently slowed, the adult is not only small but "underdeveloped", with normal or nearly-normal sized head, retarded trunk and relatively short legs. The proponent of the developmental origins theory, David Barker himself, has reported that, not only birth weight, but also body proportions at birth were also significant predictors of cardiovascular disease risk (Barker et al., 1993).

Recently, a research project conducted in our laboratory by Louise Cheng and Andrew Bailey has shown that, when faced with conditions of severe nutrient restriction, *Drosophila* larvae preferentially promote the growth of important organs, such as the brain, at the expense of others, a process known as sparing (Cheng et al., 2011; Dobbing, 1971). They subjected late-L3 larvae to starvation and compared the organ proportions at the end of development in these larvae as opposed to those in larvae that had unrestricted access to nutrients throughout. They found that most organs did not scale down with the body, with organs that are considered to be important for later survival being positively spared relative to the body. Importantly, this work uncovered a mechanism for brain sparing in which TOR/PI3K signalling activity is maintained in neuroblasts and their progeny by the nutrition-independent receptor anaplastic lymphoma kinase (Alk) and its ligand Jelly-belly (Jeb), even under conditions of nutrient restriction.

In *Drosophila*, the compelling results of classic experiments of transplantation and regeneration (reviewed by Stern and Emlen, 1999) have led to the consensus that the

upper limit on the size of appendages derived from imaginal discs is largely regulated autonomously, within the structure itself. Transplantation of wing imaginal discs into the abdomen of an adult female have shown that, even under such growth-permissive conditions, the discs do not grow more than they would in the larva. Moreover, delaying pupariation in larvae also does not cause imaginal discs to grow more than they normally would and the discs stop at the appropriate size. However, external factors such as hormones and starvation during insect development can affect the final size of the body and its constituent parts, particularly in a negative direction. In insects, during metamorphosis resources redistribute among body compartments. Holometabolous insects are, therefore, the ideal system to study how resources accumulated during larval phases are allocated. One of the few examples of diet-induced changes in organ proportions showed that rearing the grass-chewing caterpillars of *Pseudaletia unipuncta* on hard grass resulted in individuals with head masses twice as great as those of caterpillars fed soft artificial diet, even though the larvae reached the same body mass (Bernays, 1986). However, in this case the diet probably played more the role of a physical factor. Another study used butterfly wings to test whether experimentally induced changes in resource allocation to one trait during development would trigger compensatory mechanisms that alter the size of another trait (Nijhout and Emlen, 1998). The authors showed that removal of one or two hind wing imaginal discs from the larvae of *P. coenia* butterflies resulted in animals with disproportionately large forewings for their body size compared to sham-operated controls. This classic experiment showing that growth of one organ is at the expense of another with no change in overall body size hints that organs may compete for resources during development and that final size is the result of an allocation trade-off.

1.5 The long-term effects of nutrition during early life upon adult traits in insects

To date, several studies have sought to investigate the potential long-term effects of larval nutrition on adult outcomes in insects (Baldal et al., 2005; Chang et al., 2012; Jiménez-Cortés et al., 2012; Lints and Lints, 1969; Lushchak et al., 2011; Matzkin et al., 2013; Mayer and Baker, 1984; Saastamoinen et al., 2013). Towards this end, the approach most widely employed has been to increase larval density in order to limit the amount of food available to each larva (Baldal et al., 2005). However increasing larval density will also increase the amount of waste products and, therefore, toxins in the vial. For this reason, the effects of increased larval density do not necessarily reflect the effects of changes in larval nutritional status. Other studies have maintained larval density constant but tried to vary

the amount of food in the vial such that each larva would have less food available to itself. Moreover, some of these studies have only analysed newly-eclosed or young adults (Baldal et al., 2005; Lushchak et al., 2011). Since larval FB cells are present in large numbers during the first few days of adult life and only completely disappear one week after eclosion, many of the effects of lipid content/composition in young adults are likely to reflect changes in larval lipid metabolism, rather than genuine long-term effects of larval environment on the adult.

Although experimental procedures vary widely from study to study and, in consequence, findings are different and even contradicting, taken together published work indicate that environmental conditions/nutrient availability during development can have a long-term impact on the adult traits. This suggests that nutrition during early insect life may exert long-term programming effects on the adult, similarly to the effects reported in mammals. A small subset of studies have even attempted investigating the correlation between larval nutritional status and adult longevity, but findings to date have not been conclusive. The general belief is that nutritional environments that restrict larval diet and/or lengthen developmental time will have a detrimental impact on longevity. In one of the few studies that report a positive effect of altering larval nutrition on the adult, Chandrashekara et al. have reported that supplementing the larval diet with resveratrol or aleo vera decreases adult adiposity and extends lifespan in *Drosophila* (Chandrashekara and Shakarad, 2011). However, this study employed a short-lived *Drosophila* strain as starting point. Therefore, it is not clear whether the beneficial effects of supplementing the larval diet with resveratrol are also applicable to healthy, long-lived animals. Nonetheless, these findings offer further support to the idea that fruit flies can be used to model the long-term effects of nutrition during early life.

Perhaps in the most detailed set of experiments to date, Economos and Lints altered the larval growth rate, developmental times and body size by restricting the amount of yeast per larva (by varying the amount of yeast or the number of larvae per vial) or by rearing larvae at different temperatures (Economos and Lints, 1984a; 1984b). Interestingly, they found that the relationship between larval nutrition and lifespan is biphasic i.e. lifespan was maximal when each larva was proportioned 55-60 µg yeast/day, but decreased if yeast was either increased or decreased outside of this optimal range. This led the authors to propose that developmental rate cannot be the sole cause for the effects of larval environment on lifespan and, importantly, that the relationship between growth rate and lifespan is more complex than previously appreciated.

Two recent studies have used *Drosophila* to study the potential programming effects of parental diet upon offspring fitness. Valtonen et al. raised parents on either "poor" or standard diet and found that offspring of "poorly"-fed parents were larger than those of control-fed parents (Valtonen et al., 2012). Interestingly, this study showed that both maternal and paternal diets had an effect on offspring size and developmental time. In a complementary study, Buescher et al. fed adult female flies either on a high-sugar or on a control diet and compared the effects of this treatment upon F1 larval and adult offspring as well as F2 offspring (Buescher et al., 2013). The authors report a minor effect of maternal dietary sugar upon adiposity in F2 larvae. This is the first example of trans-generational metabolic programming in *Drosophila*. Moreover, Matzkin et al. fed larvae on a high-sugar diet and found that this alters the weight, protein and glycogen content of F1 larvae, compared to a low-sugar diet, even though offspring were raised on identical diets (Matzkin et al., 2013). However, this effect was only apparent in female offspring.

Interestingly, certain nutrients acquired during larval stages have been proposed to be "non-renewable" i.e. they are acquired by the larva and passed on to the adult but are not (entirely) replaced by adult-acquired nutrients (Min et al., 2006; O'Brien et al., 2004; 2002). In *Drosophila*, Min et al. (2006) have shown that one quarter of the sucrose-derived somatic carbon found in adult flies is of larval dietary provenance. The underlying mechanisms have not been characterised in detail but may include stage-specific differences in the ability to synthesise certain nutrients or absorb them from the diet. This may increase adults' dependence upon nutrient stores acquired during larval development.

1.6 A Drosophilist's toolbox

An important step forward for *Drosophila* geneticists was made with the development of the binary gene expression system GAL4/ upstream activating sequences (UAS) (Brand and Perrimon, 1993). In this system, the yeast transcriptional factor GAL4 is placed under the control of a regulatory sequence that will drive GAL4 expression in a tissue-specific manner, while the gene of interest is placed downstream of UAS. When the GAL4 and the UAS elements are brought together in the same animal, the DNA-binding domain of GAL4 will bind to UAS, while the GAL4 activation domain (GAD) will initiate transcription of the gene placed under the control of UAS. In this manner, the gene of interest in expressed in the same spatio-temporal pattern as the GAL4. GAL4 binding to the UAS can be prevented by introducing Gal80 in the system (Suster et al., 2004), which binds to GAD and prevents transcription of the gene downstream of UAS. Versatility is further increased by the

existence of temperature-sensitive versions of Gal80. Gal80 activity can, therefore, be switched on and off by a simple change in temperature.

Many of the GAL4 driver lines that are available in *Drosophila* were generated employing the enhancer trap technique, a widely used method for studying tissue-specific gene expression in flies. Classically, an enhancer trap P-element construct carries a reporter gene that is fused to a minimal promoter, which, on its own, is insufficient to drive expression of the reporter. The P-element is mobilised throughout the genome using a transposase source. The expression of the reporter will, therefore, reflect the activity of the local endogenous genomic enhancers of the region where it inserts. A targeted transposition technique, referred to as "transposon swap" has been described previously for transforming LacZ enhancer trap lines into GAL4 version (Sepp and Auld, 1999).

The availability of the whole-genome sequence of *Drosophila* has advanced functional genomic studies to identify new components of various biological programs as well as understand their function (Adams et al., 2000). Comparison with the human genome has revealed that two thirds of the genes that have been implicated in disease in humans have *Drosophila* counterparts (Chien et al., 2002). FlyAtlas is another valuable online resource that provides comprehensive gene expression patterns in multiple *Drosophila* tissues (Chintapalli et al., 2007), while Flybase is the primary database for integrated genetic and genomic data about Drosophilidae, in general, and *Drosophila melanogaster*, in particular (Drysdale FlyBase Consortium, 2008). Adding to the wealth of tools available in *Drosophila*, genome-wide RNA-mediated gene interference (RNAi) libraries were also developed and made available to the community (Dietzl et al., 2007). The RNAi transgenes are expressed using the binary GAL4/UAS system, thus enabling conditional inactivation of the target gene in a tissue-specific manner. The generation of such libraries has made unbiased, genome-wide RNAi screens possible. Of particular interest, a recent *Drosophila* genome-wide obesity screen (Pospisilik et al., 2010), resulted in nearly five hundred genes that seemed to be somewhat involved in lipid metabolism. Many of these genes had previously been implicated in fat storage and most of the hits were active in the adipose tissue as expected. A proportion of the genes, however, were active in neurons and muscle, which might point to functions in regulating eating behaviour and energy expenditure. Surprisingly, however, the results of the screen also revealed the Hedgehog signalling pathway, which is known to function as a developmental patterning pathway, as a key regulator of fat content.

1.7 Short introduction to fatty acids and their nomenclature

Lipids play important roles in biological systems. First, they are the form of long-term energy storage. Second, they play essential structural roles, as lipids are the major components of cell membranes. Third, lipids are involved in a number of cell signalling pathways, both as steroid hormones and as messenger molecules that convey signals from cell surface receptors to targets within the cell.

The simplest lipids are fatty acids, which are hydrocarbon chains that terminate with carboxylic acid groups. In biological systems, fatty acids usually contain an even number of carbons, typically between 8 and 24, with 16- and 18-carbon chains being the most common. The hydrocarbon chain is almost invariably unbranched in animal fats. Chain length and degree of saturation (i.e. number of double bonds) are important characteristics as they dictate the properties of fatty acids and the lipids derived from them. In cells, fatty acids are stored in lipid droplets in the form of tryacylgrlycerols (TAGs), which contain three fatty acids linked to a glycerol molecule.

The nomenclature of fatty acids can be confusing and has been standardised by the International Union of Pure and Applied Chemistry (IUPAC, 1997), the world authority on chemical nomenclature and terminology. There are four common naming systems:

(1) Trivial names (or common names) contain no clues to the structure of the molecule. These are historical names and typically derive from a common source where the compound is found and the source from which it was first isolated. These are also the names that are most commonly used in nutritional literature and the media.
(2) Systematic names (or IUPAC names) are strictly regulated by IUPAC and offer a detailed description of the structure, if one knows the convention. However, they tend to be long and difficult to decipher for the non-specialist.
(3) Lipid numbers (or the carboxyl-reference system) the number of carbons (i.e. chain length), the number of double bonds and, sometimes, even the position of the double bonds. Lipid numbers take the form Cx:y, where x is the number of carbon atoms in the fatty acid and y is the number of double bonds (if any). The notation C18 denotes an 18-carbon fatty acid with no double bonds, whereas C18:2 signifies that there are two double bonds.
(4) The omega-reference system indicates the number of carbons and number of double bonds, as well as the position of the double bond closest to the omega

carbon. In this system, the omega carbon is numbered 1. This system is useful in some physiological contexts, because of the differences between omega-3 and omega-6 fatty acids and the fact that the human body cannot interconvert them.

Throughout this document, I refer to fatty acids using their lipid number.

1.8 Scope and aims of this work: A *Drosophila* model of nutritional programming

The long-term programming effects of nutrition during the early life of an animal have been extensively described in humans and mammalian model organisms (Symonds et al., 2009). However, the mechanisms underlying "nutritional programming" have not yet been completely elucidated. In this thesis, I use *Drosophila melanogaster* to establish a genetically-tractable model of the long-term programming effects of nutrition during development upon adult physiology and metabolism.

More specifically, the work presented here aims to:

- investigate the proximal effects of larval dietary [yeast] upon duration of development, larval growth and sizes of internal organs, and larval lipid metabolism (Chapter 3);
- characterise the long-term effects of larval dietary [yeast] upon adult body size and organ scaling (Chapter 4), aspects of adult physiology and lipid metabolism (Chapter 5) and adult longevity (Chapter 6);
- ultimately, use this newly-established model of nutritional programming to set the bases for exploring a potential mechanism of the long-term programming effects of larval diet (in Chapter 7, I investigate the potential implication of a transferable component).

Chapter 2

Materials and Methods

2 Materials and Methods

2.1 Fly strains and husbandry

In order to minimise the differences given by genetic background heterogeneity, for most dietary manipulations experiments I used an inbred, "isogenic" *Drosophila* strain, which was kindly provided to us by John Roote from the University of Cambridge (Ryder et al., 2004). In this document I refer to this strain as w^{1118}iso. For the transposon swap (see Appendix), the following lines were used: nab ^{NP4604}-GAL4 (Maurange et al., 2008), Cyp4g1-GAL4 (Einat Cinnamon and Alex P Gould, unpublished). The midgut-driver NP1-GAL4, Δ2-3 transposase stocks, UAS-rpr and balancer stocks were obtained from the *Drosophila* Genetic Resource Centre, Kyoto. lexAop-mCD8::GFP, LG124/CyO,y^+ and LG7-3 were kindly provided by Tzumin Lee (Lai and Lee, 2006). GAL4 driver lines used in this study are: Lpp-GAL4, tub-$G80^{ts}$ (Brankatschk and Eaton, 2010); byn-GAL4, tub-$G80^{ts}$ (Takashima et al., 2008); dilp2-GAL4 (Rulifson et al., 2002). UAS lines used in this study are: UAS-FOXO (BL9575; Puig et al., 2003), UAS-$dp110^{DN}$ (Leevers et al., 1996), UAS-p60 (Weinkove et al., 1999), UAS-PTEN (Huang et al., 1999), UAS-TSC1+2 (Tapon et al., 2001), UAS-Rheb (BL9689), UAS-myr::Akt (BL5075), UAS-bmm (Gronke et al., 2005; SGF 532), UAS-Lsd2 (Gronke et al., 2003), UAS-LpR1 RNAi (TRiP line; BL27249), UAS-LpR2 RNAi (Vienna RNAi Center; KK line v107597), UAS-$S6K^{STDE}$ (BL6913). Other lines used in this study: tub-Gal80ts on 2nd and 3rd chromosomes, and balancer chromosome stocks were obtained from the Bloomington Stock Centre. Information on most of the above lines is available online from Flybase (www.flybase.org). All strains were acclimated to our laboratory food prior to initiation of the experiments. Unless otherwise specified, fly stocks were maintained on standard laboratory food (see 2.3 Dietary manipulations) at constant temperature.

2.2 Making new tissue-specific LexA/lexAop tools to complement the existing GAL4/UAS

Molecular biology. A 2.7 kb fragment of the Cg promoter and an 859 base-pair fragment of the dilp2 promoter were amplified by PCR from BACR07E07 and BACR07A05, respectively. Cloned Pfu high-fidelity DNA polymerase (Stratagene, La Jolla, CA) was used.

The following PCR primers were used (note that CACC was added to the 5' end of forward primers, which is a requirement for directional TOPO™ cloning into the Gateway entry vectors):

Dilp2 fw: CACCAACACACACACATTCATTACCCAG

Dil2 rev: TGGTTATGGGTTTACTGCTTAGGTTG

Cg fw: CACCTTCCTTCGCCCGCACACTCG

Cg rev: ATGCCCTATGCACTTAAGCCTGC

PCR products were directionally cloned into the Gateway® pENTR™ D-TOPO® entry vector (Invitrogen) following the manufacturer's instructions. The inserts were then cloned into LexA::GAD Gateway® destination vector (Diegelmann et al., 2008) using Gateway® LR Clonase™ II Enzyme Mix (Invitrogen), following the manufacturer's instructions.

$Dp110^{DN}$ cDNA was excised from pSL63 (Leevers et al., 1996) by double digestion with KpnI and XbaI at 37^{0} C for 1 hour. The 3.3 kb fragment obtained in this way was then cloned into the Gatweway®-compatible pENTR™ 1A Dual Selection Vector (Invitrogen), which had previously also been digested with KpnI and XbaI. $Dp110^{DN}$ cDNA was then cloned into lexAop Gateway® destination vector (Diegelmann et al., 2008), using Gateway® LR Clonase™ II Enzyme Mix (Invitrogen), following the manufacturer's instructions.

A 3 kb fragment of the *svp* promoter that contains an oenocyte enchancer was excised from *svp*-GAL80 (Gutierrez et al., 2007) by digesting with EcoRI. mouse S6 cDNA was excised from pKH3-mouse S6 (Roux et al., 2007) using EcoRI and SalI. *Drosophila* 4EBP cDNA, $dp110^{WT}$ cDNA, S6 cDNA and RagA cDNA were excised from HL08053, SD05105, LD31286 and GH04846, respectively (DGRC GOLD cDNA clones) with EcoRI and XhoI. The cDNAs obtained in this way were then cloned into EcoRI-, XhoI-digested Gatweway®-compatible pENTR™ 1A Dual Selection Vectors. The *svp* oenocyte-enhancer was further cloned into LexA::GAD Gateway® destination vector (Diegelmann et al., 2008) using Gateway® LR Clonase™ II Enzyme Mix (Invitrogen), following the manufacturer's instructions. Mouse-S6 cDNA and *Drosophila* 4EBP cDNA, $dp110^{WT}$ cDNA, S6 cDNA and RagA cDNA were further cloned into lexAop Gateway® destination vector (Diegelmann et al., 2008), using Gateway® LR Clonase™ II Enzyme Mix (Invitrogen), following the manufacturer's instructions. The sequences of all expression vectors were verified and the plasmids were sent to Bestgene, Inc. for injection into embryos and generation of stable transformants.

Transposon swap. A modified transposon swap technique was used in an attempt to convert GAL4 enhancer trap lines driving expression in the midgut (MyoIA-GAL4, also known as NP1-GAL4, DGRC, Kyoto), oenocytes (Einat Cinnamon and Alex P Gould, unpublished) and postembryonic CNS (nab^{NP4604}-GAL4, Maurange et al., 2008) into LexA driver lines. This technique was first described by Sepp and Auld who used it to convert LacZ into GAL4 lines (Sepp and Auld, 1999). For this, first flies were generated that contained both the GAL4 enhancer trap P-element and the LexA donor P-element. A transposase source was then introduced, in order to excise the GAL4 P-element and leave a double-stranded break. The cells' repair machinery normally tries to repair such breaks by using the homologous chromosome as a template. However, it will not be able to do that if the homologous chromosome is a balancer chromosome, so, in theory at least, it will search on the other chromosomes for the area with the highest homology and repair the break based using this as a template. In this way, rare events where the GAL4 is excised by the transposase and the break is repaired by copy-pasting the lexA P-element present in the genome can occur in the germline of animals carrying both, the GAL4 and LexA P-elements as well as an external source of transposase. These events would then result in progeny in which GAL4 has been substituted for LexA. All crosses are carried at 25^{0} C as it has been observed that transposases have optimal activity at this temperature (Lankenau et al., 1996). In total, more than 700 potential transformants were screened for each line but no successful transposition event was identified. The estimates from Sepp and Auld (1999) indicate that many thousands of potential transformants need to be screened before finding a genuine transposition event. As the project progressed and priorities changed, the generation of LexA/lexAop lines was stopped prematurely.

2.3 Dietary manipulations

Our standard laboratory food consists of 7.02 g agar, 58.5 g glucose, 66.3 cornmeal and 23.4 g autolysed yeast extract for every litre of final medium. In addition, this diet also contains the antifungals bavistan and methylparaben dissolved in 1% (v/v) ethanol. This baseline recipe is our standard laboratory fly food and is referred to as 2%-yeast fly food, throughout this document. This work has involved fly foods with autolysed yeast extract concentrations of 0.0117%, 0.117%, 0.234%, 0.585%, 1.17%, 2.34%, 4.68%, 9.36%, 14.04% and 18.72% yeast, all other ingredients staying the same. Throughout this thesis, these foods are referred to by the % of yeast they contain by the rounding up/down to the closest whole number. Glucose content of some foods was also altered. High glucose is denoted by the suffix "HG" added after the % of yeast in the diet and refers to food containing 40% (w/v) glucose, compared to the "standard" recipe which contains only

5.85% (w/v) glucose. Low glucose (LG) foods contained half the "standard" concentration of glucose i.e. 2.925% (w/v). Where glucose content of the food has been altered this is clearly indicated in the text. Fly food was aliquoted into wide polystyrene vials, i.e. 28.5 mm (O.D.) x 95mm (H), or 250 mL polyethylene bottles and allowed to set overnight. Vials/bottles were then stored at 4^0 C and used within 3 weeks (max. 2 weeks for lifespan experiments). Food bottles and/or vials were allowed to reach room temperature before being used. Vials were stoppered with Flugs® (cellulose acetate plugs for *Drosophila* vials; Deutscher Cat. No. 789035) and bottles with Droso-Plugs™ (Deutscher Cat. No. 789190).

During the early stages of this work, all dietary manipulations groups were set up in parallel, using embryos from the same collection. This would ensure that the populations of larvae/embryos used to initiate the experiment was identical. However, due to the developmental delay induced by some of the diets employed in this work, this meant that the adults eclosed on different days. Some of the most severe delays can be as long as 9 days. For lifespan experiments in particular, it is crucial to ensure that all adults are subjected to the same conditions and are fed the same batch of food. For this reason, for most of this work the different dietary manipulation groups were set up on different days (but always with embryos collected from the same parents) in order to obtain adults that eclosed within a narrow time window.

All food supplements employed in this work (such as: rapamycin, antibiotics, Orlistat, bromophenol blue etc) were added to the food while it was cooling down, when it reached ~60^0 C, and were thoroughly mixed in before the medium was aliquoted into vials. Orlistat treatment (Sigma-Aldrich) was implemented by rearing adult flies on food supplemented with 2 μM Orlistat in DMSO or vehicle only for a minimum of 7 days, as previously described (Sieber and Thummel, 2012). Rapamycin (LC Laboratories) treatment was implemented by supplementing the food with 200 μM rapamycin in ethanol or vehicle only (2.5% ethanol). Antibiotics were dissolved in ddH_2O. Bromophenol Blue powder was directly mixed into melted food.

2.4 Culturing larvae at a constant density

In order to standardise conditions during larval development, larvae were cultured at constant larval densities. For this, embryos were collected on grapejuice agar plates (25% red grape juice, 1.25% sucrose, 2.5% agar) supplemented with live yeast paste (known to stimulate egg laying in *Drosophila*) from embryo collection cages. Cages were set up with young adults (i.e. less than one week old) and maintained at 25^0 C by supplying fresh

grapejuice agar plates supplemented with live yeast paste twice per day. Adult flies were allowed to acclimatize to the "cage environment" for 3 days prior to the first embryo collection. During the early stages of this work, newly-hatched L1 larvae were collected, manually counted into groups of 50 and placed in vials. For this, embryos were collected over 3 hr periods and the plates aged for a further 18 hrs at 25^0 C. At this point, the larvae that had already hatched were removed from the plate together with the yeast paste and fresh yeast paste was supplied. Three hours later, the larvae that had hatched during this period were collected, thoroughly rinsed with PBS, counted into groups of 50 and each group gently placed in a vial with food with the aid of a soft, wet paintbrush. Larvae were handled using blunt forceps and were placed into small PBS droplets in groups of 50 in order to keep the group together while counting the rest. As a general rule, larvae were not kept in PBS for longer than 30 minutes.

The procedure described above, however, is laborious and time consuming, and, in consequence, severely limits the size of the population that one can start with. For many of the experiments described in this thesis (including lifespan experiments and lipid extractions), large numbers of adults were essential. The following further increased the number of animals one had to start with: (1) only males were used, (2) viability was reduced on certain larval diets, (3) several adult diets were analysed in parallel and (4), depending on the adult diet and the age of the animals at the time of the analysis, a proportion of the starting cohort will have already died by the time the samples were collected. For all these reasons, for most of the experiments presented here, larval density was controlled by pipetting a fixed volume of synchronised embryos onto food (Clancy and Kennington, 2001). This also eliminated the need to directly handle larvae, which Annick Sawala in the lab has recently observed can delay larval development. As females may retain fertilised embryos (Boulétreau-Merle and Terrier, 1986), a tighter synchronisation of the embryos was achieved by changing the plate at least 2-3 times before the "actual" embryo collection. Embryos were then collected over short time windows (1-1.5 hrs) during the afternoon when egg laying peaks. In order to transfer the embryos onto food, the live yeast paste was removed and the plates were washed thoroughly with PBS. The embryos were then gently detached from the plate using a clean, soft paintbrush, the suspension poured into a 15 mL Falcon tube and the embryos allowed to settle at the bottom. Most of the supernatant was then removed and the embryos were transferred into a 1.7 mL Eppendorf tube (using a 1 mL pipette tip) and aliquoted onto food. For bottles, 19 µL of embryos were pipetted, while for vials 50 embryos were counted on a clean grapejuice agar plate under a dissection microscope and carefully

transferred into the vial with a wet paintbrush. The optimal volume of embryos to pipet in a bottle was determined empirically by pipetting a range of volumes in bottles containing 2%-yeast medium. The highest volume at which larval development was not delayed nor desynchronised and the bottles looked uncrowded was chosen. Pipette tips were cut to increased the size of the tip opening (commercial wide-bore pipette tips can also be used to the same effect) and rinsed with 0.5% Triton X-100 in PBS in order to prevent the embryos from sticking to the plastic. (N.B.: The critical step in order to ensure that a consistent volume of embryos is pipetted every time is when the embryos are taken up into the pipette tip: at this step, the embryos have to be sucked up into the tip as quickly as possible i.e. by abruptly releasing the plunger.)

2.5 Determining critical weight and the time to attain it

In order to determine mass at and time to critical weight (CW) empirically, larvae were cultured in vials at 25°C and at a controlled density (see 2.4). Synchronised, newly-hatched L1 larvae were placed into vials containing food with varying [yeast]. 10 vials or more were set up for each group. Preliminary experiments determined the approximate time course to L3 on foods with different concentrations of [yeast], by floating larvae in 30% glycerol in PBS at different time points after larval hatching and staging them based on anterior spiracle morphology. Starting with mid-late L2, larvae were removed from food at different time points, weighed and placed on starvation medium (i.e. 1% agar in PBS). The time it took larvae starved starting from different time points as well as fully-fed larvae to reach WPP (white pre-pupa) stage was monitored. Critical weight was determined retrospectively based on this information. Larvae were considered to have reached CW by the beginning of the starvation period if they pupariated at the same time as (or slightly earlier than) controls that had been returned to food. If starvation caused a delay in the time to WPP, but larvae did pupariate, this indicated they had achieved minimum viable weight (MVW) before the onset of starvation.

2.6 Time to pupariation

Time to pupariation on different media was determined for synchronised larvae cultured in bottles at 25°C and at a controlled density (see 2.4). For this, pupae present in the bottle were marked in permanent pen on the bottle wall. 10 bottles were set up in parallel for each group. New pupae were counted twice-daily until no more new pupae appeared for 36 hours. For data visualisation, the total number of animals that pupated in each

bottle was set as 100% and the number of animals that had pupariated by a certain time point was plotted as a percentage of the total number of final pupae.

2.7 Wet and dry body weights of larvae and adults

Pre-wandering L3 larvae (i.e. larvae that were still in the food) were floated in 30% glycerol in PBS. Wandering L3 larvae (a distinct developmental stage that is characterised by the fact that larvae leave the food and wander on the walls of the container for 10-12 hours before settling for a dry spot to pupariate) were picked carefully from the walls of the vials/bottles with a pair of blunt forceps. Larvae were washed extensively in PBS and dried on clean Kimwipe tissues. Adult flies were lightly anaesthetised under CO_2 and weighed while still under the effect of the CO_2. Animals were transferred one by one into a pre-weighed weighing boat. If to be further used for lipid analysis by gas chromatography, pre-weighed 2 mL safe-lock Eppendorf tubes were used instead of weighing boats and, following weighing, the samples were frozen immediately on dry ice and stored at -80^0 C.

For estimating the water content of adult males, triplicate groups of 10 flies were anaesthetised under CO_2 and weighed while still under the effect of the CO_2. The samples were then desiccated at 65^0 C for three days or until their weight stabilised and re-weighed. The difference in mass between the wet weights and the corresponding dry weights was considered to be the water content of the samples.

2.8 Measurement of organ sizes and WPP volume

Tissues from animals of the specified age or developmental stage were dissected, fixed, permeabilised with 0.3% Triton X-100 in PBS and the nuclei stained with DAPI as described in section 2.10. Following the last wash, PBT was removed and the tissues incubated in VECTASHIELD® (Vector Laboratories, Petersborough, United Kingdom) for 30 minutes. The tissues were then mounted in a standardised manner in a constant volume of Vectashield with minimal squashing i.e. a constant distance between the coverslip and the slide was ensured by resting the coverslip onto two strips of Sellotape® that had previously been taped on the slide (care was taken to avoid air bubbles being trapped under the Sellotape®). 1.5 µm-spaced confocal z-stacks of whole organs were acquired with a Leica SP5 Confocal microscope. Tissue volume measurements were obtained from 3D reconstructions using ImageJ 1.40g (National Institutes of Health, USA), with the ImageJ 3D Viewer plug-in (Schmid et al., 2010), in the “surface display mode” at a constant, pre-defined threshold. Diameters of salivary gland nuclei, fat body cells nuclei, Malpighian tubules nuclei, podocytes and the cell bodies of insulin producing cells were

calculated from the average of two orthogonal measurements using Leica SP5 confocal images and the LAS AF software. For the salivary glands measurements, due to proximal-distal differences, only 1-2 nuclei that occupied similar positions in the distal third of the organ were measured per salivary gland. Volumes were estimated using the formula $^4/_3 \pi r^3$, where r= ½ diameter.

WPP volumes were estimated using the formula $\pi r^2 l$. White prepupal length (l) and diameter (2r) were calculated in μm using ImageJ1.40g and calibration of the original brightfield images, captured with a LeicaMZ16F microscope and Leica DC500 camera, using a stage graticule.

2.9 Lipid analyses

Adults were collected at the specified age under light CO_2 anaesthesia, transferred into pre-tared 2 mL safe-lock Eppendorf tubes and weighed, while still under the effect of the anaesthesia. The samples were then placed on dry ice and stored at -80^o C until lipid extraction. On average, samples comprised of 10-15 males from 2% yeast-fed larvae and 20-30 males from 0.01% yeast-fed larvae. Larvae were washed thoroughly with PBS, dried on a Kimwipe tissue and weighed. The animals were then transferred into 2 mL safe-lock Eppendorfs, placed on dry ice and stored at -80^o C until lipid extraction.

On the day of the extraction, samples were removed from -80^{o}C on dry ice and homogenised in groups of ten. The time it takes from the moment samples are thawed (when lipids are prone to oxidation and enzymes reactivate) to when they are in methanol:chloroform (which precipitates proteins and prevents lipid oxidation) is critical, so samples should be kept on dry ice until they are needed and be dealt with quickly once transferred to ice. At least two blanks containing no flies were introduced for every fifty samples and processed in parallel with the rest of the samples. Lipids were extracted following the method of Folch et al. (Folch et al., 1957). In short, 500 μL of 0.9% saline and a ball-bearing (Retsch, ϕ6 mm) were added to each sample, prior to homogenisation in the ball-mill homogeniser (Retsch, MM301) for 2 minutes at 30 Hz. The homogenate was then transferred to a 10 mL glass extraction tube (International Scientific Supplies), the ball-bearing was removed and the tube was further rinsed with 250 μL of 0.9% saline. 2:1 chloroform:methanol (v/v, 7.5 mL) containing butyrated hydroxytoluene (100 μg/mL) was added, the preparation shaken vigorously for 2 minutes and mixed on a rotary mixer for a further 15 minutes at room temperature. 1M NaCl (1.5 mL) was added, and the organic and aqueous phases were separated by centrifugation at 2000 rpm and 14OC for

10 minutes. The top aqueous phase was discarded, while the bottom solvent phase was transferred into a fresh LP4 tube (Fischer Brand, 12x75 mm) and evaporated to dryness under nitrogen at 50^{0}C.

For lipid analysis by gas chromatography, the lipid extract was further separated into fractions i.e. non-esterified fatty acids (NEFA), phospholipids (PL), cholesterol esters (CE) and triacylglycerols (TAGs). The isolation of individual lipid classes was done based on the method by Burdge et al., with modifications based on Choi et al. (Burdge et al., 2000). Briefly, total lipid extracts were dissolved in chloroform (1 mL) and applied to an aminopropyl silica column (Biotage; 100 mg packed silica per 1 mL cartridge), previously pre-conditioned with acetone (2x1 mL) and chloroform (2x1 mL), under gravity. CE and TAG fractions were eluted together with chloroform (2x1 mL) under vacuum. The fraction was dried under nitrogen at 50^{0} C. In the second stage, TAG was further separated from CE. A fresh aminopropyl silica column (Biotage) was preconditioned with hexane (4x1 mL) under vacuum. The TAG/CE extract from stage one was resuspended in chloroform (1 mL) and applied to the column under gravity. The TAG fraction was eluted with 100:5:5 hexane:chloroform:ethyl acetate (v/v/v, 2x1 mL) under vaccum and dried under nitrogen. Fatty acid methyl esters (FAMEs) were then prepared by incubation with acidified methanol. The TAG fraction isolated by solid phase extraction (SPE) were resuspended in toluene (400 µL). Methanol containing 1.5% H_2SO_4 (800 µL) was added, mixed briefly and incubated at 80^{0} C for one hours. The reaction mixture was cooled and neutralised with a solution (2 mL) containing $KHCO_3$ (0.125 M) and K_2CO_3 (0.125 M). In order to isolate the FAMEs cyclohexane (2 mL) was added, the tubes were mixed at room temperature for 15 minutes, and the aqueous and organic phases were separated by centrifugation at 2000 rpm for 10 minutes at 14^{0} C. The upper hexane layer was transferred into a fresh LP4 tube and dried under nitrogen at 50^{0} C. Fatty acid composition was determined using an Agilent 5890 GC (Agilent Technologies UK) equipped with a 30 m Innowax capillary column with 0.53 mm ID and 1 µm film thickness (Thames Restek, Saunderton, UK). Fatty acids were identified using both the retention time and fragmentation pattern with reference to lipid standards (SIGMA Aldrich, Gillingham, UK) and FAME mixtures of known composition (Thames Restek) were used as quality control material. Introduction of C15 as an internal standard and C23 as an external standard permitted quantification.

Decreasing the number of larvae per sample (from 30 to 7) resulted in the loss of low abundance fatty acids (C14:1, C17, C17:1, C18:3 and C20). Estimates of adiposity levels, however, are unlikely to be affected by this as all these fatty acids together account for

only 1-2% and the differences I report here are orders of magnitude larger than the potential error introduced by leaving out these FA species.

Initial analyses of fly fat content depended on a GC collaboration with Barbara Fielding and Leanne Hodson at the Oxford Centre for Diabetes, Endocrinology and Metabolism. However, initial attempts were plagued by high variability between biological replicates and limited reproducibility. Because of the large number of samples that this project generated, we then acquired our own GC-MS set up at NIMR. This required a period of training, technical troubleshooting and adapting the lipid extraction protocol. With regards to technical variability within experiments, together with Panayotis Pachnis and TJ Ragan, we learnt that: (1) re-extracting the leftover aqueous phase with authentic lower phase improved the recovery of several fatty acids and (2) variability decreases if the samples are kept on dry ice, rather than on ice, until the moment of homogenisation. In addition and, perhaps, not surprisingly, practice also lowered variability. As for biological variability, the three main factors appeared to be: food homogeneity, larval density and incubator humidity. We therefore controlled all three parameters for GC measurements.

2.10 Immunohistochemistry

Animals were dissected on clean depression slides at room temperature in 1x phosphate buffered saline (PBS; Gibco, Cat. No. 70011-036) and fixed in 4% w/v methanol-free formaldehyde (TAAB, Cat. No. F017) in PBS for 20 minutes at room temperature. For Lipidtox-only stainings, tissues were rinsed and washed for 10 minutes in PBS a minimum of three times and then incubated in 1:500 Deep Red Lipidtox (Invitrogen) in PBS at 4^{0}C overnight. Lipidtox was then removed and the tissues were incubated in Vectashield with DAPI (Vectorlabs) for a minimum of 30 minutes before mounting (to allow DAPI to penetrate). As per manufacturer's instructions, no washing steps are required after staining with Lipidtox, (which improved the intensity, reproducibility and homogeneity of the stainings).

If performing antibody, Phalloidin and/or DAPI stainings, following fixing, the tissues were thoroughly rinsed and washed with PBS to remove the fix, before being permeabilised and blocked for one hour at room temperature with PBS supplemented with 0.3% Triton and Normal Goat Serum. Primary antibodies were diluted in block and incubated overnight at 4^{0}C. Primaries were removed by extensively rinsing and washing (i.e. a minimum of five rinses and 10-minute washes) with 0.3% Triton in PBS. Alexa Fluor® secondary antibodies were diluted 1:500 in block and the tissues incubated in a

dark, humidity box for 2 h at room temperature. Following removal of the secondaries, tissues were rinsed and washed with 0.3% Triton in PBS for a minimum of three times and either directly mounted in Vectashield or the Triton removed by further rinsing and washing in PBS for a minimum of four times before proceeding with the Lipidtox staining- which does not work in the presence of the detergents Triton or Tween- as described above.

Rabbit anti-ACC primary antibody (Cell Signalling) was used in a concentration of 1:200 Anti-polyubiquitinated complexes primary antibody was used as described elsewhere (Demontis and Perrimon, 2010). Primary antibodies were detected with AlexaFluor®488-conjugated secondary antibodies, used at a concentration of 1:500 (Molecular Probes). Alexa Fluor®-conjugated phalloidin (Molecular Probes®) was used at a concentration of 1:100. DAPI was used at a concentration of 1:1000. Both DAPI and Phalloidin were incubated together with the secondary antibody. Antibody dilutions, DAPI and Phalloidin were prepared fresh every time. Images were acquired with a Leica SP5 confocal microscope and most images shown are projections of multiple sections. If needed, whole-image manipulations were performed in Adobe Photoshop CS5.1.

2.11 Lifespan analyses

Most experiments on wildtype animals employed the w^{1118}iso strain, which is *Wolbachia* positive (see 2.15). For lifespan experiments, embryos were collected on grapejuice agar plates supplemented with fresh yeast paste and larvae were reared in bottles at a constant density, as described in 2.4. All adults were transferred into fresh bottles containing standard 2%-yeast fly food within 24 hours of eclosion (without anaesthesia), where they were allowed to mate for a further 48 hours. In the case of flies eclosing from 0.01%-yeast bottles, the first flies to emerge were discarded and newly-eclosed adults were then collected over 24-hour periods for the next 3-4 days. The day of eclosion was marked as day 0 of adult life. After 48 hours, males and females were separated under light CO_2 anaesthesia and randomly allocated into vials at a starting density of 25 flies per vial. Flies were allowed to recover from anaesthesia in fresh, empty 50 mL Falcon tubes and were only transferred to food once awake, in order to avoid their sticking to the food. Flies were transferred into fresh vials twice weekly and deaths were scored every day to every 3 days, depending on the experiment. Adult food was freshly prepared every week or every two weeks and kept at 4^{0}C until needed. Survival data was recorded and plotted as survivorship curves using Microsoft® Excel® 2011 and statistical analysis was performed

using OASIS (Yang et al., 2011). Statistical significance between survivorship curves was assessed using the non-parametric Log-rank test.

2.12 Starvation resistance

Adults of the indicated ages were counted under brief CO_2 anaesthesia and placed into vials containing freshly prepared 1% agar in PBS in order to subject them to severe nutrient deprivation, while preventing death from dehydration. Deaths were scored several times per day until the last fly had died. Dead animals were not removed from the vial during the course of the experiments. Data were plotted as survival curves.

2.13 Collection of adult faecal material and sample preparation for analysis by ^{1}H NMR

Fly food was supplemented with 2.5% (w/v) Bromophenol Blue (BPB; Sigma-Aldrich, Cat. No. B0126), a pH-sensitive dye that cannot be absorbed nor metabolised in the fly gut (Cognigni et al., 2011) For this, fly food was gently melted in a microwave set to low power and stirred often. A set volume was then aliquoted into a beaker and the corresponding amount BPB mixed in with a metal spatula. Special care was taken to ensure that the mixture of fly food and BPB is homogenous. The food was then poured into fresh vials and allowed to set. Flies were transferred onto BPB-supplemented food 24 hrs prior to the collection of the faeces. For faeces collection, adult males that had fed on BPB-supplemented food were subjected to brief CO_2 anaesthesia (<1 min) and transferred into 10 mL clean, empty glass vials (no more than 35 flies per vial). The vials were loosely fitted with organic solvent-resistant caps and placed horizontally in an incubator set to 25℃, inside a humidity chamber to prevent dehydration. Faeces were collected over periods of no longer than 2 hours to prevent starvation and to minimise possible metabolite degradation. Following collection, adults were transferred back to food that did not contain BPB without anaesthesia. Faeces were dissolved by adding 1 mL of ddH_2O and vortexing well. Polar metabolites in the faeces were extracted using a chloroform:methanol extraction procedure based on a standard protocol for separating lipids in a solution (Bligh and Dyer, 1959). Briefly, 3.75 mL 1:2 (v/v) $CHCl_3$:MeOH were added to the vial containing the dissolved sample, followed by 1.25 mL $CHCl_3$ and then 1.25 mL ddH_2O. Samples were vortexed after each addition. Samples were then spun at 1000 rpm for 5 minutes to promote phase separation. ~90% of the aqueous (i.e. top) phase was transferred into a fresh 2 mL Eppendorf tube. In order to recover the remaining ~10%, authentic upper phase was added, vortexed, spun and ~90% of the aqueous layer

was again removed and transferred to a fresh 2 mL Eppendorf tube. This step was repeated once more. In this way, ~99.9% of the starting material should be recovered. The extracts were evaporated under vacuum overnight. All vortexing steps were done for 1 minute, which was shown to increase the quality of the extract (TJ Ragan, personal communication). NMR spectra were acquired at 298 K with a Bruker Avance III spectrometer (operating at a nominal 1H frequency of 700 MHz). The amount of faecal material collected per sample was accounted for in data analysis by normalising to the amount of BPB in each sample.

2.14 Sample collection and preparation of DNA extracts for 16S metagenomic sequencing of the gut microbiota

Dissection utensils and slides were sterilised in 10% sodium hypochlorite, thoroughly rinsed with sterile ddH_2O and dried between samples. Adult flies were anaesthesised on ice, rinsed with PBT (0.5% Triton X-100 in PBS) to facilitate their sinking in solutions, surface-sterilised in 10% sodium hypochlorite for 1 minute and thoroughly washed with sterile ddH_2O. Since this treatment does not kill the animals, surface-sterilised flies were returned on ice. Guts (i.e. whole midgut and hindgut, from proventriculus to rectum excluding the Malpighian tubules and the crop) were dissected on a depression slide in sterile *Drosophila* Ringer's solution and accumulated on ice in 160 µL of sterile lysis buffer (minus lysozyme) in a 2mL Dounce homogeniser. Samples comprised of 50 guts (for 2% flies) or 100 guts (for 0.01% flies). DNA was extracted using the DNeasy Blood and Tissue Kit (QIAGEN), following a protocol modified from the manufacturer's instructions for extracting DNA from Gram-positive bacteria and from (Wong et al., 2011a). In summary, once all guts were dissected, tissues were homogenised in the Dounce homogeniser, using first pestle A and then pestle B. The foam was allowed to clear by resting the homogenate for 2 minutes on ice. In the meantime, a solution containing 180 mg/mL egg lysozyme (Sigma Aldrich) in lysis buffer was prepared. The homogenate was transferred into ribolyser-compatible 2 mL tubes containing 0.1 mm glass beads (Scientific Industries) using a sterile glass Pasteur pipette. Lysozyme was then added to a final concentration of 20 mg/mL by adding 20 µL of the 180 mg/mL egg lysozyme in lysis buffer. Samples were spun for 1 min at 13,000 rpm to remove the foam and pulse-vortexed to resuspend. Homogenates were then incubated at 37^0 C, without shaking, for 1.5 hr with 2 x 45 seconds of bead-beating using the 0.1 mm glass beads in a cell disruptor (FastPrep 24 ribolyser, MP Biomedicals) after 45 minutes. Following bead-beating, samples were spun for 1 minute to remove foam, vortexed to resuspend and returned at 37^0 C for the rest of the incubation. Following incubation at 37^0 C, 25 µL of Proteinase K solution and 200 µL

of Buffer AL (both provided in the QIAGEN kit) were added and the samples were incubated at 56^{0} C with shaking at 1,000 rpm for 30 minutes. Following this, the glass beads were allowed to settle at the bottom and the supernatant was transferred into a fresh 1.7 mL Eppendorf tube to which 200 µL ethanol were added and thoroughly mixed. At this stage, samples were stored at -20^{0} C. Once all samples were processed in this way, samples were allowed to reach room temperature and DNA extraction was carried out as per manufacturer's instructions starting with step 4 of the QIAGEN DNeasy Blood & Tissue protocol "Purification of total DNA from Animal Tissues (Spin-Column Protocol)". The total amount of DNA in each sample was quantified using a Nanodrop 1000 and preliminary PCR tests confirmed that samples contained sufficient bacterial DNA for 16S metagenomic analysis (data not shown). DNA extracts were sent to IMGM Laboratories GmbH (Martinsried, Germany) for metagenomic analysis using the Roche 454 Junior Next Generation Sequencing platform. Variable regions V1-V3 were amplified using the universal primer pair 8F and 534R (Baker et al., 2003). *In silico* analyses revealed that this primer pair does not amplify *Drosophila* genomic or mitochondrial DNA.

2.15 Testing for *Wolbachia*

Wolbachia infection status was tested as previously described (Teixeira et al., 2008). In summary, a portion of the *Wolbachia surface protein* (*wsp*) gene was amplified using primers described elsewhere: *wsp* 81F (5′-TGGTCCAATAAGTGATGAAGAAAC-3′) and *wsp* 691R (5′-AAAAATTAAACGCTACTCCA-3′) (Zhou et al., 1998). To extract DNA, 20 adult flies were crushed in 150 µL of 25 mM NaCl, 10 mM Tris-HCl pH=8.0, 1 mM EDTA, 200 µg/ml proteinase K and incubated at 37^{0} C for 30 minutes. Proteinase K was inactivated by incubating at 95^{0} C for 5 minutes. 2 µL of the supernatant were mixed with 5 µL of ddH_2O and directly used as a PCR template. Final PCR mix contained 10 µL 2x PCR MaterMix and 1.5 µL of each 10 µM forward and reverse primers, 2 µL of the supernatant and 5 µL ddH_2O per reaction. PCR conditions were: 94^{0} C for 1 minute, 30 cycles of 94^{0} C for 1 minute, 55^{0} C for 1 minute and 72^{0} C for 1 minute, followed by 72^{0} C for 10 minutes. PCR products were separated on a 1% agarose gel containing ethidium bromide and visualized under UV light. Fly stocks were cleared of *Wolbachia* by rearing the flies on food supplemented with 50 µg/mL tetracycline for four generations. The absence of *Wolbachia* was confirmed by PCR.

2.16 Statistical analyses

Data were analysed for statistical significance using GraphPad Prism 6.0. The exact statistical test employed and the resulting p values are specified in the main body of this thesis where appropriate. All error bars represent ± 1 standard deviation, unless otherwise specified. Statistical significance is reported as: n.s. for $p>0.05$, * for $p\leq 0.05$, ** for $p\leq 0.01$, *** for $p\leq 0.001$ and **** for $p \leq 0.0001$. Statistical tests performed were: parametric Student's t-test (Figures 3.3.C, 3.6.A for podocytes and PG, 3.8, 3.9, 3.10, 4.1.B-C and 4.4.A-B), ordinary one-way ANOVA followed by Dunnett's multiple comparisons test (Figures 3.1.A, 3.4.B-C, 3.5, 3.6.A, 3.7, 4.3, 4.4.C and 4.5), 2-way ANOVA followed by Tukey's multiple comparisons test (Figures 3.1.B, 4.1.A and 4.2), 2-way ANOVA followed by Sidak's multiple comparisons test (Figures 5.12, 5.13, 5.14 and 5.15).

Chapter 3

Proximal effects of larval dietary yeast on larval growth and lipid metabolism

3 Proximal effects of dietary [yeast] on larval growth and lipid metabolism

3.1 Introduction

This chapter focuses on the proximal effects of larval diet on the larva itself. In the context of this thesis, this is important because the nutritional status of the larva impacts upon both larval and pupal growth, and the development of the imaginal tissues. The *Drosophila* larva is an exquisite model for studying growth, i.e. gain in biomass. Under standard laboratory conditions, larvae grow an impressive 150-200 fold in only four days as they develop from newly-hatched 1st instar larvae (L1) to wandering 3rd instar larvae (wL3). This renders the growing animal sensitive to even the subtlest of changes in environmental conditions, especially nutrient availability. I focused on the effects of larval dietary [yeast] on growth, as defined by a gain in biomass, and metabolism. Larvae were reared on diets with varying concentrations of yeast – considered to be the main source of proteins in the *Drosophila* diet –, while maintaining all other components constant. The exact yeast content of each diet can be found in Materials and Methods (see 2.3). In the interest of space and flow, I will use the closest whole number (by rounding up/down, as appropriate) to refer to the % of yeast in a diet for the rest of this document. In this way, 1.17% becomes 1% and 2.34% becomes 2%, while 4.68% becomes 5%.

3.2 Results: Nutrient composition of diets used in this study

In laboratory *Drosophila* diets, yeast-derived nutrients are thought to be essential for optimal growth. I considered what effect the concentration of yeast in the diet has on the three major classes of dietary macronutrients – i.e. lipids, amino acids and sugars– and which of these changes may mediate the effects of altered dietary [yeast] on larval growth and development.

3.2.1 Altering the [yeast] in the diet has little effect on the lipid content of the medium

Lipids, except for cholesterol, have been shown not to be essential for *Drosophila* development (Sang, 1956). Furthermore, cornmeal – whose concentration remained unchanged among the different diets – is rich in lipids. Therefore, changes in dietary [yeast] are not expected to impact significantly on the amount of dietary lipids. In order to

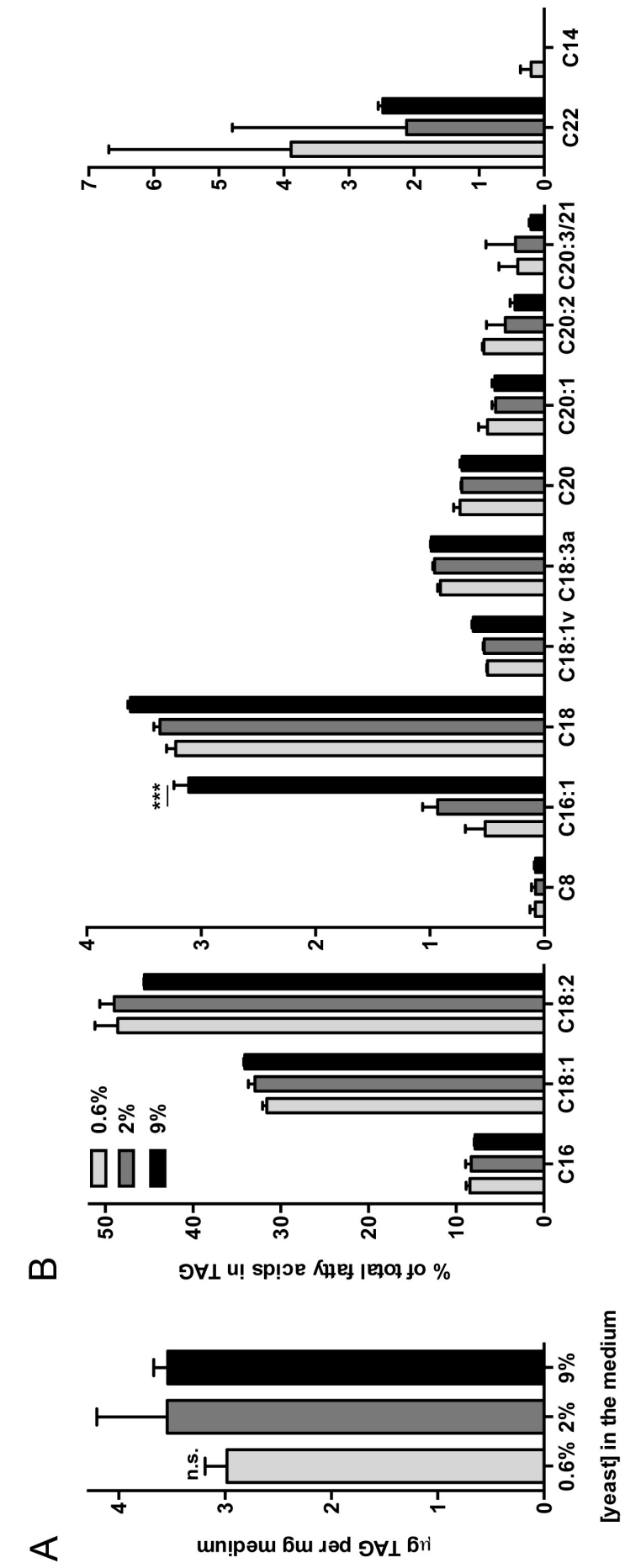

Figure 3.1: Altering dietary [yeast] in our glucose-yeast-cornmeal *Drosophila* food has little effect on dietary triacylglycerols (TAGs). (A) TAG content in diets containing 0.6%, 2% or 9% yeast. (B) Fatty acid profile of dietary TAG shown as percentage of each fatty acid relative to the total. For this and all subsequent figures, error bars represent 1 S.D. In this and all subsequent figures, statistical significance is reported as: n.s. for $p>0.05$, * for $p\leq 0.05$, ** for $p\leq 0.01$, *** for $p\leq 0.001$ and **** for $p\leq 0.0001$.

confirm this experimentally, I used gas chromatography to analyse the triacylglycerol (TAG) contents of diets containing 0.6%, 2% or 9% yeast. Despite the 16-fold difference in yeast content between the 0.6% and 9% foods, this only had a minor effect on the TAG content of the medium (Figure 3.1.A). The proportions of individual fatty acids in the medium also remained largely unaltered by manipulating yeast content, with the exception of C16:1, which increased as dietary [yeast] increased (Figure 3.1.B). This analysis revealed that, regardless of the yeast content, C18:2 makes up half of the fatty acids in dietary TAG. C18:1, the second most abundant species, accounted for >30%. C18:2, C18:1 and C16 together accounted for ~90% of the fatty acids in dietary TAG. Together, these results show that altering the concentration of dietary yeast has little consequence on the lipid content of the diet. This suggests that most TAG in our food is contributed by cornmeal.

3.2.2 Both yeast and cornmeal contribute to the dietary amino acid pool

NMR analyses of 2%-yeast food have revealed that free aminoacids are only present in minute quantities in our standard lab medium (TJ Ragan, Vanessa Tixier and Alex P Gould, personal communication). Vanessa Tixier in the laboratory has recently investigated what are the sources of amino acids in this medium. Her analyses showed that yeast has a high amino acid content (~30% w/w), but cornmeal also contains ~6% (w/w) amino acids. However, our recipe contains 6.63% cornmeal. Therefore, approximately two thirds of the amino acids in our standard 2% yeast (w/v) medium are contributed by yeast. Vanessa's analyses have shown that, surprisingly, the source for the remaining third is cornmeal (Figure 3.2.A). The importance of this observation can only be fully understood in the context of other dietary manipulation studies in *Drosophila* that do not use cornmeal in their media.

Based on the amino acid content of yeast, cornmeal and agar, I calculated the impact of altering dietary yeast – while maintaining all other components constant – on the proportions of the different individual amino acids (Figure 3.2.B). Hydroxyproline and cysteine were not detected in any of the major components of 2% yeast medium (i.e. agar, cornmeal and yeast), nor in the complete 2% medium. The relative contributions of glutamate, proline and leucine to the total amino acid pool increased when dietary yeast was reduced. While the first two are non-essential, leucine is an essential amino acid. These results show that, apart from reducing the amino acid content of the diet, reducing dietary yeast may create an amino acid imbalance. In adults, the balance of amino acids in the diet has been shown to influence lifespan and reproduction (Grandison et al., 2009a).

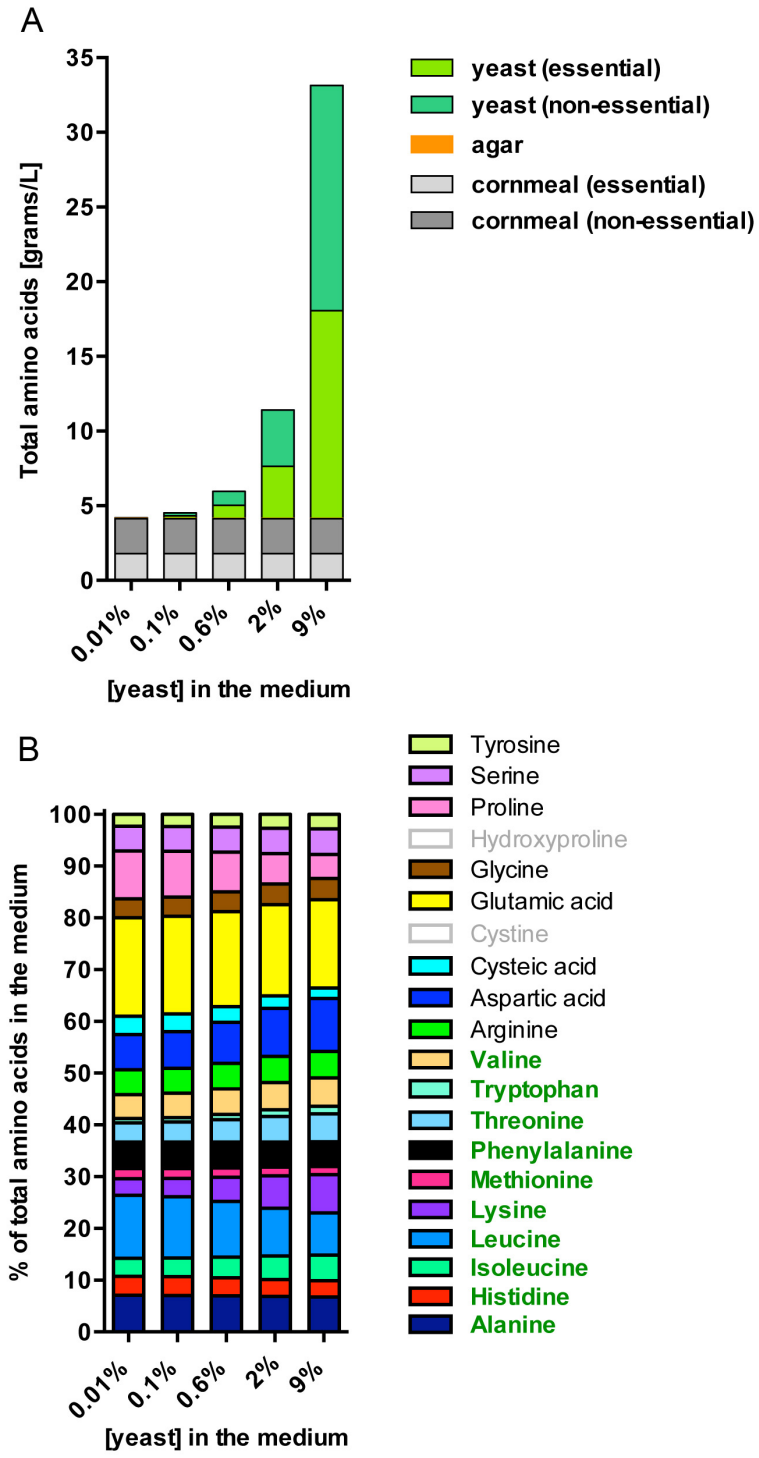

Figure 3.2: Amino acid composition of *Drosophila* diets containing different concentrations of yeast. (A) Amino acid content (g/L) of diets containing 0.01%, 0.1%, 0.6%, 2% or 9% yeast. The amounts (g/L) of essential and non-essential amino acids contributed by each yeast (green) and cornmeal (grey). (B) Relative proportions of each of the amino acids present in food as a percentage of the total. Essential amino acids are listed in green and non-essential in black. Cysteine and hydroxyproline were not detected in any of the components of the food analysed separately nor in the complete diet. Calculations derived from data provided by Vanessa Tixier.

This *in silico* exercise shows that, overall, reducing dietary yeast reduces the amino acid content of the medium and provides insight into the nature of the changes induced by altering dietary [yeast].

3.3 Dietary [yeast] effects on larval growth and duration of development

Previous studies altered larval dietary [sugar] to produce metabolic disturbances, including "obesity" (Musselman et al., 2011). I decided to take a complementary approach by varying [yeast] in the larval diet. The effects of varying dietary [yeast] during adulthood had been extensively studied and shown to affect a myriad of readouts, from reproduction and ageing to sleep-wake behaviour (Catterson et al., 2010; Partridge et al., 2005a), but no systematic studies for dietary [yeast] had been reported for the developing larva.

3.3.1 Low [yeast] larval diet delayed development

I began by assessing larval developmental time on diets with yeast concentrations on both side of 2%, our standard lab diet, i.e. ranging from 0.01% to 19%. Larval development on 0.6% or 0.2% yeast diets took 1 to 2 days longer (marked by reaching the WPP stage) as compared to media containing 5% or 9% yeast (Figure 3.3.A). Further reducing dietary [yeast] to a mere 0.01%, more than doubled developmental time: from ~140 hours (from egg laying to pupariation) on 2% yeast to a median of 350 hours on 0.01% yeast (Figure 3.3.B). While larvae reared on 2% dietary [yeast] pupariated within a relatively tight 24-hour window, larvae reared on only 0.01% dietary [yeast] pupariated over a period of >9 days, despite the two groups originating from the same synchronised population of embryos. Thus, severe reduction in dietary [yeast] not only delayed development but also desynchronised larvae. However, 40% fewer animals made it to pupariation on 0.01% compared to 2% [yeast], despite starting with the same number of embryos in both cases (Figure 3.3.C).

3.3.2 Low dietary [yeast] decreases final body size

I next assessed the effects of varying larval dietary [yeast] on body size at the end of larval development. Larvae reared on "standard" 2% yeast food grew to ~1.75 mg. A 4-fold increase in dietary yeast did not, in turn, increase wL3 body weight, while an 8-fold increase only increased wL3 weight by 5% (Figure 3.4.A-B). A 9% yeast diet did, however, accelerate development by ½ day, suggesting that, despite its lack of effect on final wL3 weight, this 4-fold increase in dietary yeast nevertheless increases the rate of growth. In stark contrast, limiting dietary yeast has immediate consequences. A 4-fold decrease in

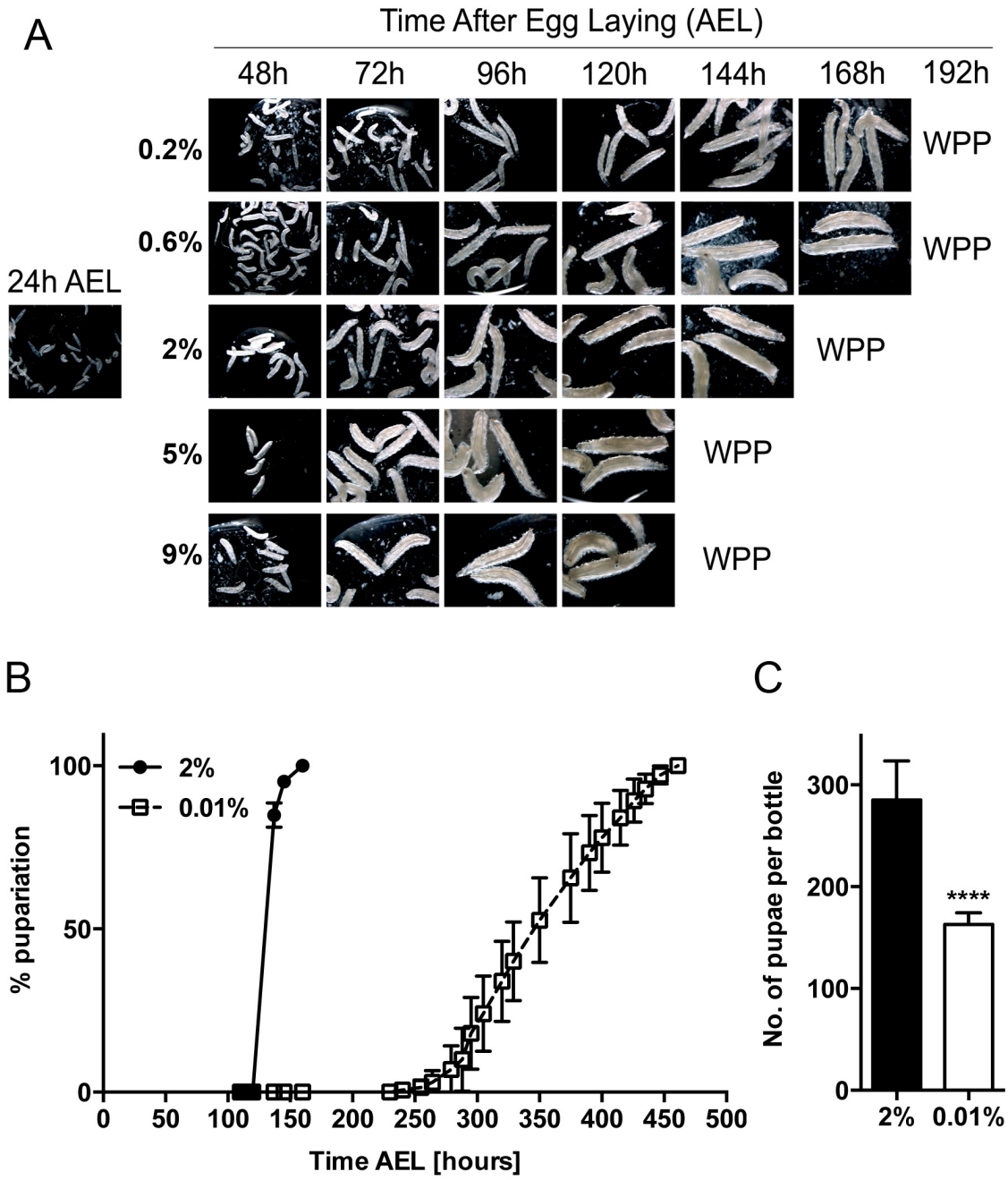

Figure 3.3: Reducing dietary [yeast] delays larval development i.e. time to pupariation as defined by reaching the white pre-pupa stage (WPP). (A) Micrographs show larvae raised on 0.2%, 0.6%, 2%, 5% and 9% every 24 h after egg laying (AEL) until pupariation. **(B)** Time course to pupariation for larvae raised on 2%- vs 0.01%-yeast diets. Proportion of larvae that have reached the WPP stage over time is shown as a percentage of the final number of pupae per bottle. **(C)** Number of animals that completed development (i.e. pupated) in bottles containing 2%-yeast food vs. bottles containing 0.01%-yeast food. All bottles were seeded with similar numbers of embryos.

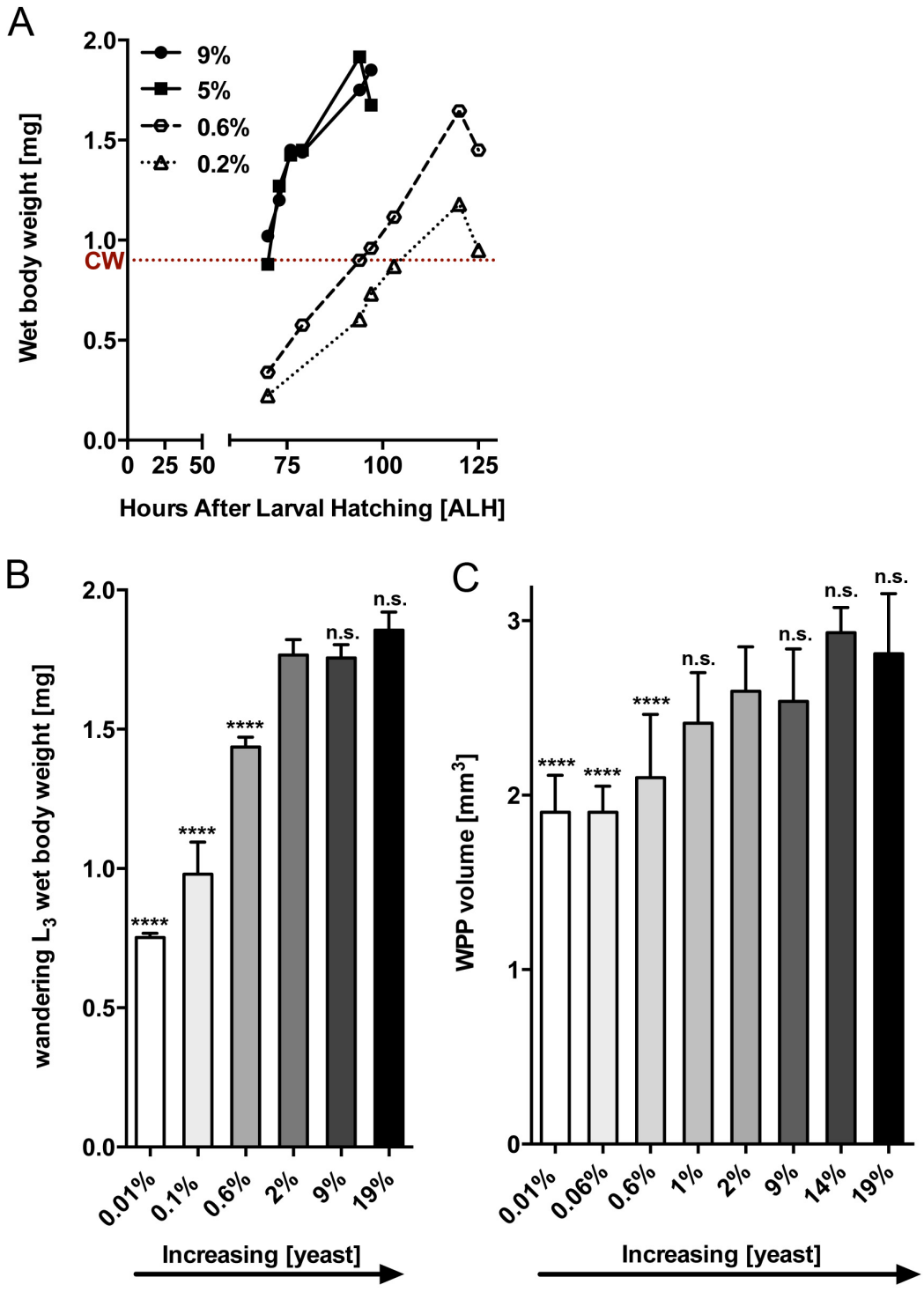

Figure 3.4: Wandering L3 (wL3) larval size correlates positively with dietary [yeast]. (A) Time course of mass gain (in mg) for larvae raised on 0.2%, 0.6%, 5% and 9% starting with 70 h after larval hatching (ALH) until pupariation. **(B)** Wet weight of wandering L3 larvae raised on diets with yeast concentrations ranging from 0.01% to 19%. All other dietary components were maintained constant. **(C)** WPP volume of animals raised on diets with yeast concentrations ranging from 0.01% to 19%.

dietary [yeast]- from 2% to 0.6%- reduced wL3 body weight by 20%, a 20-fold decrease reduced it by 45%, while a dramatic 200-fold decrease in dietary [yeast] resulted in wL3 weighing 60% less than 2%-fed controls. Considering that a 0.01% yeast diet doubled the duration of larval development, but decreased wL3 weight by 60%, this suggests that average growth rate during development was >4 times less on 0.01% than on 2% yeast (see Figure 10.2 in the Appendix). These results show that final body weight correlates positively with the % of yeast in the medium, such that wL3 body weight increases with increasing [yeast]

wL3 size was also quantified by measuring WPP volume as another measure of final body size. This was affected in a similar way to wL3 mass by larval dietary [yeast] (Figure 3.4.C). However, WPP reared on 0.01% yeast measured only ~25% less than 2% controls, whereas wL3 mass was reduced by 60%. Possible reasons for this difference include inaccuracies of volume estimation and also that the density of the animals changed due perhaps to air content.

Lowering dietary [yeast] increases the ratio of sugar:protein in the diet. Adult flies have been proposed to prioritise protein over sugar i.e. they will eat excess sugar in order to reach their protein intake target (Lee et al., 2008). In addition, the larva is a fast-growing animal that depends on dietary protein. I decided to test whether the effects of varying the concentration of dietary yeast are in fact the effects of altering the ratio of glucose:protein in the larval diet. If so, the effects of a low-yeast diet would, in fact, be the effects of excess sugar intake, rather than low protein. For this, I analysed the effects of altering the sugar:protein ratio by altering the concentration of glucose in the diet. On all diets tested, halving the glucose content (i.e. low glucose= LG) in 0.6%, 2% or 9% yeast diets had no effect on wL3 weight but sped up larval development by at least ½ day (Figure 3.5 and data not shown). This shows that lowering the sugar:protein ratio can accelerate larval development but is not sufficient to increase wL3 weight. In contrast, increasing the glucose content in the larval diet from 5.85% to 40% (w/w) delayed, or even halted, larval development (data not shown). I refer to this 40% glucose medium as high glucose diet (i.e. HG). The severity of the impact of HG on larval development depended on the yeast content of the medium. On a diet containing 9% yeast, HG delayed larval development by 4 days and decreased wL3 body weight by 20% (Figure 3.5). On a diet containing 2% yeast, HG delayed development by more than 2 weeks and only a small proportion of the animals completed development (data not shown). Finally, HG in combination with 0.6% dietary yeast completely arrested larval development: larvae remained alive for longer than 2 weeks after egg laying, but morphological analyses revealed that these animals never

developed past the first larval instar. The results of increasing dietary glucose seem to support the idea that increasing the glucose:protein ratio of the diet is detrimental to larval development. However, the sugar:yeast ratio in the 0.6%HG diet is 68.4:1 (40% glucose vs. 0.585% yeast), much lower than that in 0.01% , for which the ratio is 500:1 (5.85% glucose vs. 0.012% yeast). The fact that larvae can complete development on a 0.01% yeast diet but cannot progress past L1 on a 0.5%HG diet demonstrates that high absolute concentrations of dietary glucose are detrimental to larval development. The detrimental effects of high glucose can, however, be partially rescued by a concomitant increase in protein.

3.3.3 CW is independent of dietary [yeast]

An interesting question is whether CW (critical weight) is sensitive to nutrition. I found that all larvae weighed 0.89 ± 0.018 mg (Table 3.1), by the stage at which they were able to pupariate without delay upon removal of nutrients. Given that (a) mass at CW did not change and that (b) once CW is attained, time to WPP is fixed regardless of nutrient availability, then body weight at the end of development will depend on the rate of growth during the TGP (terminal growth period). Based on this, one can calculate the rates of growth before and after CW for each diet (see Figure 10.2 in the Appendix). This indicates that pre-CW growth rate increases with increasing dietary [yeast], but TGP mass gain plateaus at ~2% yeast. It is interesting to note that, despite the dramatic effects of dietary [yeast] on larval growth and developmental time, metamorphosis lasted approx. 4 days in all cases. Interestingly, 0.01% yeast-fed larvae only attained a mass of ~0.75 mg at wL3, considerably less than CW. This indicates that these larvae must have reached minimum viable weight (MVW), but not CW.

Table 3.1: Developmental time and mass at critical weight (CW) and wandering L3 stage (wL3) for larvae raised on food containing 0.2%, 0.6%, 5% or 9% yeast

	Time from…				Mass at….	
[yeast] in the food	**L_1 hatching to CW**	**CW to WPP**	**WPP to eclosion**	**hatching to eclosion**	**CW [mg/larva]**	**wL_3 stage [mg/larva]**
0.2%	≤104 h	≥36 h	~ 4 days	~ 10.5	0.87	0.95
0.6%	≤96 h	≥35 h	~ 4 days	~ 9.5 days	0.9	1.39
5%	≤68 h	≥32 h	~ 4 days	~ 8.5 days	0.88	1.67
9%	≤68 h	≥32 h	~ 4 days	~8 days	0.91	1.83

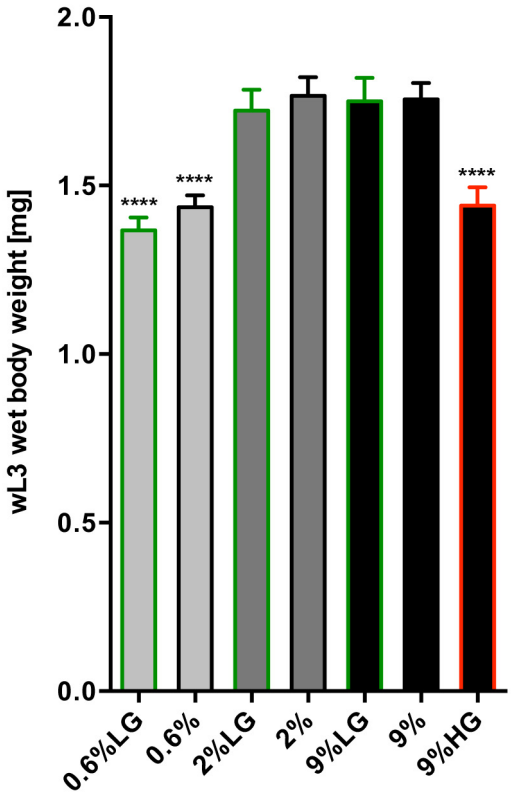

Figure 3.5: Wet weights of wL3 larvae raised on diets containing 0.6%, 2% or 9% yeast in combination with either low, "standard" or high glucose. "Standard" glucose = 5.85% (w/v) glucose; low glucose (LG) = 2.9% (w/v) glucose; high glucose (HG) = 40% (w/v) glucose.

3.4 Allometric scaling of larval organs with body size

3.4.1 Organ sparing as a function of [yeast]

When comparing between species, it has long been known that, as body size changes, the sizes of different organs often change allometrically with it- that is, they change in a non-isometric fashion (Huxley, 1932). Among animals of the same species, however, it is a common misconception that "*external factors such as nutrient availability and crowding can alter overall animal size, but individual body parts scale reproducibly to match the body even with challenges from a changing environment.*" (Neto-Silva et al., 2009) or that by "*[c]oordinating cell proliferation with morphogens and nutrients [...] organs scale appropriately with body size*" (Parker, 2011). In the lab, work done by Louise Cheng and Andrew Bailey has revealed that if post-CW larvae are subjected to severe nutrient restriction (NR), organs such as the wing disc and the brain will continue to grow, despite the overall body and other internal organs, such as oenocytes or the fat body, having stopped (Cheng et al., 2011).

Having seen the effects of larval dietary [yeast] on overall body growth, I next examined how the sizes of six different tissues correlated with overall body size at the end of development (i.e. WPP stage), for larvae subjected to under-, normal and over-nutrition – as defined by the yeast content of the larval diet, setting 2% yeast as "normal". As polyploid tissues grow by endoreplicative cycles, rather than by mitotic cell division, and given that nuclear size is proportional to nuclear DNA content, the nuclear volume of polyploid cells can, therefore, be used as a proxy to assess tissue growth. I used nuclear volume to assess the growth of the salivary glands, podocytes and ecdysone-producing cells of the prothoracic gland (PG). To measure the sizes of IPC cell bodies, I marked these cells by driving membrane-tagged GFP (i.e. UAS-CD8::GFP) with dilp2-GAL4 and estimated their volume from orthogonal measurements of their diameters. Brain and wing disc volumes were measured from 3D reconstructions of confocal stacks.

I found that, as dietary [yeast] was reduced and final body size dropped, the growth of organs such as the CNS or the wing disc was protected, at the expense of organs such as the salivary glands (SGs) and, surprisingly, the IPCs (Figure 3.6.A-B). This indicates that selective organ sparing is triggered by low [yeast] as well as by NR. More importantly, the data also show that each organ has its own "threshold for sparing". For example, both the brain and the wing disc were positively spared relative to the body down to 0.1% dietary [yeast]. In fact, down to 0.6% yeast, their absolute sizes remained unchanged, despite

body weight dropping by 20%. When larvae were reared on 0.1% yeast, brain and wing disc sizes decreased by ~20%, but this was still less than the ~45% reduction in body weight. However, only the brain continued to be positively spared when dietary yeast was dropped to 0.01%, with the reduction in wing disc size now being in line with that seen for the overall body weight (Figure 3.6). That is, when [yeast] was reduced from 0.1% to 0.01% the wing disc passed from positive to neutral sparing.

In contrast, dietary [yeast] had an immediate effect on the sizes of the SGs and IPCs: the 20% drop in body weight on 0.6% yeast was accompanied by a ~40% decrease in the sizes of both organs (Figure 3.6). Larvae reared on 0.01% dietary [yeast] weighed 60% less than controls, but their SGs and IPCs were >75% smaller than those of animals reared on 2% yeast. The SGs and IPCs were, therefore, negatively spared relative to the body even when the reduction in dietary [yeast] was modest. Interestingly, the sizes of IPCs and SGs both increased by 7-8% when dietary [yeast] was increased above our standard 2%, but this effect was not statistically significant. To better understand the surprising behaviour of the IPCs upon reducing dietary [yeast], I quantified SG and IPC volumes for larvae that had been raised on HG (i.e. high glucose) versions of the 2% and 9% diets, as a means to decrease body size without altering the concentration of yeast in the diet. These experiments revealed that IPC and SG volumes of larvae raised on the HG diets were similar to those of animals raised on a 0.6% yeast diet (see Figure 10.3 in the Appendix). Knowing that these two high-glucose diets decreased wL3 size to the same extent as lowering [yeast] to 0.6%, these results demonstrate that the size of the IPCs is tightly linked with larval growth and cannot be explained as a function of dietary [yeast].

Podocyte and ecdysone-producing cells of the prothoracic gland (PG) sizes were only quantified on the control diet (i.e. 2% yeast) and the diet with the lowest [yeast] (i.e. 0.01% yeast), so their "sparing dynamics" remain unknown (Figure 3.6). When larvae were reared on 0.01% dietary [yeast], the size of podocytes dropped by 80% – i.e. they were negatively spared to an extent similar to that seen for the IPCs and SGs –, while the size of the ecdysone-producing cells remained in line with body weight – i.e. it dropped by ~60%, suggesting neutral sparing. More experiments, however, will be needed to test whether these cells are neutrally spared across all yeast concentrations.

In summary, the analysis of organ sizes at different yeast concentrations demonstrates that each organ has its own threshold for sparing. Therefore, whether an organ is positively, neutrally or negatively spared depends on the severity of the insult. The data

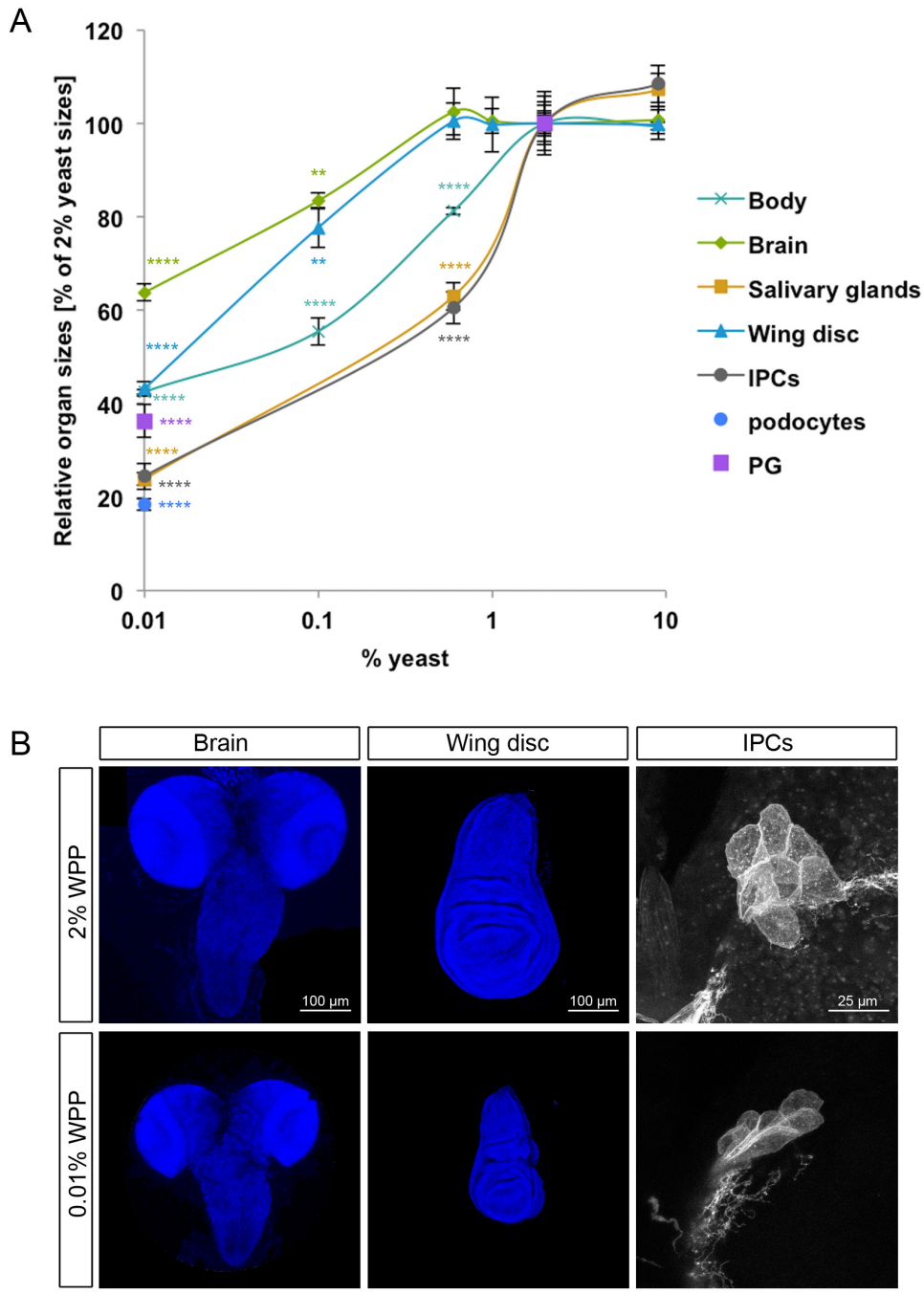

Figure 3.6: Alterations in dietary [yeast] triggers differential organ sparing. (A) Relative wL3 body weight and organ sizes at the end of development (i.e. WPP stage) of brain, wing disc, salivary glands, IPCs (insulin-producing cells), podocytes and ecdysone-producing cells of the prothoracic gland (PG) dissected from animals raised on dietary [yeast] ranging from 0.01% to 9%. Body and organ sizes of 2% animals were set as 100%. Body/organ sizes of larvae raised on all other diets are reported as percentage of the size of body/organs of animals raised on 2%-yeast diet. Error bars show ± 1 S.E.M. **(B)** Confocal micrographs show WPP brains, wing discs and IPCs of larvae reared on 2%-yeast diet (top) vs. 0.01% yeast diet (bottom) imaged at the same magnification. Brains and wing discs were stained with DAPI (blue). IPCs were marked by overexpression of UAS-CD8::GFP (shown in grayscale) under the control of the IPC driver, dilp2-GAL4.

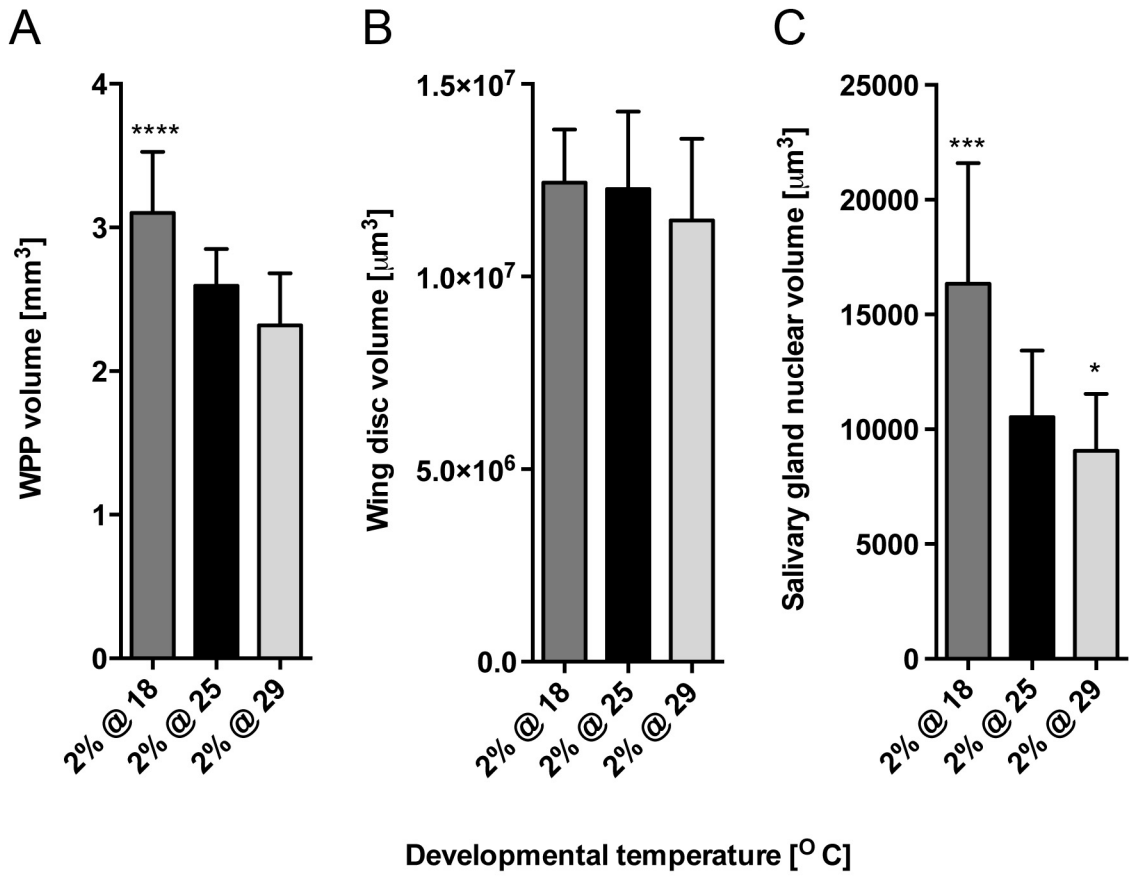

Figure 3.7: Altering the rate of growth by varying environmental temperature during development also alters organ proportions. (A) WPP volume of larvae raised on a 2%-yeast diet at 18, 25 or 29℃. **(B)** WPP wing disc volumes of larvae raised on a 2%-yeast diet at 18, 25 or 29℃. **(C)** WPP salivary gland nuclear volumes of larvae raised on a 2%-yeast diet at 18, 25 or 29℃.

also allow us to construct an organ sparing hierarchy, such that CNS > wing disc > body = ecdysone-producing cells > IPCs = SGs ≥ podocytes.

3.4.2 Altering the rate of growth using temperature also alters organ proportions

The rate of development in insects is known to be influenced by temperature (Edgar, 2006), so I used this environmental parameter as a means of altering the rate of growth independent of diet. This allowed me to study whether altered rate of growth *per se* could impact on organ proportions. For these experiments, I maintained dietary yeast constant at 2% and placed larvae at either 18^{0}C, 25^{0}C or 29^{0}C. As expected, larval development was extended by ~9 days at 18^{0}C compared to 25^{0}C. Further increasing the temperature to 29^{0}C only accelerated development by ~½ day. WPP volume correlated inversely with the duration of development (Figure 3.7.A). The differences in WPP volume were also mirrored in the size of the salivary glands (Figure 3.7.C). However, despite the different developmental rates and body sizes, wing discs grew to the same absolute size, regardless of developmental temperature (Figure 3.7.B). Therefore, altering developmental rate and final body size independent of diet composition is sufficient to alter organ proportions. This demonstrates that the organ sparing hierarchy observed with altered dietary [yeast] is maintained across different developmental insults.

3.5 Effects of dietary [yeast] on larval lipid metabolism

I next investigated the potential effects that changing larval dietary [yeast] may have on lipid metabolism. Lipids were extracted from whole wL3 animals reared on different diets by chloroform:methanol extraction and separated into the four main lipid classes by solid phase extraction: triacylglycerols (TAG), non-esterified fatty acids (NEFA), cholesterol esters (CE) and phospholipids (PL). Gas Chromatography-Mass Spectrometry (GC-MS) of the fatty acid methyl esthers (FAMEs) was then used to quantify fatty acids (FAs) in the TAG, CE and NEFA fractions. As a readout for larval adiposity (i.e. stored fat content) I used the sum of all FAs in the TAG and CE fractions, the main forms of stored fat. NEFA were used for estimating free fatty acids.

3.5.1 Low dietary [yeast] increases larval adiposity and alters lipid composition

In a first set of lipid experiments, adiposity levels of larvae reared on 0.6% vs. 9% dietary yeast were compared. Low dietary [yeast] decreased body weight but increased the adiposity (= TAG+CE per mg wet body weight) of wL3 larvae (Figure 3.8.A-B). It is

interesting that, similarly to the trend observed for positively spared organs, absolute TAG content per animal was spared under these moderately low dietary yeast conditions (data not shown). Animals reared on 0.6% dietary yeast displayed an increase of 40% in TAG content relative to their body weight compared to wL3 animals reared on 9% dietary yeast; both groups, however, contained 80-90 μg TAG per larva.

I then compared 2% vs. 0.01% (Figure 3.9). While absolute TAG quantities per larva dropped from ~90 μg for larvae raised on 2% to ~50 μg for larvae raised on 0.01% (data not shown), relative adiposity levels of 0.01%-fed wL3 larvae were still >45% higher than those of the 2%-fed controls (Figure 3.9.B). In summary, TAG quantifications reveal that both moderate (i.e. 0.6%) and extreme (i.e. 0.01%) yeast restriction regimes increase wL3 adiposity by 40-45%.

In contrast to the dramatic changes seen in stored fat content the levels and composition of NEFA in wL3 larvae reared on 0.01% dietary or on 2% dietary yeast were very similar (Figure 3.10.A). Reducing larval dietary [yeast] from 2% to 0.01% yeast did appear to significantly increase the proportion of C14 and decrease those of C16 and C18:2 in wL3 NEFA (Figure 3.10.B). NEFA have been correlated with altered metabolic states and disease in mammals. However, overall, wL3 NEFA levels were very low and highly variable (individual data points are shown on Figure 3.10.A), which may have obscured more subtle changes.

GC data gives information on the amount of each fatty acid, so I analysed the contribution of individual fatty acid species to the total TAG pool in wL3 (Figures 3.8.C and 3.9.C). Regardless of dietary [yeast], over 90% of the total fatty acids in TAG were represented by only five fatty acid, namely: C14:0 (myristic acid), C16:0 (palmitic acid), C16:1 (palmitoleic acid), C18:1 (oleic acid) and the ω-6 fatty acid C18:2 (linoleic acid). However, this analysis revealed that the increase in TAG content on low [yeast] was accompanied by altered fatty acid profiles. Reducing dietary yeast to 0.6% or 0.01% had little effect on the proportions of saturated fatty acids, with the exception of C18, which was lowered in both cases. Surprisingly, reducing dietary yeast to 0.6%, decreased the percentage of the mono-unsaturated fatty acids C16:1 and C18:1, whereas reducing it to 0.01% increased the relative contributions of both species. Furthermore, rearing larvae on a 0.6% yeast diet doubled the proportion of C18:2 in TAG, while rearing them on a 0.01% yeast diet reduced C18:2 in TAG by ~40%. The polyunsaturated ω-6 fatty acid C18:2 cannot be synthesised by mammals or *Drosophila* and so must be derived from the diet (Cook and McMaster, 2002; Draper et al., 2000; Rapport and Samuelson, 1983).

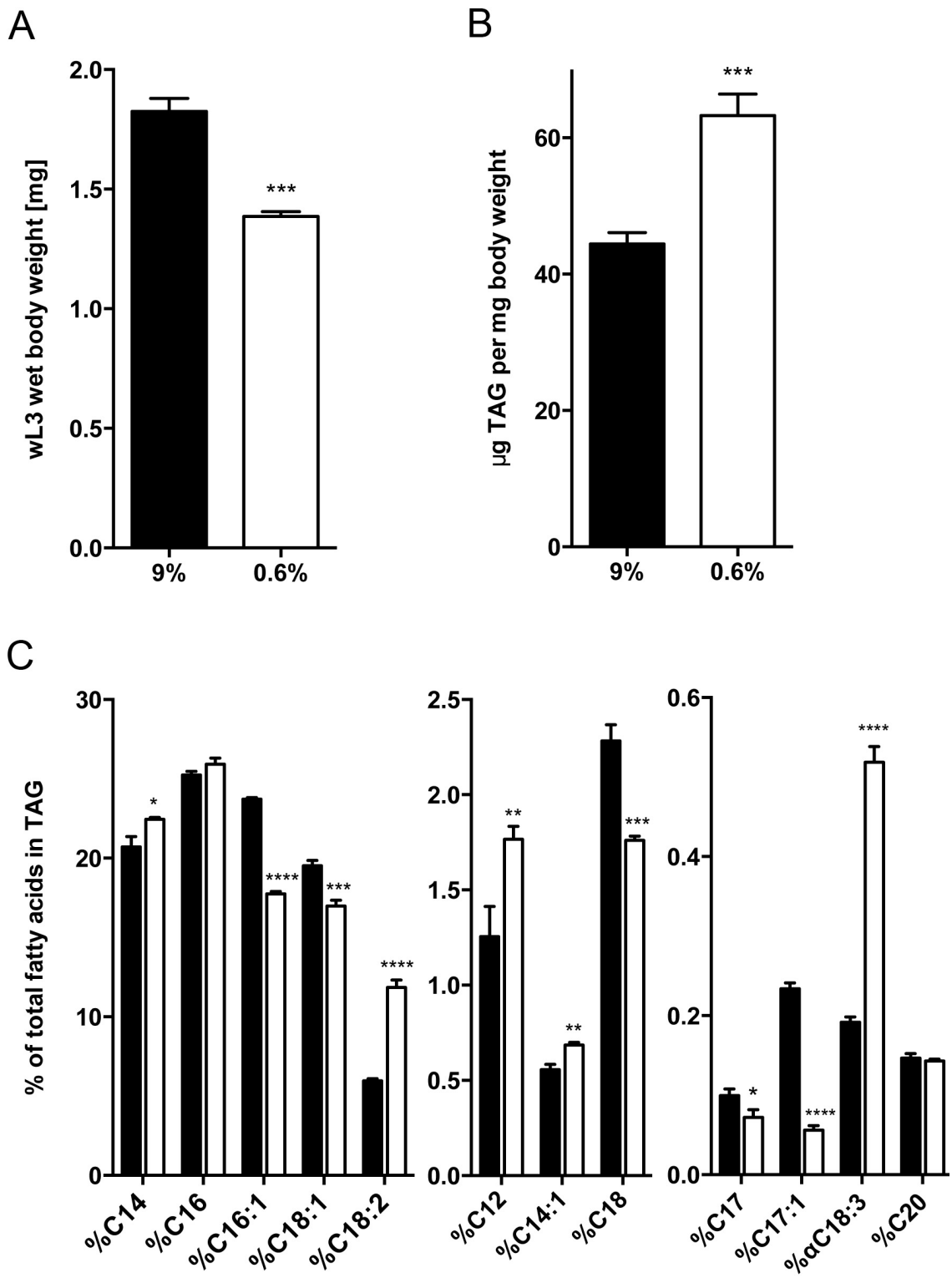

Figure 3.8: Decreasing larval dietary [yeast] from 9% to 0.6% decreases wL3 body weight (A), increases adiposity (µg TAG+CE fatty acids/mg body weight) (B) and alters the fatty acid composition in stored fat i.e. TAG+CE fractions (C). Individual fatty acids are shown as a percentage of the sum of all fatty acids detected in the TAG+CE fraction per sample.

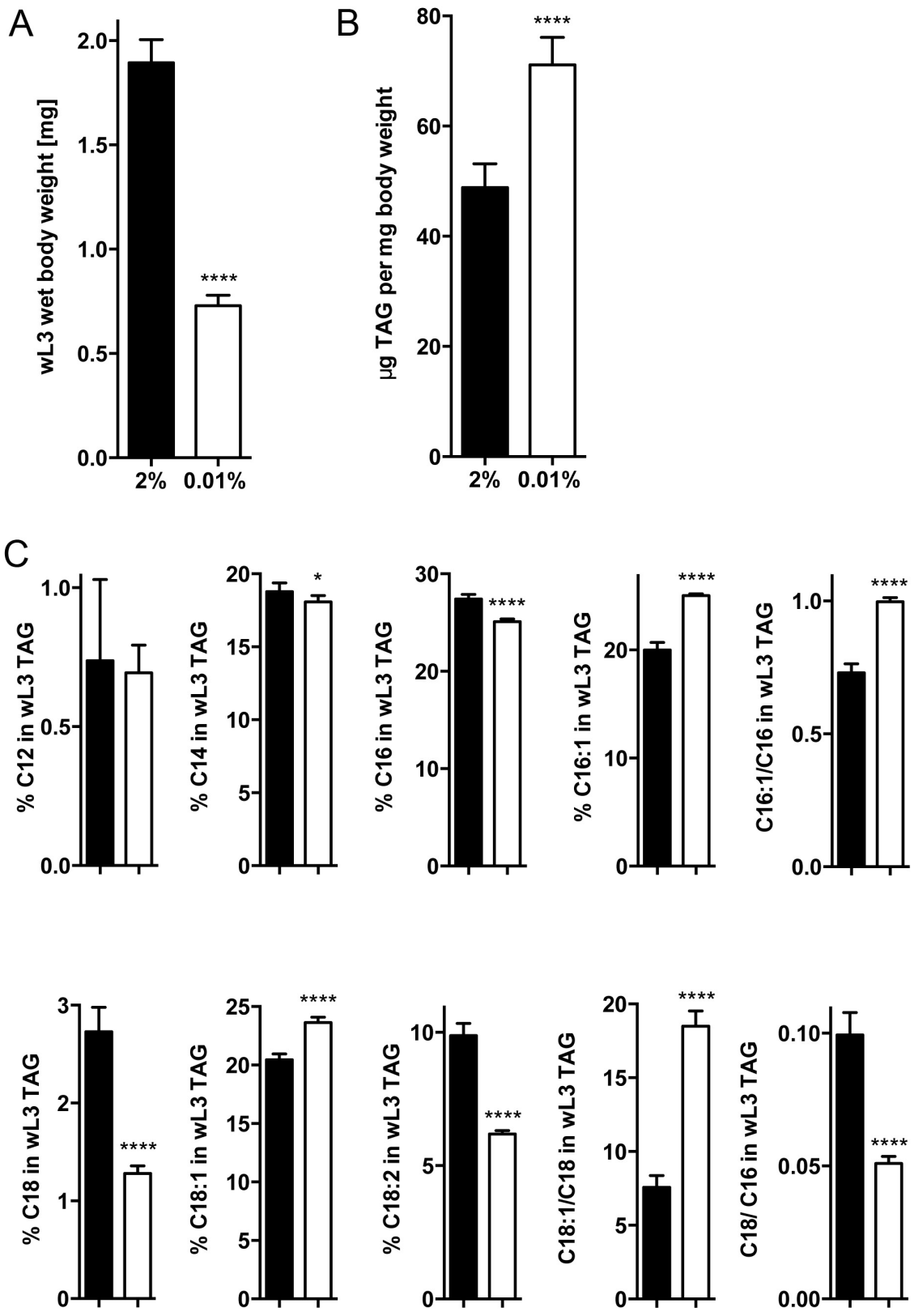

Figure 3.9: Decreasing larval dietary [yeast] from 2% to 0.01% dramatically decreases wL3 body weight (A), increases adiposity (µg TAG+CE fatty acids/mg body weight) (B) and alters the fatty acid composition in stored fat i.e. TAG+CE fractions (C). Individual fatty acids are shown as a percentage of the sum of all fatty acids detected in the TAG+CE fraction per sample.

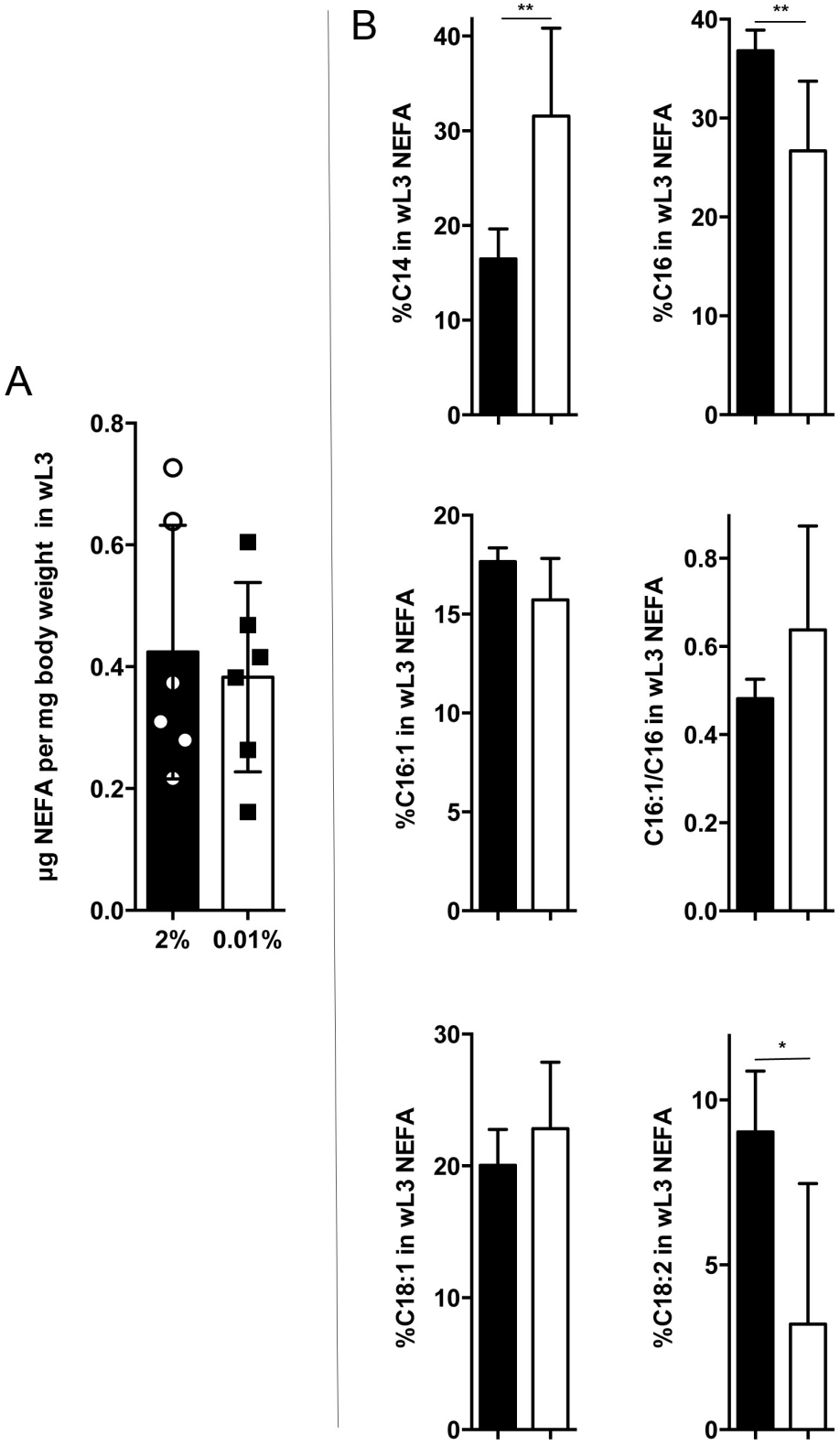

Figure 3.10: Decreasing larval dietary yeast from 2% to 0.01% does not affect the NEFA content of wL3 larvae (A) but alters NEFA fatty acid composition (B). Individual fatty acids are shown as a percentage of the sum of all fatty acids detected in the NEFA fraction per sample.

C16 is the major product of *de novo* fatty acid synthesis. Elongases can then use C16 to produce FAs with 18 carbons and longer. In mammals, the ratio of mono-unsaturated fatty acids to their saturated precursors is referred to as the desaturation index and has been proposed to correlate with the activity of stearoyl-CoA desaturase (the enzyme that introduces to double bond in C16 to produce C16:1; Flowers and Ntambi, 2008). Further insight can, therefore, be gained from comparing, not the % of each fatty acid relative to the total, but the ratios of meaningful pairs of fatty acids. In mammals, the desaturation index and the "elongation index" (i.e. the ratio of C18/C16) are considered to be predictive of hepatic fatty acid synthesis and, therefore, risk of developing non-alcoholic fatty liver (Flowers, 2009). When examining the desaturation (C16:1/C16) and elongation (C18/C16) indices, once again, moderate vs. severe yeast restriction had contrasting effects: 0.01% dietary yeast increased the desaturation index by 36% (Figure 3.9.C and data in Figure 3.8.C), while 0.6% dietary yeast decreased it by 30%. Both dietary yeast reduction regimes lowered the "elongation index" C18/C16 to different extents i.e. 0.6% dietary yeast reduced the C18/C16 ratio by 25%, while the 0.01% yeast diet halved it (Figure 3.9.C and data in Figure 3.8.C).

The results of lipid analyses by GC-MS show that low yeast larval diets have dramatic effects upon the amount and profile of stored fat in wL3 larvae but not upon that of NEFA. However, despite the fact that both moderate (i.e. 0.6%) and severe (i.e. 0.01%) yeast reductions increased adiposity relative to body mass to a similar extent, the two are not equivalent with respect to fatty acid composition and, so, may have different metabolic outcomes.

3.5.2 Low [yeast] larval diet induces ectopic accumulation of lipids

It is unclear how increased adiposity predisposes to metabolic abnormalities, such as insulin resistance, diabetes or cardiovascular disease. In mammals, one of the theories that attempts to explain the connection between the two, postulates that increased adiposity *per se* is not the main culprit, but the inability of the adipose tissue to keep expanding to accommodate more lipids (Mittendorfer, 2011). When this limit is reached, lipids "spill over"/"overflow" from the adipose tissue, which results in an increased flux of fatty acids to and ectopic lipid accumulation in non-adipose tissues (Savage, 2009). Ectopic lipid accumulation causes lipotoxicity and has been associated with insulin resistance and organ dysfunction (Samuel and Shulman, 2012). This would explain why too much body fat (obesity) and too little (lipodystrophy) have the same metabolic consequences. To test whether increased adiposity was associated with "lipid overflow", larvae reared on 0.01%

yeast, which had 45% more TAG per mg body weight than 2% controls (see Figure 3.9.B), were analysed in more detail. Analyses of the fat bodies of 2% vs 0.01% yeast-fed larvae revealed no apparent morphological defects even though TAG levels differed. Using LipidTOX (a neutral lipid stain) ectopic lipid droplets were found to accumulate in several of the internal organs of larvae reared on 0.01% yeast. These include muscles (body wall and perivisceral), podocytes, salivary glands and ring gland (Figures 3.11 and 3.12, and data not shown). This strongly suggests that 0.01% dietary yeast increased the flux of lipids and/or lipid precursors to non-adipose tissues.

3.6 Genetically-induced lipid overflow replicates low [yeast] "lipodystrophy"

Mice with lowered lipolysis rates (i.e. heterozygous for hormone-sensitive lipase) gain as much weight/fat as controls when fed on a high-fat, obesogenic diet, but do not develop the associated metabolic complications (Girousse et al., 2013). In a converse experiment, I asked whether increasing lipolysis in the adipose tissue of control-fed animals would be sufficient to induce metabolic complications similar to those observed in animals fed a very low yeast diet. By increasing lipolysis I attempted to mimic a situation of "lipid overflow" i.e. of increased lipid flux from adipose to non-adipose tissues.) For this, the lipase brummer (bmm), the *Drosophila* homolog of mammalian ATGL (Gronke et al., 2005), was overexpressed specifically in the fat body of 2%-fed larvae, by using the fat body driver Lipophorin-GAL4 (Lpp-GAL4). Overexpressing Bmm in the larval fat body did not affect wL3 body weight and did not delay development – as Lpp>bmm larvae reached wL3 at the same time as their UAS-bmm control siblings reared in the same vial, and the proportion of each genotype present in the vials corresponded to what one would expect based on Mendelian segregation (data not shown). However, this manipulation drastically reduced the size of the lipid droplets in fat body cells, with some cells being completely depleted of lipids, providing proof that overexpressing Bmm in the larval fat body did increase lipolysis (Figure 3.13.C-D). I found that mimicking a "lipid overspill" by overexpressing Bmm in the fat body of 2%-fed larvae was sufficient to induce a dramatic accumulation of lipid droplets ectopically in ring gland (Figure 3.13.A-B) and salivary gland cells (Figure 3.13.C-D), reminiscent of the effects of 0.01% dietary [yeast]. This demonstrates that increased flux of lipids to non-adipose tissues is sufficient to induce the accumulation of lipid droplets ectopically. It would be interesting to see if this also resulted in ectopic lipid droplets in muscles and whether this manipulation alone is enough to make the animals insulin resistant.

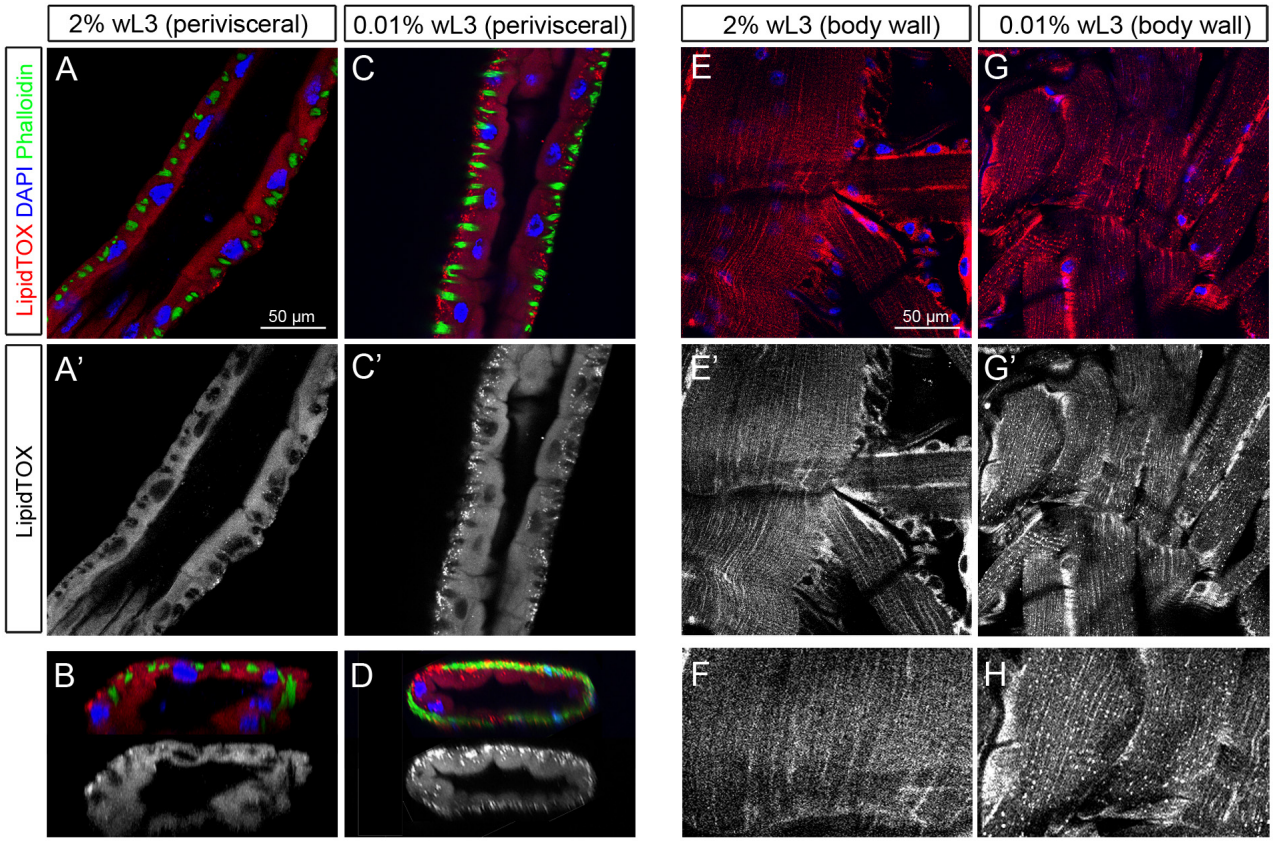

Figure 3.11: 0.01% larval dietary yeast induces the accumulation of lipid droplets ectopically in wL3 muscles. (A, A', C, C') Lipid droplets (LipidTOX punctae) accumulate in perivisceral muscles (green), but not in gut enterocytes, of 0.01%-fed larvae. Confocal micrographs show sections of the wL3 anterior midgut from larvae raised on 2%-yeast (A, A') vs. 0.01%-yeast diets (C, C'). Shown in **(B)** and **(D)** are transversal cross sections of the guts shown in (A) and (C), respectively. **(E, E', G, G')** Confocal micrographs of muscle preps show that lipid droplets (LipidTOX punctae) accumulate in body-wall muscles of wL3 0.01%-fed larvae but not of 2%-fed larvae. Shown in **(F)** and **(H)** are magnifications of (E') and (G'). Nuclei were stained with DAPI (blue).

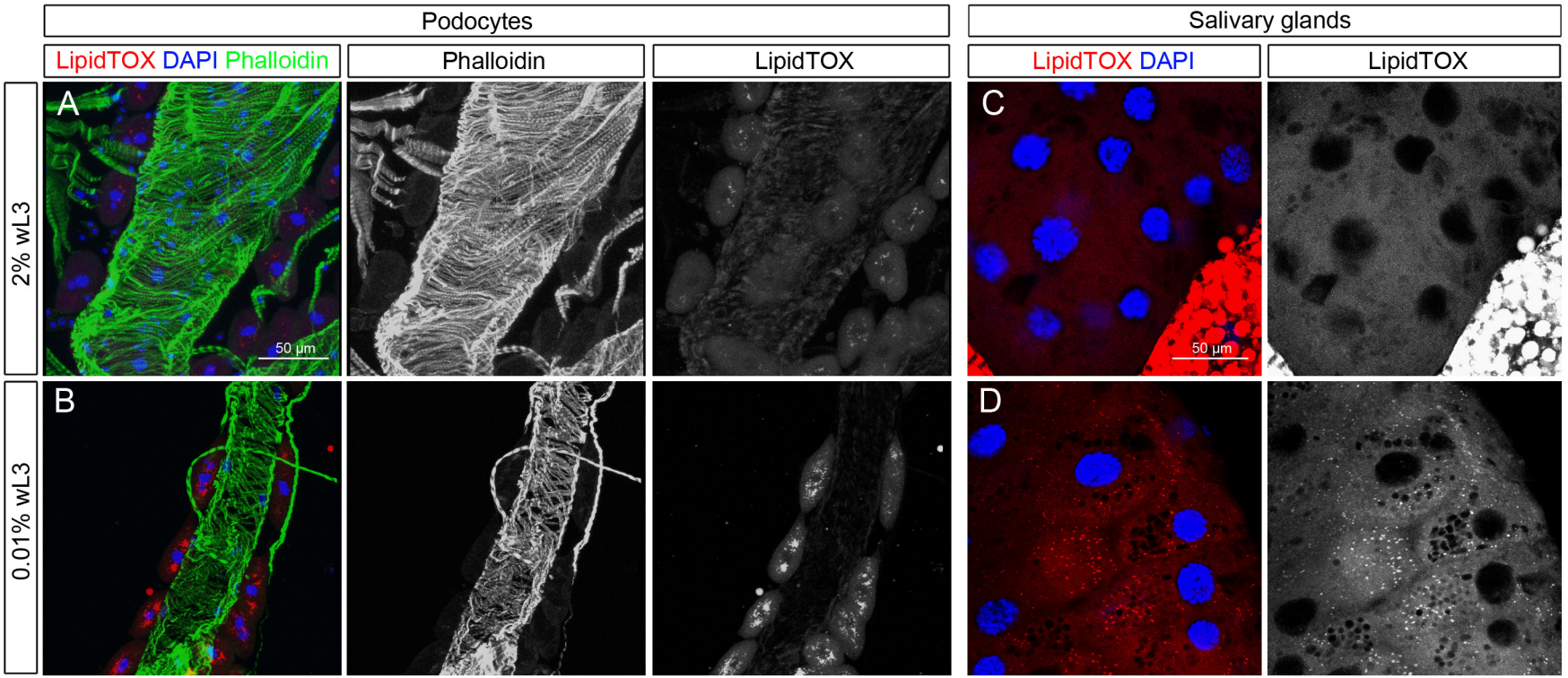

Figure 3.12: 0.01% larval dietary yeast induces the accumulation of lipid droplets ectopically in wL3 podocytes (A-B) and salivary glands (C-D). (A-B) Confocal micrographs show the dorsal vessel (cardiac muscles are stained with Phalloidin) and the podocytes, which adhere to it, on either side. **(C-D)** Confocal micrographs show high magnification images of salivary gland cells. The fat body that adheres to the salivary glands can be seen in (C; lower right corner). Lipid droplets (LipidTOX punctae) accumulate within the podocytes and salivary gland cells of 0.01%-fed wL3 but not those of 2%-fed wL3 larvae. No lipid droplets are found within the dorsal vessel, regardless of dietary [yeast]. Nuclei were stained with DAPI (blue).

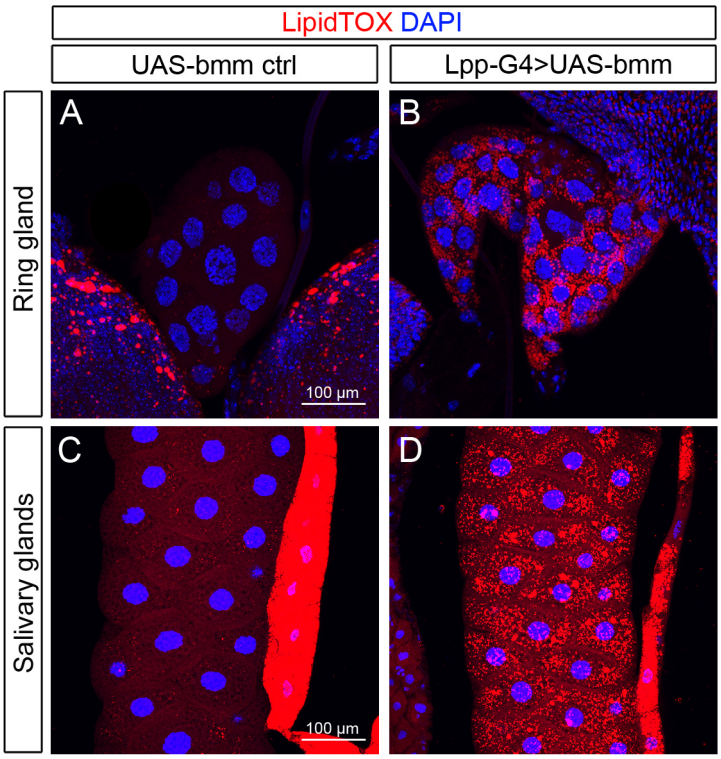

Figure 3.13: Increased lipolysis by Bmm overexpression in the fat body of 2%-fed larvae is sufficient to induce ectopic lipid droplet accumulation in the ring gland (A-B) and salivary glands (C-D). (A-B) Confocal micrographs show that lipid droplets (red; stained with LipidTOX) fill ring gland cells of Lpp>bmm animals. No lipid droplets, however, can be detected in the ring gland of UAS-bmm controls raised in the same vial. The brain lobes, which accumulate lipid droplets during L3, can be seen flanking the ring gland in (A). **(C-D)** Confocal micrographs show that lipid droplets (red; stained with LipidTOX) accumulate in the salivary glands of Lpp>bmm animals but not those of UAS-bmm controls. The fat body that adheres to the salivary glands is also shown for both experimental animals and UAS-bmm controls (on the left of the salivary glands in both images). Note that fat body cells of Lpp>bmm larvae appear "shrunken" and contain fewer and smaller lipid droplets, with some cells being completely depleted of lipid droplets.

3.7 Discussion

In this chapter, I have shown that alterations in dietary [yeast] have profound effects on developmental time, body size, organ scaling, fat storage, lipid composition and lipid distribution. I also provide evidence in support of the idea that critical weight in *Drosophila* is more than a theoretical developmental stage and it is an actual mass that remains remarkably constant over a wide range of [yeast]. These findings are in agreement with classical studies that have suggested that CW is determined by genotype, but not by environmental conditions (reviewed by Edgar, 2006). For experimental work, this can be a valuable means of accurately determining the developmental stage of larvae. Moreover, analyses of food composition revealed that cornmeal – an often-overlooked component of laboratory *Drosophila* diets – contributes a third of the total aminoacids found in our standard 2%-yeast fly diet. Presumably, this explains why larvae completed development on 0.01% yeast food, while other labs have reported similar developmental delays and decreased body size on diets containing as much as 0.6% yeast. Alternatively, differences between different labs could be explained by the differences between the different yeasts on the market (Bass et al., 2007).

Throughout this chapter, the effects reported are for male and female larvae pooled together. We have recently learned that the two genders do not always respond in the same manner to experimental manipulations (TJ Ragan and Andrew Bailey, personal communication), so by analysing mixed populations of males and females pooled together more subtle changes may have been obscured or effects of different intensities in the two genders will have been levelled to an average.

3.7.1 When resources are scarce, most will go to few

Measurements of internal organ sizes revealed that each internal organ has its own "yeast threshold" for sparing, above which the growth of the organ will be protected/prioritised. When this threshold is reached, organ growth is no longer spared. While the size of "essential" organs, such as the brain, is tightly regulated and the last to be sacrificed, organs such as the salivary glands are plastic and are the first ones to be affected by changes in the diet. That the salivary glands are negatively spared was predictable. Less expected was their relative gain in size upon increasing dietary [yeast]. Taken together, the data suggest that the salivary glands act as a deposit for excess nutrients in times of plenty and are the first to be sacrificed in the event of scarcity.

The strong negative sparing shown by tissues as critical for growth as the IPCs was initially surprising. However, this may be explained by the fact that an animal faced with low nutrient availability during extended periods of time would need to keep the insulin signalling of most tissues as low as possible. Work from our lab has shown that the important tissues are buffered against these systemic changes (Cheng et al., 2011). This would also serve a purpose in case a nutrient-deprived animal suddenly encountered a nutritional "oasis", in order to prevent the "less important" organs from taking up the very first nutrients that rush into the circulation. If this nutritional abundance persists, then the animal will have had enough time to increase its insulin production and the rest of the tissues will also benefit from the newfound resources. If it is only transitory, then this ensures that feeding the essential organs will have been prioritised. This theory is based on the assumption that if IPC size is exquisitely sensitive to dietary [yeast], this may help match their output (i.e. Dilp secretion) to nutrient availability and thus contribute to the tight link between growth and [yeast]. In order to test this, one would need to establish whether IPC size correlates with Dilp production and secretion levels. Suggesting that this may indeed be so, increasing IPC size by overexpressing a constitutively active form of dp110, i.e. $dp110^{CAAX}$, results in increased levels of circulating Dilp2 and boosts larval growth (Cheng et al., 2011).

Based on the data I present in this chapter, one can establish the following larval organ sparing hierarchy: CNS > wing discs > body = ecdysone-producing cells > IPCs = SGs ≥ podocytes. One could complete this picture by expanding the analyses to other organs, such as gut, Malpighian tubules, oenocytes and other imaginal discs, to better understand the complex link between the size of the body and the size of its constituent parts.

3.7.2 Are the effects of altering the concentration of yeast in the diet the result of altering the ratios of the different macronutrients in the diet?

Several studies have suggested that the ratio of sugar to yeast is critical, rather than absolute concentrations of the two in the diet (Lee et al., 2008; Skorupa et al., 2008). As a developing animal, the larva is likely to prioritise protein over carbohydrates or lipids. It is believed that if an animal feeds on a diet with a set ratio of sugar:protein and if this animal prioritises protein over sugar, then it will eat until its protein needs are met, regardless of the sugar it ingests. For a diet with a high sugar:protein, this means that it will eat a lot more sugar before reaching its protein intake target. This excess sugar can have devastating consequences. However, in my experiments the ratio of glucose:yeast in 0.01%-yeast food is 500:1, whereas the same ratio in food containing 0.6%-yeast and 400

grams of glucose per litre is only 68.4:1. Despite the latter's apparently more favourable glucose:yeast ratio, larval development does not proceed past the 1st larval instar stage on this food, while larvae complete development and enter metamorphosis when fed on a 0.01% yeast food. This demonstrates that, at least for larval development, absolute amounts are important. The confusion has also been fuelled by the fact that the effects of high dietary glucose can be partially rescued by concomitantly increasing the concentration of yeast. However, my results show that the converse does not apply. That is, the effects of a high glucose diet cannot be replicated by lowering [yeast] to achieve the same glucose:yeast ratio in a constant glucose background. This suggests that the effects of dietary composition are complex and go beyond the ratio of sugar:protein. The results presented here, however, do not rule out the possibility that the ratio of dietary sugar:protein can have consequences upon metabolism. In future, it would be interesting to test the effects of diets containing the same glucose:yeast ratio, but different amounts of glucose and/or yeast, on larval lipid metabolism.

3.7.3 The low-yeast larva as a model for metabolic disease

Here, I focused on studying the effects of lowering dietary [yeast] – within limits that sustain larval development – upon larval metabolic homeostasis. Previous studies have focused on the effects of high sugar diets (Musselman et al., 2011; 2013) or short periods of starvation (Cheng et al., 2011; Géminard et al., 2009). In a complementary approach, I show that two dietary yeast restriction regimes of different intensities both lead to a similar increase in overall body fat content. However, the two diets (i.e. 0.6% and 0.01%) have very different effects on lipid profile, developmental time and body size, suggesting that they are not equivalent. Moreover, a dramatic reduction of dietary yeast from 2% to 0.01% induces the accumulation of lipid droplets ectopically in non-adipose tissues such as salivary glands, ring gland and muscles (body wall as well as visceral ones). It would be interesting to test whether 0.6% also induces ectopic lipids in non-adipose larval tissues. Should it not, moderate vs. extreme restriction in dietary yeast could offer an useful paradigm in which to dissect the causes and consequences of ectopic lipid droplet accumulation. In terms of developmental delay, I showed that the effects of restricting dietary yeast are not equivalent to the effects of increasing dietary glucose. However, it would be interesting to compare the metabolic effects of a 0.01%-yeast larval diet with those of a high-sugar diet, previously described to also increase larval adiposity (Musselman et al., 2011).

Similarly to the effects of low yeast diets, mutations in IIS components result in reduced size, developmental delay and increased lipid accumulation in young adults (Böhni et al., 1999; Broughton et al., 2005; Clancy et al., 2001; Goberdhan and Wilson, 2003; Slack et al., 2010; Tatar et al., 2001; Werz et al., 2009). Although lipid measurements are mostly reported in young adults, i.e. ~3 days after eclosion, this is likely to reflect differences in larval adiposity levels, because part of the larval fat body will still be present in the young adult and adult metabolism is unlikely to have impacted significantly upon fat stores within only 3 days. Interestingly, not all manipulations/genetic backgrounds that reduce body size result in increased adiposity. Parisi et al. have recently reported that knocking down the lipid metabolic enzyme Desat1 (the fly homolog of stearoyl-CoA desaturase) in the larval fat body results in stunted growth but decreases adiposity (Parisi et al., 2013). This suggests that increased adiposity is not an inevitable consequence of decreased body size. Rather, increased adiposity appears to be a specific effect of lowered insulin signalling. Part of the mechanism underlying this link has recently been elucidated in *Drosophila* by Xu et al. who showed that IIS signalling controls the activation of fatty acids for beta-oxidation via *pudgy*, an Acyl Co-A synthetase that is under the transcriptional regulation of FOXO (Xu et al., 2012).

To date, ectopic lipid droplet accumulation in *Drosophila* larvae has only been reported in mutants for *dSeipin* (Tian et al., 2011). Interestingly, the human homolog of *dSeipin* is *BSCL2* (Berardinelli-Seip Congenital Lipodystrophy 2), mutations in which cause the most severe form of human lipodystrophy. *dSeipin* mutants have a lean phenotype (due to decreased fat storage in the fat body) and accumulate lipid droplets ectopically in the salivary glands. This is similar to the effects of 0.01% larval dietary yeast and provides evidence in support of the idea that impaired/lower storage capacity in the fat body can lead to the accumulation of lipids ectopically. In future, one could test whether increasing the fat body storage capacity in 0.01%-fed larvae (for example by increasing IIS signalling in fat body cells) can resolve the ectopic lipids. Conversely, the prediction is that decreasing the storage capacity in control 2%-fed larvae would also result in ectopic lipid droplets. Suggesting that the ability of the fat body to store lipids plays an essential role, Musselman et al. have recently reported that preventing fat body lipid synthesis resulted in lethality on an "obesogenic" high sugar diet (Musselman et al., 2013). Conversely, increasing fat storage (by knocking down *king tubby* in the fat body) improved glucose homeostasis in high sugar-fed larvae (Musselman et al., 2013).

Bmm overexpression in the fat body of 2%-fed larvae revealed that increased lipolysis is sufficient to induce the accumulation of lipid droplets ectopically in wL3 salivary glands

and ring gland. It would be interesting to perform the converse experiment of lowering lipolysis in the fat body of 0.01%-fed larvae and test whether this too is sufficient to abrogate the accumulation of lipids in non-adipose tissues. Preliminary ^{1}H NMR analyses of larval haemolymph have revealed that a 0.01%-yeast diet induces marked changes in the concentrations of haemolymph polar metabolites, including trehalose, lactate and several amino acids, compared to the control 2%-yeast diet (data not shown). Surprisingly, these analyses have further shown that increasing lipolysis in the fat body of control 2%-fed larvae – which I found is sufficient to induce ectopic lipid accumulation in salivary glands and ring gland – also replicates part of the changes in haemolyph metabolite profile observed in larvae raised on 0.01% dietary yeast. These observations complement those reported by Girousse et al. (2013) in mice. Moreover, these preliminary results suggest that the connection between increased lipid flux, ectopic lipid droplets and metabolic homeostasis is conserved between flies and mammals. In future, this model could be used in conjunction with the powerful genetic tools available in *Drosophila* to study the still-intriguing connection between increased adiposity and associated metabolic complications.

It has been argued that, when dietary [yeast] is reduced, flies partially compensate for this by increasing food intake (Carvalho et al., 2005). Food lipid analyses revealed that C18:2 makes up half of the FAs in dietary TAG and that it mostly originates from cornmeal, a dietary component that remained constant between the different diets. In addition, flies cannot synthesise, elongate or desaturate C18:2 (Draper et al., 2000; Rapport and Samuelson, 1983; Shen et al., 2010). This means that all the C18:2 present in stored TAG must be derived from absorption in the gut. In light of this, the changes in relative C18:2 proportions in the TAG fractions from the different groups (i.e. 0.6%-yeast doubled the percentage of C18:2 in TAG compared to 9%-yeast, while 0.01%-yeast halved it compared to 2%-yeast) may suggest that, for animals reared on 0.6% yeast the increase in adiposity could be due to increased uptake of dietary fats, while for animals reared on 0.01% yeast, the increase in adiposity may be the result of increased *de novo* synthesis. This could be addressed by supplementing larval diet with ^{13}C-labeled glucose or ^{13}C-labbeled acetate and quantifying incorporation into lipids on low- vs. control/high-yeast diets. Further comparing the fatty acid composition of the food with that in wL3 lipid stores provides insight into larval lipid metabolism. In food lipids, C16:1 accounts for 1-3% of the total fatty acids, while C14 accounts for less than 0.2% of the total. Strikingly, in wL3 TAG, each C16:1 and C14 account for 20% of the total fatty acids. This has two implications. Firstly, that the proportion of C16:1 and, therefore, the desaturation index (i.e. C16:1/C16) is

likely to reflect the activity of fly Desat1. This suggests that the C16:1/C16 ratio may be a valid readout for the activity of this enzyme in larvae. Secondly, that a significant proportion of larval lipid stores are likely to be derived from *de novo* lipid synthesis.

In summary, the work in this chapter shows that dietary yeast restriction during *Drosophila* larval development has dramatic consequences on larval size, development and lipid metabolism. Specifically, I show that lowering the concentration of yeast in the larval diet reduces final body size and growth rate, delays development, alters body proportions/organ sparing, increases adiposity and alters the fatty acid composition of stored fat. 0.01%-induced increase in adiposity also correlated with the accumulation of lipid droplets ectopically in non-adipose tissues, reminiscent of "lipid overflow" phenotypes. Importantly, these effects cannot be explained (fully) as a function of altering the glucose:yeast ratio in the diet.

Chapter 4

Effects of larval diet on adult size and organ scaling

4 Effects of larval diet on adult size and organ scaling

4.1 Introduction

In this chapter I set out to investigate how altered larval growth and development translates into the adult. During metamorphosis, most larval tissues are eliminated by apoptosis and adult structures form from imaginal tissues that are specified during embryogenesis. These tissues then grow and develop during larval life. Therefore, the role of the larva is not only to acquire sufficient energy to support metamorphosis, but also to incubate the tissues from which the future adult will form. In this sense, one could say that the larva is like the "mother of the adult", or that the larva acts as a "placenta" for the developing and growing imaginal tissues. In light of this link between larva and adult, I examined the impact of larval dietary [yeast], not only on adult body size but also on the relative sizes of adult external structures and some of the internal organs.

In females, there is a complicated relationship between reproduction and metabolism, as females invest a considerable amount of their resources into the production of eggs (Cognigni et al., 2011; Djawdan et al., 1996; Min et al., 2006). Because of this, effects can often be exaggerated or muted – for example, embryos are rich in nutrients that can be resorbed should the need arise –, which adds an extra layer of complexity for studies of metabolism. In order to avoid the potential confounding effects of altered reproduction on adult metabolism, males, which invest far less resources in reproduction, were used for most of this work. However, key results were confirmed in females to rule out male only effects.

In order to establish a *Drosophila* model for the long-term effects of inadequate nutrition during development, larvae were reared on diets with varying concentrations of yeast, while maintaining all other components constant. All adults were collected within 24 hours of eclosion and treated equally. This experimental approach allowed us to assign effects observed in the adult to larval dietary [yeast]. In the text, but especially on the figures- where space is limited-, I refer to adults by the diet they were reared on as larvae such that "n% adults" refers to adult animals that were reared on a diet containing "n%" yeast during larval development.

4.2 Results: Larval dietary [yeast] programmes body mass during adulthood

To assess the impact of larval dietary [yeast] on adult size, I tested 5 larval diets, containing 0.01%, 0.1%, 0.6%, 2% and 9% yeast, on a fixed 9% yeast adult diet. Larvae reared on low dietary [yeast] gave rise to small adults that weighed significantly less than those from control larvae fed on 2% yeast (Figure 4.1.A). The largest reduction was seen for the lowest yeast, i.e. 0.01%. These adults weighed 40% less than control 2% adult males. These differences were not transient "developmental hangovers" present only at eclosion, as they were maintained throughout adult life, regardless of the adult diet. Neither was the difference in body weight due to differences in water content, as revealed by comparing 3-day old animals' dry weights to their wet body weights (Figure 4.1.B). Female body weight was also significantly decreased by low larval dietary [yeast], with the weight of 9-day old 0.01% females being half that of same-age 2% females (Figure 4.1.C). Hence, both genders show similar effects of larval dietary yeast. Taken together, these results show that larval dietary [yeast] programmes adult body mass.

The weight of newly-eclosed adult flies decreased sharply during the first 24 hours of adult life, but only for flies that had fed on 0.6% dietary yeast or above during larval development (Figure 4.2.A-B). This effect was stronger if the larvae were reared at 29^{O}C (Figure 4.2.B), as compared to 25^{O}C (Figure 4.2.A), which was the temperature used for most of this work, unless otherwise noted. Despite the fact that newly-eclosed adults started off with similar weights, adults from larvae reared at 29^{O}C lost up to 20 % of their eclosion body mass during the ensuing 24hrs, as compared to the 9-13% lost by their counterparts reared at 25^{O}C as larvae. Adult animals reared on low-yeast larval diets (i.e. 0.06% or 0.01% yeast), however, maintained or even increased their eclosion weight during the same interval.

4.2.1 The "cost of metamorphosis"

Adult weights at two days correlated with the final weight of the larva, but not, as one might expect, in a linear fashion (Table 4.4.1). Animals reared on diets containing down to 0.6% yeast lost approximately half of their body mass during metamorphosis. However, animals reared on food containing 0.01% yeast only lost a third. I refer to this readout of how much of the larval mass is transferred on to the adult animal as the "cost of metamorphosis". These results are slightly affected by, but do not change, if wet body weights at eclosion (instead of those at two days) are used for the calculation (data not

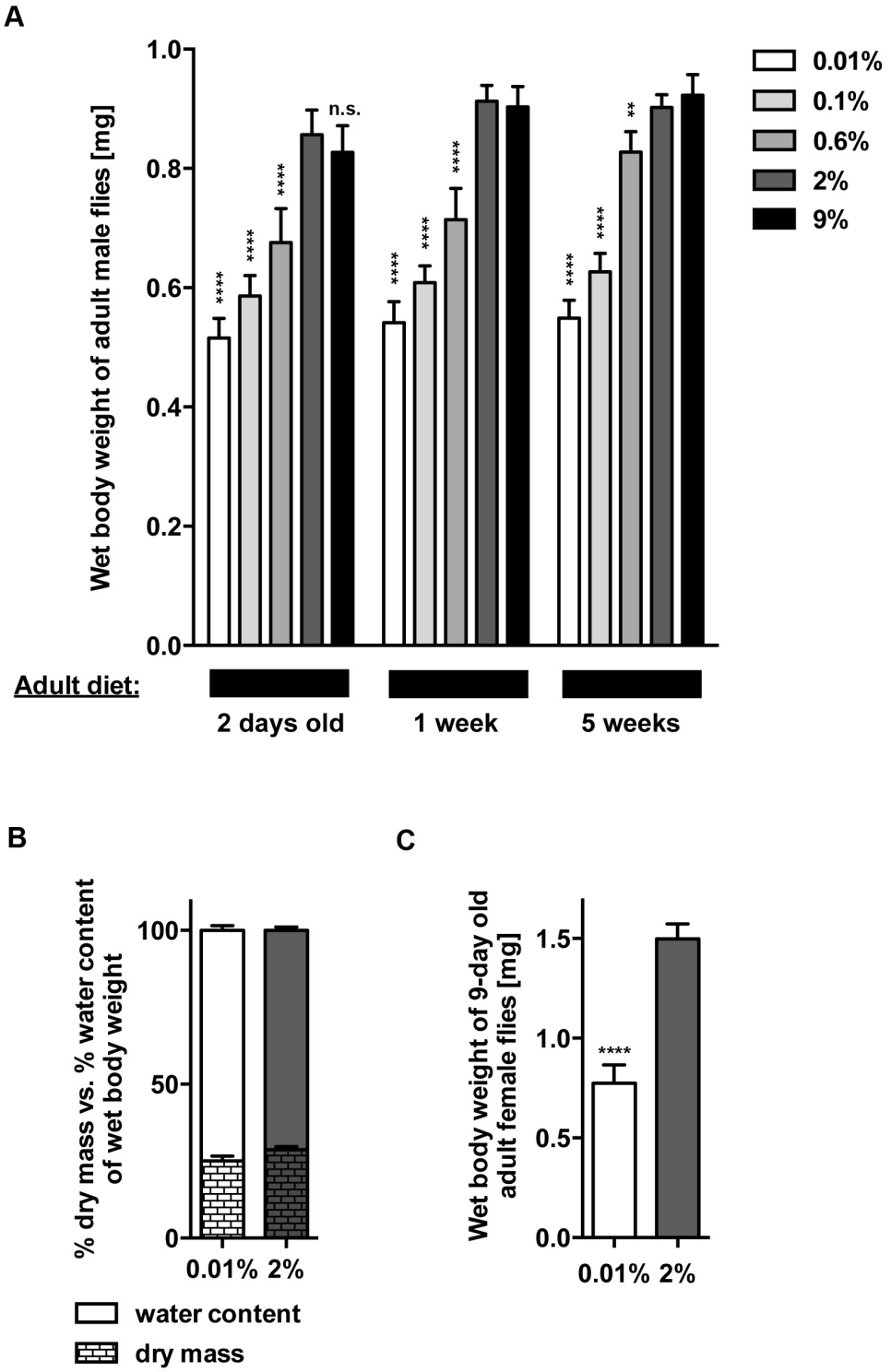

Figure 4.1: Larval dietary [yeast] programmes adult body weight. (A) Histogram shows wet weights of adult male flies that were raised on diets containing varying concentrations of yeast as larvae. Adult male weight was assessed at 2 days, 1 week and 5 weeks after eclosion. All adults were fed on a 9%-yeast diet. **(B)** Body composition of 3-day old males flies, from larvae raised on 0.01% or 2%-yeast diet, in terms of water content vs. dry mass (both show as percentage of wet weight). **(C)** Wet weights of 9-day old adult female flies that were raised on either a 0.01% or a 2%-yeast diet as larvae.

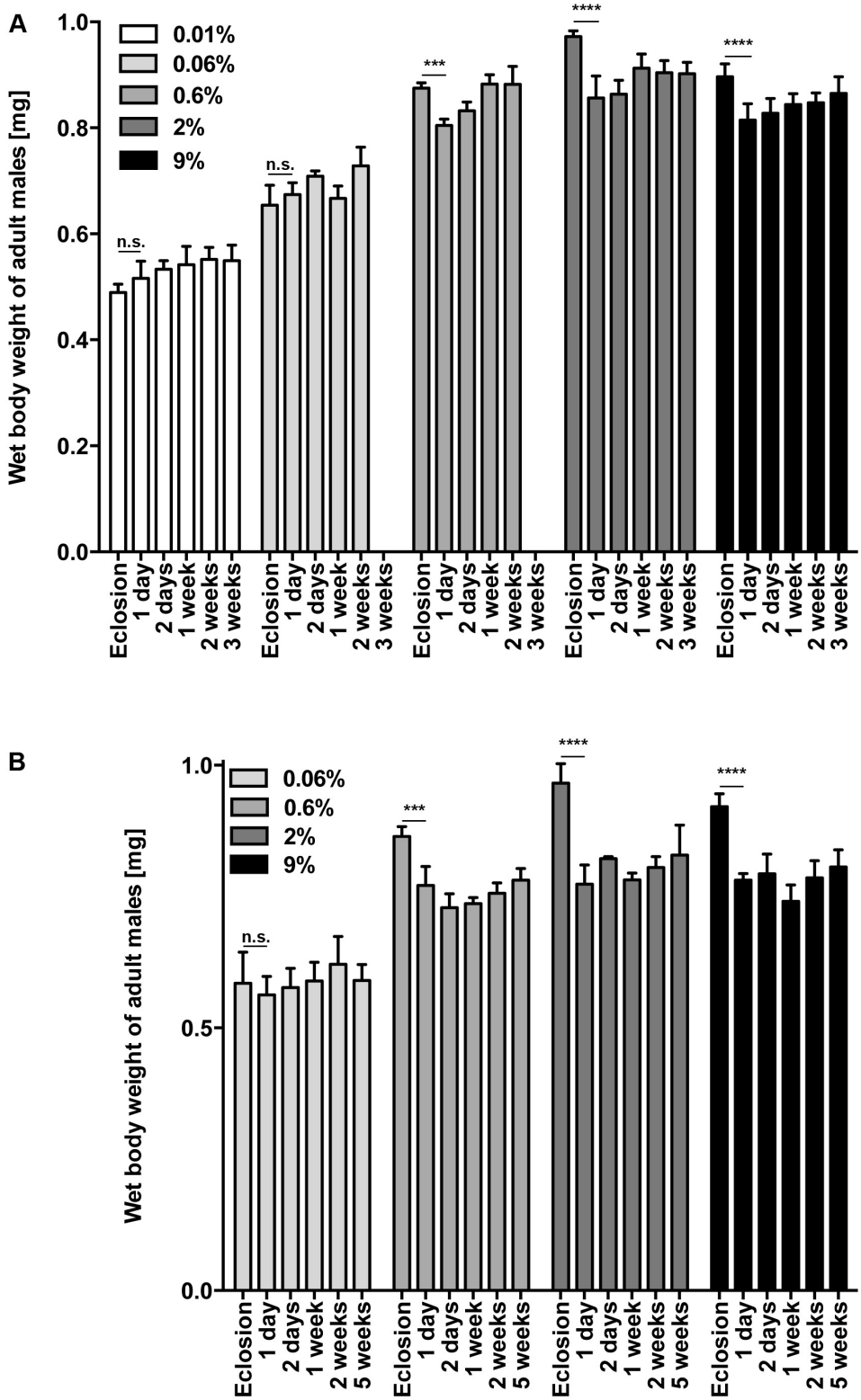

Figure 4.2: The weight of newly-eclosed adult male flies decreases sharply during the first 24 hours of adult life. This phenomenon is absent in adults from undernourished larvae (i.e. larval dietary [yeast] below 0.6%). **(A)** and **(B)** show wet weights of adult males from eclosion up to 5 weeks of life. Larvae were reared on diets containing varying yeast concentrations (from 0.01% to 9%). All adults were maintained on a 9%-yeast diet. **(A)** Larvae and adults were raised at 25℃. **(B)** Larvae were raised at 29℃ and adults were transferred to 18℃ 36-48 h after eclosion.

shown). This suggests that larval undernutrition can lead to animals more efficiently transforming larval body mass into adult mass.

Table 4.4.1: The "cost of metamorphosis". Larvae raised on a 0.01%-yeast diet lose only a third of their wL3 weight during metamorphosis, unlike larvae raised on diets containing 0.6%, 2% 9% yeast, which lose half of their wL3 weight during metamorphosis.

Larval diet	**0.01%**	**0.6%**	**2%**	**9%**
Average weight at wL3 [mg]	0.75	1.43	1.76	1.75
Average weight at eclosion [mg]	0.5	0.67	0.85	0.82
⇒ % weight at eclosion/ weight at WPP	66.7%	46.8%	48.3%	46.9%
⇒ "Cost of metamorphosis"	33.3%	53.2%	51.7%	53.1%

4.3 Disc-derived head and thoracic structures are positively spared

To measure the effects of larval [yeast] on adult organ size, I used 10-day old males that fed on 9% yeast food since eclosion, unless otherwise stated. Measurements of adult wing area corroborated the observations made in the Chapter 3 for the larval wing disc volume (see 3.4.1), consistent with most disc proliferation occurring in the larva. I found that reductions in larval dietary [yeast] down to 0.6% did not reduce absolute wing area (Figure 4.3.A). When larval dietary [yeast] was dropped to 0.2%, however, absolute wing area decreased by 15%, but was still positively spared, relative to body weight which was reduced by ~30% (Figure 4.3.G). I then asked whether this reduction in absolute wing area, was due to decreased cell number, decreased cell size, or both. Quantifications of cell density in a pre-defined area of the wing established that there was no difference in cell density (Figure 4.3.B). This indicates that cell number and, therefore, proliferation, not cell growth, is primarily altered. Eye areas, although more variable, followed the same pattern as the wings in terms of both, absolute numbers and allometry (Figure 4.3.C and H). The area of the scutellum and the distance between the eyes at the posterior base of the antennae were also measured as proxies for thorax size and head size, respectively. As for wing and eye areas, reducing larval dietary [yeast] down to 0.6% had no effect on their absolute sizes, suggesting that head size and thorax size were also positively spared (Figure 4.3.D-E and I). Lastly, the absolute length of the femur decreased slightly, but significantly, when larval dietary [yeast] was decreased from 2% to 0.6% (Figure 4.3.F). Nonetheless, in terms of allometry, the femur too fell into the category of a positively spared structure (data not shown).

Together, these results show that the reduction in overall body size, observed in adults fed on low [yeast] larval diets, is not accompanied by a similar shrinkage of all body parts. The sizes of all head and thoracic structures tested (all derivatives of imaginal discs) were positively spared relative to the reduction in body weight. Assuming that the whole is the sum of the parts, we can infer that the size of the only remaining adult structure, the abdomen, is negatively spared by low larval [yeast]. Because of its irregular shape and non-rigid exoskeleton, no direct measurements of the overall size of the abdomen were made. Nevertheless, this line of thought led me to investigate the sizes of organs found inside the abdominal cavity.

4.4 The adult fat body and Malpighian tubules are not positively spared

The sizes of the internal organs inside the abdomen were measured in 10-day males that had fed on either 0.01% or on 2% [yeast] as larvae. Body weight of 0.01% adult males was reduced by ~40%, compared to that of age-matched 2% males (Figure 4.4.A). Beginning with abdominal fat body, using nuclear volume as a proxy for cell size, the nuclear volume was reduced by 40%, indicating neutral sparing of the fat body (Figure 4.4.A). In contrast, for Malpighian tubules the nuclear volume was 65% smaller in 0.01% males compared to 2%, showing that as adult body size decreases, Malpighian tubules are negatively spared (Figure 4.4.A). Hence, the internal organs of the abdomen are spared less than disc-derived external structures of the head and thorax.

4.5 The IPCs continue to grow during adulthood

The IPCs are one of the key regulators of growth and metabolic homeostasis in the fly. As part of the CNS, the IPCs are one of the few "organs" that carry over from the larva to the adult i.e. there are no separate larval and adult cell populations. As such, it is possible that alterations in larval diet could affect adult metabolic homeostasis directly via long-term programming of the IPCs. At the end of larval development, the IPCs showed strong negative sparing, even with only moderate reductions in dietary [yeast] (see 3.4.1). In adult males, I found that the IPC cell soma volume was reduced by ~60% as a result of decreasing larval [yeast] from 2% to 0.01% (Figure 4.4.A). This shows that the IPCs are still negatively spared in the adult. Hence, the negative sparing suffered by IPCs in larvae is not corrected during subsequent metamorphosis.

I then measured IPC soma volumes in 4-week old animals fed from eclosion on either a 0.6% yeast, 0.6%HG (0.6% yeast food containing high glucose i.e. containing 400 grams glucose/L; HG=high glucose) or 9%HG diet. These analyses revealed that negatively

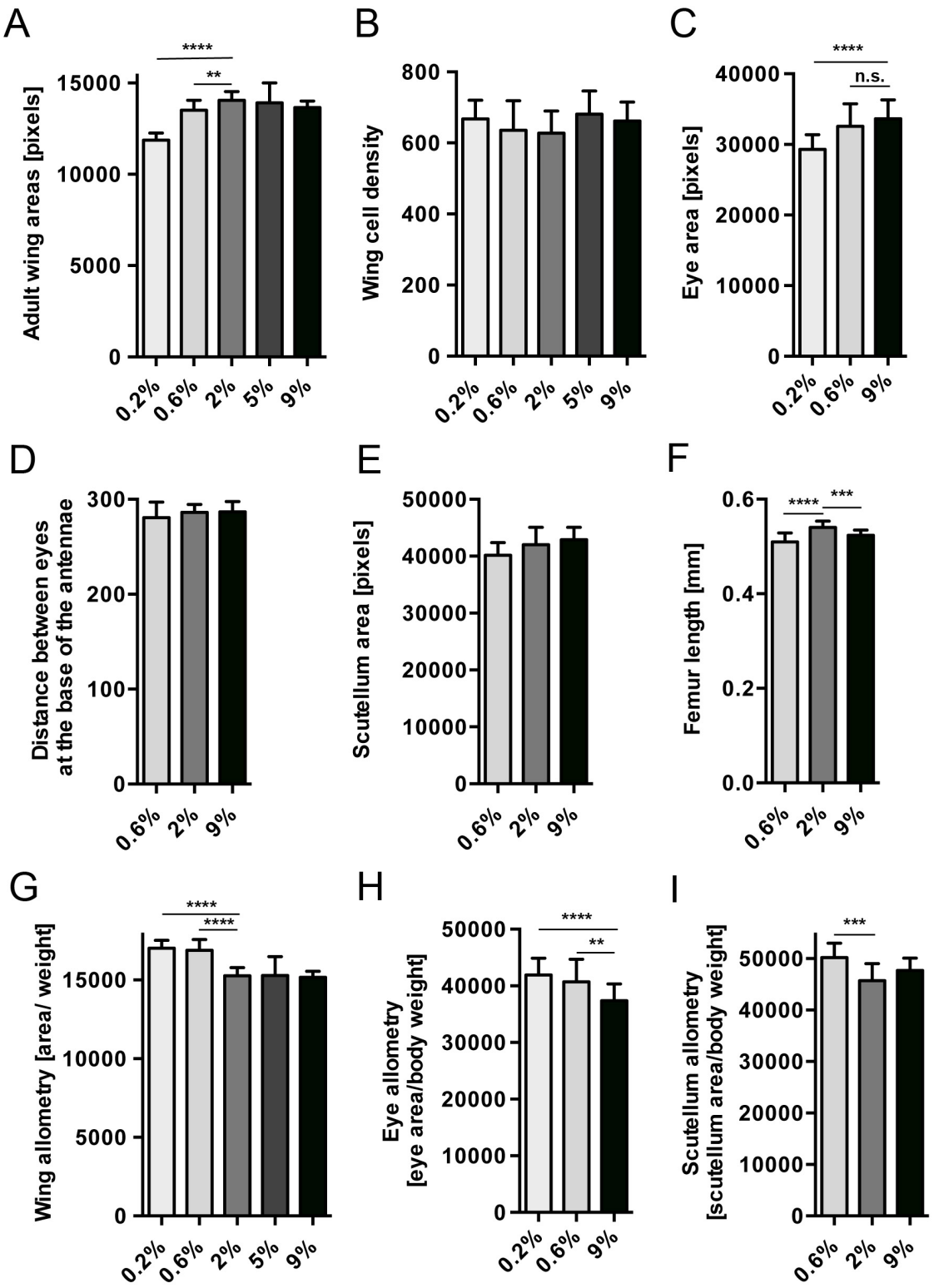

Figure 4.3:Larval nutrition programmes adult external organ proportions. (A and C-F) Measurements of external disc-derived adult structures for adults from larvae raised on low-, control- or high-yeast diets. **(B)** Lack of an effect of larval diet on cell density quantified in a pre-defined area of the wing. **(G-I)** Effects of larval dietary [yeast] on of wing, eye and scutellum allometry, respectively, i.e. size of each structure relative to overall adult body size.

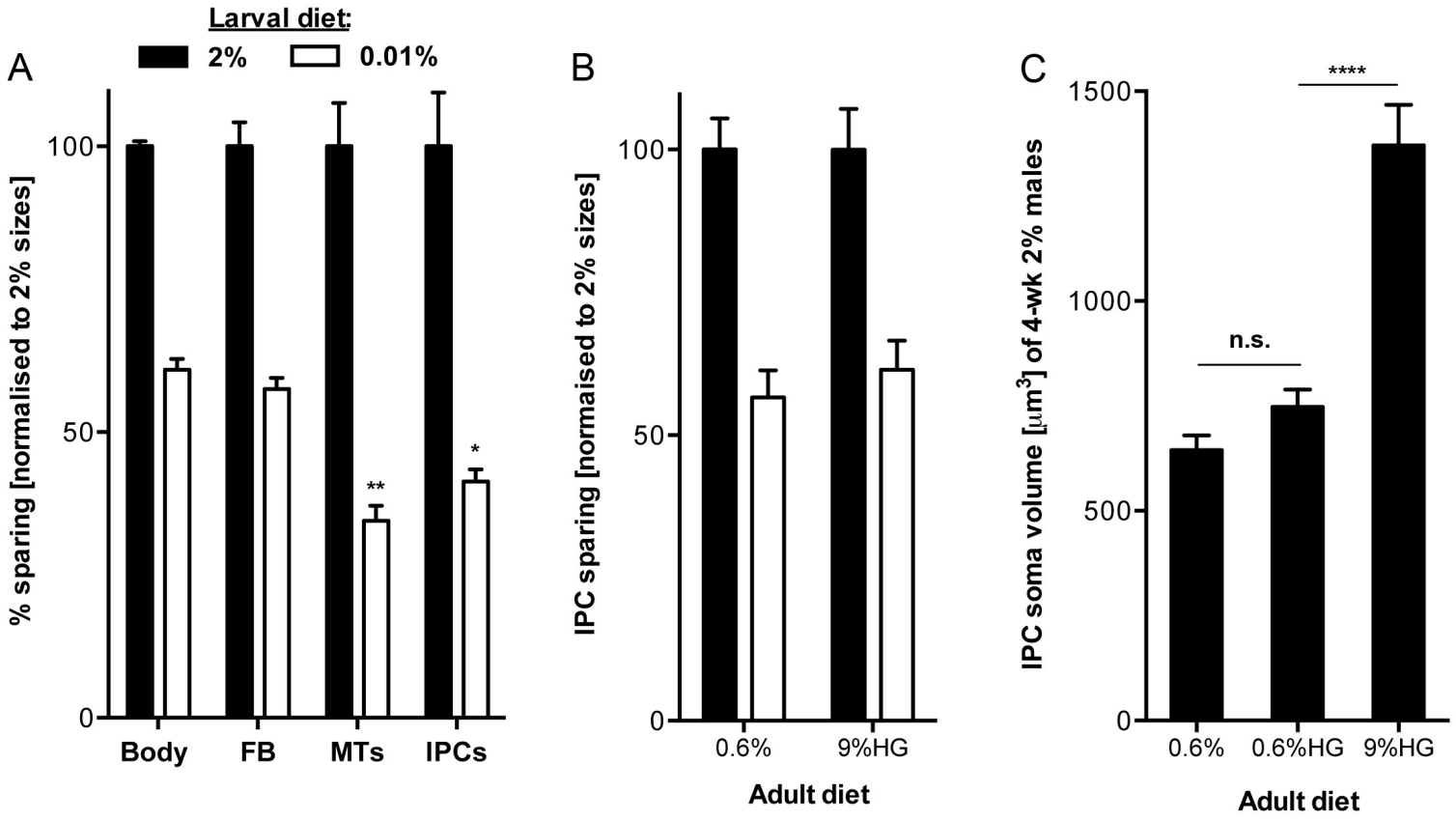

Figure 4.4:Larval dietary [yeast] programmes adult internal organ sparing. (A) Histogram shows the body weight and volumes of fat body (FB), Malpighian tubules (MTs) and insulin-producing cells (IPCs) of 10-day old 0.01% males (white bars) as a percentage of body and organ sizes of age-matched 2% males (black bars). **(B)** Histogram shows the IPC soma volume of 4-week 0.01% males fed on either a 0.6%-yeast diet or a 9%HG diet since eclosion, as a percentage of the IPC soma volume of age-matched 2% males fed on the same adult diet. **(C)** Histogram shows the IPC soma volume (in μm^3) of 4-week old 2% males fed on either a 0.6%-yeast diet, a 0.6%HG diet or a 9%HG diet since eclosion. HG = high glucose i.e. diet contains 40% (w/v) glucose. In this figure, error bars represent 1 S.E.M.

spared adult IPCs catch up in relative size perfectly, such that they acquire neutral sparing relative to body weight within one month of eclosion (Figure 4.4.B). These experiments also revealed that the absolute size of 4-week adult IPCs depends on the adult diet (Figure 4.4.C). Increasing the glucose content of the adult diet had no effect on IPC soma volume. However, increasing [yeast] from 0.6% to 9% in the adult diet doubled the volume of the IPCs by 4 weeks of age. Intriguingly, the IPCs of 0.01% adults only grew to 60% of the size of 2% control animals' IPCs, regardless of whether adults had fed on a 0.6% or a 9%HG diet. In *Drosophila*, the concept that internal adult organs continue to grow and that this growth is modulated by adult diet is new. Similar responsiveness to the adult nutritional environment was recently described for the gut in young adults (O'Brien et al., 2011), but the IPCs seem to respond to adult diet much slower than the gut.

4.6 Low larval dietary yeast did not affect adult fertility (nor fecundity)

I next sought to understand whether, despite 0.01% adults being viable, their very small body size affected the animals' fertility/fecundity. In *Drosophila*, the most straightforward assay for this is to count the number of eggs females lay per day. For this, I mated 0.01% and 2% 1-week females to young 2% males, allowed them to mate for 72 hours and then quantified egg laying over three consecutive 24-hour periods. The pooled results of this analysis are presented in Figure 4.5. A 9%HG (i.e. 9% yeast + high glucose) adult diet decreased egg laying significantly compared to a 0.6% yeast diet, regardless of larval diet. This indicates that, as defined by dietary restriction/fecundity studies from other labs (Bass et al., 2007), the 9%HG diet is toxic with respect to fecundity. Larval dietary [yeast], however, did not affect egg laying significantly, regardless of whether adults were fed on 0.6% yeast food or on 9%HG food.

To test whether male fertility was affected by larval dietary yeast, 0.01% and 2% 1-week females were also mated with young 0.01% males and the numbers of eggs laid by the two groups of females were compared with those laid by their siblings mated with 2% males. Male larval dietary [yeast] had no impact on the number of eggs that females laid over three consecutive 24-hour periods (Figure 4.5). All embryos (i.e. collected from the four different groups of females) were then aged for 28 hours at 25°C, but no difference was observed in the number of larvae hatching from embryos fertilised by 2% males compared to those fertilised by 0.01% males (data not shown). Therefore, larval dietary [yeast] appears to have no significant effect upon male fertility, even at the 0.01% level.

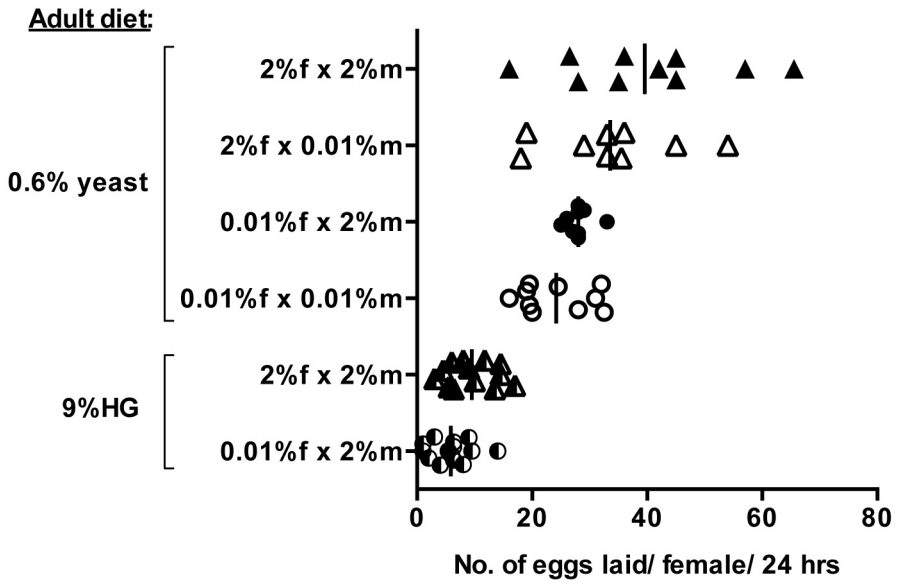

Figure 4.5: Severe yeast restriction during larval life does not impact upon adult female fecundity or male fertility. Graph shows average number of eggs laid per female over 24 h. 1-week 2% and 0.01% females (labelled as 2%f and 0.01%f, respectively), fed on either a 0.6%-yeast or a 9%HG adult diet, were mated with young males (2% m refers to 2% males and 0.01%m to 0.01% males). Egg laying at 25℃ was quantified over three consecutive 24-hour periods. Individual data points and mean are shown for each group.

4.7 Discussion

Here I show that larval dietary [yeast] programmes adult body weight, not only at eclosion but throughout adulthood. Newly-eclosed males lost 10-20% of their body mass during the first 24 hrs of adult life, an effect that was absent for animals that were undernourished during development. This could be attributed to post-eclosion diuretic water loss. Water content has been proposed to facilitate insect metamorphosis, but males have been found to excrete water upon eclosion to facilitate flight (Bushman et al., 1987; Folk et al., 2001). Although the duration of metamorphosis was not affected by larval dietary [yeast], this may indicate that part of the differences in wL3 weight between undernourished and control larvae may be due to differences in water content. Alternatively, a smaller pupa will have a higher surface to volume ratio, which could result in smaller pupae losing water at a faster rate than bigger ones. To distinguish between these possibilities, one could assess the water content of wL3 reared on low-yeast vs. high-yeast foods. In addition, the dramatic weight loss experienced by newly-eclosed flies during the first 24 hours of life tells a cautionary tale about assessing the weight of newly-eclosed adults when their weight has stabilised.

Surprisingly, animals reared on 0.01% larval dietary yeast lose only one third of their wL3 body mass during metamorphosis, significantly less than animals reared on 0.6% and above, which lose half of their wL3 body mass by 2 days after eclosion. The bases for this difference are unknown, but may be explained by either decreased water content of 0.01%-fed wL3 larvae or the increased adiposity of 0.01%-fed wL3. The latter will have equipped 0.01% larvae with more energy for metamorphosis, which could lead to 0.01% animals needing to use less of their lean body mass to support this energy-demanding, non-feeding stage. These results also suggest that there is no direct relationship that one can use to estimate final larval body based on the size of the adult, as the relationship between the two can, and does change, with larval nutritional status.

Adults from larvae raised on low-yeast diets were smaller and weighed significantly less at eclosion, as well as throughout adult life. It is sometimes assumed that the mass of the adult insect is set once the animal reaches maturity because the insect body is enclosed in a stiff exoskeleton. The bounding exoskeleton defines insects' adult body size in the same way that the mammalian skeleton defines an animal's length/height. However, the differences observed in adult body size are not because adults cannot gain weight. The adult abdomen, in particular, is very flexible with an elastic pleura linking the tergites and

sternites. Indeed, I observed that adults from larvae raised on 0.6% yeast increased their weight by as much as 20% by 5 weeks of age, compared to their weight at 2 days after eclosion.

My results show that adults raised as larvae on low [yeast] diets are not simply isometrically scaled versions of those raised on standard 2% yeast. Instead, disc-derived structures of head and thorax are positively spared, while a subset of abdominal organs, such as the Malpighian tubules, are negatively spared. This is in line with negative sparing observed in larval podocytes when larvae were reared on 0.01% (see 3.4.1). The Malpighian tubules and the podocytes form a two-component filtering/excretory system, equivalent in function to the mammalian kidneys. This could have important physiological consequences during adulthood on water balance and excretion. To test the physiological relevance of the negative sparing of the excretory system, one could start by comparing the resistance to starvation of adults raised on low-yeast food as larvae to that of adults raised on control 2%-yeast food as larvae.

Surprisingly, my results show that the IPCs can and do grow during adulthood, in a manner that is dependent on the composition of the adult diet. At the time, it had not yet been shown that any adult organ has the potential to continue growing. Since then, research from David Bilder's laboratory has described that the adult gut undergoes dramatic growth during the first days after eclosion and that this growth is nutrient-dependent (O'Brien et al., 2011). However, the study by O'Brien et al. used relatively young adults, i.e. less than 1 week old. These animals are still "maturing" and the larval fat body is yet to be eliminated (Aguila et al., 2007). Here I took a step further and showed that nutrition can still regulate the size of the IPCs of a fully "matured" animal. My results, together with those of O'Brien et al. (2011) argue for the importance of measuring organ sizes at different time points. In the larva, Dilp secretion from larval IPCs is controlled remotely via a yet unidentified signal secreted from the FB in response to amino acid sensing (Géminard et al., 2009). Much less is known about adult IPCs. Since the adult fat body was neutrally spared and the size of fat body cell nuclei does not appear to change during adulthood (P. Pachnis and A.P. Gould, personal communication & data not shown), it would be interesting to test how the initial negative sparing of the IPCs and their changes in size during adulthood correlate with output/Dilp production. Moreover, it would also be interesting to measure the size of the adult gut, as another organ that is responsive to adult nutrition, and establish whether it too changes from negative/neutral sparing to positive sparing during adulthood. The data presented in this chapter demonstrates that the distinction between whether organs are negatively, neutrally and

positively spared is critically dependent upon adult age and adult diet. The larval (see Chapter 3) and adult data support the idea that there is a sparing hierarchy with a "threshold for sparing" for each organ. To date, we have no data on whether this hierarchy is fixed, or whether it changes depending upon the sparing stimulus i.e. impaired growth due to different causes, such as low yeast, high dietary sugar, low cornmeal, low oxygen or drugs/toxins.

Finally, despite their much smaller body size, egg laying was not compromised in 0.01% females compared to 2%. This suggests that any metabolic/physiological changes that these animals may display are not due to altered investment into reproduction. Importantly, male fertility was also unaffected by larval diet. This shows that despite the dramatic effect low [yeast] larval diets have on body size, adult function is not fundamentally compromised. However, fecundity analyses revealed that the high content of glucose used for the "high glucose" foods in this work are "toxic" with respect to female fecundity by the definition of Bass et al., as they decrease egg laying (Bass et al., 2007).

Chapter 5

Effects of larval diet on adult lipid metabolism

5 Effects of larval diet on adult lipid metabolism

5.1 Introduction

Most detailed studies of physiology and metabolism in *Drosophila* have focused on the larva rather than the adult. However, the physiology of the adult is expected to be in stark contrast to that of a developing, fast growing larva. In this chapter, I begin to investigate the effects of larval dietary [yeast] on adult physiology and lipid metabolism. The larval fat body is still present in the adult up to 7 days after eclosion (Aguila et al., 2007) and the adult fat body (and not only) undergoes dramatic changes during the first week of adult life (Johnson and Butterworth, 1985). Therefore, to focus on the physiology of mature adults, animals of older than 7 days will tend to be used. Physiological and metabolic outcomes were assessed in adults fed on one of four adult diets since eclosion: 0.6% yeast, 0.6% high glucose (0.6%HG), 9% yeast or 9% yeast high glucose (9%HG). The 0.6% yeast and 9% yeast diets contained "normal" glucose i.e. 5.85% (w/v), the amount present in the *Drosophila* foods used in the lab. HG diets contained 40% (w/v) glucose.

5.2 Results: Low larval dietary [yeast] induces ectopic lipid accumulation in the adult hindgut

In Chapter 3, I showed that larval dietary yeast restriction increased larval adiposity and induced the accumulation of excess lipids ectopically in non-adipose tissues (see 3.5.2). To begin to understand the potential long-term effects of larval [yeast] upon adult metabolism, lipid distribution among internal adult organs was investigated. One- and two-week 0.01% and 2% adults fed on one of the four adult diets were dissected and stained with the neutral lipid stain LipidTOX.

5.2.1 Lipids accumulate in the hindgut of 0.01% adults, regardless of the adult diet

LipidTOX stainings revealed the accumulation of neutral lipids in the form of lipid droplets in several non-adipose tissues of the adult (data not shown). These include the testes, male accessory glands, proventriculus, posterior midgut and rectal ampulla. It is likely that some of these lipid droplet accumulations, such as those in the posterior midgut, are influenced by adult diet. However, this was not examined further as lipid droplets at all of these sites were not modified by larval diet. No lipid droplets were observed in adult muscles, regardless of larval or adult diet (data not shown).

Surprisingly, 0.01% larval dietary yeast induced the accumulation of sizeable amounts of lipids in the hindguts of 1-week males, regardless of adult diet, while no lipids were found in the hindguts of age-matched 2% males (Figure 5.1.A-B), on any of the four adult diets. Co-staining with LipidTOX and Phalloidin confirmed that the lipids accumulated in hindgut enterocytes and not in the perivisceral muscle cells that surround the hindgut (Figure 5.1.C-D). Lipid droplets were also detected in the hindguts of once-mated 1-week 0.01% females but were absent from the hindguts of age-matched 2% females (data not shown). This indicates that larval dietary [yeast] programming of ectopic lipids in the adult hindgut reflects a mechanism common to both genders. The hindgut is the third and most posterior segment of the *Drosophila* gastrointestinal tract. The Malpighian tubules, which are part of the fly's excretory system, filter haemolymph and empty their contents into the intestinal tract at the midgut to hindgut junction. The hindgut, therefore, processes both the digestive products from the midgut and the products of haemolymph filtration. These are excreted together through the posterior end of the hindgut as waste matter.

The observations from Chapter 3 that 0.01% yeast larval diet induced ectopic lipid accumulation in several larval organs (even though no ectopic lipids were detected in the larval hindgut; see 3.5.2), prompted the possibility that the ectopic lipids present in the hindguts of 0.01% adults could be the result of carry over from larvae. However, dissections and lipid stainings performed on hindguts from newly-eclosed males established that adult hindguts contained no lipids at eclosion, regardless of larval diet (Figure 5.2). This shows that adult hindgut lipid droplets first appear during adulthood and represent a genuine long-term effect of larval dietary [yeast] on adult metabolism. The stability of this adult phenotype was further addressed by investigating whether ectopic hindgut lipids are transient or persistent. Analyses of 2- and 3-week 0.01% adult males and beyond indicate that ectopic hindgut lipids represent a chronic alteration in 0.01% adults' physiology (Figure 5.3 and data not shown).

5.2.2 Ectopic lipids also appear in the hindgut of 2% males with ageing

To understand the longitudinal progression of the hindgut lipid accumulation, guts of 2% and 0.01% males fed on different adult diets were assayed at different ages. During these ageing experiments, it emerged that 2% males progressively accumulated hindgut lipids with ageing (Figure 5.4). Interestingly, the age at which 2% males started to display excess hindgut lipid accumulation varied with adult diet, such that by 3 weeks, hindgut lipid droplets abounded in 2% males fed on 9%HG (Figure 5.4.A), while only small amounts of

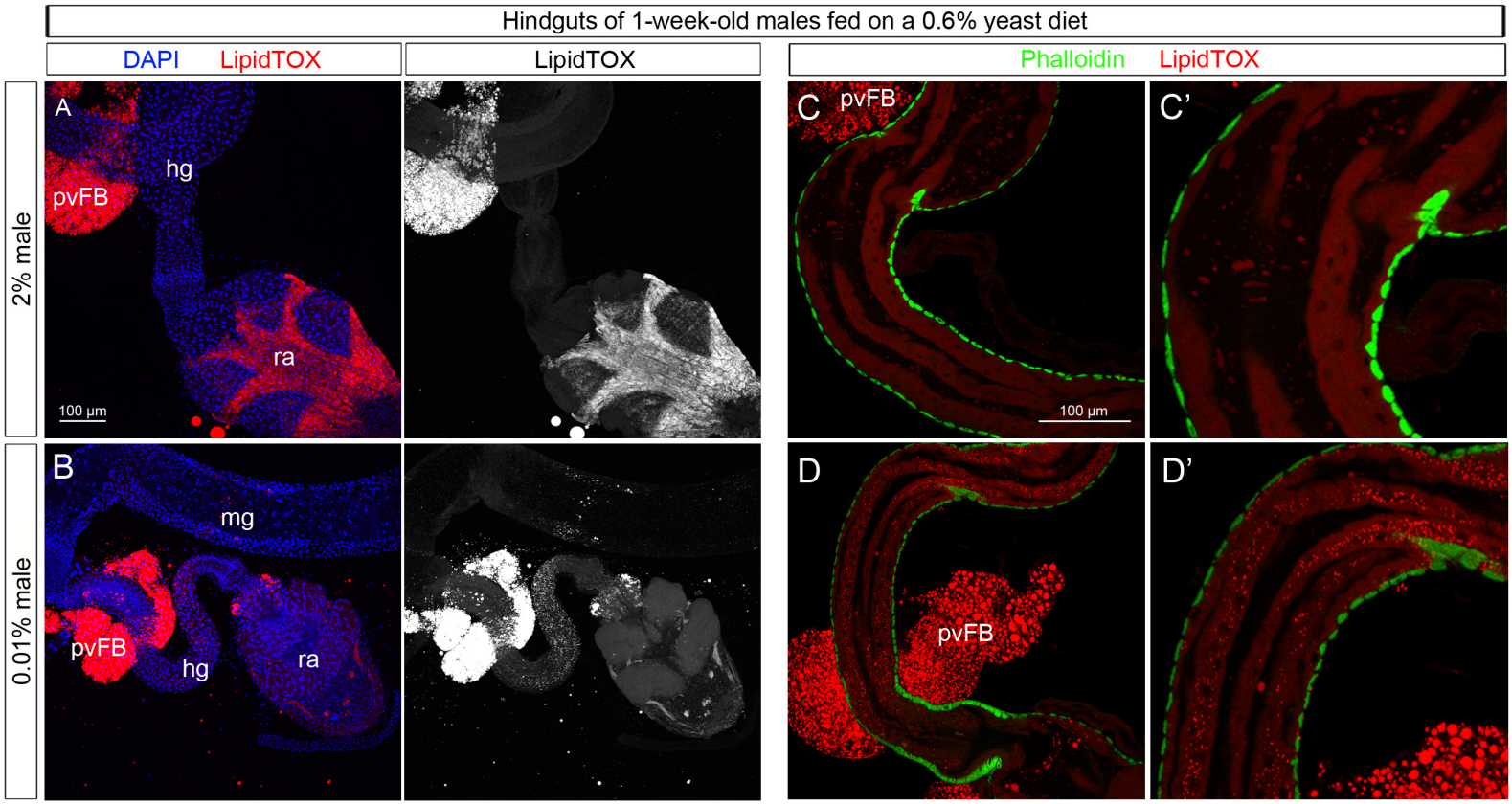

Figure 5.1: 0.01% larval dietary yeast induces the accumulation of lipid droplets in adult hindgut enterocytes. (A-B) Confocal micrographs of 1-week 2% (A) and 0.01% (B) male hindguts stained for neutral lipids with LipidTOX. Adults were fed on a 0.6%-yeast diet. Lipid droplets (LipidTOX punctae) accumulate within the hindguts of 0.01% males but not those of age-matched 2% controls. **(C-D)** Lipid droplets (LipidTOX punctae; red) accumulate within hindgut enterocytes, not hindgut perivisceral muscles (visualised by Phalloidin staining; green). **(C')** and **(D')** show higher magnifications of (C) and (D), respectively. Nuclei were stained with DAPI (blue). mg = midgut; pvFB = perivisceral fat body ring; ra = rectal ampulla; hg = hindgut

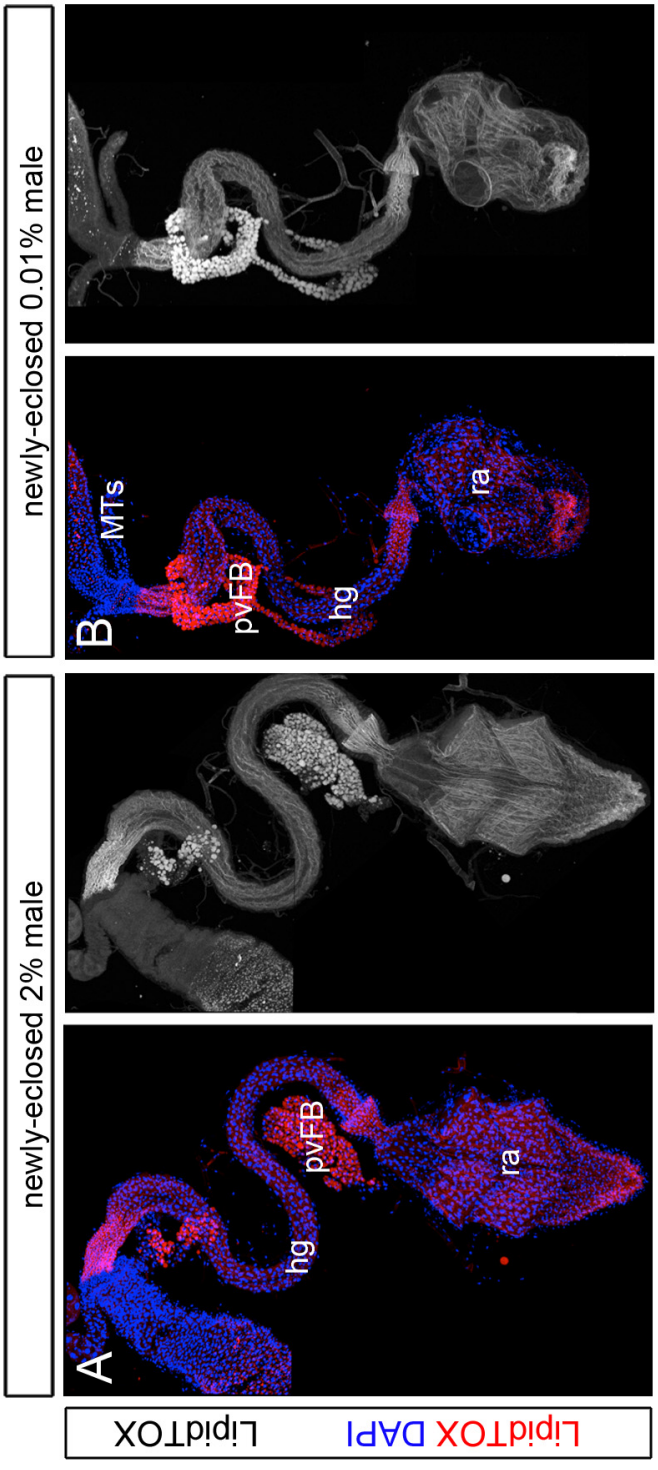

Figure 5.2:Hindgut lipid droplets are not present at eclosion, regardless of larval diet.
(A-B) Confocal micrographs of newly-eclosed 2% and 0.01% male hindguts stained with DAPI (blue) and the neutral lipid dye, LipidTOX. hg = hindgut; pvFB = perivisceral fat body ring; ra = rectal ampulla; MTs = Malpighian tubules.

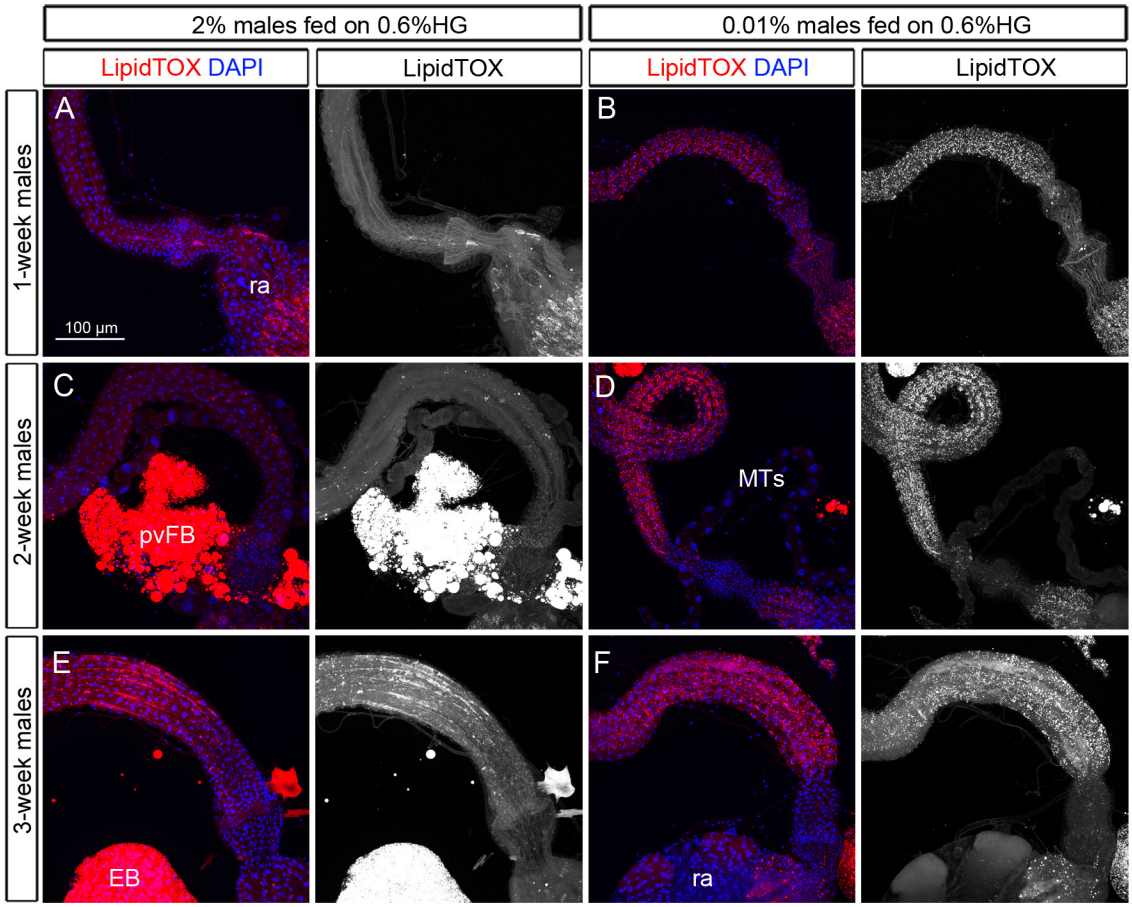

Figure 5.3: Hindgut lipid droplets persist at 2 and 3 weeks after eclosion. (A,C) No lipid droplets can be detected 2% male hindguts at 1 nor 2 weeks of age. **(E)** The hindgut becomes increasingly autofluorescent with age and displays highly-autofluorescent longitudinal "patches". However, no obvious accumulation of lipid droplets can be detected in 3-week 2% hindguts, either. **(B, D, F)** Lipid droplets (LipidTOX punctae) are already abundant at 1 week in the hindgut of 0.01% males and persist at 2 and 3 weeks after eclosion.

Nuclei were stained with DAPI. Lipid droplets were detected by staining with the neutral lipid stain, LipidTOX.

pvFB = perivisceral fat body; ra = rectal ampulla; MTs = Malpighian tubules; EB = ejaculatory bulb.

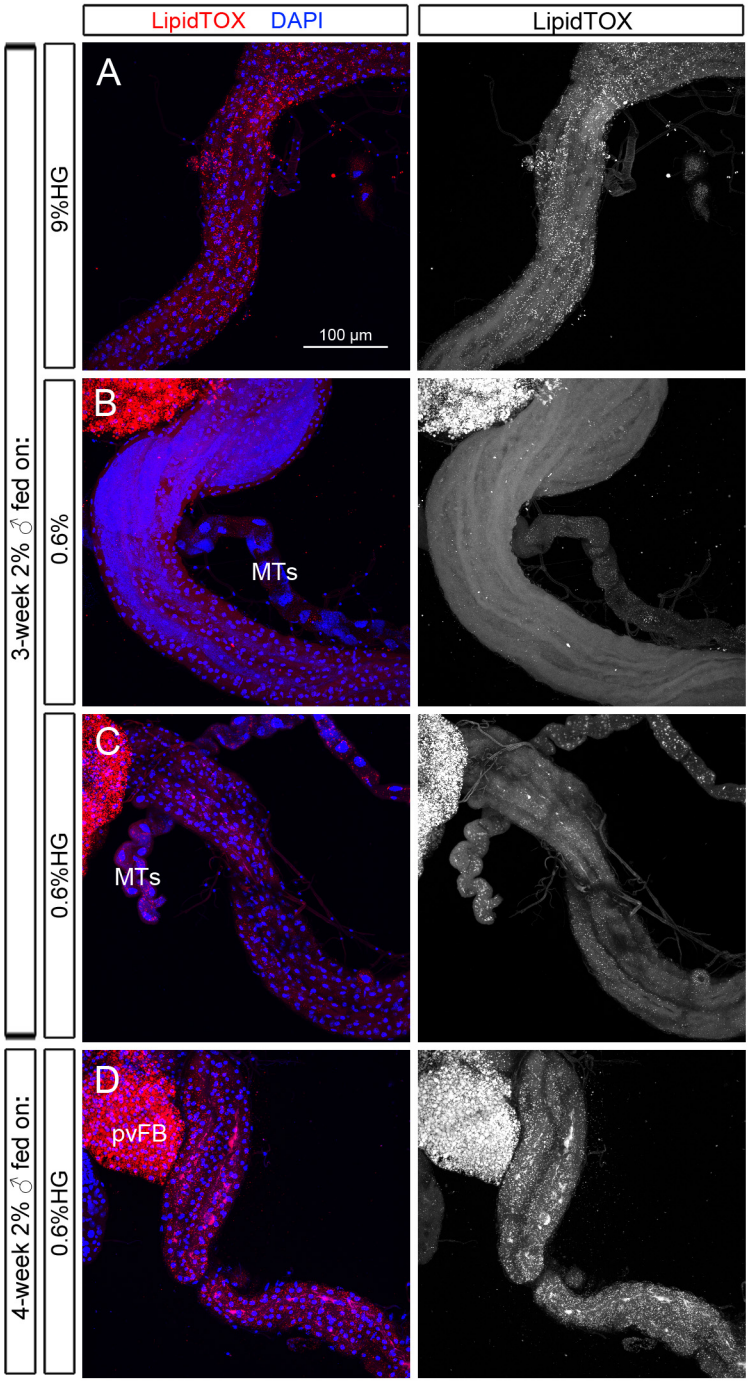

Figure 5.4: Hindgut lipid droplets also accumulate in 2% male hindgut enterocytes with ageing. The age of onset of 2% hindgut lipid droplet accumulation depends on adult diet. **(A)** Hindgut lipid droplets are present in the hindguts of 2% males fed on a 9%HG diet at 3 weeks of age. **(B)** No hindgut lipid droplets are present in 3-week old 2% males fed on a 0.6%-yeast diet. **(C)** Almost no hindgut lipid droplets are present in 2% males fed on a 0.6%HG diet at 3 weeks of age. **(D)** Hindgut lipids appear in 2% males fed on a 0.6%HG diet by 4 weeks of age.

Nuclei were stained with DAPI (blue). Lipid droplets were detected by staining with the neutral lipid stain, LipidTOX.

pvFB = perivisceral fat body; MTs = Malpighian tubules

hindgut lipids were detected in their siblings fed on 0.6%HG (Figure 5.4.C) and no lipids were detected in the hindguts of those fed on 0.6% yeast (Figure 5.4.B). By 4 weeks, however, lipid droplets filled the hindguts of 2% males fed on 0.6%HG as well (Figure 5.4.D). These findings show that larval and adult diet interact to determine the age-of-onset of ectopic hindgut lipids. They also indicate that hindgut lipid accumulation is an age-related metabolic trait relevant to adults raised as larvae on standard as well as extreme diets.

5.3 Where do hindgut lipids come from?

Several scenarios could lead to the accumulation of lipids in the hindgut. One simple possibility is that, because of their smaller body size and, therefore, smaller midguts, 0.01% flies would digest food incompletely. This could allow excess diet-derived lipids to reach and be taken up by hindgut enterocytes.

In order to test whether hindgut lipids originated from dietary lipids, the adult food was supplemented with 2 μM Orlistat. Orlistat is an over-the-counter, weight loss drug that lowers the absorption of dietary lipids by inhibiting digestive lipases (Henness and Perry, 2006) and that has been reported to work in *Drosophila* (Sieber and Thummel, 2009; Subramanian et al., 2013). In my experiments, Orlistat supplementation of the adult diet had no effect on 0.01% males' hindgut lipids (Figure 5.5.A-B). This suggests that hindgut lipids are not diet-derived. However, the effect of Orlistat supplementation on midgut lipid droplets was not obvious either, in contrast to previous reports (Figure 5.5.C-D). Given this discrepancy, I sought other approaches to finding the source of hindgut lipids.

To test whether hindgut enterocytes might take up dietary glucose rather than lipids, I used 2-NBDG (2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-d-glucose). 2-NBDG is a fluorescent glucose analog that can be taken up by cells and phosphorylated, but cannot be metabolised any further (Zou et al., 2005). This was used in two different types of experiments: (1) *in vivo* supplementation of the food or 1% agar/PBS, and (2) *in vitro* supplementation of Schneider's insect medium or PBS in which dissected guts were incubated for short periods of time. Guts were visualised immediately, without fixation, using a confocal microscope. The *in vivo* approach was designed to determine whether hindgut enterocytes take up dietary sugars from the gut lumen and, if so, whether larval dietary [yeast] affected this. I experimented with a range of experimental parameters and setups, but the 2-NBDG signal proved difficult to detect above the natural autofluorescence of gut cells and against the background of gut contents. The *in vitro*

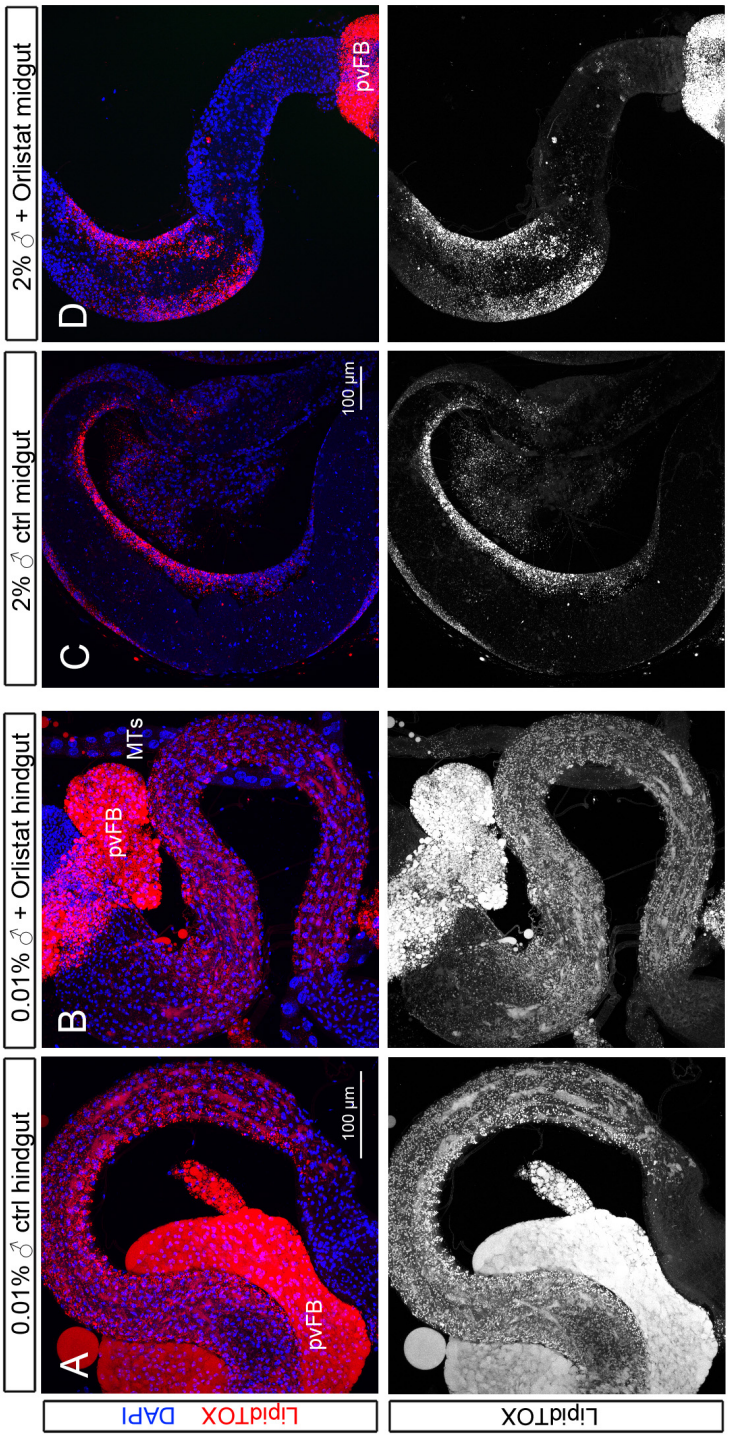

Figure 5.5: Orlistat treatment did not abrogate lipid droplet accumulation in 0.01% hindguts, but neither did it affect midgut lipids (A-B) Adult treatment with 2 µM Orlistat since eclosion did not affect hindgut lipid droplet accumulation in 10-day 0.01% males. **(C-D)** However, Orlistat treatment did not affect midgut lipid droplet accumulation either, in contrast to previous reports (Sieber et al., 2009; Subramanian et al., 2013). Nuclei are stained with DAPI (blue) and neutral lipids with LipidTOX (red).

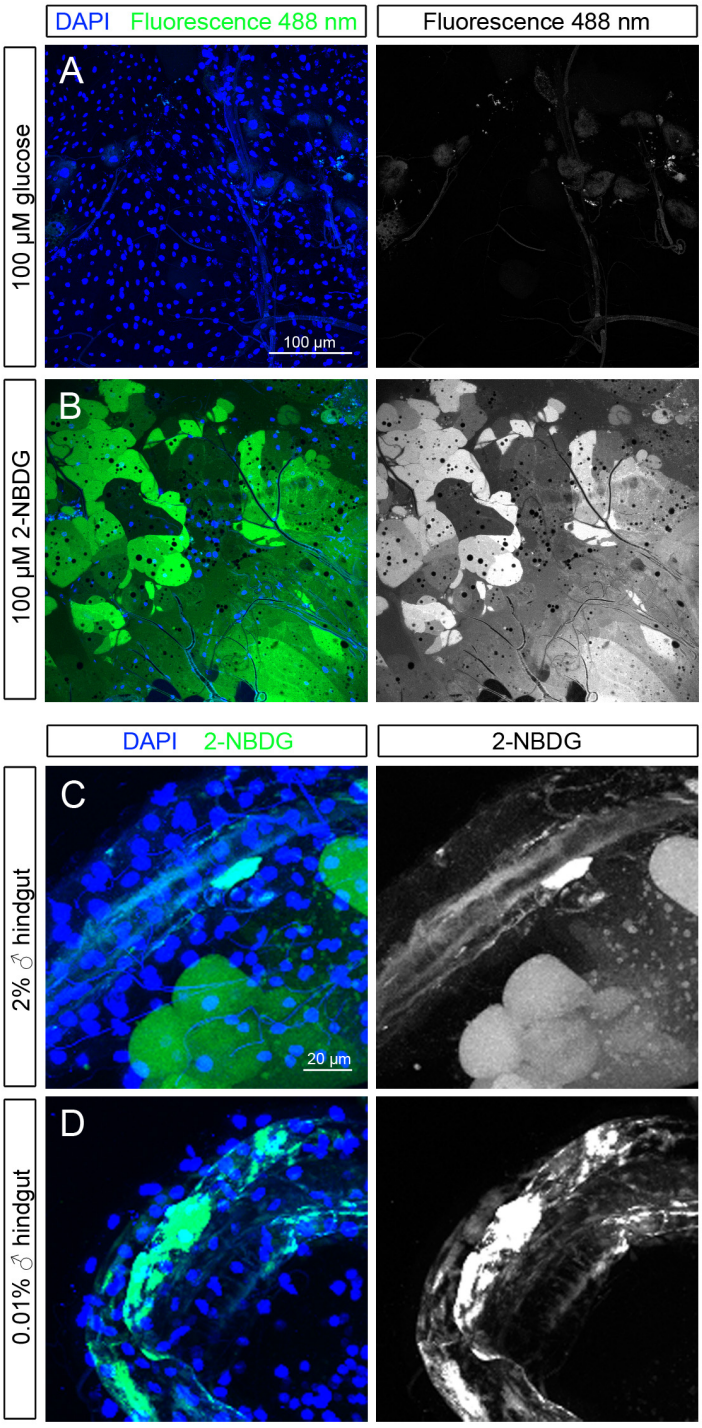

Figure 5.6: No evidence was found for glucose uptake by hindgut enterocytes. (A-B) *Drosophila* adult fat body cells can take up the fluorescent glucose analog 2-NBDG. A strong, but heterogenous signal was detected in abdominal fat body cells after 10 minutes of incubation with 100 µM 2-NBDG in PBS. **(C-D)** No 2-NBDG signal was detected in hindgut 2% or 0.01% hindgut enterocytes from animals fed for 3 h on 1% PBS in agar supplemented with 2-NBDG. Strong fluorescence was, however, detected in the gut lumen, showing that animals had ingested the 2-NBDG. Nulcei are stained with DAPI (blue). The 2-NBDG signal was detected using a confocal microscope as emission at 540 nm following excitation with the 488 nm laser.

approach involved short (15-30 minutes) organ cultures to address whether the hindgut was capable of taking up circulating glucose from the haemolymph. Strong fluorescence was detected in fat body cells following incubation with 100 μM 2-NBDG in PBS (Figure 5.6.A-B), providing proof-of-principle that 2-NBDG can be taken up by *Drosophila* tissues. Interestingly, the pattern of 2-NBDG uptake by fat body cells was strikingly heterogeneous, with some cells displaying a strong signal adjacent to cells displaying weak or no fluorescence. Throughout both *in vitro* and *in vivo* experiments, no evidence was found for glucose uptake by hindgut cells from the gut lumen (Figure 5.6.C-D) or from the haemolymph (data not shown). This suggests that de novo lipogenesis from glucose in hindgut enterocytes may not be the principal mechanism of lipid accumulation.

To gain further insight into the mechanism of accumulation of ectopic hindgut lipids, I adopted a genetic approach. The potential autonomous or systemic role(s) of the IIS, TOR and lipid metabolic pathways were tested. In order to allow larval development to progress unperturbed and to manipulate gene expression specifically in the adult, I made use of a temperature-sensitive variant of Gal80 (i.e. $Gal80^{ts}$) (reviewed in (Elliott and Brand, 2008)). This system allows one to restrict the transcriptional activation of the UAS transgene by a simple temperature switch from $18^{0}C$, the permissive temperature, (i.e. $Gal80^{ts}$ is active and UAS activation is impeded) to $29^{0}C$, the restrictive temperature (i.e. $Gal80^{ts}$ is inactive and GAL4 can drive expression of the UAS transgene). The results of the genetic manipulations were analysed in 10- to 14-day old males, an age that should allow sufficient time for the genetic manipulations to take effect, but by which point control 2% animals would not yet accumulate large amounts of hindgut lipids. However, since most adults were reared at 29℃ – which accelerates ageing compared to 25℃ (Miquel et al., 1976) – 2% males of some control genotypes had already started to accumulate small amounts of hindgut lipids. However, the results reported below refer only to dramatic changes compared to the controls. UAS controls are shown as these animals were reared in the same vial as experimental animals during both, larval and adult stages.

5.3.1 Hindgut lipids appear to be synthesised in the hindgut, but not from glucose

In Chapter 3, I found that 0.01% larval dietary yeast increased the flux of lipids/lipid precursors towards non-adipose tissues, which resulted in the accumulation of lipid droplets ectopically in several internal larval organs (see 3.5.2), reminiscent of "lipid overflow" situations described in mammals (Savage, 2009). Given this, I asked whether hindgut lipids of 0.01% adults might result from a similar "lipid overflow/overspill" from

the adult fat body. Lowering lipolysis in the fat body of adult 0.01% males, by overexpressing the *Drosophila* perilipin homolog Lipid storage droplet-2 (Lsd2; also known as Perilipin 2 or Plin2 (Gronke et al., 2003; Teixeira et al., 2003), had no effect on hindgut lipids (Figure 5.7.A-B). Moreover, blocking the uptake of lipophorin-coupled lipids from circulation by hindgut cells, by knocking down the lipophorin receptors LpR1 and LpR2 (Parra-Peralbo and Culi, 2011), did not abrogate, nor even reduce, the amount of hindgut lipids present in 0.01% male hindguts (Figure 5.7.C-F). Moreover, a genetically-induced increase in circulating lipids, by overexpressing the lipase Bmm (Gronke et al., 2005) in the fat body of 2% males, did not result in an accumulation of ectopic hindgut lipids (Figure 5.7.G-H). Taken together, the results suggest that the ectopic lipid droplets accumulating in the hindguts of 0.01% adults do not result from "lipid overflow" from storage sites to non-adipose tissues.

I next tested the alternative hypothesis that ectopic hindgut lipids are produced by *de novo* lipogenesis. Knocking down acetyl CoA carboxylase (ACC), the enzyme that catalyses one of the first steps of *de novo* lipogenesis (Barber et al., 2005), dramatically reduced ectopic lipids in 0.01% adults' hindguts (Figure 5.8.A-B), suggesting that hindgut lipids are the result of *de novo* lipid synthesis in hindgut enterocytes. Anti-ACC antibody appeared to stain hindgut enterocytes (Figure 5.9), but expression levels were much lower than in the rectal ampulla or in the fat body (Figure 5.9 and data not shown). Overall, no difference was observed in ACC levels between 0.01% and 2% adults. To further dissect the origin of hindgut lipids, the identity of the potential substrates was addressed. Pyruvate dehydrogenase (PDH) controls the entry of pyruvate into the TCA cycle from where it can be converted into cytoplasmic acetyl-CoA, a substrate for *de novo* lipid synthesis. ATP citrate lyase (ACL) links both glucose and glutamate metabolism to cytoplasmic acetyl-CoA synthesis and, thus, with lipid biosynthesis (reviewed by Fritz and Fajas, 2010). Reduction of PDH or ACL activity, by RNAi overexpression, in adult 0.01% hindguts had no effect on the accumulation of ectopic hindgut lipids (Figure 5.8.C-F), suggesting that hindgut lipids are not synthesised from glucose or glutamate.

In summary, the 2-NBDG and genetic experiments together indicate that ectopic hindgut lipids, programmed by a 0.01% yeast larval diet, are synthesised from acetyl-CoA in hindgut enterocytes. The source of acetyl-CoA, however, is not clear, but does not seem to correspond to direct enterocyte uptake of dietary glucose nor perhaps glutamate.

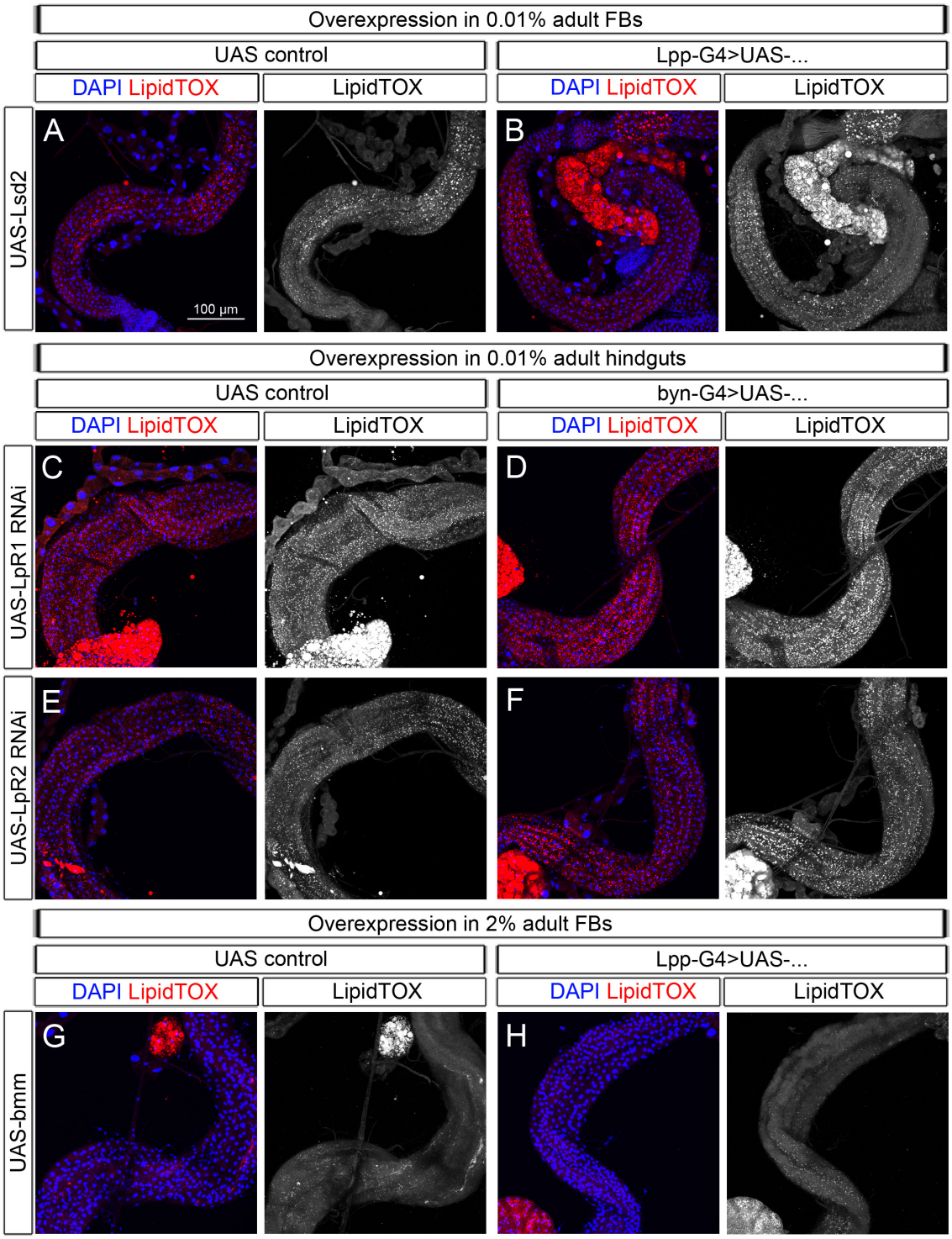

Figure 5.7: 0.01% hindgut lipid droplets do not result from lipid uptake by hindgut enterocytes from the haemolymph. (A-B) Lowering lipolysis in the 0.01% adult fat body, by overexpressing Lsd2, had no effect on hindgut lipid droplets (LipidTOX punctae). **(C-F)** Preventing uptake of lipophorin-coupled lipids from the haemolymph, by knocking down LpR1 or LpR2 in the hindgut, had no effect on 0.01% hindgut lipids. **(G-H)** Increasing lipolysis in the fat body, by overexpressing Bmm, failed to induce the premature accumulation of hindgut lipid droplets in 2% males. Nuclei are stained with DAPI (blue) and neutral lipids with LipidTOX (red).

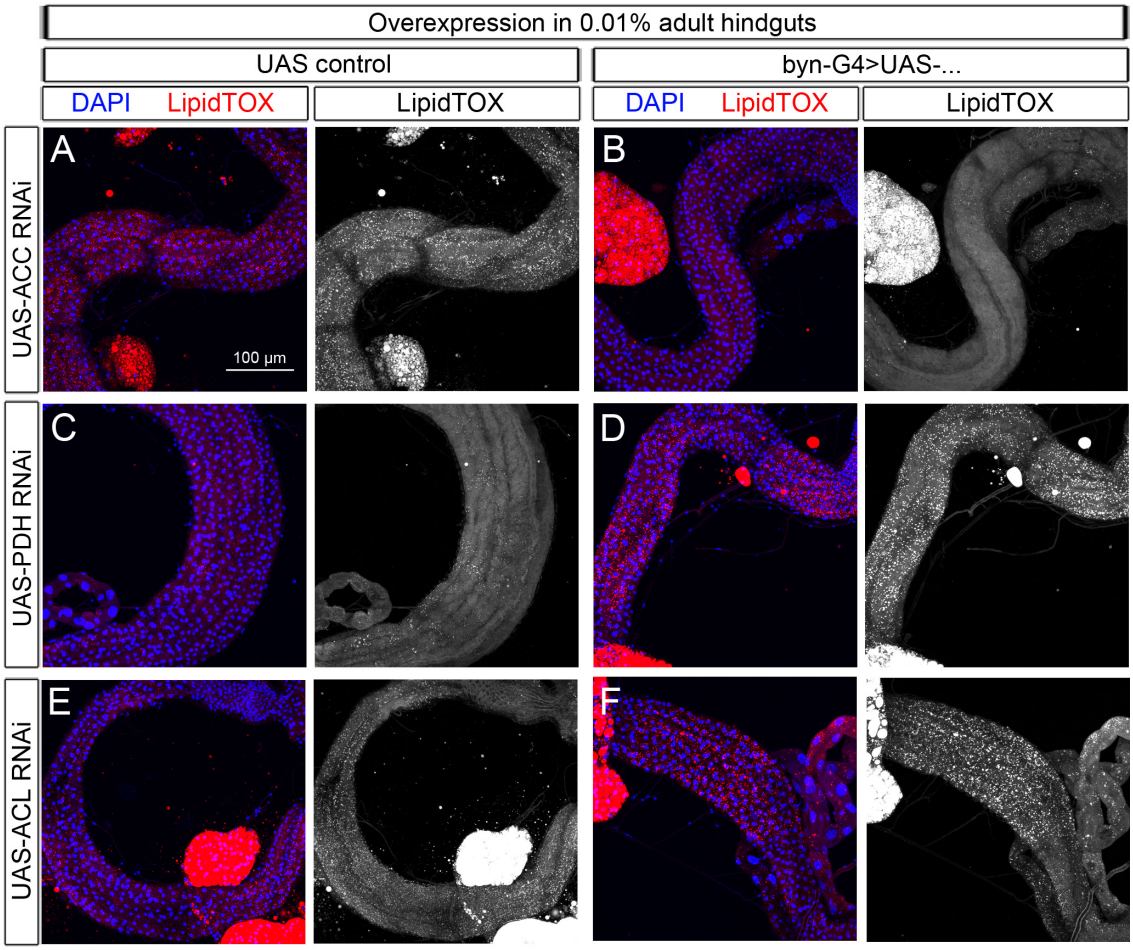

Figure 5.8: 0.01% hindgut lipid droplets result from *de novo* lipid synthesis in hindgut enterocytes, but perhaps not from glucose or glutamate. (A-B) Preventing/lowering *de novo* fatty acid synthesis, by knocking down ACC (acetyl-CoA carboxylase) in 0.01% hindgut enterocytes abrogates the accumulation of hindgut lipid droplets. **(C-F)** Preventing/lowering the channelling of glutamate and/or glucose into fatty acid synthesis, by knocking down PDH (pyruvate dehydrogenase) or ACL (ATP citrate lyase), had no effect on 0.01% hindgut lipid droplets. Nuclei are stained with DAPI (blue) and neutral lipids with LipidTOX (red).

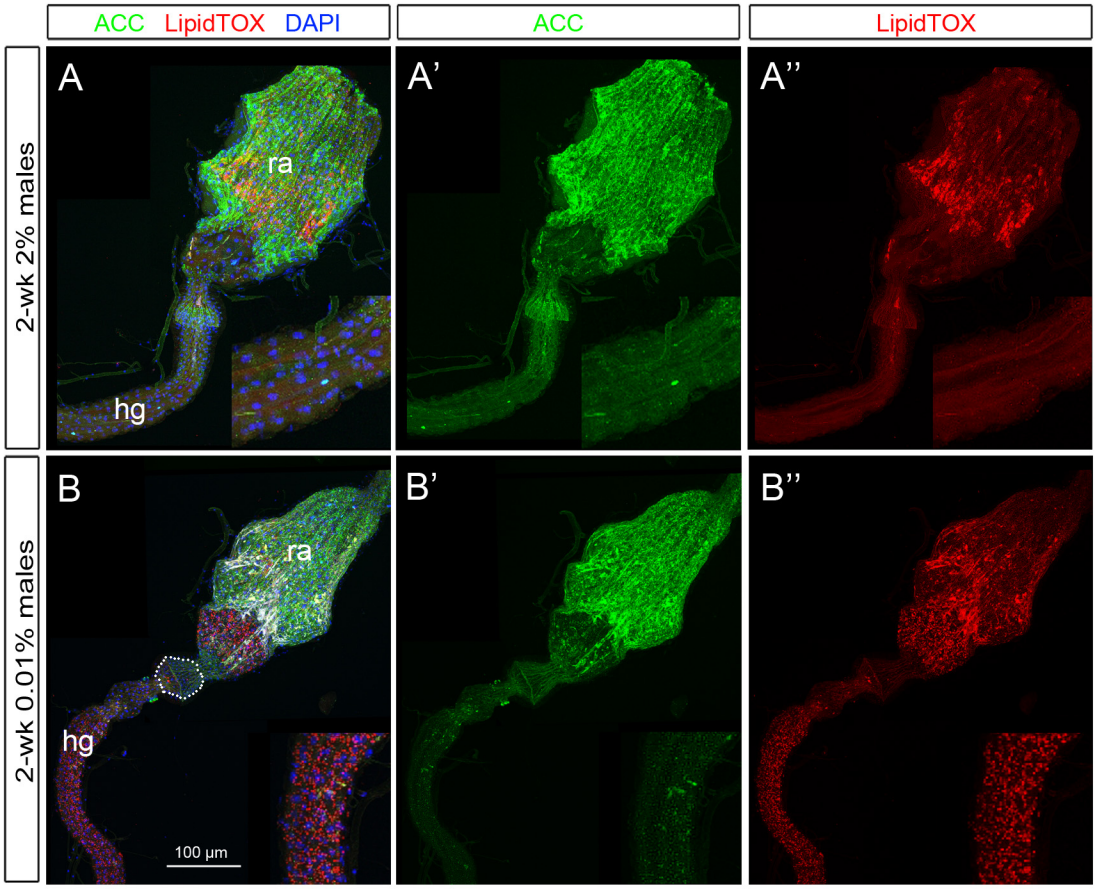

Figure 5.9: ACC is expressed in the adult hindgut, but its levels do not appear to be modified by a 0.01%-yeast larval diet. (A-B) ACC appears to be expressed in adult male hindgut enterocytes but at much lower levels than in the rectal ampulla. Nuclei are stained with DAPI (blue), neutral lipids with LipidTOX (red) and anti-ACC antibody staining is in green. hg = hindgut; ra = rectal ampulla. Note: Outlined region in (B) marks the dilp2-GAL4 expression domain within the hindgut (see Discussion for details).

5.3.2 Reducing IIS signalling in the hindgut is sufficient to induce ectopic hindgut lipids

In mammals, the accumulation of lipids in non-adipose cells is correlated with cell autonomous insulin resistance (reviewed by Samuel and Shulman, 2012). To investigate whether there is a causal relationship between IIS pathway activity levels in the hindgut and accumulation of lipid droplets, I manipulated members of the IIS pathway specifically in the adult hindgut, using byn-GAL4 (Takashima et al., 2008). Down-regulating IIS signalling by overexpressing the p60 subunit of PI3K (Weinkove et al., 1999) in the hindgut of 2% adults was sufficient to induce the accumulation of ectopic hindgut lipids in 10-day old animals (Figure 5.10.A-B). PTEN, a negative regulator of the pathway (Goberdhan et al., 1999), and $dp110^{DN}$, a dominant negative form of the dp110 subunit of PI3K (Leevers et al., 1996), both had similar effects (data not shown). Overexpression of the downstream IIS negative effector FOXO (Jünger et al., 2003; Kramer et al., 2003; Puig et al., 2003) also replicated the results obtained with more upstream members of the pathway (Figure 5.10.C-D), suggesting that the effects of lowered insulin signalling on hindgut lipids are mediated via FOXO. However, downregulating TOR signalling by overexpressing the inhibitor complex TSC1+2 (Tapon et al., 2001) did not replicate the effects of reduced insulin signalling (Figure 5.10.E-F). This suggests that the effect on hindgut lipid metabolism is specific to the IIS pathway. Together, these results show that impaired insulin signalling is sufficient to autonomously induce the accumulation of ectopic lipid droplets in the adult hindgut.

Activating the TOR pathway, by overexpressing the positive regulator Rheb (Patel et al., 2003; Stocker et al., 2003), in the hindguts of 0.01% males abrogated the accumulation of excess hindgut lipids (Figure 5.10.G-H). Preliminary results suggest that the same effect results from increased IIS signalling via overexpression of a constitutively active, myristoylated Akt (myr::Akt Stocker et al., 2002); data not shown). Taken together, these results suggest that low IIS signalling in the hindgut during adult stages is sufficient to recapitulate the programming effects of 0.01% larval dietary [yeast] on hindgut lipids.

5.3.3 Genetic manipulations of the adult IPCs

The IPCs of 0.01% adult were negatively spared for part of adulthood, raising the possibility that their reduced size/activity might underlie the ectopic hindgut lipids (see 4.5). I tested whether lowering IPC activity/size of 2% adults would be sufficient to induce hindgut lipids. Genetically blocking IPC activity in 2% adults, by overexpressing a

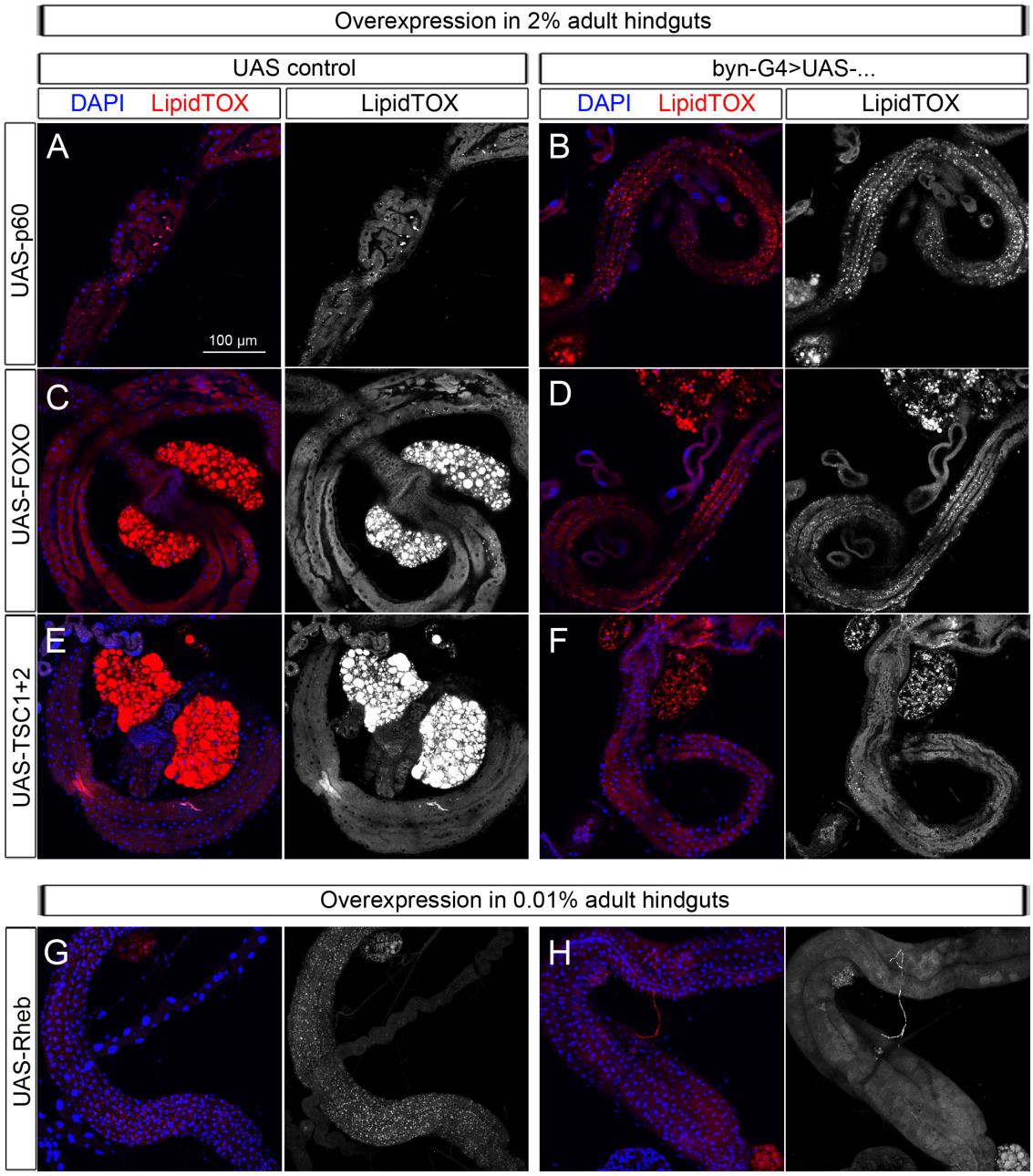

Figure 5.10: Lowering IIS signalling autonomously induces the accumulation of lipid droplets in the 2% hindgut at young ages. Lowering IIS signalling in the 2% adult hindgut by overexpressing p60 **(A-B)** or FOXO **(C-D)** is sufficient to induce the accumulation of hindgut lipid droplets. **(E-F)** However, inhibiting TORC1 signalling, by overexpressing the TSC1+2 complex, in hindgut enterocytes is not sufficient to induce the accumulation of lipid droplets in 2% male hindguts. **(G-H)** Rheb overexpression in the 0.01% adult hindgut abrogates the accumulation of lipid droplets. Nuclei are stained with DAPI (blue), neutral lipids with LipidTOX (red).

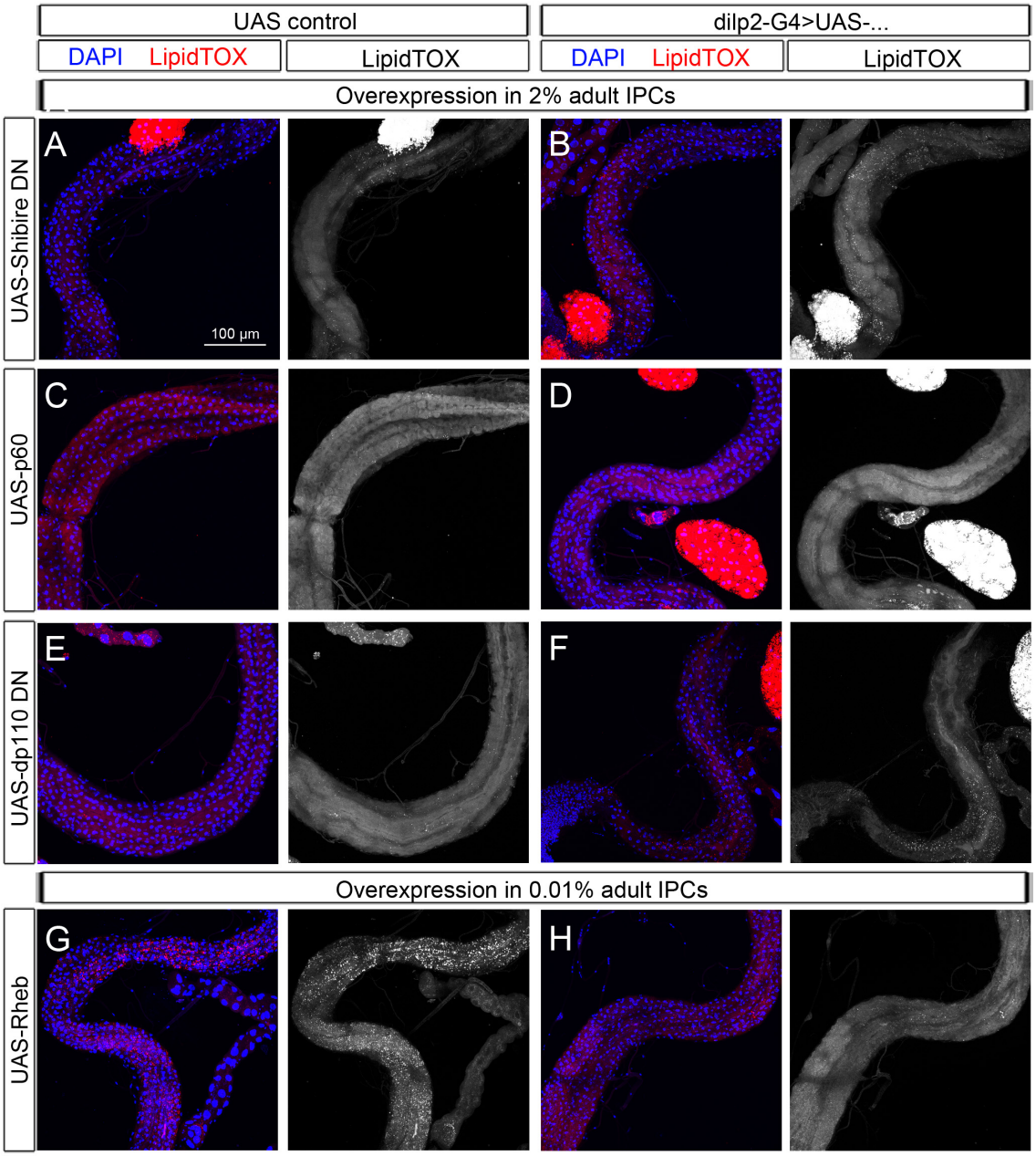

Figure 5.11: The IPCs may regulate 0.01% hindgut lipid droplet accumulation via systemic effects. (A-F) Blocking IPC function, by overexpressing Shibire^{DN}, or lowering IIS within the IPCs, by overexpressing p60 or dp110^{DN}, failed to induce the accumulation of hindgut lipid droplets in 2% adults. **(G-H)** IPC Rheb overexpression abrogated the accumulation of hindgut lipid droplets in 0.01% adults. Nuclei are stained with DAPI (blue), neutral lipids with LipidTOX (red).

dominant negative form of Shibire (Shibire^{DN}; Moline et al., 1999), the *Drosophila* homolog of dynamin, failed to induce ectopic hindgut lipids (Figure 5.11.A-B). Moreover, lowering IIS signalling in IPCs by overexpressing p60 or dp110^{DN} was also not sufficient to induce ectopic hindgut lipids (Figure 5.11.C-F). In mice, Rheb overexpression in pancreatic β-cells increases cell mass and insulin secretion (Hamada et al., 2009). Overexpressing Rheb in the IPCs of 0.01% animals abrogated hindgut lipids (Figure 5.11.G-H). This suggests that IIS/TOR signalling within the IPCs regulates the accumulation of lipids ectopically in non-adipose tissues, such as the hindgut, via systemic effects. It will be interesting to test whether increased signalling via the IIS pathway in 0.01% adult IPCs is also sufficient to abrogate the accumulation of ectopic lipids in the hindgut and which TOR components downstream of Rheb are responsible for mediating this effect. Taken together, the results of the adult IPC manipulations suggest that low IIS/TOR signalling is necessary but not sufficient for the accumulation of ectopic lipids in the adult hindgut.

5.4 Larval dietary [yeast] interacts with the adult diet to regulate adult lipid metabolism

Stored fat in the form of lipid droplets is mainly composed of TAG and CE (Fujimoto et al., 2008). Therefore, the adiposity measurements presented here represent TAG plus CE levels together, but, in the interest of space, are referred to as "TAG levels" because CE make up less than 5% of the lipids in *Drosophila* (Parisi et al., 2011).

5.4.1 Low larval dietary [yeast] decreases NEFA levels in adults fed on 0.6%HG, but not other adult diets

I analysed NEFA levels in 3.5-week 0.01% and 2% adults fed on either 0.6%, 0.6%HG and 9%HG since eclosion. Of the three, 0.6%HG was the only adult diet, which, in combination with low larval dietary yeast, altered adult NEFA levels (Figure 5.12.A). When fed on 0.6%HG, 0.01% males had 30% less NEFA as compared to 2% males. Analyses of individual fatty acid proportions in the NEFA fraction revealed that 0.01% larval dietary yeast significantly increased the percentages of C18 and C18:3 in adult NEFA, across the three adult diets, when compared to their corresponding 2% controls (Figure 5.12.B). As with overall NEFA levels, some of the effects of larval dietary [yeast] were only apparent on the 0.6%HG adult diet. In the case of NEFA fatty acids, 0.6%HG increased the percentage of C18:2 and lowered that of C16:1 in 2% adults, but both effects were absent in 0.01% animals (Figure 5.12.B). Although the mechanisms underlying the changes in total NEFA and in C16:1 and C18:2 proportions are not yet clear, the 0.6%HG diet behaves

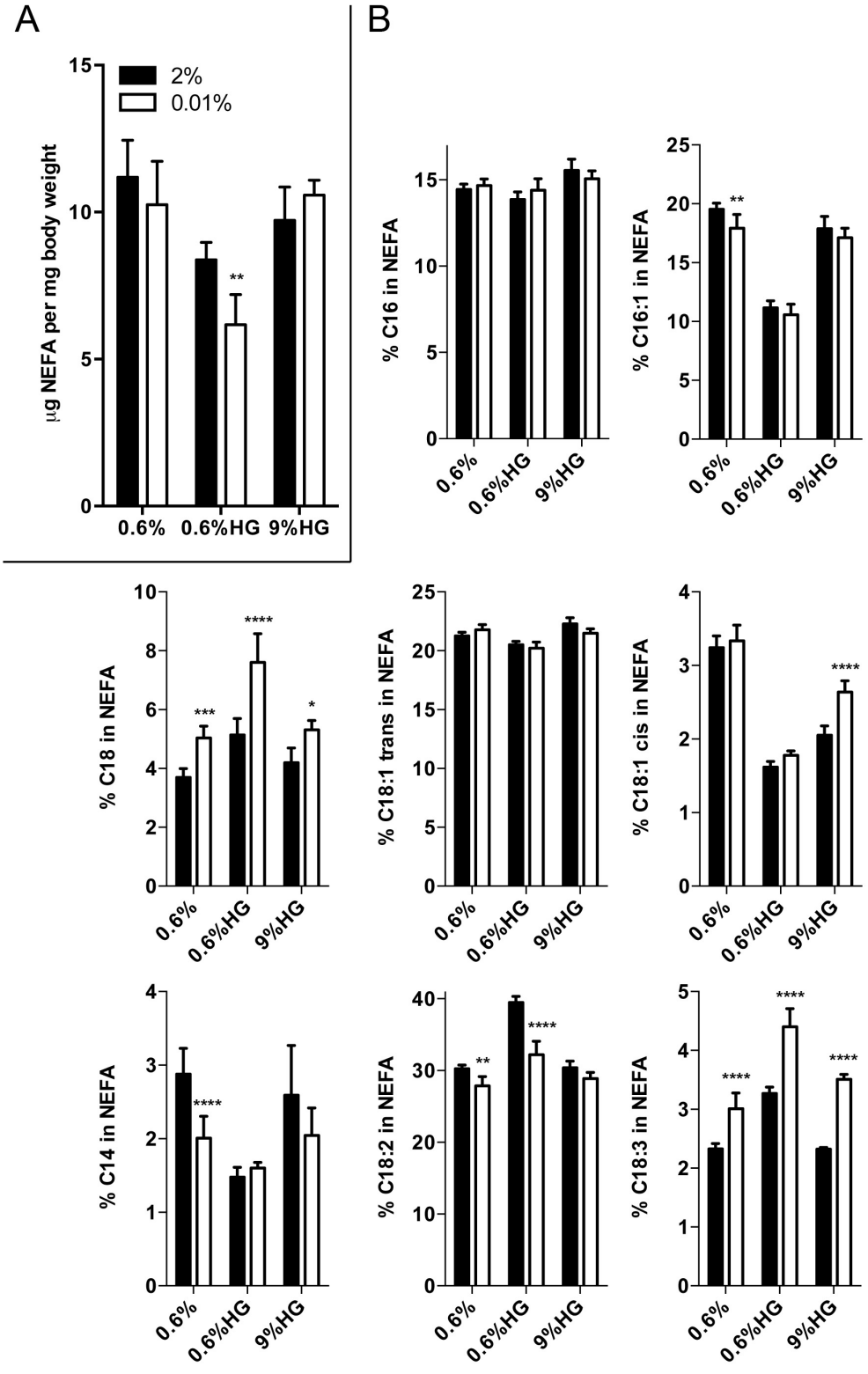

Figure 5.12: 0.01%-yeast larval diet interacts with a 0.6%HG adult diet to alter adult NEFA. **(A)** NEFA content of 3.5-week 2% (black bars) and 0.01% (white bars) males fed on one of three adult diets: 0.6%-yeast, 0.6%HG or 9%HG. **(B)** Fatty acid profile in NEFA fraction of 3.5-week 2% (black bars) and 0.01% (white bars) males. Individual fatty acids are shown as a percentage of the sum of all fatty acids detected in the NEFA fraction per sample.

differently from the others. Increased C18:2, a fatty acid derived exclusively from the diet (most of it from the cornmeal) may reflect increased food intake on the 0.6%HG diet.

5.4.2 Low larval dietary [yeast] increases the adiposity of adults fed on 0.6%HG, but not other adult diets

Adiposity (i.e. μg TAG per mg body weight) was analysed in 0.01% and 2% adult males that had fed on one of four adult diets since eclosion: 0.6%, 0.6%HG, 9% or 9%HG (Figure 5.13.A). Surprisingly, I found that 0.01% larval dietary [yeast] increased the adiposity of adults fed on 0.6%HG, as compared to 2% larval dietary [yeast], but had no effect when combined with any of the other three adult diets i.e. 0.6% yeast, 0.6%HG or 9% yeast diets. When fed on 0.6%HG since eclosion, 0.01% adult males displayed more than doubled (~150% increase) adiposity levels at 2 weeks and a 60% increase at 3.5 weeks of age. However, the effect disappeared by 5 weeks of adult life. One possibility is that 0.6%HG may increase larval TAG carryover onto the adult via the larval fat body. To test this hypothesis, one would need to measure adiposity at eclosion and during the first week of adult life on 0.6%HG vs. one of the other adult diets.

The 0.6%HG adult diet was also the only of the four adult diets to affect significantly adult weight. Figure 5.13.B shows the weights of 2-week 0.01% and 2% males that were fed on each of the four adult diets since eclosion. While adult weight remained stable across three different adult diets among males belonging to the same larval dietary regime, adults fed on 0.6%HG weighed ~10% less than their siblings fed on any of the other three adult diets – a small, but highly reproducible and statistically significant difference. Given that 0.6%HG did not lower adult adiposity, the decreased weights of adults fed on this diet suggests that 0.6%HG reduces lean body mass, possibly via muscle wasting.

The adiposity measurements revealed that adult male TAG content tends to decrease with age, regardless of larval dietary [yeast]. The exception, however, is when adults are fed on 9%HG (Figure 5.13.A). The tendency to decrease in adiposity with age has been documented previously in *Drosophila* (Johnson and Butterworth, 1985) and is also reflected in a progressive reduction in the size and density of fat body lipid droplets with age (data not shown & Panayotis Pachnis, personal communication). Interestingly, I observed that lipid droplets in the fat bodies of old adults (i.e. 7-week old males fed on a 0.6% yeast diet since eclosion) become autofluorescent, regardless of larval diet (see Figure 10.4 in the Appendix). This likely reflects an age-related accumulation of lipofuscin,

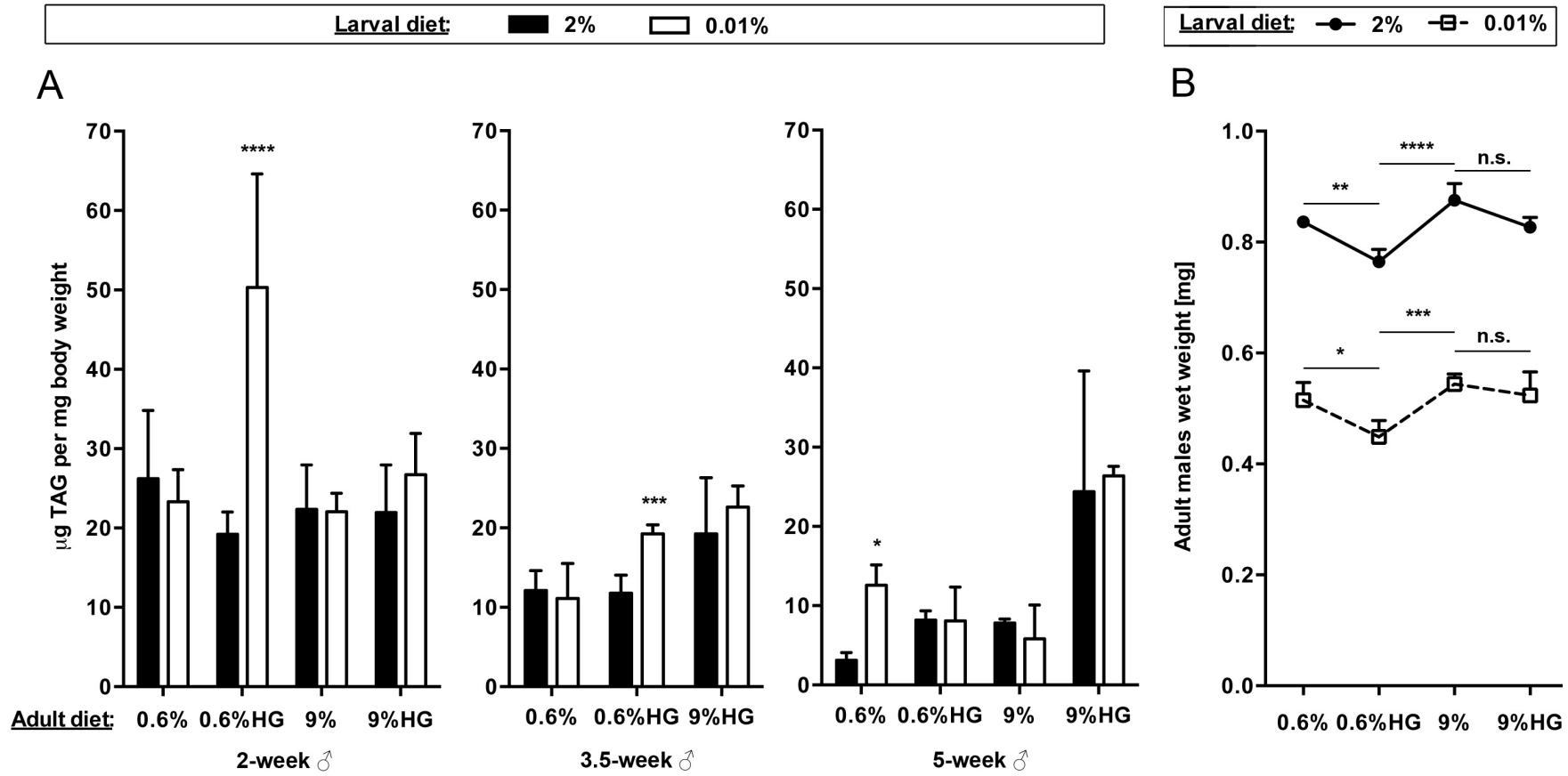

Figure 5.13: Larval dietary yeast restriction interacts with adult diet to programme adult lipid metabolism. (A) Histograms show adiposity measurements (i.e. µg TAG+CE fatty acids per mg of body weight) of 2-week, 3.5-week and 5-week males that were raised on either a 2%- or a 0.01%-yeast diet as larvae and were fed on one of four adult diets: 0.6%-yeast, 0.6%HG, 9%-yeast or 9%HG. 0.01%-yeast larval diet interacts with 0.6%HG to more than double the adult adiposity at 2 weeks and increase it by 70% at 3.5 weeks. This effect is lost by 5 weeks of age. Overall, adiposity decreases with age, except for adults fed on a 9%HG diet. **(B)** 2-week adult male weights [mg]. A 0.6%HG adult diet decreases adult weight, compared to any of the three other adult diets tested.

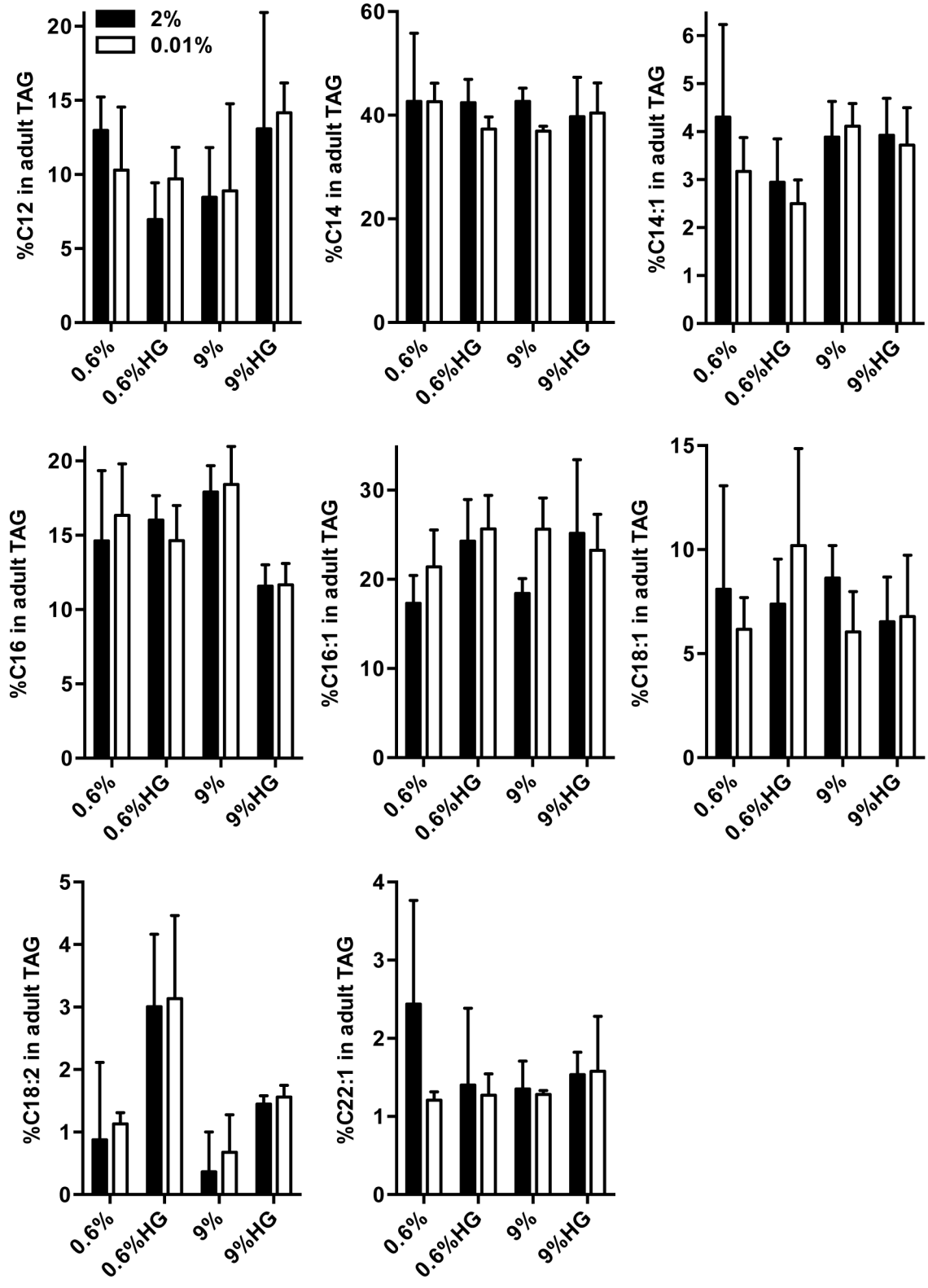

Figure 5.14: TAG+CE fatty acid profile of 2-week 2% (black bars) versus 0.01% (white bars) males. Adults were fed on one of four diets: 0.6%-yeast, 0.6%HG, 9%-yeast and 9%HG. Individual fatty acids are shown as a percentage of the sum of all fatty acids detected in the TAG+CE fraction in each sample.

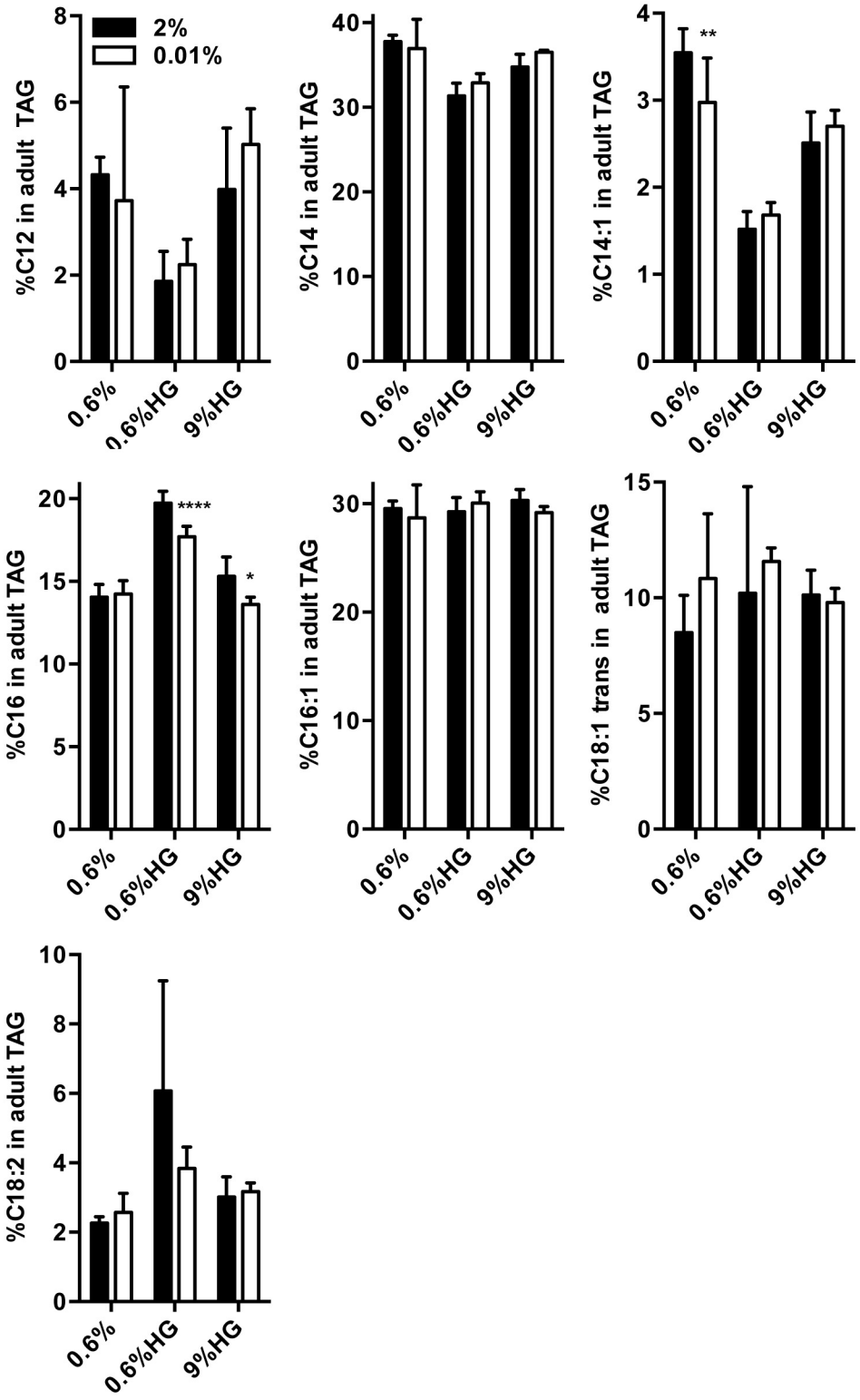

Figure 5.15: TAG+CE fatty acid profile of 3.5-week 2% (black bars) versus 0.01% (white bars) males. Adults were fed on one of four diets: 0.6%-yeast, 0.6%HG, 9%-yeast and 9%HG. Individual fatty acids are shown as a percentage of the sum of all fatty acids detected in the TAG+CE fraction in each sample. Note that error bars in this and all other figures represent 1 S.D., unless otherwise specified.

ill-defined autofluorescent material that has been associated with vertebrate ageing (Brunk and Terman, 2002; Jung et al., 2010).

Despite the general trend of an overall decline in adiposity with age, its magnitude is influenced by both the larval and the adult diet. For example, 0.01% and 2% adults that were fed on 0.6% yeast both lost half of their body fat between 2 and 3.5 weeks of age, but 0.01% adults maintained their fat content at 5 weeks, while 2% adults lost a further 75% of their 3.5-week body fat by 5 weeks. This differential age-associated reduction in TAG levels induced apparent differences in adiposity levels that were not present at 2 weeks, suggesting that diets that may appear to be "obesogenic" in older adults, may, in fact, reflect reduced fat loss, rather than adult fat gain.

The marked changes in adult male adiposity, however, were not accompanied by major alterations in the proportions of individual fatty acids in TAG at 2 or 3.5 weeks. Of the adult diets tested, 0.6%HG stood out again, being the only of the four adult diets tested that significantly altered the relative proportions of several fatty acids in TAG (Figures 5.14 and 5.15), but this was regardless of whether the larval diet was 0.01% or 2%. Of note, the percentage of C18:2 was higher on 0.6%HG, perhaps reflecting increased food intake of this exclusively diet-derived fatty acid.

Taken together, the adiposity measurements of 0.01% and 2% adults fed on the different diets demonstrate that low larval dietary [yeast] interacts with adult diet to programme adiposity and adults' response to dietary composition.

5.4.3 Increased adiposity induces ectopic lipid droplets in podocytes by three weeks of age

In vertebrates, excess adiposity has been linked with lipid overflow and thus the accumulation of lipids ectopically in non-adipose tissues. Knowing that the fat body cells of 0.01% adults scale down isometrically with the body (see 4.4), while adiposity more than doubled when these adults were fed on 0.6%HG, I sought to understand the mismatch between reduced storage capacity and increased TAG reserves. Scrutiny of the adult internal organs revealed that increased adult adiposity of 0.01% adults on 0.6%HG was accompanied by an excess accumulation of ectopic lipid droplets in podocytes, but not in muscles by 3 weeks of age (Figure 5.16.A-B and data not shown). No such lipid accumulation was detected in the podocytes of adults fed on 9%HG, the other high glucose diet used in this work, regardless of larval diet (Figure 5.16.C-D). This shows that the accumulation of lipid droplets ectopically in podocytes resulted from the specific

interaction between larval and adult diet that increased adult adiposity. However, the increased adiposity of 0.01% males was not accompanied by cardiac lipid accumulation (Figure 5.16.A). One possible explanation is that the increase in TAG reserves of 0.01% males on a 0.6%HG diet exceeds the adipose tissue storage capacity. This may then result in lipids spilling over from the adipose tissue into the circulation, from where they are taken up into non-adipose tissues such as podocytes.

To test this experimentally, I increased lipolysis in the fat bodies of 2% adults by overexpressing the lipase Bmm, in an attempt to mimic a "lipid overspill" situation. Bmm decreased fat body lipid droplet size and resulted in a peri-nuclear relocalisation of the remaining lipid droplets, suggesting that, as expected, lipids were indeed mobilised from the fat body (Figure 5.17; Gronke et al., 2005). Interestingly, this genetic manipulation was sufficient to induce the accumulation of ectopic lipid droplets in podocytes (Figure 5.17). This demonstrates that an increase in circulating lipids levels alone (i.e. without being accompanied by increased adiposity) is sufficient to induce the accumulation of lipids in non-adipose tissues, such as the podocytes, and offers evidence in support of the possibility that the ectopic lipids found in the podocytes of 0.01% adults may have resulted from a "lipid overflow" from the fat body. However, Bmm overexpression did not result in ectopic cardiac lipids (Figure 5.17), as previously reported for a fly model of high fat diet-induced increase in adult adiposity (Birse et al., 2010). This may suggest that different mechanisms may operate in the high adult dietary fat model compared to the one presented here, opening the exciting possibility that increased adiposity itself is an endpoint that bears less consequence on the effects on animal physiology than the aetiology of increased adiposity. Alternatively, since Birse et al. measured cardiac TAG content on dissected hearts and since the podocytes adhere to the heart, it is possible that the increase in cardiac TAG content they report may actually be an increase in podocyte TAG content. The authors do mention that "[c]are was taken to remove from the heart as much adipose tissue and other heart associated cells as possible", but did not confirm that lipid accumulation could actually be detected in the heart by performing neutral lipid stainings on dissected organs.

5.5 Discussion

This chapter showed that larval dietary [yeast], not only alters adult body proportions and size (as shown in Chapter 4), but also has profound long-term effects on adult lipid metabolism. Moreover, the programming effects of larval [yeast] on adult adiposity

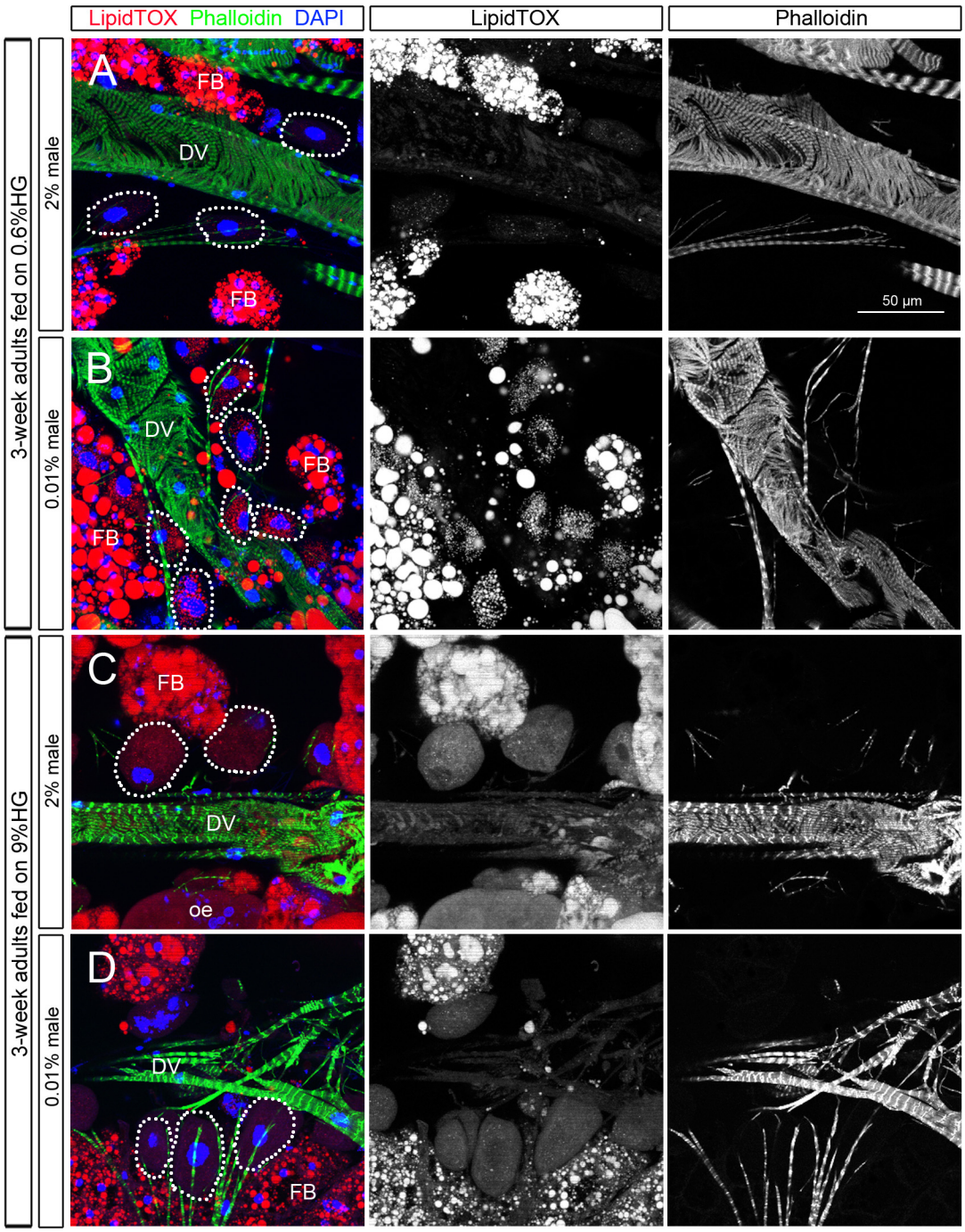

Figure 5.16: Increased adiposity induces the accumulation of lipid droplets ectopically in adult podocytes but not muscles or dorsal vessel. Lipid droplets (LipidTOX punctae) accumulate within the podocytes (outlined) of 3-week 0.01% males fed on 0.6%HG **(B)**, but not those of age-matched 2% males fed on 0.6%HG **(A)** nor of 3-week 0.01% or 2% males fed on 9%HG **(C-D)**, the other high glucose diet used in this thesis. However, no lipid droplets were detected in the dorsal vessel (DV), regardless of larval or adult diet. Nuclei are stained with DAPI (blue). Neutral lipids are visualised by LipidTOX staining (red) and muscles (cardiac and body wall) by Phalloidin staining (green). Podocytes are outlined. FB = fat body; DV = dorsal vessel; oe = oenocytes.

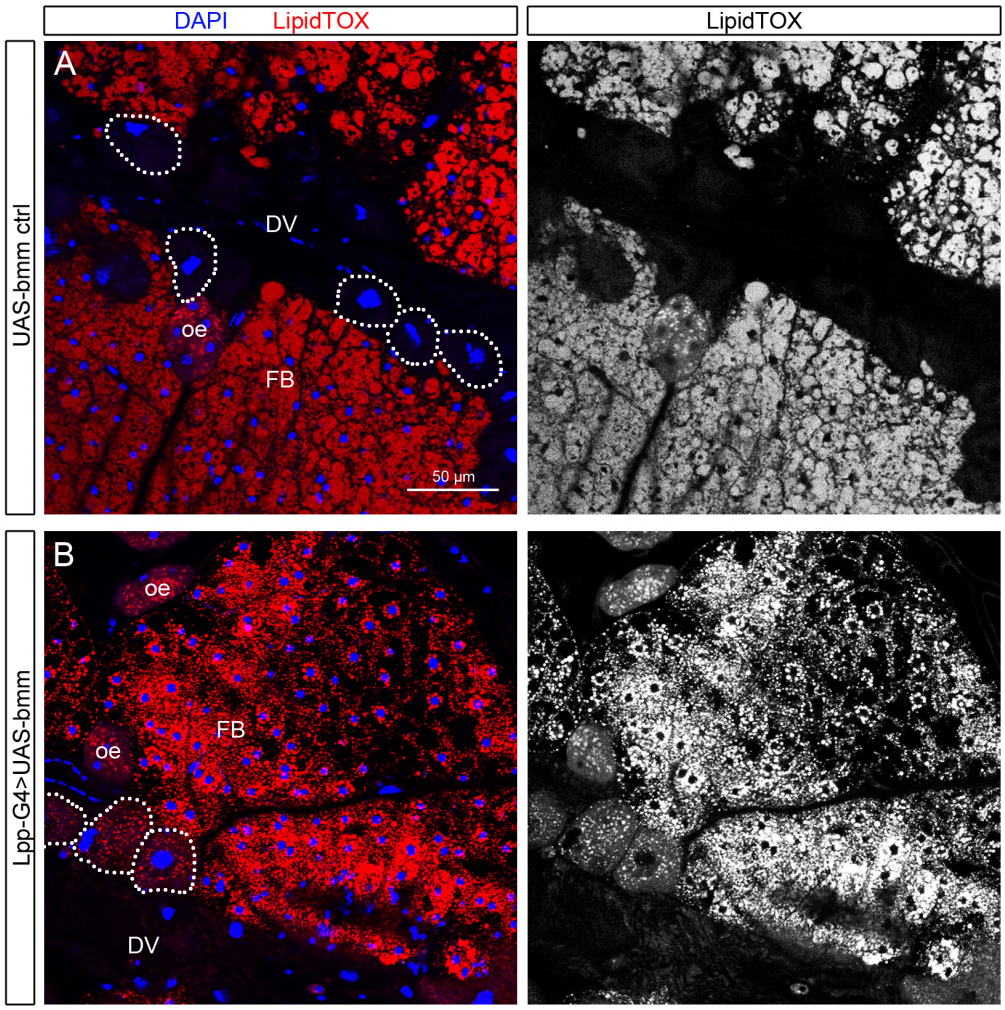

Figure 5.17: Increasing lipolysis in the 2% adult fat body is sufficient to replicate the accumulation of ectopic lipid droplets in podocytes. (A) No lipid droplets (LipidTOX punctae) can be detected in the podocytes (outlined) of UAS-bmm ctrl males. The fat body (FB), instead, is filled with large lipid droplets. **(B)** Bmm overexpression in the 2% adult fat body is sufficient to induce the accumulation of lipid droplets (LipidTOX punctae) in podocytes (outlined), but not within the dorsal vessel (DV). Note that Lpp>bmm decreases the amount and size of lipid droplets in the fat body (FB), indicative of increased lipolysis. Nuclei are stained with DAPI (blue). Neutral lipids are visualised by LipidTOX staining (red). Podocytes are outlined. FB = fat body; DV = dorsal vessel; oe = oenocytes.

involve important interactions with the adult diet. In this sense, the 0.01% adult is "at risk" of high adiposity, but this risk is only realised on the 0.6%HG adult diet.

5.5.1 Larval diet-induced accumulation of lipid droplets in non-adipose tissues

I found that low larval dietary [yeast] induced the accumulation of lipid droplets in the adult hindgut by 1 week of age already. Hindgut-specific genetic manipulations suggested that lipids accumulating within adult 0.01% hindguts were not taken up from the haemolymph (evidence from knocking down LpR1&2 in the 0.01% adult hindgut, plus Bmm overexpression in the 2% adult fat body). Instead, ectopic hindgut lipids appeared to be the result of *de novo* lipid synthesis occurring in the hindgut, but probably not from glucose or glutamate (evidence from knocking down ACC, PDH and ACL in the 01% adult hindgut). Further experiments exploring other sources of lipid precursors such as acetate would be interesting.

I further demonstrated that lowering IIS by overexpressing p60, $dp110^{DN}$, PTEN or FOXO in the 2% adult hindgut was sufficient to induce the accumulation of lipid droplets in this organ, while increasing IIS/TOR in the 0.01% adult hindgut abrogated the accumulation of hindgut lipid droplets. However, suppressing TOR by overexpressing TSC1+2 in the hindguts of 2% adults was not sufficient to induce the accumulation of lipids ectopically. This suggests that low TOR signalling is necessary, but not sufficient, while low IIS signalling is both necessary and sufficient. The effects of manipulating IIS signalling in the hindgut could potentially be mediated by direct effects on IIS relevant to nutrient uptake or to autonomous effects on cell size. One reason why decreasing hindgut cell size may result in the accumulation of lipids in this tissue could be that smaller cells have a decreased capacity to process lipids/lipid precursors, which might result in a back-log and, consequently, in the detectable accumulation of neutral lipids. To test this would require genetic manipulations that increase hindgut cell size without altering IIS signalling.

Interestingly, I found that Rheb overexpression in 0.01% adult IPCs abrogates hindgut lipids. Preliminary results suggest that overexpressing an activated form of S6 kinase does not have the same effect, suggesting that the effects of Rheb overexpression are not mediated via S6 kinase (data not shown). However, it must be noted that the IPC driver line used in these experiments (i.e. dilp2-GAL4; Rulifson et al., 2002) also drives expression in a small region of the hindgut (outlined in Figure 5.9). This is unlikely to account for the results obtained using this driver for two reasons. First, the dilp2-G4

hindgut expression does not encompass the domain of hindgut lipids. And second, genetic manipulations did not have the same effect with this driver and with the hindgut-specific byn-GAL4 driver – for example, compare dilp2-G4>UAS-p60 (Figure 5.11.C-D) with byn-G4>UAS-p60 (Figure 5.10.A-B). To confirm this, experiments could be repeated with a second IPC driver (i.e. dilp2-3-GAL4; Ikeya et al., 2002), whose expression I have confirmed is restricted to the IPCs. Taken together, the results presented here suggest that increased TOR/IIS signalling in the IPCs of 0.01% adults is sufficient to prevent ectopic lipid droplets accumulating in the hindgut. To better understand the bases of this systemic effect on hindgut lipids, it would be interesting to test whether the adult IPC genetic manipulations affect the size/output of the IPCs.

Feeding 0.01% males on 0.6%HG doubled their adiposity. Interestingly, increased adiposity was accompanied by accumulation of lipids ectopically in the podocytes. Genetically increasing lipolysis in the fat body of 2% control males was sufficient to induce the accumulation of ectopic lipid droplets in podocytes. This replicates the lipid droplet accumulation within the podocytes of 0.01% adult animals, but without a concomitant increase in adiposity. However, the same endpoint (i.e. lipid droplets in 0.01% adult podocytes) could still reflect different underlying mechanisms. As well as increased circulating lipid levels, alternative possibilities include an increase in circulating sugars or a systemic effect of increased adiposity upon insulin secretion, thus resulting in lowered insulin signalling in peripheral tissues. The latter possibility seems less likely as it would also have resulted in an accumulation of lipids in the hindgut, which was not the case. Whatever the precise mechanism, the effects of overexpressing Bmm in the fat body of 2% adults demonstrates that increasing circulating lipids without increasing adiposity is sufficient to give fatty podocytes. The fact that lipids accumulated in podocytes, rather than other internal organs, may reflect the podocytes' role, as part of the fly's excretory system, in continuously filtering the haemolymph. These findings provide evidence in favour of the hypothesis that it is the lipid overflow from the major fat storage depot, rather than the amount of stored fat *per se*, that leads to metabolic disorders.

5.5.2 The "hindgut hypothesis"

I found that 0.01% induces the accumulation of lipid droplets specifically in the adult hindgut, regardless of adult diet and, therefore, adiposity. Moreover, I showed that this lipid accumulation does not result from the passive uptake of lipids from the haemolymph, but is the result of *de novo* lipid synthesis in hindgut enterocytes. In humans and mammalian model systems, gastric bypass surgery has immediate beneficial effects on

glucose homeostasis through mechanisms that are independent of weight loss. It has been proposed that this is the result in alterations in gut hormones. The mechanisms underlying these effects are not yet completely understood, but, based on the evidence to date, two theories have been formulated (reviewed by Thomas and Schauer, 2010). The "foregut hypothesis" suggests that the proximal part of the intestine, in response to nutrients, secretes a yet-unknown factor that promotes insulin resistance. Therefore, surgeries that bypass this intestinal segment improve metabolic health by eliminating this detrimental "factor". The "hindgut hypothesis" was first proposed by Mason in 1999 and maintains that the surgical shortening of the intestine results in L-cells (found in the ileum and colon) being exposed to (higher levels of) undigested nutrients. This, in turn, stimulates their secretion of glucagon-like peptide-1 (GLP-1), which leads to improved glucose homeostasis after bariatric surgery (Mason, 1999). Potentially, this may suggest a very interesting correlation between the hindgut and metabolic homeostasis, which has never been explored in *Drosophila*. In the case of 0.01% adults, one could speculate that their decreased size would correlate with shorter guts that, in turn, could lead to higher levels of undigested nutrients reaching the distal gut/hindgut. While this is highly speculative, one could start by studying what effects the smaller body size of 0.01% adults has on the size/length of the gut compared to 2% adults and how this correlates with intestinal transit times.

5.5.3 The danger of the single time point

Through adiposity measurements at 3 different ages and for adults fed on four different diets I showed that, overall, adult TAG levels decrease with age. This was true for both 0.01% and 2% males fed on three out of the four adult diets tested. Despite the widely different lifespan outcomes for adults fed on the different diets, at 2 weeks there were no differences in the adiposity levels of 2% males fed on any of the four adult diets tested. Between 2 and 3.5 weeks and again between 3.5 and 5 weeks, the adiposity of adults on the different diets decreased to different extents. This resulted in significant differences in the adiposity of 2% males at 3.5 and at 5 weeks, depending on the diet they fed on. Therefore, if one were to assess adiposity of 5-week 2% males, one would conclude that high glucose diets are "obesogenic" as they increase adult adiposity at this time point. This explains why high sugar:protein diets have previously been reported to be "obesogneic" in *Drosophila* (Skorupa et al., 2008). However, as previously mentioned, the differential adiposity of 2% adults at later time points does not results from fat gain, as the term "obesogenic" would imply, but rather from differences in the rate of age-associated decrease in fat content. We do not yet know whether this differential decrease in adiposity

with ageing is due to impaired lipolysis/lower utilisation or to increased deposition- either via *de novo* synthesis or accumulation of dietary lipids. Whatever the mechanism, it is clear that *Drosophila* studies should be careful not to use the word "obesogenic" unless referring to a diet that actually increases adult TAG content between two time points. Most *Drosophila* studies of metabolism to date have focused on a single end point and generalised from that. The adult adiposity measurements presented in this thesis underscore the importance of studying adult lipid metabolism as a dynamic process and warn of the pitfalls of generalising based on a snapshot. Because "the problem with single stories is not that they are untrue, but that they are incomplete" (Adichie, 2009).

Moreover, if the "obesogenic" potential of a diet is to be analysed, then a measure that is not influenced by adult diet itself should be used as a denominator. Several studies have reported TAG levels relative to the animals' protein content (Al-Anzi et al., 2009; Navarro et al., 2010; Skorupa et al., 2008). However, the latter parameter changes with adult diet and, in fact, appears to be positively correlated with the concentration of yeast in the adult diet (Skorupa et al., 2008). Therefore, by defining adiposity as a function of a variable trait that is itself under the influence of adult dietary composition, one can mistakenly report effects on adiposity when only the denominator has changed and, conversely, overlook "obesogenic" conditions because they affect both fat content and the denominator to a similar extent. For example, a condition that decreases protein content or muscle mass but has no effect on stored fat content will seem to have an "obesogenic" effect. Conversely, a dietary manipulation that promotes increased fat storage alongside increasing protein content will appear to have no effect on adiposity levels.

In the course of these studies I noticed that ageing increases the heterogeneity of both hindgut lipids and adiposity. This effect is more obvious when assessing hindgut lipids, for which individual animals are analysed, than when quantifying adiposity, for which lipids from 10 or more adults are averaged per sample. One possibility is that this phenomenon reflects the heterogeneity in individual "survival trajectories". One would expect that each animal's individual position along the population's lifespan curve will dictate its physiological state. To test this, one would need to be able to know whether individual 2% flies that accumulated hindgut lipid droplets were "closer to dying" than their siblings displaying fewer hindgut droplets. Shahrestani et al. have proposed a method to distinguish between female flies that are in the "death spiral" (i.e. 6-15 days prior to death) and same-aged flies that are not approaching death (Shahrestani et al., 2012). This is based on a rapid decline in fecundity and "time spent in motion" experienced by the former, and can allow the experimenter to discern those animals that are close to death.

5.5.4 Summary

In summary, the results presented in this chapter demonstrate a long-term programming effect of low larval dietary [yeast] on adult lipid metabolism. I find fixed effects of larval diet – i.e. that are not modified by the composition of the adult diet, such as the accumulation of lipid droplets in the hindguts of 0.01% adults from an early age – as well as effects that result from the interaction between larval and adult diets, such as the dramatic increase in adiposity displayed by 0.01% males that are fed on 0.6%HG. In addition, I find that lipid droplets also accumulate in the hindguts of control 2% adults with ageing. This suggests that hindgut lipid droplet accumulation is a previously uncharacterised biological phenomenon that is part of the normal ageing process. Finally, I show that the increased adiposity of 0.01% males correlates with ectopic lipid droplet accumulation in adult podocytes. Increasing the breakdown of lipids in the 2% adult fat body was sufficient to replicate this effect, without an increase in adiposity. Taken together, the results suggest that podocyte lipid droplets may results from a "lipid overflow" situation caused by the dramatic increase in fat content seen in 0.01% males fed on 0.6%HG. This is the first demonstration that increased adiposity can lead to the accumulation of lipid droplets ectopically in non-adipose tissues in *Drosophila*. The "increased adiposity/ectopic podocyte lipid droplets" model could, together with the larval model, be employed to study the much-debated relationship between ectopic lipids, insulin resistance and organ dysfunction (reviewed by Samuel and Shulman, 2012; Szendroedi and Roden, 2009). In contrast to podocyte lipid droplets, I showed that hindgut lipid droplets found in 0.01% adults result from active synthesis of lipids in hindgut enterocytes themselves and do not merely reflect passive uptake and storage of lipids from circulation. In the future, it would be interesting to study the physiological relevance of lipid droplet accumulation within hindgut enterocytes. Given that the hindgut is known to function in ion and/or water resorbtion, it could be informative to prevent or induce hindgut lipid droplets and assess the effect this has on faecal water content (as previously described by Cognigni et al., 2011) or desiccation resistance, for example.

Chapter 6

Effects of larval diet on lifespan

6 Effects of larval diet on lifespan

6.1 Introduction

Undernutrition during development at first came under scrutiny because it correlated with increased risk of dying from cardiovascular disease in humans (reviewed by Barker, 2007). This was also confirmed in mammalian model organisms (reviewed by Martin-Gronert and Ozanne, 2012). However, Ozanne and coleagues have shown that mice from mothers fed on a low-protein diet during gestation had decreased lifespan only if they were allowed to catch up in growth during lactation (Ozanne and Nicholas Hales, 2004). Animals that did not catch up during lactation stayed small and lived longer. This suggests that the timing of undernutrition during development is critical in determining whether lifespan is shortened or lengthened. Thus, low protein diets at some developmental stages may even prove beneficial for long-term animal survival. In *Drosophila*, Tu and Tatar have shown that depriving *Drosophila* larvae of yeast during L3 results in small, but viable adults that, however, do not live longer than their "fed-throughout" controls (Tu and Tatar, 2003). This further strengthens the idea that timing is an essential determinant of the long-term effects of undernutrition.

The 1990s saw the discovery of the first genes that extend lifespan when mutated. In *Drosophila*, *methuselah* (*mth*), which encodes a G protein-coupled receptor, was identified from a screen of P-element insertions for long-lived strains (Lin et al., 1998). Many mutations that affect lifespan are now known. These include mutations in genes encoding for InR, Chico, FOXO, members of the TOR signalling pathway, Dilps, rpd3 histone deacetylase, EcR, superoxide dismutase, Sirtuins or IGFBP7 have all been shown to extend fly lifespan (Alic et al., 2011; Clancy et al., 2001; Gronke et al., 2010; Kapahi et al., 2004; Lombard et al., 2011; Simon et al., 2003; Sun et al., 2012; Tatar et al., 2001). Moreover, whole-body (or, less common, organ-specific) genetic manipulations that alter signalling through pathways such as the PI3K, TOR or JNK signalling pathways can also alter lifespan (Biteau et al., 2010; Broughton et al., 2005; Demontis and Perrimon, 2010; Giannakou et al., 2004; Hwangbo et al., 2004; Hyun and Hashimoto, 2011; Rana et al., 2013; Rera et al., 2011; Wang et al., 2003; Zid et al., 2009).

In *Drosophila*, adult dietary [yeast] and amino acids have well-studied and dramatic effects on the survival patterns of laboratory-bred flies. Restricting the [yeast] in the adult diet extends *Drosophila* lifespan. Importantly, the effect of dietary restriction (DR) cannot be

explained by calorie restriction (Lee et al., 2008; Mair et al., 2005). As detailed in the General Introduction (see 1.5), several studies have attempted to address the potential effects of larval environmental conditions upon adult survival, but have mostly been unsuccessful in revealing a significant role for larval diet in determining adult longevity (Chandrashekara and Shakarad, 2011; Zwaan et al., 1991; 1992), with the notable exception of a classical series of experiments performed by Economos and Lints who concluded that there is an narrow range of larval dietary [yeast] within which lifespan is optimised (Economos and Lints, 1984a; 1984b). In their experiments, going above or below this optimum decreased lifespan. The general dogma, however, has been that developmental time correlates negatively with lifespan i.e. that delaying development will shorten lifespan. The experimental approach taken in this thesis differed in several essential aspects from previously reported studies. For example, the approach most commonly employed had been to increase larval density, while maintaining the amount/composition of food constant, in order to limit nutrient availability per larva. However, this method will also lead to an accumulation of waste products/toxins in the vial. Effects detected in this way, therefore, will not necessarily be caused by changes in larval nutritional status. Moreover, by comparison with available literature, my results suggest that the effects observed with restricting dietary [yeast] are caused by changes in larval food composition and not just delayed development/stunted growth. In this chapter, I investigate the effects of lowering the concentration of yeast in the larval diet upon adult longevity.

Median lifespan, also known as 50% survival is the time point by which half of the population has died. Mean, or average lifespan is the arithmetic mean of the survival. Median lifespan is usually reported, as opposed to mean, because it is less sensitive to highly skewed distributions (for example, a small proportion of the population living unusually long will influence the mean much more than the median). For this reason, in the text, I report the effects as changes in median lifespan. Lifespan statistics, for the most important findings, including mean and median lifespan as well as survival at 25%, 75% and 90%, are available in the Appendix. Statistical analyses were carried out in OASIS (Yang et al., 2011). Statistical differences between the groups were calculated using the non-parametric Log-rank test (also known as Mantel-Cox test). Lifespan varies between the different laboratory strains of wild-type *Drosophila* (Grandison et al., 2009b; Mockett et al., 2012). However, longevity of the w^{1118}iso wild-type strain used throughout this work compared favourably with those reported for long-lived, outbred wild-type strains, such as Dahomey.

6.2 Results: Larval dietary [yeast] programmes adult longevity

To test whether larval dietary [yeast] affects adult lifespan, larvae were reared on diets containing 0.01%, 0.1%, 0.6% or 2% yeast. All newly-eclosed adults were then transferred onto 2% yeast food within 24 hours of eclosion and allowed to mate for a further 48hrs (referred to in the literature as "once-mated"), before being separated into genders and placed in vials at a fixed density of 25 flies per vial. I recorded the survival of adults from each of the four larval dietary [yeast] groups on four different adult diets: 0.6% yeast, 0.6%HG, 9% yeast and 9%HG, i.e. 16 groups in total. The 0.6% and the 9% yeast adult diets were chosen to simulate "DR vs. high yeast" conditions, as diluting yeast in the adult diet is known to increase lifespan. I also decided to record lifespan on high glucose versions of the 0.6% and 9% yeast diets i.e. 0.6%HG and 9%HG, respectively, as adult dietary glucose content is thought to alter lifespan, although the mechanism is not clear (Bruce et al., 2013; Lee et al., 2008; Skorupa et al., 2008). During the course of the lifespan studies, it became clear that w^{1118}iso is positive for the maternally transmitted endosymbiont *Wolbachia*, which is estimated to be present in more than a third of laboratory stocks. For details, please see Appendix.

6.2.1 Lifespan is determined by the interaction between larval and adult diets

These experiments confirmed the findings of other labs that adult DR extends lifespan (Appendix Figure 10.5). Surprisingly, however, I found that DR extended 2% males' median lifespan by ~50%, while 0.01% males responded to DR with an increase in median lifespan of only 20%. 0.01% and 2% adults also responded differently to high dietary glucose (Appendix Figure 10.5). High glucose had no effect on 2% male lifespan when combined with high yeast (i.e. 9% yeast). However, when added to a diet containing only 0.6% yeast, high glucose shortened median 2% lifespan by ~25%, compared to the 0.6% containing "standard" glucose. In contrast, feeding 0.01% males on a 0.6%HG diet as adults, reduced their lifespan to half, compared with a 0.6% diet. Surprisingly, however, when combined with a high yeast diet, high glucose actually increased 0.01% male median lifespan by 16%. Together with the adiposity data in Chapter 5, these results demonstrate that larval dietary [yeast] programmes adults' response to dietary composition.

Importantly, lifespan experiments revealed that low larval dietary [yeast] increases male lifespan significantly, in a dose-dependent manner, when adults are fed on all diets except 0.6%HG (Figure 6.1.A-D). Interestingly, 0.6%HG was also the only of the four adult diets tested which doubled the adiposity of 0.01% males and induced ectopic lipid accumulation

in internal adult organs other than the hindgut (see 5.4.3). Thus, there is a clear correlation between the increased adiposity and shortened lifespan of 0.01% males. This would be an interesting link to follow up at mechanistic level. The two most extreme low [yeast] larval diets both had similar effects, extending male median lifespan on all adult diets, except 0.6%HG. The extent of the increase depended on adult diet; 0.01% larval [yeast] extended median lifespan dramatically by >100% compared to 2% larval [yeast], when adults were fed on 9%HG (Figure 6.1.A). Moreover, when adults were fed on 9% yeast food, 0.01% adult median lifespan was increased by ~90% (Figure 6.1.B). Feeding adults on 0.6% yeast food brought the cohorts' survival curves closer together, such that 0.01% larval [yeast] extended median lifespan by only 20% compared to 2% larval [yeast] when adults were kept on a 0.6% yeast medium (Figure 6.1.C).

To control for variability between experiments, I performed an independent set of experiments (Figure 6.2) – employing 9%HG, 0.6% yeast and 0.6%HG as adult diets. These reproduced the large effects of larval dietary [yeast] on male lifespan. 0.01% larval dietary [yeast] resulted in a 126% increase in median lifespan compared to 2% larval dietary [yeast] when adults were fed on 9%HG, 33% when adults fed on 0.6% yeast food, but no extension when adults fed on 0.6%HG.

Throughout the course of these experiments I observed that feeding larvae on a 0.6% yeast diet resulted in more variable effects on adult lifespan (Figures 6.1.C and 6.2.B). The survival curves of adults from 0.6%-fed larvae grouped close to those of 2% adults when adults were fed on the 0.6%HG diet. However, in the two independent experiments, the survival curves of 0.6% adults fed on the 0.6% adult diet grouped either with those of 2% adults (Experiment 1, see Figure 6.1.C) or with those of those of 0.01% adults (Experiment 2, see Figure 6.2.B). This shows that modest reductions in larval dietary [yeast] are not sufficient to induce a robust effect on adult lifespan, perhaps explaining why previous attempts from other labs have been unsuccessful in finding an effect of larval dietary composition on adult lifespan.

As most *Drosophila* studies of lifespan to date have analysed females, I also tested this gender. I observed that 0.01% vs 2% larval dietary yeast extended the lifespan of females by 50% when the adults fed on 9%HG (Figure 6.3.A). In contrast, when adult females fed on 0.6%HG, low larval dietary [yeast] failed to increase female lifespan (Figure 6.3.B). These results show that female and male lifespan are programmed in a similar manner by larval dietary [yeast].

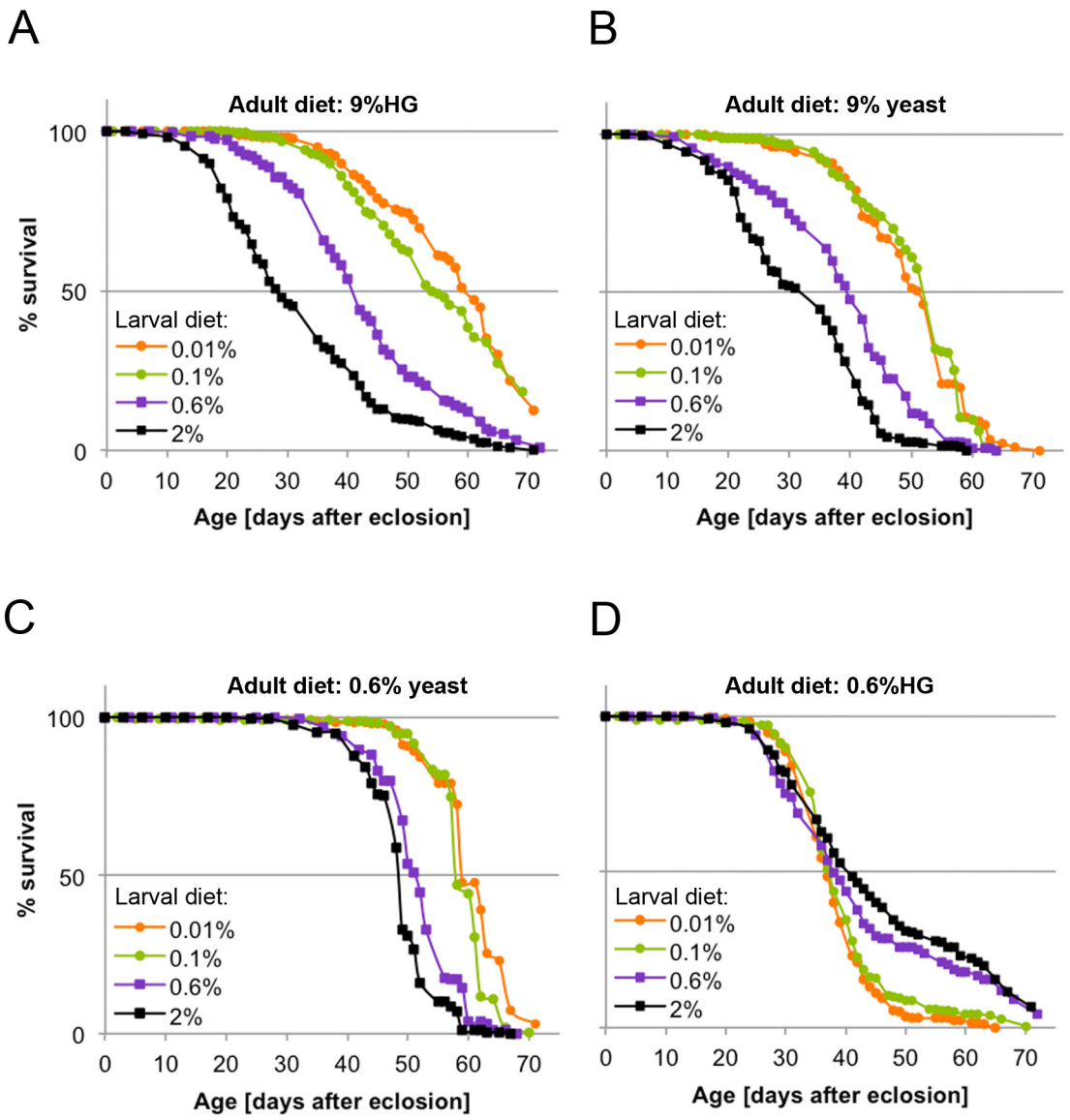

Figure 6.1: Low larval dietary [yeast] interacts with adult diet to programme male lifespan. **(A-D)** Survivorship curves of adult male flies that were raised on a 0.01%-, 0.1%-, 0.6%- or 2%-yeast diet as larvae and were fed on one of four adult diets: 9%HG **(A)**, 9%-yeast **(B)**, 0.6%-yeast **(C)** or 0.6%HG **(D)**. 0.01% larval dietary yeast doubles male median lifespan compared to 2% when adults are fed on a 9%HG diet **(A)** and increases it by ~90% when adults are fed on a 9%-yeast diet **(B)** and by 20% when adults are fed on a 0.6%-yeast diet **(C)**. However, no difference in median lifespan was observed when adults were fed on a 0.6%HG diet **(D)**. 0.6%HG was also the only of the four adult diets to increase 0.01% male adiposity levels (see Chapter 5). Statistics available in the Appendix.

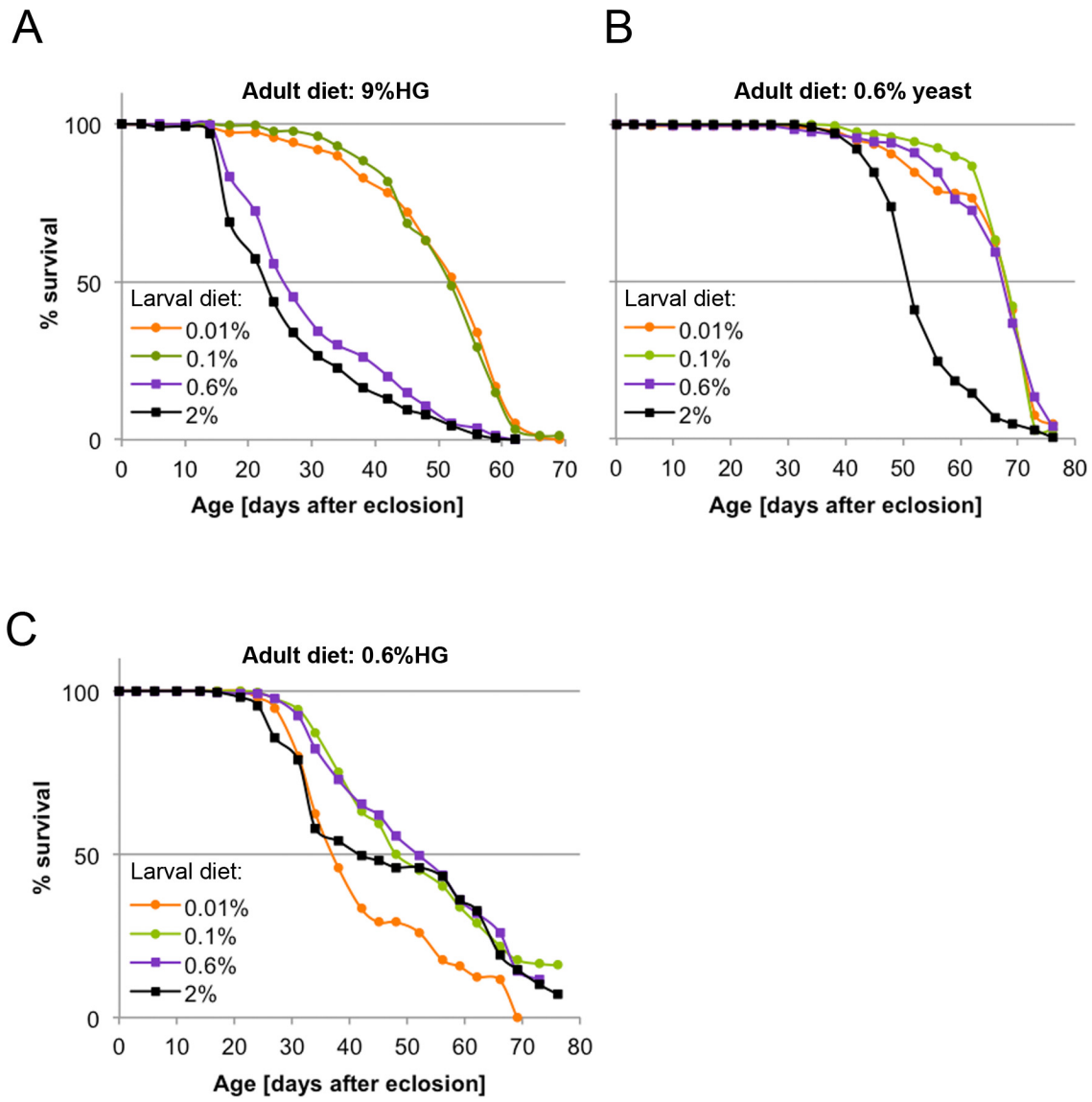

Figure 6.2: The dramatic adult-diet dependent effects of 0.01% larval dietary yeast on male lifespan were confirmed in an independent set of experiments. 0.01% larval dietary yeast extended male median lifespan by 126% when adults were fed on a 9%HG diet **(A)** and by 33% when adults were fed on a 0.6%-yeast diet **(B)**, compared with 2% larval dietary yeast. However, feeding adults on a 0.6%HG abrogated the 0.01% larval diet-induced extension in male median lifespan **(C)**. Statistics available in the Appendix.

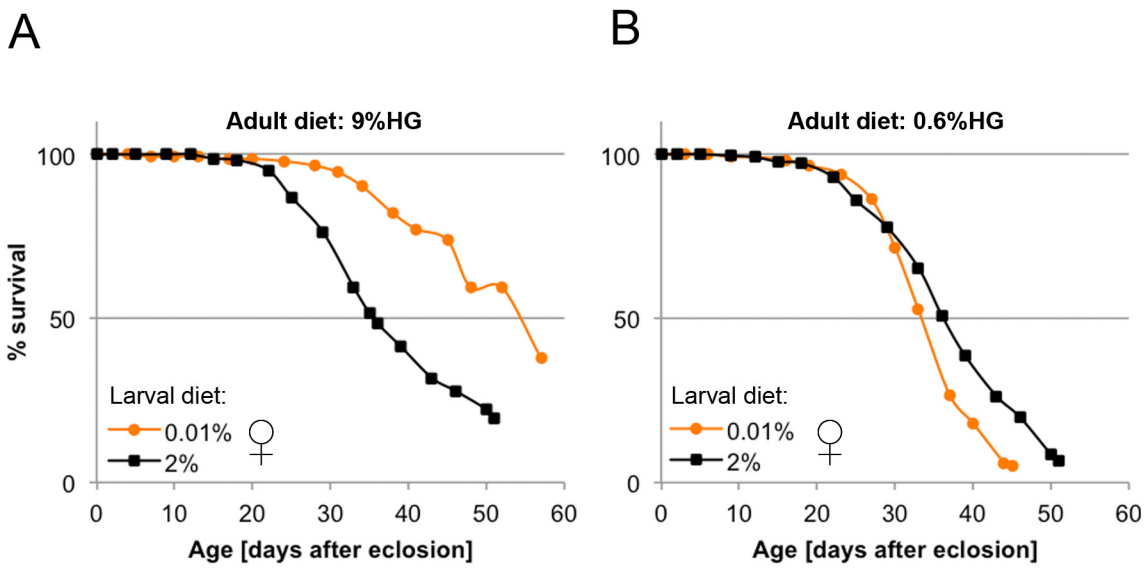

Figure 6.3: Larval dietary [yeast] also programmes female lifespan. 0.01% larval dietary yeast also extends the median female lifespan when adults are fed on a 9%HG diet **(A)**, but this effect is abrogated when adults are fed on a 0.6%HG diet **(B)**.

Tables 10.2, 10.3 and 10.4 in the Appendix present how my results compare with previous dietary or genetic manipulations that have been reported to extend *Drosophila* lifespan. Although the data presented in these tables does not represent an exhaustive collection of all the interventions/manipulations that can extend *Drosophila* lifespan, it brings together some of the most important findings in the field of *Drosophila* lifespan research and places the results presented here in context. By comparison, it becomes obvious that the magnitude of the 0.01%-induced increase in median lifespan is large and greater than seen for most adult DR or genetic manipulations/ mutants to date. The important finding that larval dietary [yeast] extends adult lifespan forms the basis of most subsequent work in this thesis, which aimed to better understand the nutritional programming of lifespan.

6.2.2 "Catch-up" abrogates the lifespan-extending effects of 0.01% larval dietary [yeast]

In mice, catch-up growth abrogates the beneficial effects of a low protein maternal diet on offspring lifespan (Ozanne and Nicholas Hales, 2004). In order to test whether the same holds true in our *Drosophila* model of low yeast diet during development, I investigated the effects of allowing undernourished larvae to catch up in growth/size during L3. This also addressed the question of whether there is a critical window during larval development when the lifespan-extending effects of low larval dietary [yeast] are established. Building on the findings of Tu and Tatar (2003), who yeast-deprived larvae during L3, I decided to test whether undernutrition during L1 and L2 larval development might be sufficient to replicate the lifespan-extending effects of undernutrition throughout larval development. For this, larvae were allowed to develop on 0.01% dietary [yeast] for the first two larval instars and then swapped onto 2% yeast food around the L2/L3 transition. Adults were fed on either 0.6% yeast or on 0.6%HG and their survival curves were compared to those of 0.01% and 2% animals. Preliminary experiments showed that, when swapped from "poor" to "rich" food at the beginning of L3, larvae undergo significant catch-up growth and the weight of the eclosing adults is approaching that of animals that had been fed on "rich" food throughout development (data not shown). This is in accordance with the observation that CW remained stable across the different larval diets i.e. final body size is dictated by growth during the TGP (see 3.3.3). I found that allowing larvae to catch-up during L3 abolished the lifespan-extending effects of low larval dietary [yeast]. Thus, "catch-up" males adopted similar survival curves to animals that had been reared on 2% yeast food throughout larval development (Figure 6.4). Hence, catch-up growth did not have a detrimental effect on *Drosophila* lifespan (i.e. catch-up adults fared no worse than 2% controls). Moreover, these findings demonstrate that yeast

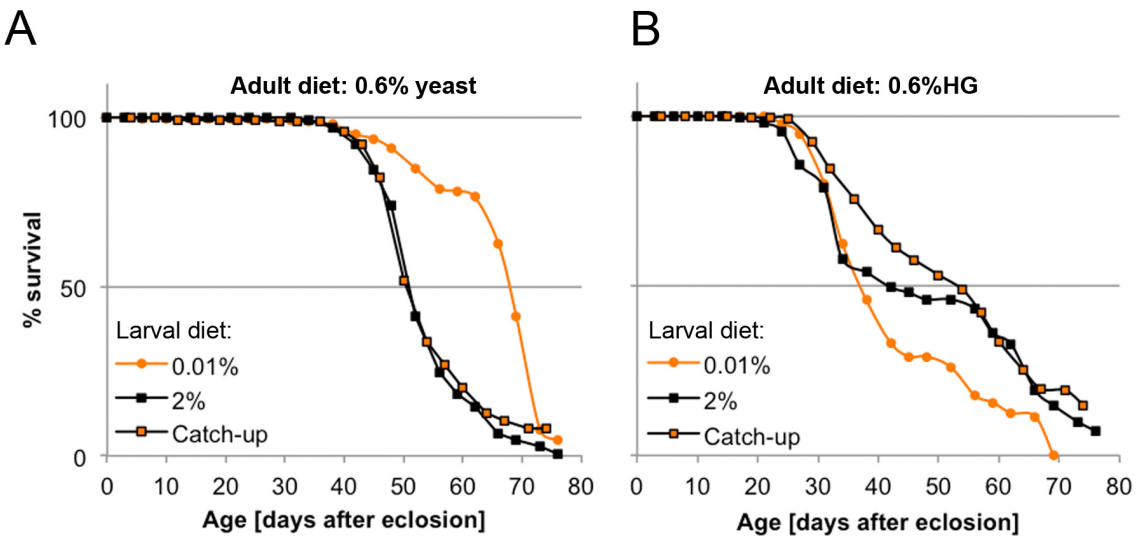

Figure 6.4: Catch-up growth during L3 abrogated the lifespan-extending effects of 0.01% larval dietary yeast. Transferring larvae from 0.01%- to 2%-yeast food at the L2/L3 transition gave rise to adult flies that adopted similar survival patterns to those that had been on 2%-yeast food throughout larval development. 0.01% males displayed a 33% increase in median lifespan when adults were fed on 0.6%-yeast, compared to both 2% and "catch-up" males **(A)**. "Catch-up" males displayed a 20% increased in median lifespan compared to 0.01% and 2% males, but the difference between the survivorship curves was significant only compared with 0.01% males **(B)**.

restriction during only the early phase of larval development is insufficient to program adult longevity.

6.3 Larval dietary [yeast] has only minor effects on adult starvation resistance

Resistance to various types of stress has been linked with lifespan extension (Finkel and Holbrook, 2000; Martin et al., 1996). Hence, the resistance to starvation was tested for 3.5-week 0.01%, 0.6% and 2% males that had been fed on 9% yeast. A minor decrease in 0.01% adult resistance to starvation, compared to that of 0.6% and 2% adults, was observed (Figure 6.5.A). However, only the difference between adults from 0.01% and 0.6% larval [yeast] was statistically significant (Log-Rank test, p=0.0149). Although the trend was still present in 6-week old males, the difference between 0.6% and 0.01% adults had lost statistical significance (Figure 6.5.B). Metabolic stores (both carbohydrate and lipid) have been linked with resistance to starvation (Djawdan et al., 1998). This raises the possibility that the increased adiposity of 0.01% adults fed on 0.6%HG could render them less sensitive to starvation. Surprisingly, however, despite 2-week old 0.01% males fed on 0.6%HG having double the adiposity of 2% controls, these animals showed a small, but statistically significant decrease in resistance to starvation compared to 2% males, at 1 week of age (Log-Rank test, p=0.0005; Figure 6.5.C). I conclude that, although larval dietary [yeast] can programme adult adiposity, it does not appear to have a major effect on adult starvation resistance.

6.4 The role of TOR signalling in larval [yeast] lifespan programming

The IIS and TOR pathways, the two main signalling pathways that match nutrient availability and growth in the developing organism, are also the two main pathways that have been proposed to control DR lifespan extension from yeast to mammals (Tatar, 2011). In order to dissect their involvement in a specific lifespan phenotype, one can alter signalling through these pathways pharmacologically or genetically.

In order to test whether the lifespan extending effects of 0.01% larval dietary [yeast] are mediated via TOR signalling, I adopted a pharmacological approach. Rapamycin is a bacterial product and a potent inhibitor of TOR (Heitman et al., 1991; Johnson et al., 2013). In mice, rapamycin extends lifespan, even when treatment starts later in life (Harrison et al., 2009). In *Drosophila*, supplementing the adult diet with rapamycin has been shown to increase lifespan by lowering signalling via the TORC1 branch and independently of the IIS pathway i.e. rapamycin did not affect pAkt levels and further extended the lifespan of long-

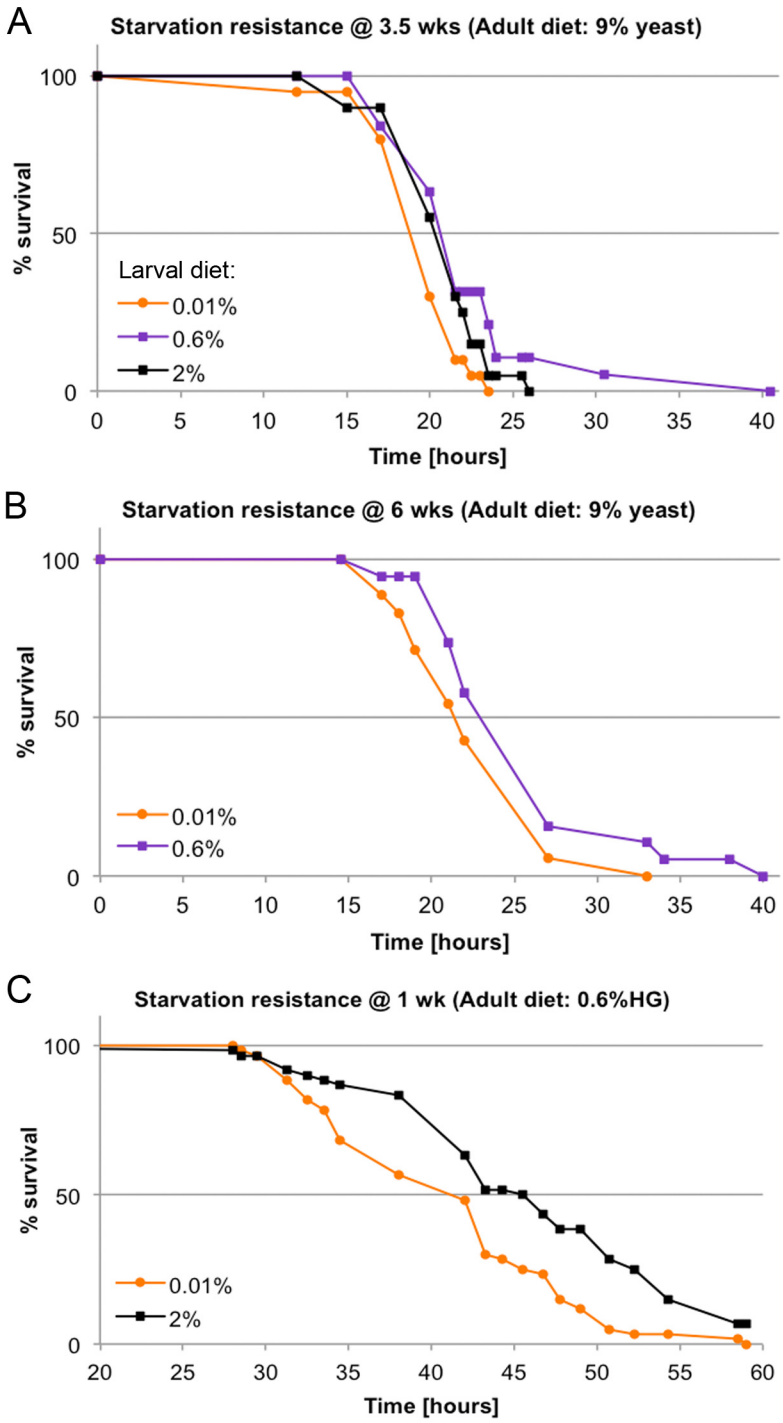

Figure 6.5: Low larval dietary yeast has only minor effects on adult resistance to starvation. **(A-B)** Resistance to starvation of 3.5-week and 6-week males raised on a 0.01%-, 0.6%- or 2%-yeast larval diet and fed on 9%-yeast adult diet. 0.01% larval dietary yeast slightly reduced adult resistance to starvation. **(C)** Resistance to starvation of 1-week old 2% vs. 0.01% males fed on 0.6%HG, the adult diet that increased 0.01% adult adiposity. Surprisingly, 0.01% males were less resistant to starvation.

lived IIS mutants (Bjedov et al., 2010). Based on the findings of Bjedov et al. (2010), we reasoned that, if the lifespan extending effects of 0.01% larval dietary [yeast] were mediated via decreased TORC1 signalling, then administering rapamycin to the adult diet should extend the lifespan of 2% flies but not that of 0.01% flies. On the other hand, if the effects of larval dietary [yeast] on lifespan were independent of TORC1, then rapamycin should have a similar effect on the lifespan of both groups.

Supplementing the 9%HG or the 0.6% yeast adult diet with 200 μM rapamycin increased the median lifespan of 2% adult males by 6% compared to ethanol vehicle controls (Figure 6.6). This is in agreement with the results of Bjedov et al. (2010). In the case of 0.01% flies, however, supplementation of the adult food with vehicle only (i.e. 2.5% v/v ethanol) dramatically reduced lifespan. When 0.01% adults were fed on 0.6% yeast food supplemented with EtOH, the lifespan-extending effects of 0.01% larval dietary [yeast] were abrogated (Figure 6.6.A). In addition, when adults were fed on 9%HG supplemented with EtOH, the increase in median lifespan between 0.01% and 2% flies was reduced to 50% (Figure 6.6.B; the differences recorded in other experiments that used 9%HG food without EtOH ranged from 90% to 150%). These unexpected, but interesting results suggest that 0.01% larval dietary [yeast] may increase adults' sensitivity to ethanol. Because of this, the effects of rapamycin on the lifespan of 0.01% adults could not be established. Interpreting the results from adults fed on 9%HG was further complicated by the fact that ethanol supplementation appears to make the surface of the food stickier. Presumably because of their small size, 0.01% adults stuck to it more frequently, which may have resulted in a proportion of the flies dying of "unnatural" causes. For these experiments, adults were fed on food supplemented with rapamycin (dissolved in ethanol) or with ethanol only, but not on the original diet (i.e. minus vehicle). Before embarking on a new set of rapamycin experiments, it will be interesting to establish the effects of ethanol supplementation on the lifespan of 2% and 0.01% adults, as well as on other aspects of their physiology.

6.5 Muscle accumulation of polyubiquitinated complexes with ageing remains unaltered by larval dietary [yeast]

In *Drosophila*, muscle function decline with age has been shown to correlate with the progressive accumulation of protein aggregates that can be visualised by antibody staining with an antibody to polyubiquitinated complexes (Demontis and Perrimon, 2010). Increasing the activity of the transcription factor FOXO or that of 4E-BP in adult muscles specifically, delays this process and extends lifespan, while loss of FOXO accelerates it and

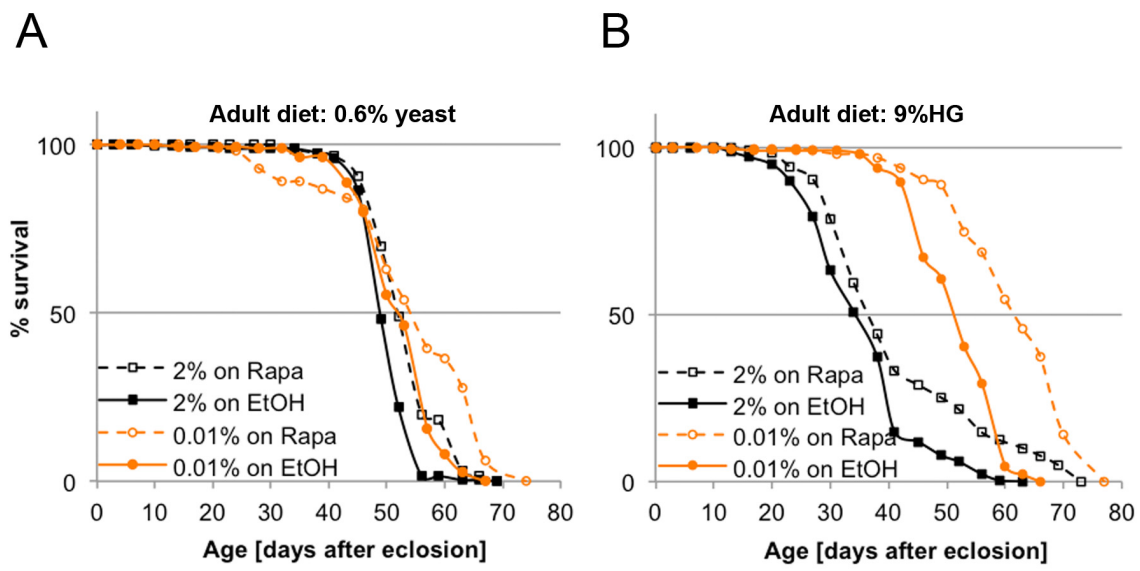

Figure 6.6: Testing the role of decreased TOR signalling in mediating the effects of a low-yeast larval diet on male lifespan by rapamycin treatment. (A-B) Supplementing the adult diet with 200 µM rapamycin extended the 2% male median lifespan by 6% compared to vechicle (i.e. 2.5% v/v EtOH) only. This is in accordance with reported findings (Bjedov et al., 2010). However, EtOH supplementation alone dramatically shortened 0.01% male lifespan, such that: ethanol supplementation abrogated the lifespan extension previously observed in 0.01% males fed on a 0.6%-yeast diet compared to 2% males **(A)** and reduced the difference in lifespan between 0.01% and 2% males fed on a 9%HG to only ~50% **(B)**. In experiments where adult food was not supplemented with EtOH, 0.01% males median lifespan was extended by 90 to 150% compared to that of 2% males, when adults were fed on a 9%HG adult diet.

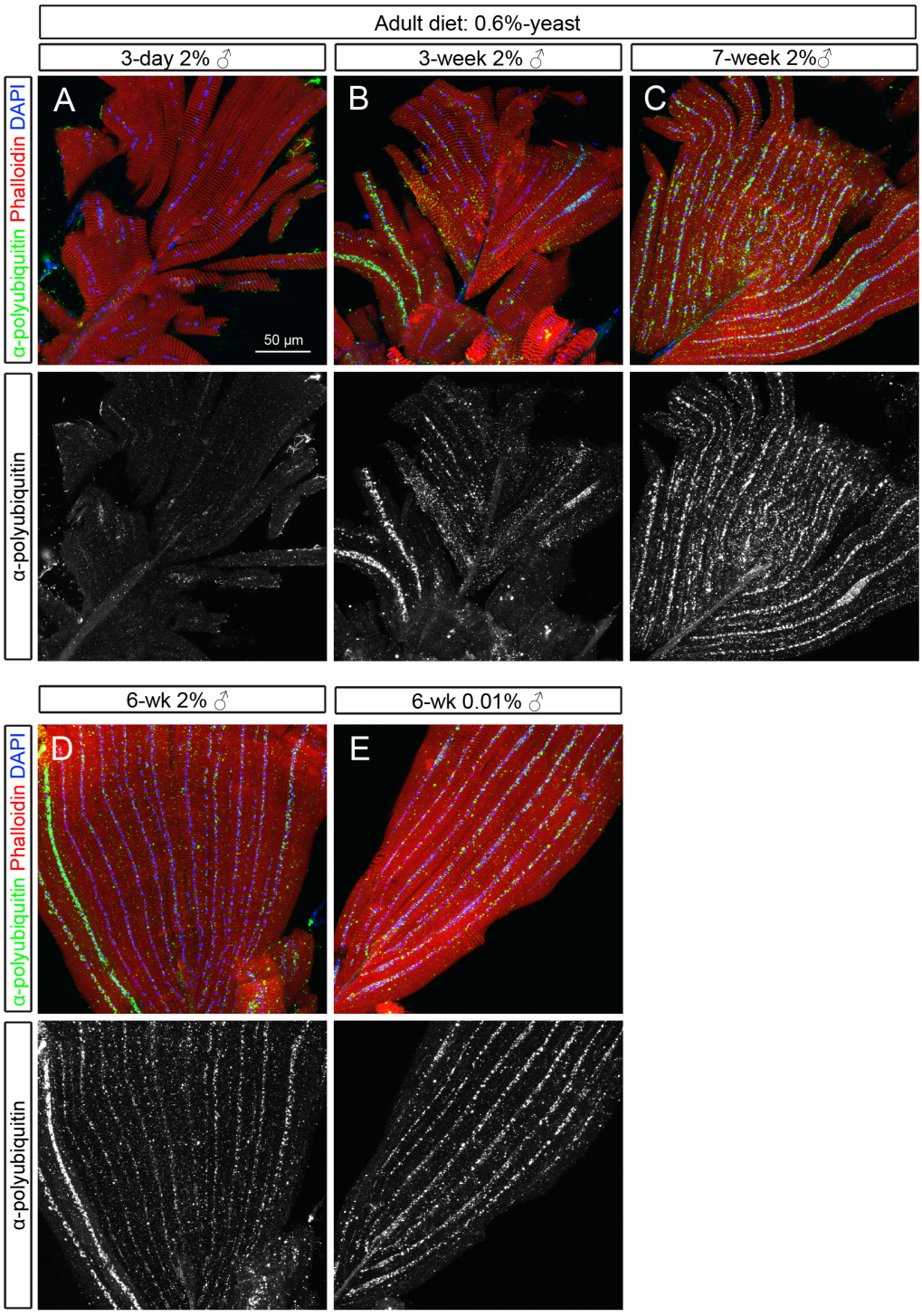

Figure 6.7: 0.01% larval dietary yeast does not delay the accumulation of polyubiquitinated aggregates in muscles with ageing. (A) Confocal micrographs of 3-day, 3-week and 7-week 2% males fed on a 0.6%-yeast diet confirmed that polyubiquitinated complexes (green) accumulate in adult muscles (red) with ageing. **(B)** Polyubiquitinated complexes accumulated to the same extent in the muscles of 6-week old 2% vs. 0.01% males fed on a 0.6%-yeast diet. Nuclei are stained with DAPI (blue), polyubiquitin protein aggregates are in green and muscles are visualised by Phalloidin staining (red).

shortens lifespan (Demontis and Perrimon, 2010). This led Demontis et al. to propose that the accumulation of polyubiquitinated protein aggregates in muscles is correlated with the ageing process, not just with choronological age. Therefore, I tested whether the accumulation of polyubiquitinated complexes in adult muscles was delayed in long-lived 0.01% males. Consistent with the previous study, the polyubiquitin signal increased dramatically with age in control 2% flies fed on 0.6% yeast (Figure 6.7.A-C). Few aggregates were present in the muscles of 3-day old males, whereas their numbers and size increased by 3 weeks of age and became abundant by 7 weeks. However, no difference was found in the extent of the accumulation of protein aggregates in the muscles (Figure 6.7.D-E) – or adipose tissue (data not shown) – of 6-week old 0.01% vs. 2% adults fed on 0.6% yeast. Given their different lifespans, this suggests that, in contrast to FOXO/4E-BP genetic manipulations, the accumulation of polyubiquitinated complexes in adult *Drosophila* tissues in my nutrient programming model is primarily a function of chronological age, not ageing *per se*.

6.6 Discussion

The work presented in this chapter demonstrates that larval diet can have dramatic effects on adult longevity. I have showed that low larval dietary [yeast] interacts with adult diet to extend lifespan on 3 of the 4 adult diets tested. Interestingly, the dietary history of the adult has been shown to have no long-lasting consequences on fly survival i.e. if adults are switched from a low- to a high-protein diet at mid-life they immediately adopt the mortality rates of animals that have been on the high-protein diet throughout (Mair et al., 2003). This makes the effects of larval dietary [yeast] on lifespan all the more striking. The low-yeast larval diet-induced differences in median lifespan largely reflect the delay in the onset of rapid mortality (i.e. the inflection point on the survival curve), rather than changes in the slope of the survival curves. Adult IPC ablation has been reported to have a similar effect i.e. age-related mortality started later, but then proceeded at the same rate as in controls (Broughton et al., 2005).

In longevity research, an important distinction is that between lifespan and healthspan (i.e. healthy ageing) (Tatar, 2009). Therefore, it is important to establish whether an intervention extends lifespan by virtue of making animals sick for longer or whether it slows down senescence and prolongs the period an animal is in good health. However, heathspan is poorly defined in humans, let alone in model organisms. Despite the ill-defined terminology, one can still use different readouts to assess animal fitness. In *Drosophila*, the most widely reported such readouts are activity levels and climbing ability.

The later is assessed in a "negative geotaxis assay" and is used for testing age-related locomotor decline (Gargano et al., 2005). The former requires specially designed insect activity monitors, which we are in the process of acquiring. Both of these methods could be used to assess whether long-lived 0.01% flies stay active and "fit" for longer than their 2% counterparts. This would provide insight into the factors that mediate the dramatic increase in lifespan induced by low larval dietary yeast. In the quest for understanding whether an intervention/manipulation that extends lifespan actually delays senescence, genuine markers of ageing, rather than chronological age, would also be invaluable in determining the "ageing status" of an animal.

Interestingly, feeding 0.01% adults on a 0.6%HG diet abrogated the larval diet-induced extension in lifespan. The dependence of a lifespan effect upon adult dietary composition is not a particularity of this study. Indeed, the few studies that have extended their observations to a series of adult diets also find that the magnitude, and even direction, of the effect depends on adult diet. Resveratrol, which has been shown to increase lifespan in flies and mice (reviewed in (Chung et al., 2012; Lam et al., 2013), does not affect the longevity of wild-type males or females fed on standard-sugar/low-yeast or high-sugar/low-yeast diets, but extends the lifespan of females fed on a low-sugar/high-yeast or on high-fat diets (Wang et al., 2013). Moreover, the effects of many of the genetic manipulations or mutations that affect lifespan have been shown to depend on the adult diet. Clancy et al. showed that *chico*$^{-/-}$ females are longer-lived than controls but not when kept on food dilutions of 0.65 times or lower (Clancy, 2002). Overexpressing TSC2 ubiquitously during larval and adult stages can extend male median lifespan anywhere between 5% to 95% depending on adult dietary [yeast] (Kapahi et al., 2004). Lifespan extension is larger when recorded on adult foods with higher [yeast] because an increase in adult dietary [yeast] is less detrimental to the longevity of flies overexpressing TSC2. Furthermore, Indy (I'm not dead yet) heterozygotes are longer-lived than wildtype on a "normal", but not on a low-yeast food, suggesting that the effects of DR and those of mutating *Indy* on lifespan may act via overlapping routes (Wang et al., 2009). However, the effect of Indy on lifespan is controversial. Indy mutations extend lifespan when placed in a CantonS or yw background but not when placed in a w^{1118} background (Neretti et al., 2009; Toivonen et al., 2009; Wang et al., 2009). Similar adult diet-dependent lifespan effects have been reported for altered expression of/mutations in superoxide dismutase 1 (SOD1), 4E-BP, proteins in the mitochondrial electron transport chain and others (Sun et al., 2012; Zid et al., 2009).

Larval dietary [yeast] alters the adults' response to adult dietary composition. In my experiments, DR (i.e. reduction in adult dietary [yeast] from 9% to 0.6%) extended 2% males' median lifespan by ~70%, while 0.01% males responded to DR with an increase in median lifespan of only 20%. If larval dietary [yeast] and DR extended lifespan via non-overlapping mechanisms, then both 2% and 0.01% flies should have responded to DR in the same manner. The fact that larval dietary [yeast] modified the adults' response to DR suggests that adult DR and low larval dietary [yeast] extend lifespan by, at least, partially overlapping/interacting mechanisms. However, the fact that DR further extended the lifespan of long-lived 0.01% flies shows that these mechanisms, though potentially overlapping, are not identical.

One interesting question is whether there is a critical window during larval development when larval diet acts to establish its long-term effects on adult longevity. Together with the findings of Tu and Tatar (2003), the results of the "catch-up growth" experiments I present in this chapter demonstrate that a restricted period of undernutrition during L1+L2 or L3 alone is not sufficient to induce long lasting effects on adult survival. However, there are important differences between my experiments and those of Tu and Tatar. For example, they (a) present the lifespan of males and females together (b) fed adults on a diet supplemented with live yeast and (c) deprived L3 larvae of yeast completely. Finally, their experimental paradigm lowered female egg laying. Therefore, it would be interesting to perform the converse experiment of undernourishing larvae during late development using the same conditions used in the experiments presented here. This may help to establish whether larval undernutrition acts to extend adult lifespan during a specific developmental window. The experiments of Tu and Tatar also suggest that reduced adult size (and perhaps altered body proportion) is not by itself sufficient to programme adult lifespan.

Rapamycin extended the median lifespan of 2% adults by ~6%, as previously published (Bjedov et al., 2010), regardless of whether the adults were fed on 9%HG or 0.6% yeast. This limited effect together with the fact that the effects of larval dietary [yeast] on lifespan depended on the adult diet, while the effect of rapamycin did not, suggest that the effects of larval dietary [yeast] on lifespan are not mediated by altered TORC1 signalling. During the rapamycin experiments, it also emerged that 0.01% adult flies, despite longer lived, may be more sensitive to ethanol. This could be related to altered lipid metabolism as McKechnie and Geer found a correlation between tissue 18-carbon fatty acids levels and resistance to ethanol in Drososphila larvae (McKechnie and Geer, 1993). Moreover, it is known that dietary ethanol affects the composition of lipids in *Drosophila* larvae (Geer

et al., 1986). It would be interesting to characterise the effects of supplementing the adult diet with varying concentrations of ethanol on 0.01% vs. 2% lipid content and fatty acid profile. These findings also raise the possibility that low larval dietary [yeast] may have detrimental effects on adult xenobiotic metabolism. In the future, it would be interesting to characterise 0.01% adults' resistance to ethanol as well as other xenobiotics.

Surprisingly, I found that despite 0.01% males fed on 0.6%HG having increased fat stores, they appear to be more sensitive to starvation. This findings, together with the fact that these males have 30% less NEFA compared to 2% controls fed on the same adult diet, suggests that fat mobilisation may be impaired in 0.01% adults. To test this, one could adopt different strategies. First, one can starve 0.01% vs. 2% males and assess lipid mobilisation from the fat body at different time points either by LipidTOX stainings or by GC analyses of their lipid content. One could also assess the expression levels of *bmm* and *akhr* (adipokinetic hormone receptor) under fed and starved conditions. Finally, one could assess basal and starvation-induced lipolytic activity of cytosolic fat body cell extracts, as previously described (Gronke et al., 2007). If lipid mobilisation is impaired in 0.01% adults, perhaps this can explain their increased adiposity.

The ratio of protein to sugar in the diet has been suggested to play an important role in the metabolic outcome of different diets (Lee et al., 2008; Skorupa et al., 2008). In the context of lifespan, Lee et al. tested 28 different combinations of sucrose to yeast and found a correlation between dietary sugar:protein ratio and lifespan in mated Canton-S females: lifespan increased with increasing sugar:protein (Lee et al., 2008). While a useful proof of principle for the potential applications of the "Geometric Framework" (Raubenheimer and Simpson, 1997), this study only used lifespan data for 36 individually-housed females per dietary treatment. Perhaps this also explains the remarkably short lifespans observed in this study, regardless of adult dietary treatment: the longest median lifespan was ~25 days. Although an interesting concept, the application of the Geometric Framework in *Drosophila* needs perfecting. The study by Lee et al. (2008) has, however, for the first time, proposed a clear answer in the debate of whether adult flies compensate for nutrient dilution by overeating.

The magnitude of the effects I report here are larger than most genetic manipulations and dietary interventions reported in the literature to date (see Tables 10.2, 10.3 and 10.4). This may be due to a series of reasons, not least because most studies have only recorded adult lifespan on one adult diet. As I show here, the effects of larval dietary [yeast] on adult longevity are highly dependent on adult nutrition. This underscores the importance

of characterising the effects of an intervention on a range of environmental conditions in order to reveal its true effects and to better understand the underlying mechanisms. The different effects of DR on the lifespan of 2% vs. 0.01% adults together with the rapamycin results and the recent findings of Na et al. that the lifespan-extending phenotype of *chico*$^{-/-}$ is abrogated on a high sugar diet (Na et al., 2013), all suggest that the effects of low yeast larval diet on adult lifespan may in part be mediated via lowered IIS signalling.

Chapter 7

Microbiota: the missing link?

7 Microbiota: the missing link?

7.1 Introduction

As flies age, the surface of the food they feed on becomes increasingly sticky and shiny- especially true for "standard" glucose foods. This effect, which becomes apparent within one day of placing the animals in the vial, is an age-dependent effect and does not occur in empty vials. In vials containing young flies food surface remains matte for at least several days. While the exact reasons for the changes in food surface texture have never been formally investigated, it is thought to reflect fly-associated microbes that spread onto the food. Indeed, in support of this theory, the external and internal bacterial load of flies increases with age and the cuticle of aged flies is covered with structures resembling bacterial biofilms, which disappear under germ-free conditions (Ren et al., 2007). This would also explain why this phenomenon is not observed for young flies. Although difficult to capture in an image, I have observed that this effect appears later and is much less pronounced in vials housing 0.01% males compared to those housing 2% males. The surface texture of the food for high-glucose adult diets was only minimally affected by the presence of flies in the vial, yet the largest difference in adult lifespan was observed when adults fed on 9%HG. This suggests that differences in food surface "stickiness" alone cannot explain the differences in lifespan induced by larval dietary yeast (see Chapter 6). However, this led me to investigate the possibility that larval diet may alter adults' microbial load/ types of microbes associated with the adult, which, in turn, can impact upon host metabolism and/or longevity (reviewed by Ottaviani et al., 2011; Tremaroli and Bäckhed, 2012).

In mammals, bacteria in the colon metabolise complex carbohydrates and other undigested nutrients to produce short chain fatty acids (SCFAs), which can then be taken up by colonocytes (reviewed by Macfarlane and Macfarlane, 2003; Tremaroli and Bäckhed, 2012). In Chapter 5, I showed that 0.01% larval dietary yeast induces the accumulation of lipid droplets specifically within adult hindgut enterocytes by 1 week of age. This correlation is intriguing and provided further incentive to analyse the role that the microbiota may play in mediating the long-term effects of larval nutrition. Cho et al. have recently showed that long-term treatment with low doses of antibiotics alters the murine colonic microbiota, colonic SCFAs and adiposity (Cho et al., 2012). The mechanistic link between the microbiota and host metabolism is yet to be elucidate. However, it has

been reported that the effects of the microbiota on host adiposity are modulated by the SCFA receptor Gpr41, which is expressed in a subset of enteroendocrine cells (Samuel et al., 2008). Moreover, axenic mice (i.e. that lack microbiota) are protected against the effects of an obesogenic diet (Bäckhed et al., 2007; Rabot et al., 2010). Conversely, diet-induced obesity is linked with alterations in the distal gut microbiota (Turnbaugh et al., 2008).

The composition of the microbiota changes with age, a phenomenon documented in a wide range of organisms, from humans (Biagi et al., 2010; Hopkins and Macfarlane, 2002; Mariat et al., 2009; O'Toole, 2012) and rodents (McKnite et al., 2012) to flies (Wong et al., 2011a). Whether the microbiota affects lifespan is still to be determined as this topic has only recently captured the attention of the scientific community (Ottaviani et al., 2011). If so, this would open up the exciting possibility that certain mutations or dietary regimes could affect animal longevity via changes in the composition of the gut microbiota. Given the time course of longevity analyses and the inherent technical difficulties of maintaining animals gnotobiotic throughout the duration of the experiment, little is known in mammals, at the moment.

Drosophila has a much simpler gut microbiome than mammals, with 4-5 species accounting for >90% of the total (Chandler et al., 2011; Wong et al., 2011a). In larvae, the gut microbiota have been shown to modulate growth through IIS/TOR dependent mechanisms (Shin et al., 2011; Storelli et al., 2011). Moreover, microbiota are essential for larval growth under conditions of low dietary yeast. The few *Drosophila* studies that have addressed the potential effect of the microbiota upon lifespan report conflicting results: either that microbiota enhance lifespan (Brummel et al., 2004), or that they have no effect (Ren et al., 2007; Ridley et al., 2012). It may be that certain bacterial species promote longevity (i.e. their presence is beneficial), while others have detrimental effects on adult survival (i.e. lifespan would be extended in their absence by removing their deleterious effects).

In *Drosophila*, embryos are free of bacteria (with the exception of intracellular endosymbionts such as *Wolbachia*). Females deposit bacteria on the outside of the embryo as they lay it and newly-hatched larvae acquire their microbiota by feeding on the chorions. During metamorphosis, while the larval gut is histolysed and the adult gut is formed, bacterial titres decrease. Upon adult eclosion, bacteria present inside the pupa, i.e. inherited from larval stages, colonise the gut to establish the adult microbiota. Given that research into the links between microbiota and host physiology is still in its early days,

very few studies have addressed the possibility that nutrition during early life may "programme" adult metabolic homeostasis through long-lasting effects upon microbiota composition (reviewed by Kaplan and Walker, 2012). In this chapter, I set out to investigate the hypothesis that larval diet may cause long-lasting changes in the composition of the gut microbiota, which, in turn, can interact with adult diet to impact on adult metabolism and/or longevity. In theory, this is one possibility that could explain how larval and adult diets may interact to give different outcomes.

7.2 Results: Antibiotics alter adult adiposity

To study whether altered adult gut microbiota could mediate the increased adiposity of 0.01% males fed on 0.6%HG, adult food was supplemented with antibiotics. Literature on the topic of antibiotic treatment in *Drosophila* is scarce and the approaches taken vary widely (Brummel et al., 2004; Ren et al., 2007; Ridley et al., 2012; Storelli et al., 2011). Based on this limited information, a cocktail of four broad-spectrum antibiotics – i.e. ampicillin, erythromycin, kanamycin and tetracycline – was chosen as a starting point. I used GC-MS to analyse the lipid content and profile of 10-day old 0.01% and 2% males fed on 0.6%HG ± 400 μg/mL of the antibiotic cocktail. Due to a technical difficulty, adiposity measurements of control 2% flies fed on 0.6%HG were only generated for one sample. However, this data point was in the range of previous measurements and the validity of the results was further confirmed by the fact that, as expected, the adiposity levels of 0.01% males were twice that of their 2% counterparts (see 5.4.2). Supplementing the adult diet with antibiotics, almost doubled the adiposity of 2% males, but had little effect on the already-high adiposity levels of 0.01% males (Figure 7.1.A). In other words, antibiotic treatment brought the adiposity of 2% flies up in the range of that of 0.01% flies. The antibiotic treatment, however, did not affect the overall profile of fatty acids in stored TAG (Figure 7.1.B). Whether this antibiotic-induced increase in 2% animals' adiposity levels also resulted in ectopic lipid accumulation in non-adipose tissues – such as podocytes, as seen for 0.01% flies fed on 0.6%HG – remains an open question. This shows that antibiotic treatment has the potential to alter adult fly metabolism, similarly to mammalian studies (Cho et al., 2012). The results suggest that alteration in adult-associated microbial communities may underlie the increased adiposity displayed by 0.01% males fed on a 0.6%HG adult diet. In future, it would be interesting to experiment with a range of concentrations as well as different antibiotic cocktails. It would also be informative to assess the effects of adult antibiotic treatment upon internal and external bacterial loads.

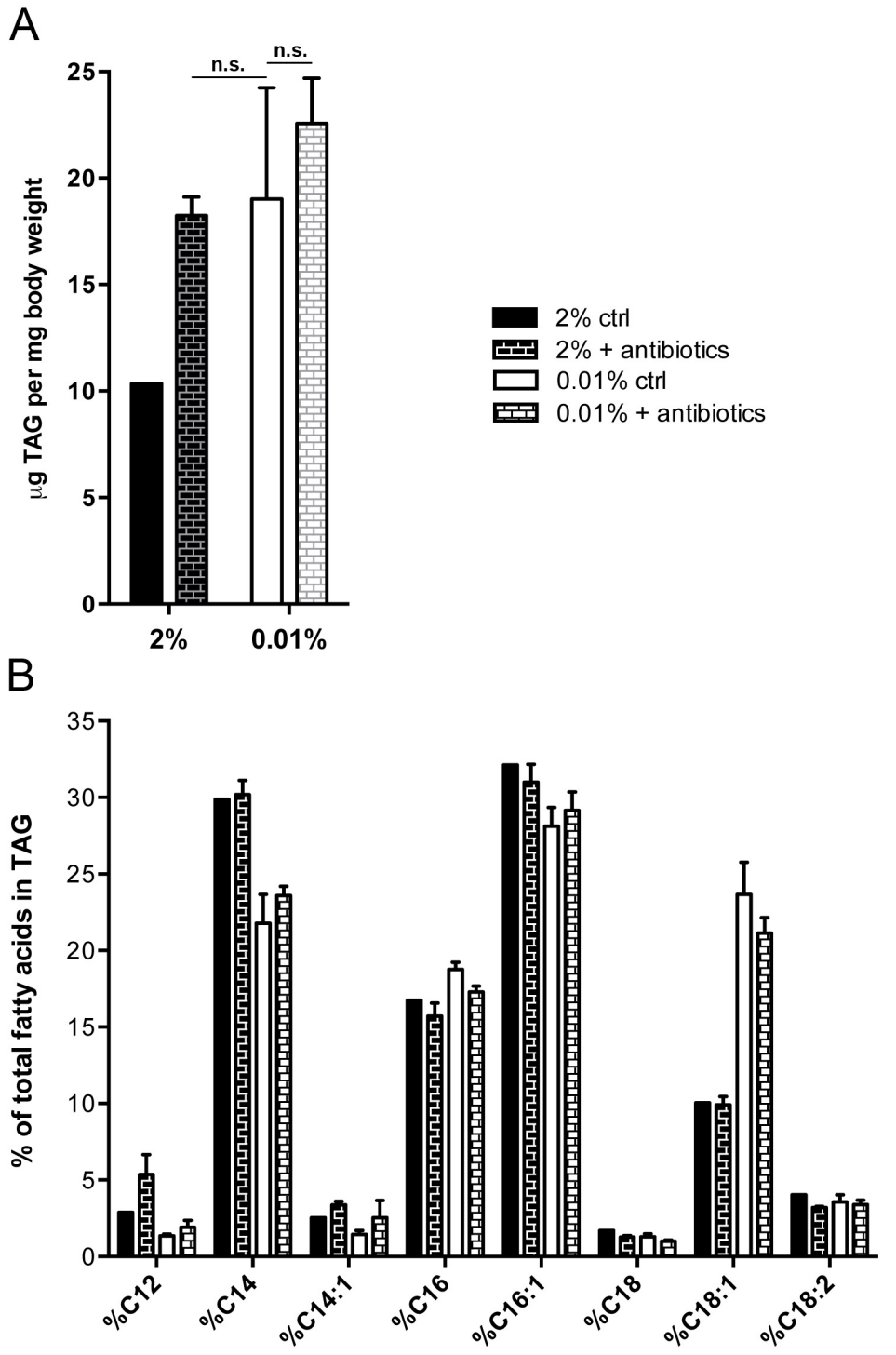

Figure 7.1: Antibiotic treatment can increase adult adiposity. (A) Supplementing the adult diet with 400 μg/mL of a four-antibiotic cocktail (i.e. kanamycin, erythromycin, tetracycline and ampicillin) doubled the adiposity of 10-day old 2% males, but had no significant impact on the already-high 0.01% male adiposity. **(B)** Antibiotic treatment did not alter the fatty acid profile in adult male TAG+CE fraction.

7.3 Adult gut microbiota may be influenced by larval diet

7.3.1 Development of a method for faecal analysis

To begin to study what effects altered larval dietary yeast may have upon adult gut microbiota, I decided to study the faecal metabolite profile of 0.01% vs. 2% adults by ^{1}H NMR, in collaboration with TJ Ragan. This could potentially provide insight into gut microbial metabolic activity, as well as potential differences in intestinal absorption or excretion. In mammals, differential metabolic activities of gut bacteria are reflected in altered metabolite concentrations in the faeces. In *Drosophila*, characteristics of the excreta (such as frequency, pH and dilution) have been linked with females' reproductive and metabolic status (Cognigni et al., 2011).

The principal challenge with analysing the metabolic composition of *Drosophila* faeces was to find a way to measure (or normalise) the amount of material in each sample. For ^{1}H NMR analyses of fly haemolymph, the volume of haemolymph extracted per sample is accounted for in the data analysis by extracting the haemolymph in PBS containing a known concentration of ^{13}C-formate and correcting for the "dilution factor" of ^{13}C-formate in the final sample. This approach could not be employed for the analysis of fly faeces for a number of reasons. The main reason is that this "dilution approach" only works for liquid samples and would not have provided information about the volume or amount of solid material in the sample. To normalise to the amount of "material" collected, I used dietary Bromophenol Blue (BPB). BPB is a pH-sensitive dye that absorbs red light and was chosen because it cannot be broken down or absorbed by the flies' digestive system (Cognigni et al., 2011). Further favourable properties of BPB in this case were: (1) the fact that we found it partitions completely in the aqueous phase during chloroform:methanol extraction and (2) that its NMR spectrum did not overlap with the spectra of any of the other metabolites present in fly faeces. Experiments comparing the excreta of adults fed on BPB-supplemented food with those of adults fed on control food revealed that BPB supplementation did not affect the polar metabolite profile of fly faeces (data not shown). The quantifications presented here are, therefore, based on data normalised to the amount of BPB in the sample.

Obtaining suitable amounts of material for metabolomic analysis from such small animals presented challenges of its own. The method that was finally chosen involved placing flies in empty glass vials and collecting faeces over periods of no longer than 2 hours, in order to prevent starvation/dehydration and to minimise possible metabolite degradation (see

2.13 for details). The advantage of this method was that faeces were collected directly into the same vials that were then used for choloroform:methanol extraction of polar metabolites. In addition, flies were only minimally interfered with, which meant that the same flies could be used for collections at different time points or could be kept for other assays. Following collection, the adults were transferred back onto food that did not contain BPB. 24 hours later, the blue coloration completely disappeared from the guts of 2% animals fed on 9%HG. Interestingly, however, the guts of 0.01% males fed on 9%HG still contained variable amounts of blue food (data not shown), suggesting that intestinal transit times too may fall under the programming effect of larval diet.

7.3.2 Larval diet, adult diet and age all alter faecal polar metabolite profiles

Comparing the faecal metabolic profiles of 0.01% and 2% males fed on 9%HG (the adult diet on which the lifespan difference was greatest), it emerged that faeces of 2-week 0.01% males contained significantly less glucose relative to BPB than those of age-matched 2% controls (Figure 7.2.A). Ageing also affected the composition of the faeces, with 2% males having higher relative faecal glucose levels at 4 week than at 2 weeks (Figure 7.2.A). This demonstrates that the composition of adult faeces also falls under the programming effect of larval nutrition. Furthermore, as faeces glucose levels increased with age, these results suggest that 2-week 0.01% males have a "younger" faecal metabolic profile as compared to 2-week 2% males. This is in accordance to the former being much longer-lived on 9%HG. Taken together, the results open the exciting possibility that the metabolic profile of adult fly faeces could be used a non-invasive means of obtaining insight into fly metabolic status. Feeding adults on 0.6%HG (which doubled the adiposity of 0.01% males) had the opposite effect i.e. higher glucose levels were detected in the faeces of 2-week 0.01% males than in those of 2-week 2% males (Figure 7.2.A). These results reinforce the lifespan and adiposity measurements presented in Chapters 5 and 6, which suggested that larval dietary [yeast] altered adults' response to adult diet. Interestingly, betaine was also detected in the faeces (Figure 7.3.A). This compound was not detected in the food (data not shown), suggesting that faecal betaine results from bacterial fermentation within the gut. Betaine levels in the faeces were not altered by larval diet, but were dramatically influenced by adult diet. A 9%HG adult diet increased faecal betaine levels by ~20-fold as compared to a 0.6%HG diet at both 2 and 4 weeks of age, and regardless of larval dietary yeast. Taken together, these results show that larval diet interacts with adult diet to programme faecal glucose content.

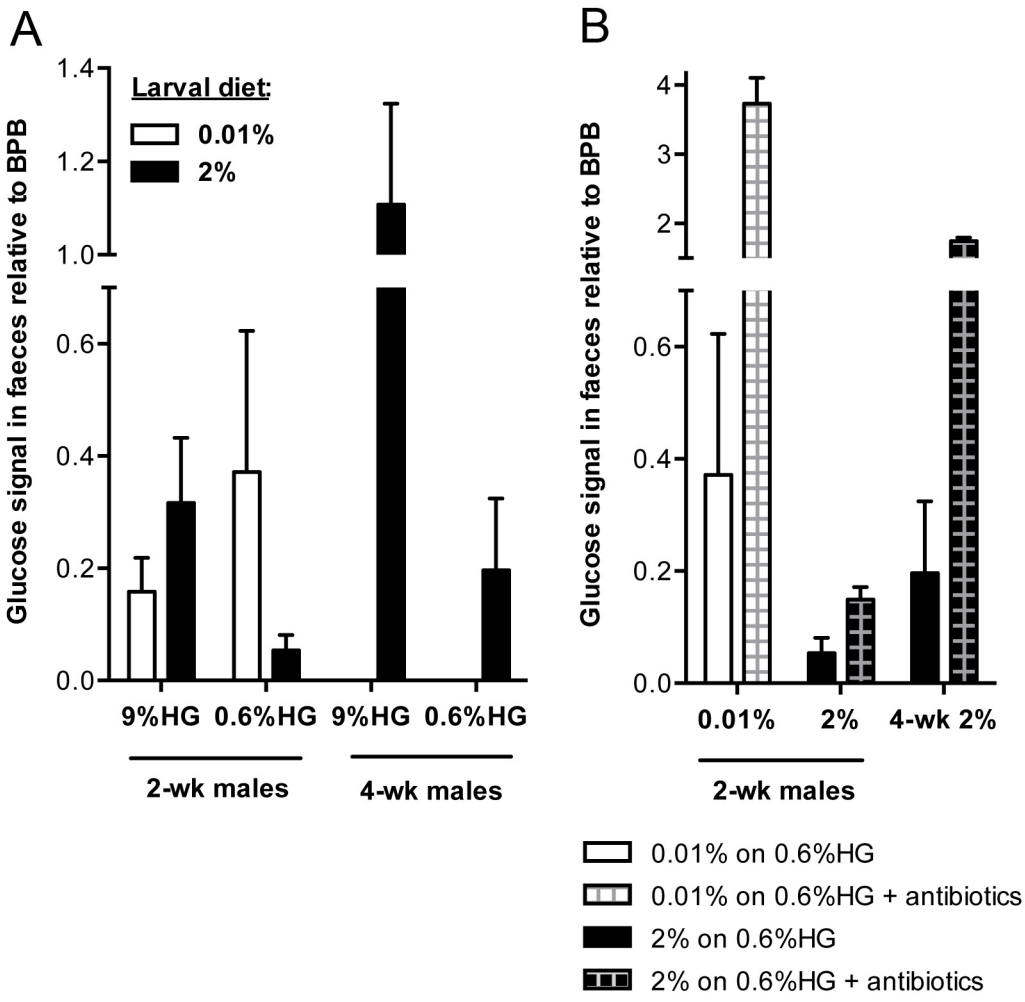

Figure 7.2: Larval diet programmes glucose content of adult faeces, an effect that can be replicated by adult antibiotic treatment. (A) 0.01% larval dietary yeast, compared to 2%, decreased slightly the glucose content of 2-wk male faeces, when adults were fed on a 9%HG diet. In contrast, 0.01% larval dietary yeast increased the relative glucose content of 2-wk adult faeces, by ~4-fold when adults were fed on a 0.6%HG diet. Glucose levels in 2% adult faeces increased ~3-fold from 2 weeks to 4 weeks of age, for both a 0.6%HG and a 9%HG adult diets. **(B)** Supplementing a 0.6%HG adult diet with 400 µg/mL of a four-antibiotic cocktail (i.e. kanamycin, erythromycin, tetracycline and ampicillin) increased relative faecal glucose levels by ~10-fold for 2-wk 0.01% males and 4-wk 2% males and by 2.8-fold for 2-wk 2% males. Flies were maintained on food ± antibiotics throughout adulthood. To account for differences in the amount of faeces collected per sample, the amount of glucose was normalised to the BPB signal in each sample. As in most other figures, error bars represent 1 S.D.

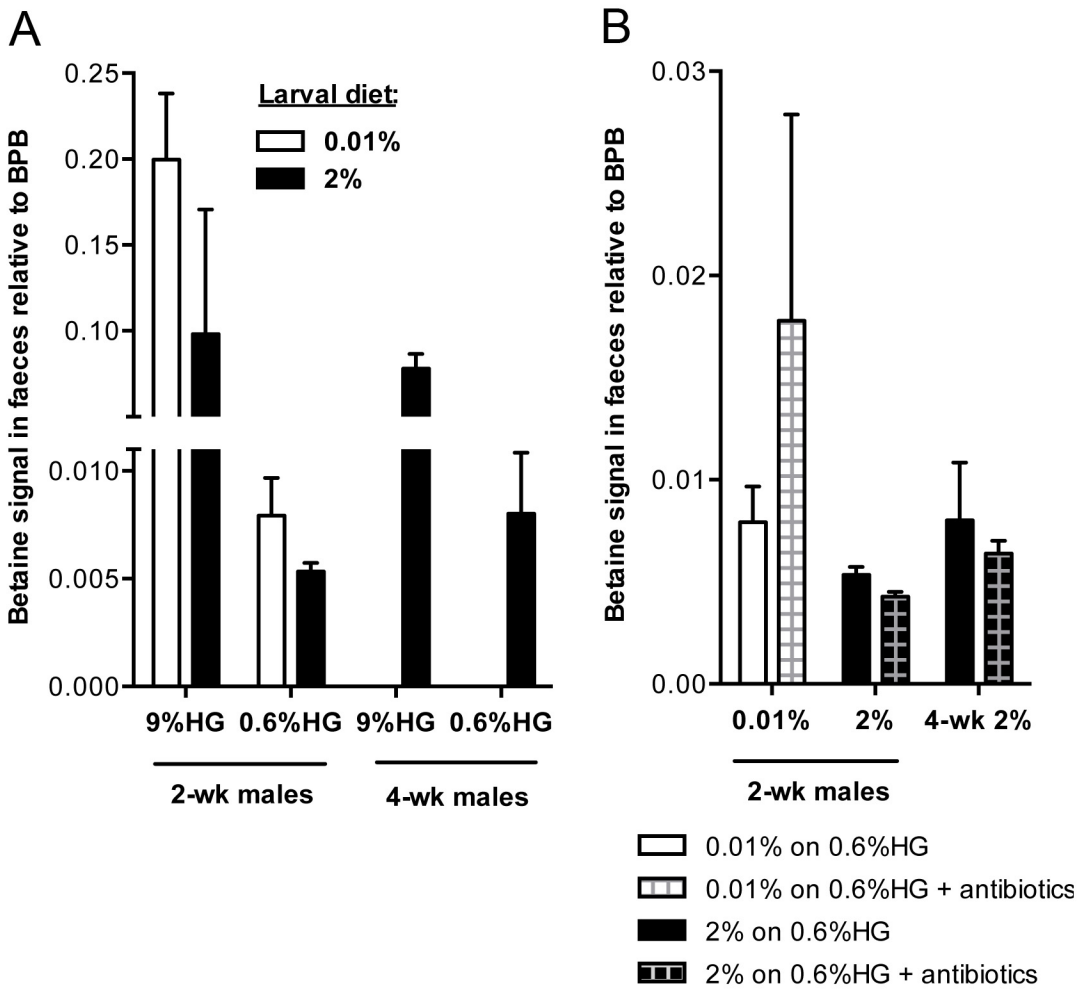

Figure 7.3: Betaine faecal content is altered by adult, but not larval diet. (A) A 9%HG adult diet increased faecal betaine levels by 20-fold at 2 weeks of age and by ~13-fold at 4 weeks of age. Relative faecal betaine levels were not affected significantly by larval diet. Moreover, 2% male faecal betaine levels did not change significantly between 2 and 4 weeks of age. **(B)** Supplementing a 0.6%HG adult diet with 400 µg/mL of a four-antibiotic cocktail (i.e. kanamycin, erythromycin, tetracycline and ampicillin) increased relative betaine levels in the faeces of 2-wk 0.01% males but had no effect on the relative levels of betaine in the faeces of 2-wk nor 4-wk 2% males**.** Flies were maintained on food ± antibiotics throughout adulthood. To account for differences in the amount of faeces collected per sample, the amount of glucose was normalised to the BPB signal in each sample.

7.3.3 Adult antibiotic treatment mimics larval programming of faecal carbohydrate content

To begin to understand the possible contributions of gut microbial metabolic activity on the composition of faeces, adults fed on a 0.6%HG diet were treated with 400 μg/mL of the four-antibiotic cocktail mentioned above. Interestingly, antibiotic treatment altered the faecal metabolic profile of 2- and 4-week adults, causing a dramatic 10-fold increase in the faecal glucose levels of 2-week 0.01% males and those of 4-week 2% males, and a 2.8-fold increase in faecal glucose levels of 2-week 2% males (Figure 7.2.B). *Lactobacillae* represent one of the major classes of bacteria in the *Drosophila* gut microbiome (Wong et al., 2011a). These bacteria use hexoses, such as glucose, to produce lactic acid. The dramatic increase in the amount of glucose present in the faeces upon antibiotic treatment could potentially reflect alterations in the composition of the gut microbiota and/or reduced bacterial counts. Either option could have resulted in reduced utilisation of glucose by gut bacteria and, therefore, increased presence in the faeces. Alternatively, antibiotics could influence the take up of sugars in the intestine, but there is little scientific evidence in the literature to support this possibility.

The composition of the *Drosophila* gut microbiota reported in the literature (Chandler et al., 2011; Ren et al., 2007; Shin et al., 2011; Storelli et al., 2011; Wong et al., 2011a) varies widely between strains and laboratories (presumably because of the differences in food composition; see Table 10.1). Towards testing the hypothesis that changes in the gut microbiota may link larval dietary [yeast] with altered adult metabolism and/or longevity, we decided to characterise the adult gut microbiota for the w^{1118}iso strain used in this work and under the experimental conditions for which the long-term effects of larval diet were characterised. To do this, I used 454 pyrosequencing of the V1-V3 variable regions of the 16s rRNA gene using the 8F and 534R universal primers (Baker et al., 2003). This method is independent of culturing the bacteria and thus allows unbiased sampling of the bacterial community present in a sample. The samples originated from 3-week old 2% and 0.01% flies fed on either 0.6%HG or on 9%HG as adults i.e. four groups in total. These two adult diets were chosen because: (a) 9%HG was the adult diet on which the lifespan extension was maximal, while (b) 0.6%HG was the only adult diet on which 0.01% males showed doubled adiposity and no extension in lifespan. 3-week old adults were selected as, by this age, the survival curves of the two populations had just started to separate, but had not diverged enough for ageing to affect the results. For this analysis, the original w^{1118}iso line was used, which is *Wolbachia* +ve , as this was the strain used for most experiments described here. Preliminary 16S metagenomic analysis produced 197,292

reads distributed among 12 samples. A predominance of *Wolbachia* impeded statistical analysis, but by removing these reads, the presence of Firmicutes, Proteobacteria and Actinobacteria were confirmed (data not shown). These are the three bacterial phyla that are reported to make up the majority of gut microbiota in 3- to 5-week old males (Wong et al., 2011a). Interestingly, these results also suggested that 2% flies fed on 9%HG harbour increased levels of *Lactobacillus brevis*. An additional round of metagenomic sequencing is needed to confirm this, but the predominance of *Wolbachia* in the results – despite the gut not being a tissue heavily infected with *Wolbachia* – suggests that the high sugar diets used here might dramatically lower bacterial counts.

7.4 The role of transferable components in mediating the effects of larval diet on lifespan

Larvae reared on 0.01% yeast food supplemented with 50 µg/mL tetracycline survived for more than 10 days, but did not progress past the first larval instar (data not shown). Supplementation of the larval diet with 50 µg/mL of tetracycline has been shown to reduce gut microbiota by >90% (Ridley et al., 2012). This observation corroborates the findings of two previous studies that have shown that the gut microbiota is essential for *Drosophila* larval development on "poor" (i.e. low [yeast]) diets (Shin et al., 2011; Storelli et al., 2011).

Given the importance of the microbiota in supporting larval development on low [yeast] diets, in order to study the role of the microbiota in linking larval dietary [yeast] to adult longevity, one would ideally allow larval development to proceed undisturbed and eliminate the microbiota only in adult flies. In an attempt to achieve this, I experimented with supplementing the adult diet with antibiotics and compared their lifespan to that of identically reared animals fed on food without antibiotics. Diet supplementation with 50 µg/mL or 400 µg/mL of the antibiotic cocktail had no significant effect on the median lifespan of 2% flies (Figure 7.4.A and C). By the time the experiments were stopped, no difference could be detected in the survival patterns of 0.01% adults either (Figure 7.4.B-C).

7.4.1 Co-housing experiments demonstrate that lifespan is transferable

As even high concentrations of antibiotics do not remove all microbes, I adopted a different experimental paradigm, investigating whether lifespan is "transferable" (via faeces or otherwise). I analysed the lifespan of long-lived 0.01% males that were co-housed with their shorter-lived 2% counterparts. The advantage of this approach is that it

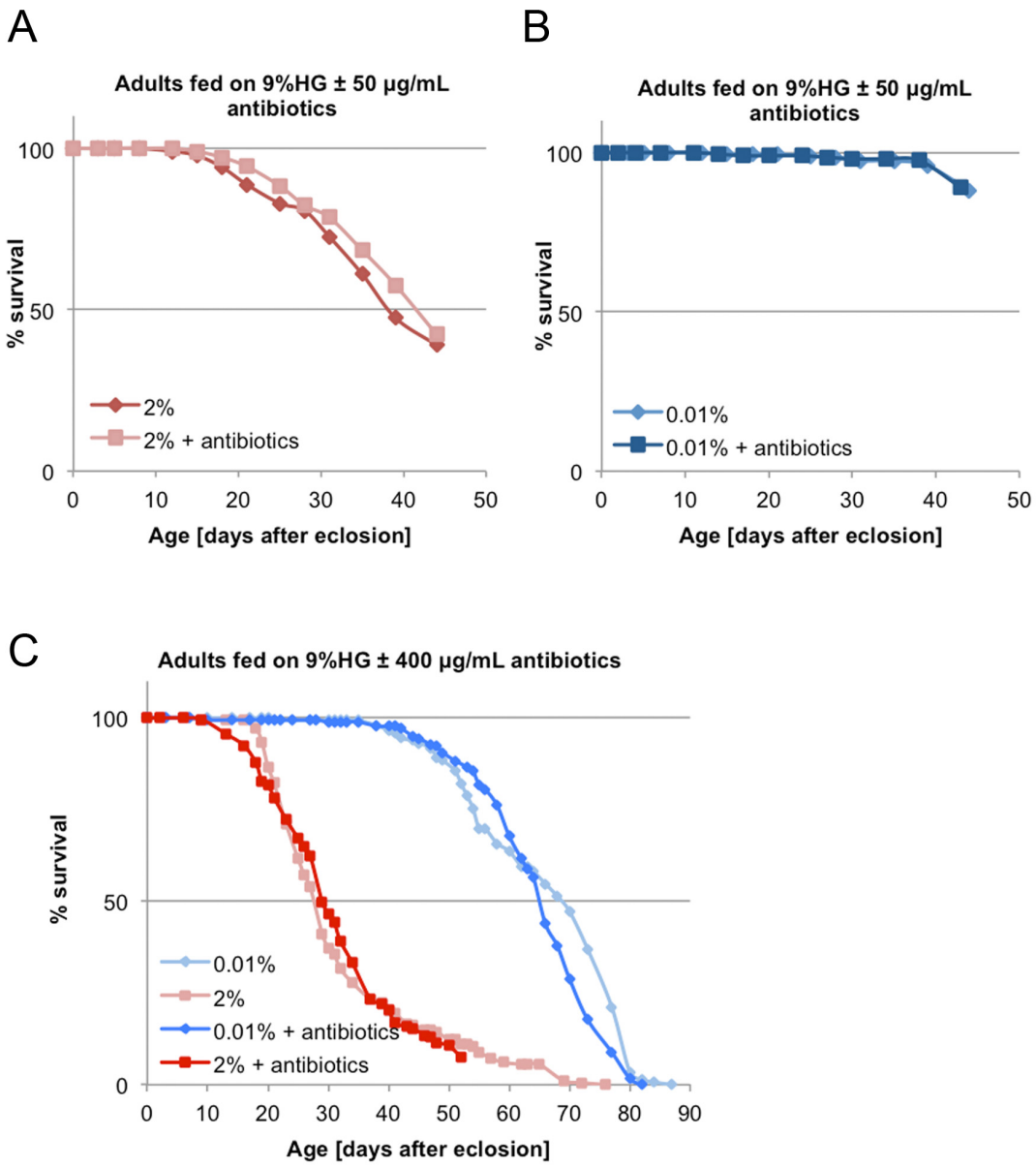

Figure 7.4: Antibiotic treatment had no effect on male lifespan (for adults fed on a 9%HG diet), regardless of larval diet. Supplementation of a 9%HG diet with 50 µg/mL of a four-antibiotic cocktail (i.e. kanamycin, erythromycin, tetracycline and ampicillin) had no effect on the lifespan of 2% males **(A)** nor on that of 0.01% males, by the time the experiment was stopped **(B)**. Supplementing a 9%HG diet with 400 µg/mL of the antibiotic cocktail also failed to elicit a change in the lifespans of 2% and 0.01% males **(C)**.

covers all social/transferable effects and is not limited to differences in microbiota. In a first experiment, I combined 15 males of each 0.01% and 2% per vial (i.e. 30 flies per vial in total) and compared their survival to that of 0.01% and 2% controls housed separately (also at a density of 30 flies per vial). Co-housing experiments were made possible by the pronounced difference in body size between 0.01% and 2% adults. The survival curves of 0.01% vs. 2% males housed separately diverged as expected for adults fed on 9%HG. In this particular experiment, 0.01% flies showed an extension of 110% in median lifespan compared to 2% controls (Figure 7.5.A). Co-housing 0.01% and 2% flies extended the lifespan of 2% flies and shortened that of the previously long-lived 0.01% flies (Figure 7.5.A). The median lifespan of 2% flies that were co-housed with 0.01% flies was extended by 54% compared to that of 2% control flies, while the median lifespan of co-housed 0.01% flies was ~half that of 0.01% controls and, therefore, similar to that of 2% controls (only a 7% increase compared to 2% controls). This surprising finding suggests that co-housing can swap over the relative longevities of 0.01% vs. 2% flies.

In the lifespan literature, it is common practice to house the animals at the same initial density, which is why I used 15+15/vial for co-housing experiments and 30/vial for controls, in order to compare between flies that had been housed at the same density. However, we reasoned that introducing a second control of 2% flies housed separately at an initial density of 15 flies/vial would be necessary in order to try to understand the strikingly large increase in 2% lifespan induced by co-housing. I repeated the co-housing experiment described above with this added control and confirmed the initial finding that co-housing extends the lifespan of 2% flies and reduces that of 0.01% flies. In this second experiment, at a fixed density of 30 flies/vial, 0.01% controls displayed a 145% increase in median lifespan compared to 2% controls, while co-housing extended 2% median lifespan by 110% but decreased 0.01% median lifespan to almost half again (Figure 7.5.B). Surprisingly, however, decreasing the initial density from 30 to 15 flies per vial, increased the median lifespan of 2% flies housed separately by 125%, thus bringing the longevity of 2% flies to levels similar to that of co-housed 2% animals and 0.01% controls (Figure 7.5.B). The fact that the survival curve of 2% flies co-housed with 0.01% (15 of each) was similar to that of 15 2% flies housed on their own, opens the possibility that the increased longevity of co-housed 2% flies may be largely attributable to a density mechanism. However, 0.01% and 2% flies co-housed in the same vial displayed strikingly different survival patterns, demonstrating the existence of “intrinsic” differences between both types of fly that do not appear to be shared equally.

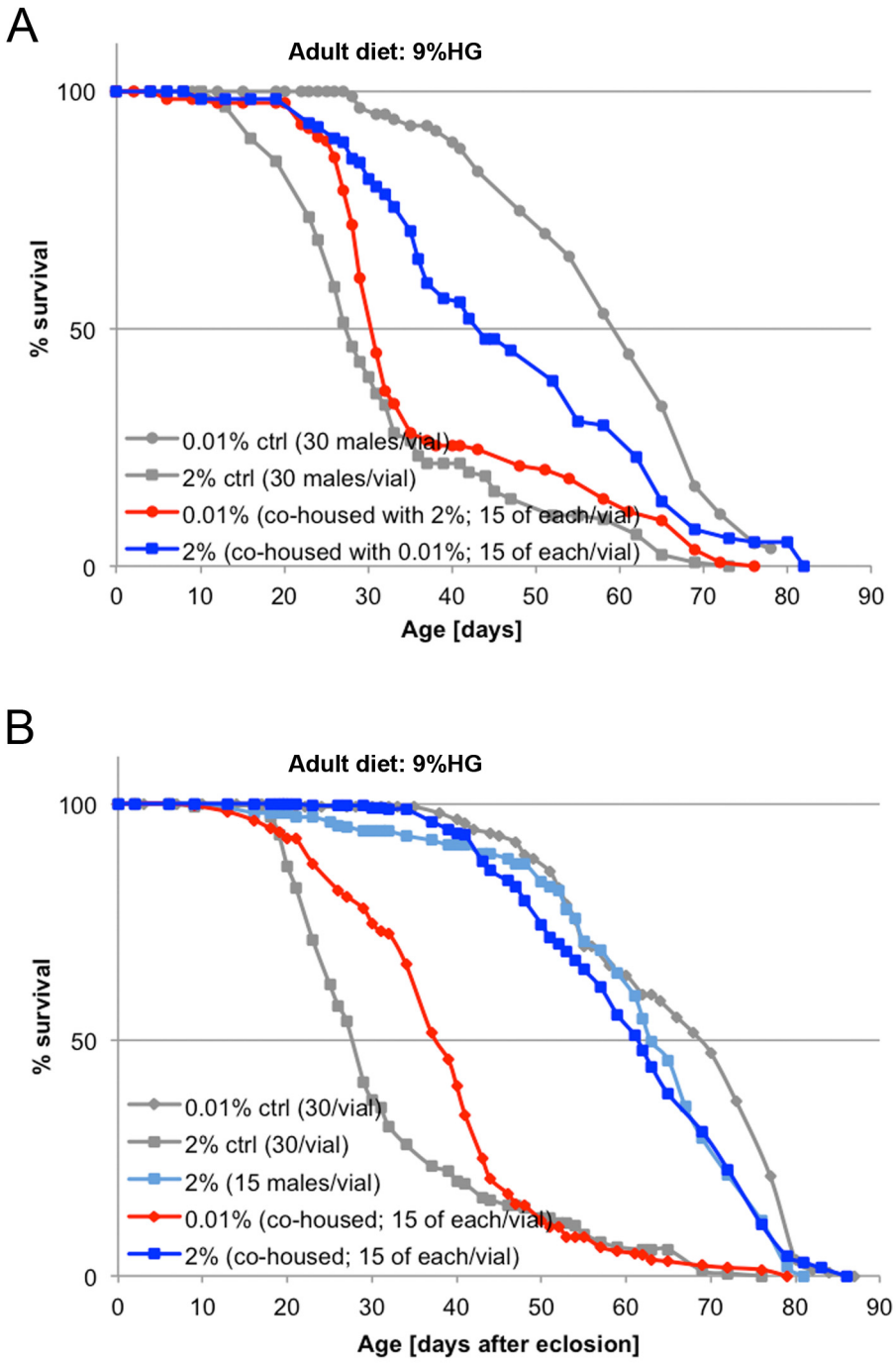

Figure 7.5: "Co-housing" reverses the effects of larval diet on male lifespan. 0.01% larval dietary yeast dramatically extends the lifespan of adult flies housed separately (at a density of 30/vial) from 2% control flies (also housed at an initial density of 30/vial). However, co-housing 0.01% and 2% male flies together in the same vial (15 of each/vial) dramatically shortens the lifespan of 0.01% males (almost by half) and extends the lifespan of the previously shorter-lived 2% males. **(A)** and **(B)** show two independent experiments. In the experiment shown in **(B)** an additional control was introduced i.e. 2% males housed separately at an initial density of 15/vial. Co-housed 2% males and 2% males housed separately at a density of 15 per vial had similar lifespans. Statistics available in the Appendix.

7.4.2 Housing density contributes to "lifespan transfer" effects

Following the surprising observation that the lifespan of 2% control flies feeding on 9%HG as adults was doubled by simply decreasing the number of flies from 30 to 15 per vial, I explored the effects of adult density on lifespan in more detail. For this, I recorded the survival patterns of adults maintained at different densities per vial. All adults were fed on 9%HG – the diet on which the lifespan difference between 0.01% adults and controls was biggest. The longevity of 2% flies housed at various initial densities was tested. Decreasing density of 2% males from 30 to 15 per vial again resulted in a dramatic 110% increase in lifespan (Figure 7.6.A), thus replicating the initial density experiment (see Figure 7.5.B in previous section). Further reducing the initial density to 7 or 3 flies per vial had no additional benefit (Figure 7.6.A).

In the case of 0.01% flies, their survival was tested at initial densities of 3, 7, 15, 30, 60 and 90 flies per vial. Housing density had dramatic effects on the longevity of 0.01% males as well. Housing 0.01% flies at densities of 60 and 90 per vial halved their median lifespan compared to the "control" density of 30 flies per vial (Figure 7.6.B), resulting in survival patterns similar to those of 2% flies housed at 30 per vial (also see Appendix Figure 10.6). For the long-lived 0.01% flies, decreasing their density from 30 to 15 flies per vial increased median lifespan by a further 17%, while further decreasing it to 7 or 3 flies per vial brought no additional benefit (Figure 7.6.B).

In summary, decreasing the initial densities of 0.01% or 2% flies changed their survival curves from convex to concave, but the inflection point differed: the results suggest that this change occurs at an initial density between 30 and 15 for 2% flies, and between 60 and 30 for 0.01% flies. However, for both 0.01% and 2% males, maximal longevity was recorded for adults housed at an initial density of 15 per vial.

7.5 Discussion

The results presented in this chapter demonstrate that the long-term effects of low larval dietary yeast are mediated, at least in part, via a transferable component. This is reflected in altered faecal metabolic profile. Furthermore, adult antibiotic treatment doubled the adiposity of 2% males but not that of 0.01% males and, thus, replicated the effects of 0.01%-yeast larval diet on adult adiposity. Further experiments are needed to establish the role of the microbiota in mediating the effects of early-life nutrition on adult metabolic homeostasis as well its potential role in mediating the effect of adult diet on longevity. The

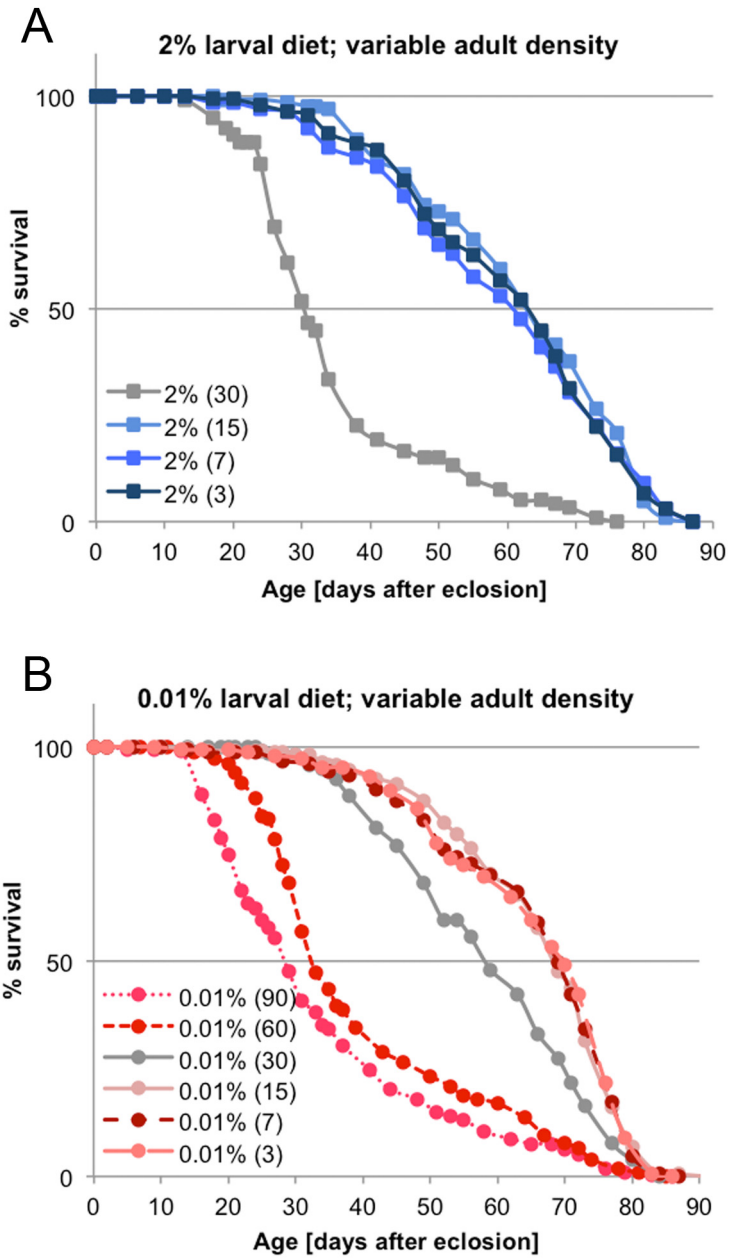

Figure 7.6: Housing density effects on 0.01% and 2% male lifespan. Housing density had dramatic effects on adult male lifespan. **(A)** Reducing the initial density of 2% males per vial from 30 to 15 doubles 2% male median lifespan. Further decreasing the initial density from 15 to 7 or 3 has no additional benefit. **(B)** Increasing the initial density of 0.01% males from 30 to 60 or 90 per vial halves 0.01% median lifespan, while decreasing it from 30 to 15 extends 0.01% male median lifespan by a further 17%. Further decreasing the initial density from 15 to 7 or 3 has no additional benefit. Statistics available in the Appendix. Also see Appendix for A and B overlaid.

results presented here can be used as a promising starting point for future investigations into the links between early diet, adult microbiota and adult metabolic perturbations.

7.5.1 Faecal metabolic profile as a peephole into fly metabolic status

In flies, what goes in has been the topic of heated controversies (Carvalho et al., 2005; Fanson et al., 2012; Lee et al., 2008; Skorupa et al., 2008; Wong et al., 2009). Up until now, little attention has been paid to what goes out. Studying faecal composition has the potential to provide invaluable insight into metabolic activities along the gastrointestinal tract (such as digestion, absorption and microbial fermentation) as well as excretion. In mammals, metabolite concentrations in the faeces are used as a non-invasive way of studying microbial metabolic activities within the gut (Jacobs et al., 2008). Indeed, faecal levels of several metabolites, including SCFAs, have been shown to correlate with microbial fermentation. Therefore, I decided to implement a method that would provide information about the metabolites present in the faeces and their abundance. Together with TJ Ragan, we have been successful in collecting, extracting and quantifying metabolites present in adult male faeces. These data have not been completely analysed as many of the metabolites present in the faeces are not easily identifiable. However, these analyses have revealed that faecal relative glucose concentrations are higher in the shorter-lived adults i.e. 2% males when fed on 9%HG, but 0.01% males when fed on 0.6%HG. Suggesting that there may indeed be a correlation between ageing and faecal glucose content, glucose content increased in the faeces of 2% males from 2 to 4 weeks of age. The increased concentration of glucose in faeces could be due to decreased counts of bacteria that metabolise hexoses, which would lead to less glucose being used up in the gut and more being excreted. In addition, antibiotic treatment may have favoured the colonisation of the gut by fungi. Little is known about the *Drosophila* intestinal fungal flora, but increased colonisation by fungi could further hinder the establishment of a bacterial flora. An immediate experiment would be to assess the effect of high dietary sugar on the number of CFUs per gut. To date, the effect of high sugar diets on the *Drosophila* microbiota has not been investigated. Interestingly, betaine was also detected in adult fly faeces, but this compound appeared not to be altered by larval diet. Instead, faecal betaine levels were ~20-fold higher in 2-week males as well as 4-week males fed on a 9%HG diet, as compared to those fed on a 0.6%HG diet. Betaine is produced by bacterial fermentation of phosphatidylcholine in the guts and, together with choline, trimethylamine N-oxide, is proposed to predict risk of cardiovascular disease in humans (reviewed by Burcelin et al., 2011; Howitt and Garrett, 2012). Taken together, data from profiling the polar metabolite concentrations in faeces by NMR suggests that larval diet interacts with adult diet to

programme adult faecal metabolite profile, in a similar way as reported for lifespan in Chapter 6 and for adiposity in Chapter 5. In future, it would be interesting to determine which of the metabolites present in faeces are of microbial origin and focus analyses on changes in the levels of specific metabolites that result from bacterial fermentation within the gut lumen. This could be done by comparing the faecal metabolic profile of germ-free flies to that of "conventional" controls.

7.5.2 Antibiotic treatment affected adult adiposity, but not lifespan

Adult antibiotic treatment replicated the increase in adiposity induced by the interaction between 0.01%-yeast larval diet and a 0.6%HG adult diet. This is similar to the findings of Cho et al. who reported that long-term treatment with sub-therapeutic doses of an antibiotic cocktail increases adiposity in mice (Cho et al., 2012). Cho et al. also showed that increased adiposity correlated with altered colonic microbiota composition. Eliminating the microbiota has been reported to have the opposite effect i.e. render mice resistant to diet-induced obesity (Rabot et al., 2010). The effects of adult antibiotic treatment on the fly gut microbiome have not been characterised. However, my results suggest that, perhaps instead of eliminating the microbiota, the antibiotic treatment employed here may have altered the relative composition of the microbial communities associated with adult flies. It is possible that species that were least affected by these four antibiotics became dominant. Indeed, despite the broad spectrum of the antibiotics used, mycobacteria are resistant to all four of them. It will be interesting to measure adiposity at different ages and on different antibiotics.

Despite the dramatic effect that antibiotic treatment had on adult adiposity, treatment with 50 µg/mL or 400 µg/mL had no effect on the lifespan of 0.01% nor 2% fed on a 9%HG diet. Potentially, this may be because antibiotic treatment would only affect the bacterial flora, but not yeasts or viruses. To test whether the lifespan of 2% adults fed on 9%HG is determined by the interaction between microbiota and adult diet, one could compare the lifespan of germ-free 2% flies with that of identically-reared, "conventional" animals.

7.5.3 Co-housing suggests lifespan may in part be programmed via a transferrable component

Surprisingly, I found that 0.01% and 2% flies co-housed in the same vial "swapped" survival trajectories. That is, in co-housing experiments the lifespan of 2% males almost doubled, whereas that of 0.01% males was reduced to half. During their seminal series of

"experimental studies on the duration of life in *Drosophila*", Pearl et al. came to the conclusion that the density the adults were housed at affected their longevity (Pearl and Parker, 1922; Pearl et al., 1927). They housed flies in 1 oz. vials on banana-agar medium and found that the animals lived longest when the initial density was 35 flies per vial. Increasing or decreasing the number of flies per vial progressively shortened lifespan. This intriguing effect has seen little attention since. Graves and Mueller proposed that population density affects lifespan either by dietary restriction or "*through alterations in the quality of the environment brought on by the presence of large numbers of individuals*" (Graves and Mueller, 1993). However, the effects of density in the study by Pearl et al. (1927) were less dramatic than the ones reported here. This may suggest that the effects of adult population density on lifespan depend upon the adult diet used. In future, this could be tested by repeating the "density lifespan" experiments but using different adult diets. Should the effects of housing density on lifespan be modulated in function of adult dietary composition, this would suggest that the effects of housing density on lifespan are attributable to changes in gut content/microbiota. To test this experimentally one could test whether the dramatic effects of housing density on lifespan are still maintained for germ-free animals.

Since 0.01% adult are much smaller than 2%, one way to interpret the results of the lifespan experiments is that they simply reflect the amount of units of fly mass per vial. If one considers that each control 2% adult male represents on mass unit, then each 0.01% adult male would represent only 0.6 mass units (as 0.01% adults weighed 40% less than their 2% counterparts). In mass units, 15 0.01% males would then be the equivalent of 8 2% males, while 15 2% males the equivalent of 25 0.01% males (see cartoon in Figure 7.7). The hypothesis that the "units of mass per vial" cause the differences observed in lifespan would predict that 0.01% flies should reach their full survival potential when housed 25 per vial and going below this number should have no additional effect on lifespan. However, just as 2% flies, 0.01% males lived longest when housed 15 per vial. The "mass units" hypothesis also does not explain why decreasing initial density below 15 has no additional benefit. Alternative interpretations of the co-housing experiments involve fungi, viruses, pheromones or aggression. The shortened lifespan of co-housed 0.01% animals could also be due to increased aggression from 2% flies. If possible, it would be interesting to perform the co-housing experiments using a setup that prevents animals from physically interacting, but allows the spread of bacteria/fungi/viruses/pheromones/etc. between the two populations. It remains

intriguing, however, that, despite being exposed to exactly the same conditions, co-housed 0.01% and 2% flies show such dramatic differences in their lifespan.

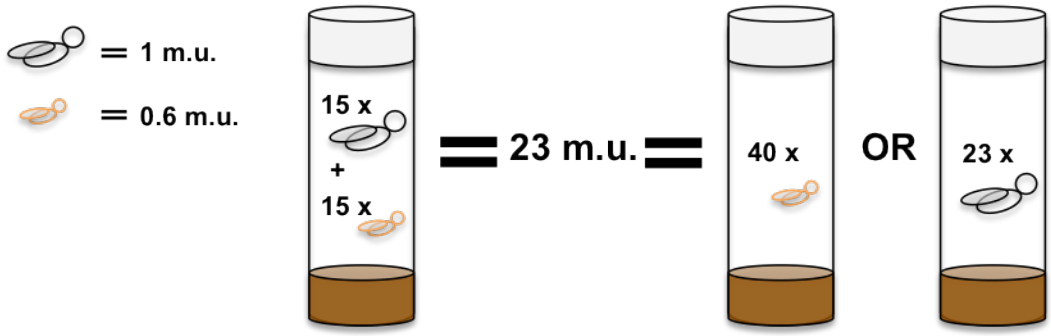

Figure 7.7: The "mass units (m.u.) per vial" hypothesis. 2% adult flies represented in **black**. 0.01% adult flies represented in **orange**. See text for details.

Chapter 8

Discussion

8 Discussion

A vast amount of literature links early life nutrition to health outcomes during adulthood in humans and mammalian model organisms, including mice, rats, guinea pigs, dogs and sheep. Despite this, the underlying cellular and molecular bases of this phenomenon are not yet well understood (reviewed by Martin-Gronert and Ozanne, 2012). Through the work presented in this thesis I show that, despite more than 500 million years of evolutionary divergence between *Drosophila* and mammals, the programming effects of nutrition during development are conserved. I demonstrate that diet composition during larval stages interacts with adult diet to determine adult lifespan and adiposity in *Drosophila*. This work establishes a new and genetically-tractable model for dissecting the mechanisms underlying the developmental origins of adult health and disease in humans. It also brings new insights into aspects of *Drosophila* metabolism and physiology that were not previously characterised. For example, I find that the accumulation of lipid droplets in hindgut enterocytes is a normal feature of ageing in flies fed on a control diet, rather than only the result of the more extreme dietary manipulations employed in this study. I also found that increased adiposity is associated with ectopic lipid deposition in non-adipose tissues, during both larval and adult stages. I further show that this can be replicated in control animals by increasing lipolysis in the fat body i.e. by genetically inducing a spillover of lipids from their major storage depot into the circulation. I now discuss the potential implications of the findings arising from this work and how each could be tested experimentally. Finally, I propose a speculative working model for how low yeast during larval development may programme adult metabolism and lifespan in *Drosophila*.

Many of the larval and adult dietary manipulations presented in this study involved changing the concentrations of autolysed yeast in the diet, while leaving other components constant. Bass et al. have argued that high concentrations of yeast extract are not suitable for adult dietary restrction (DR) experiments as they can have "toxic" detrimental effects on both female lifespan and fecundity (Bass et al., 2007). The authors proposed that lowering the concentration of yeast extract in the adult diet might therefore extend lifespan by relieving toxicity rather than by reflecting a genuine dietary restriction effect. However, I have observed that increasing the dietary [yeast] within the range used for the diets in this thesis work actually increases female fecundity (i.e. 0.6% yeast compared to 9% yeast adult diets) and is, therefore, not toxic by the definition of Bass et al. Furthermore, if the yeast concentrations used in our diets had been toxic, one would also

expect that larval growth and development would have been inhibited. Instead, within the range of 0.01% to 16%, raising the [yeast] in the larval diet accelerated development and increased mass gain (see Chapter 3). I conclude that, unlike the very high yeast extract concentrations used by Bass et al., concentrations of autolysed yeast in the range used in these thesis are not toxic to *Drosophila* larvae or adults.

8.1 Markers of age vs. ageing

In Chapter 6 I found that, despite very large differences in median lifespan, polyubiquitinated complexes accumulated in muscles to approximately the same extent by six weeks in both 0.01% and 2% adults. Polyubiquitinated complexes have previously been shown to increase with ageing and they have been proposed as a marker of ageing as their accumulation is delayed by genetic manipulations that extend lifespan (Demontis and Perrimon, 2010). However, these genetic manipulations were muscle-specific or whole body mutants potentially affecting muscles in a cell autonomous manner. Therefore, this evidence alone does not prove that there is a systemic effect on polyubiquitinated aggregates in muscles as would be expected for a marker of the global ageing process. Indeed, my results suggest that polyubiquitinated aggregates in muscles might be indicative of chronological age rather than ageing. In order to study genuine ageing effects – rather than phenomena that occur with chronological age, but are unrelated to organismal ageing – markers that correlate with longevity under different experimental conditions and are under systemic, not just organ-autonomous control, need to be defined. Nucleolar fragmentation, which results from the loss of heterochromatin with ageing, has been proposed to be one such marker (Larson et al., 2012). Due to technical difficulties, however, the nucleolar fragmentation status in 0.01% vs. 2% adults was not determined in this thesis.

Throughout the course of this work, I found that the age at which hindgut lipid droplets first appeared in 2% animals varied with the adult diet (see Chapter 5). Hence, adults fed on 9%HG are the first to display hindgut lipid droplets (at 3 weeks of age), followed by adults fed on 0.6%HG (at ~ 4 weeks). However, hindgut lipid droplets had not even accumulated by 4 weeks in adults fed a 0.6% yeast diet. In Chapter 6, I showed that the median lifespan of 2% adults is shortest on a 9%HG adult diet, intermediate on a 0.6%HG adult diet and longest on a 0.6% yeast adult diet. This indicates a negative correlation between the age of onset of hindgut lipid droplets and the longevity of 2% adults. However, it must not be overlooked that the two diets on which 2% males were found to accumulate hindgut lipids with ageing were both high glucose diets. To test if there is a

more general negative correlation, across many different diets, between lifespan and the age-of-onset of hindgut lipid droplets in 2% adults,, additional analysis is needed for older animals fed on 0.6% yeast, as well as for animals fed on 9% yeast i.e. a "normal" glucose diet, which had the same effect on survival trajectories as 9%HG. If the more general correlation holds true, then one would predict that hindgut lipid droplets should accumulate in 2% adults fed on a 9% yeast diet at the same rate as those on a 9%HG diet, but that they should only start accumulating at ~6 weeks of age on a 0.6% yeast diet. In Chapter 5, I also showed that impaired PI3K signalling in hindgut enterocytes is sufficient to induce the premature accumulation of hindgut lipid droplets. Therefore, if hindgut lipid droplet accumulation is a *bona fide* marker of ageing in 2% adults, it may reflect impaired peripheral insulin sensitivity or progressive IPC failure with age (also see 8.6)

8.2 Origins of ectopic lipid droplets in larval organs and adult podocytes

During these studies, significant differences have emerged between larval and adult lipid metabolism. For example, adults store less TAG than larvae but have much higher NEFA levels. In the larva, lipids such as TAG provide an energy store for the subsequent non-feeding stages of development from prepupa into newly eclosed adult. Therefore, low wL3 NEFA levels could reflect very low rates of lipid mobilisation from the larval fat body store. In both the larva and the adult, lipid stores reflect combined synthesis from sugars plus absorption of dietary lipids, processes involving the fat body and the midgut. Lipids synthesised in or absorbed by the midgut are transported to the fat body in the form of diacylglycerol (DAG), coupled with lipophorin in lipoprotein complexes (Palm et al., 2012) and so do not contribute to NEFA. Instead, the NEFA fraction is thought to be largely composed of circulating free fatty acids resulting from lipolysis in the fat body. Thus, both the NEFA and DAG fractions contribute to circulating lipids and thus might also contribute to the ectopic lipid droplets that can form in non-adipose larval and adult organs under certain dietary manipulations. Evidence of an increased flux of lipids to non-adipose tissues was presented in the case of 0.01% larval dietary [yeast]. This induced the accumulation of lipids ectopically in several larval non-adipose organs as well as much later on in podocytes, when adults were fed on 0.6%HG. This is reminiscent of mammalian "lipid overflow" (Savage, 2009) and could be replicated genetically by mobilising FB lipid stores (Lpp>bmm; see Chapter 3 and Chapter 5), which is expected to increase haemolymph free fatty acids. Surprisingly, however, the adult diet (0.6%HG) that doubled the adiposity of 0.01% males and induced ectopic podocyte lipid droplets also lowered whole-body NEFA at 3.5 weeks, compared to the 0.6% and 9%HG adult diets. In addition, on a 0.6%HG diet, I found that 0.01% males had ~30% less NEFA than 2% males.

Therefore, even though ectopic lipid droplets in the larval organs and adult podocytes of 0.01% animals can be replicated by genetic manipulations that increase fat body lipolysis, their appearance in dietary manipulations does not always correlate with increased NEFA. Perhaps, diet-induced lipid overspill occurs not only from the fat body in the form of NEFA but also from the midgut in the form of DAG. Alternatively, the underlying diet-dependent mechanism of ectopic lipid droplet accumulation may not involve the overspill of lipids but could reflect increased circulating sugars which could then be taken up and converted into ectopic lipid droplets by *de novo* lipogenesis in peripheral organs. This may especially be the case in larvae for which preliminary ^{1}H NMR analyses suggest a significant increase in circulating trehalose in 0.01% larvae compared to 2% (I. Stefana, T.J Ragan and A.Gould, data not shown). ^{1}H NMR analyses of adult haemolymph, however, indicate no change in circulating sugar levels (data not shown). This suggests that the underlying causes for the ectopic lipids in larvae and in adult podocytes may be different.

Taking advantage of the advanced genetic tools available in *Drosophila*, the precise cause of the ectopic lipid accumulation in larvae and adults could in future be addressed by: (1) lowering lipolysis in the FB of 0.01% animals (by overexpressing Lsd2 or bmm RNAi); (2) blocking the uptake of circulating DAG by non-adipose tissues (by knocking down LpR1 and/or LpR2); (3) blocking lipid synthesis in non-adipose tissues (by knocking down enzymes such as ACC or PDH); (4) blocking the synthesis of MAG or DAG in non-adipose tissues (we do not yet know how to block directly the uptake of free fatty but this approach would distinguish between increased flux of free fatty acids vs. increased flux of DAG); (5) blocking the export of lipids from the midgut (by FB knock down of LTP- a FB-derived ApoB family protein, needed in the midgut for the loading of DAG onto Lpp (Palm et al., 2012)).

8.3 Ectopic lipid droplets as a protective mechanism

I have shown that rearing larvae on a 0.01% yeast diet reduces growth, increases wL3 adiposity and leads to the ectopic accumulation of lipids in non-adipose tissues, including salivary glands, ring gland, muscles and podocytes (see Chapter 3). However, these larvae are still able to undergo metamorphosis and to eclose into viable adults. Even as early as one week of age, these 0.01% adults have accumulated extensive hindgut lipid droplets. In mammals, ectopic accumulation of lipids in non-adipose tissues strongly correlates with insulin resistance and organ dysfunction (reviewed in (Samuel and Shulman, 2012; Yki-Järvinen, 2002). It may therefore seem counterintuitive that 0.01% adults, which have large amounts of hindgut lipid droplets, are also longer lived on 3 of the 4 adult diets

tested than are control 2% adults. However, it has been shown that it is not lipid accumulation *per se*, but the accumulation of reactive lipid metabolism intermediates, such as ceramides or DAG, that impairs insulin sensitivity. Hence intracellular stores of neutral lipids such as TAG are considered metabolically inert and may in fact represent a protective mechanism. This is based on mouse studies showing, for example, that inhibition of mitochondrial acyl-CoA:glycerol-sn-3-phosphate acyltransferase (mtGPAT), the enzyme loading the first fatty acyl-CoA onto the glycerol backbone (i.e. the first and committed step in TAG synthesis), leads to an accumulation of fatty acyl-CoA yet prevents insulin resistance (Neschen et al., 2005). Conversely, hepatic overexpression of GPAT in chow-fed rats leads to fatty liver and liver insulin resistance (Nagle et al., 2007). Therefore, whereas the ectopic accumulation of lipids usually reflects an underlying increase in the precursors of TAG, an improved ability to "dispose" of these precursors in a neutral lipid store may protect the animal and confer a survival advantage. If this were the case for 0.01% adults, then a prediction is that hindgut-specific inhibition of TAG synthesis (e.g by knock down of DGAT, the enzyme that catalyses the synthesis of TAG from DAG) would reduce hindgut lipid droplets and have detrimental effects on longevity and/or metabolic health. Under more standard dietary conditions, in 2% males, I found that the age at which hindgut lipids first start to accumulate was dependent upon adult diet. This too could represent a protective mechanism during ageing that counteracts potential increases in TAG precursors within hindgut enterocytes.

8.4 Small body size alone does not account for the low larval [yeast] extension of lifespan

Throughout the animal kingdom there is a general tendency for lifespan to increase as size does. However, within the same species, it has been proposed that, big animals die young (Kraus et al., 2013). I have shown that rearing larvae on low yeast diets results in small adults that live considerably longer than 2% controls when fed on 3 of the 4 adult diets tested (from 20 to 150% increases in median lifespan; see Chapter 6). This raised the possibility that there might be a "small fly" explanation for adult longevity.

In perhaps the first study to address the effects of larval environment on adult lifespan, Alpatov starved 60-hr larvae to generate small adults and concluded that the lifespan of small and normal-sized flies were similar (Alpatov, 1930). Seven decades later, Tu and Tatar deprived larvae of dietary yeast during L3, which generated adults that were as small as the 0.01% adults described in this thesis (Tu and Tatar, 2003). They too reported that this larval treatment had no effect on adult lifespan. In fact, animals that were yeast-

deprived during L3 actually displayed increased early mortality rates, opposite to the effect I observe with low larval dietary [yeast] throughout development (see Chapter 6). Taking a different approach, Baldal et al. found that high larval density – which decreases wL3 and adult body sizes – did not affect mean lifespan in males or females in *Drosophila melanogaster* or in *Drosophila ananassae* and it decreased mean male lifespan in *Drosophila willistoni* (Baldal et al., 2005). At the same time, Khazaeli et al. addressed the relationship between body size and lifespan by generating small and normal-sized populations of 29 laboratory strains of *Drosophila melanogaster* (Khazaeli et al., 2005). Small flies were generated by restricting the amount of food available per larva in the vial. They found that, overall, small and normal-sized flies of the same strain (including w^{1118}) had very similar lifespans. These previous studies together indicate that small body size, induced by dietary or other environmental manipulations, does not itself account for extended lifespan. This also strongly suggests that body size is not sufficient to explain the underlying lifespan extension mechanism in my 0.01% larval yeast model.

Mutations in components of the PI3K signalling pathway have been shown to extend lifespan. As insulin signalling also controls larval growth and development, these mutants are often smaller than wild type animals. However, studies into the effects of mutating the insulin receptor substrate Chico on fly lifespan concluded that heterozygous $chico^{-/+}$ males have the same size as their wild type controls but display increased lifespan (Clancy et al., 2001). Homozygous $chico^{-/-}$ males, however, are smaller than controls and have reduced lifespan. Grönke et al. generated specific null mutants for Dilps 1-7 and assessed their developmental time, body weight and lifespan, among other readouts (Gronke et al., 2010). $dilp2^{-/-}$ extended lifespan by ~10% alone or in combination with $dilp3^{-/-}$. The body weight of *dilp2* and also *dilp2-3* double mutant flies was reduced by only 5-11%. However, the *dilp2-3,5* combined mutant, which decreased adult female body weight by 42% (i.e. to a similar extent as 0.01% larval dietary yeast) and delayed larval development by 10-17 days, showed no change in median lifespan in a *Wolbachia*-free background, under standard lab conditions, i.e. 1x sucrose-yeast food used in the Partridge lab (see Table 10.1 for food compositions). Interestingly, regardless of *Wolbachia* status, the *dilp2-3,5* combined mutant failed to show a normal response to DR i.e. the lifespan of this mutant was not changed by altering adult dietary [yeast]. In consequence, *dilp2-3,5* combined mutants were longer-lived than wild types at high yeast concentrations. This is similar to the effects I found for 0.01% adults, which are longer-lived on 9% yeast adult diets because this high [yeast] does not shorten 0.01% male lifespan to the same extent as it does for 2% males. Taken together, the published dietary and genetic manipulations

indicate clearly that the effects of low larval dietary [yeast] on adult longevity reported in this thesis cannot be explained solely by reduced body size. In future, it would be interesting to use the isogenic w^{1118} strain to generate flies of a similar size to 0.01% adults (for example, by post-CW starvation or by transferring 2% larvae to 0.01% at the beginning of L3) and to compare their lifespan to that of 0.01% and of 2% adults under the same environmental conditions used in this thesis.

8.5 Correlations between adiposity and lifespan

Chapter 5 showed that 0.01% males that were fed on 0.6%HG as adults had double the adiposity at 2 weeks and a 70% increase in adiposity at 3.5 weeks compared to age-matched 2% controls. By 3 weeks, the increased adiposity of 0.01% males also correlated with the accumulation of lipid droplets in podocytes. Importantly, 0.6%HG was also the only of the four adult diets tested on which 0.01% larval dietary yeast did not extend male or female lifespan. While four adult diets is not a large enough sample to explain fully this correlation, it is nevertheless intriguing. Hence, despite their potential to live long on the "right" diet, 0.01% adults feeding on 0.6%HG may die prematurely of metabolic complications associated with increased adiposity. As a first step towards understanding the possible underlying mechanism, it would be interesting to establish whether the TAG increase in 0.01% males on 0.6%HG is due to increased lipid synthesis, decreased mobilisation, increased lipid/glucose uptake from the diet, or a combination of the above. One could also correlate survivorship curves on different adult diets with “adiposity curves” throughout adulthood. *bmm* mutants, which have increased adiposity but impaired lipid mobilisation, could be used to test the effect of increased adiposity on lifespan in the absence of an increase in the levels of circulating lipids. Conversely, one could knock down *bmm* in the fat body to test the effects of increased circulating lipids without increased adiposity. I have shown that this manipulation is sufficient to replicate the podocyte lipid droplet accumulation associated with increased adiposity. It would also be interesting to test whether preventing the excessive increase in adiposity of 0.01% on 0.6%HG would be sufficient to rescue their lifespan to similar extents as observed in their siblings fed on the other three adult diets.

In mammals, there is no doubt that too much body fat can be fatal. The dangers of having too little are much less publicised, but can be equally detrimental. In fact they can be considerably more difficult to treat – in part because of our limited understanding of conditions such as lipodystrophies and anorexia, in part because there is only limited scope for lifestyle interventions. In flies, a causal link between body fat content and

survival trajectories has never been formally demonstrated. In fact, for all its benefits, using *Drosophila* to study the mechanisms of ageing and lifespan determination is complicated by a lack of understanding of the underlying causes of mortality. Long-lived adult flies that carry mutations in components of the IIS/TOR pathways display increased adiposity (Böhni et al., 1999; Broughton et al., 2005; Clancy et al., 2001; Slack et al., 2010; Tatar et al., 2001; Werz et al., 2009). However, *brummer* or *akhr* (adipokinetic hormone receptor) mutants, both of which display increased adiposity, are slightly shorter-lived or have a normal lifespan, respectively (Gronke et al., 2005; 2007). In addition, inhibiting the transsulfuration pathway reduces TAG content but also abrogates the DR-mediated extension in lifespan (Kabil et al., 2011). Hence, based on results reported in the literature, it is difficult to define clearly the role of body fat in fly lifespan. The different studies have used very different diets (see Table 10.1 for food compositions across different labs), have assessed animals' adiposity at very different ages and differ in the way adiposity is reported. For example, adiposity can be reported as any of the following: whole-body TAG content relative to whole-body protein content, fat body TAG content relative to fat body protein content, TAG content per fly, TAG content per µg wet body weight or TAG content per µg dry body weight. If colorimetric kits are used that measure TAG indirectly by measuring the amount of glycerol produced upon hydrolysing TAG into fatty acids and glycerol, then nmoles of glycerol can be reported over any of the previously mentioned denominators. Moreover, very few studies report adiposity at more than one age. Importantly, methods to quantify adiposity vary widely, with most labs opting for commercial colorimetric kits, whose reliability for measuring TAG levels in *Drosophila* has been questioned (Al-Anzi and Zinn, 2010; Hildebrandt et al., 2011).

8.6 Larval diet alters the proportions of adult IPCs and other organs

Chapter 3 demonstrated that altering the concentration of larval dietary yeast not only affects overall larval body size but also the relative proportions of internal organs (including brain, wing disc, salivary glands and IPCs). This work revealed that each of the internal larval organs has its own "yeast threshold" for sparing, above which its growth will not be compromised, despite changes in overall body size. Altered larval organ scaling then translates into altered organ scaling in the adult (see Chapter 4). Despite the clear link between growth rate during development and final organ proportions, the potential link between altered organ proportions and adult physiology has yet to be explored.

One interesting case of relative organ size concerns IPCs, the cells that themselves regulate systemic growth by secreting Dilps into the circulation. Chapter 3 showed that larval IPCs

undergo strong negative sparing upon reduction of dietary [yeast]. Consequently, 0.01% adult IPCs presumably start off negatively spared and remain negatively-spared for at least 10 days after eclosion (see Chapter 4). Surprisingly, however, I found that IPCs grow during adulthood such that, by 4 weeks of age on either a 0.6% or a 9%HG adult diet, their relative size (relative to body mass) in 0.01% males catches up with that of 2% males. Furthermore, the size of 2% adult IPCs at 4 weeks depends upon the concentration of yeast in the adult diet, but not upon that of glucose (see Chapter 4). Similarly to adult IPC size, Dilp release from the larval IPCs is not stimulated by high-glucose or high-fat diets, but rather depends on dietary amino acids (Géminard et al., 2009). Seen in context with the data of myself and others (Bruce et al., 2013; Grandison et al., 2009a; Lee et al., 2008; Partridge et al., 2005b; Skorupa et al., 2008) showing that adult yeast/protein tends to reduce lifespan, one possible interpretation is that the size of the IPCs could, in fact, be closely correlated with ageing and that the differences in IPC sizes between 0.6% and 9%-fed adults reflect different rates of ageing. This hypothesis would predict that the IPCs of 0.6%-fed adults would carry on growing and eventually reach the size of those of 9%-fed adults. If IPC size does turn out to be causally linked with ageing, then the lifespan extension of 0.01% flies compared to 2% adults could be due to the time it takes for 0.01% adult IPCs to catch up.

One possible mechanism to explain how IPC size can be linked with ageing would be that the size of the IPCs correlates with their output, i.e. Dilp synthesis and secretion. In order to test whether bigger IPCs result in increased levels of haemolymph Dilps, one would need to quantify circulating Dilps. Antibodies against Dilps 2, 3, 4 and 5 (the main Dilps produced by the IPCs) exist and have been successfully used in immunohistochemistry by us and others (Bai et al., 2012; Géminard et al., 2009; Gronke et al., 2010). However, the Dilp2 antibody available to us does not appear to work in immunoblotting protocols (Géminard et al., 2009). Nevetheless, Dilp secretion into haemolymph can be measured by overexpressing a Flag-tagged form of Dilp2 in the IPCs (Honegger et al., 2008). Employing this method, a previous study from our lab has shown that increasing IPC soma volume in larvae by 150% (by overexpressing PI3K) increased the levels of Flag::Dilp2 in larval haemolymph by 50% (Cheng et al., 2011). It will be interesting to test this approach for the adult IPCs. If IPC size correlated with Dilp production, it would explain why the effects of high dietary glucose are only apparent at lower yeast concentrations. At high [yeast] adult IPCs are large enough to deal with the increased glucose load, but this increased insulin production/signalling shortens lifespan. On a low yeast-high glucose diet, adult IPCs stay smaller and are, therefore, unable to sufficiently increase Dilp production in

response to the high glucose load. According to this speculative model, high dietary yeast could shorten *Drosophila* lifespan by promoting growth of the IPCs and, consequently, increasing Dilp production and thus peripheral insulin signalling. In contrast, low yeast diets do not increase IPC size to the same extent and thus increase lifespan by maintaining lower levels of Dilp production. This "relative IPC size" working model makes two testable predictions. First, preventing adult IPC growth in response to dietary [yeast] should extend lifespan of 2% adults. And second, forcing adult IPC size to increase prematurely should reduce the lifespan of 0.01% adults.

Several mammalian studies have shown that perinatal malnutrition programmes β-cell development and insulin production in the adult animal (Fujisawa et al., 2007; Garofano et al., 1998; 1999; He et al., 2012; Reusens and Remacle, 2006). The specific protocols, mouse/rat strains and results often vary, which can probably be explained in part by the exact adult diet used by each lab. This is an important aspect when comparing findings across different studies because, as shown in this thesis for *Drosophila*, even the effects of the same dietary manipulation during development will have different outcomes depending on the adult diet. However, taken together the overwhelming number of mammalian studies demonstrate that nutrition during development programmes β-cell function throughout adulthood. Hence, one component of the nutritional programming mechanism in both mammals and *Drosophila* may involve long-term effects upon the relative size/activity of the major systemic source of insulin signalling.

8.7 Gut microbiota may regulate hindgut lipids as well as lifespan

Genetic manipulations in Chapter 5 show that hindgut lipid droplets in 0.01% adults require *de novo* lipid synthesis in enterocytes (evidence from byn-G4>UAS-ACC RNAi), rather than from the uptake of circulating free fatty acids (evidence from Bmm overexpression in the 2% fat body and Lsd2 overexpression in the 0.01% fat body) or DAG (evidence from byn-G4>UAS-LpR1&2 RNAi). Surprisingly, however, hindgut lipids do not appear to be synthesised from glucose or glutamate, two common substrates for fatty acid synthesis (evidence from byn-G4>UAS-ACL RNAi and byn-G4>UAS-PDH RNAi). Therefore, the substrate(s) for hindgut lipid synthesis remains to be identified. In mammals, bacteria in the colon metabolise complex carbohydrates and other undigested nutrients to produce short chain fatty acids (SCFAs), such as acetate, lactate and butyrate, which are then taken by colonocytes (Macfarlane and Macfarlane, 2003). This presents the exciting possibility that the accumulation of lipid droplets specifically in the adult hindgut could be the result of uptake of microbial-derived SCFAs from the gut lumen. The *Drosophila* genome encodes

for putative acetyl-CoA transporters (such as CG9706), so it would be worthwhile testing whether 0.01% hindgut cells can take up acetate or acetyl-CoA directly. Given that the microbiota of the adult fly at eclosion are inherited from the larva, hindgut lipid droplets could, therefore, be "programmed" by a change in the composition of the larval gut microbial community induced by low larval dietary [yeast].. If hindgut lipids are indeed derived from bacterial SCFAs, then one would expect them to be abrogated in germ-free animals. However, microbiota are essential for larval development under conditions of low dietary yeast (Shin et al., 2011; Storelli et al., 2011) and I found that 0.01% larvae fail to develop under germ-free conditions. Instead, it would be possible to test whether hindgut lipid droplets still accumulate in germ-free 2% males with ageing. Alternatively, to study whether 0.01% hindgut lipids depend on the presence of a microbiota, one could use antibiotics to try to eliminate the microbiota during the adult stage only. However, I found that antibiotics had no effect on 0.01% or 2% lifespan on a 9%HG adult diet. This lack of an effect, together with ^{1}H NMR analyses of fly faeces from adults fed on food ± antibiotics (see Chapter 7 and data not shown), suggests that antibiotic supplementation of the adult diet alone is not sufficient to eliminate the microbiota. Intriguingly, the two studies that reported adult antibiotic treatment to affect fly lifespan, both employed rifamycin (Bonnay et al., 2013; Brummel et al., 2004), an antibiotic that was not part of the cocktail employed here or by others (Ren et al., 2007; Ridley et al., 2012; Storelli et al., 2011). Rifamycin targets mycobacteria specifically. Mycobacterial infections are notoriously hard to treat because members of this genus are resistant to most broad-spectrum antibiotics.

In Chapter 7, I made the intriguing observation that the lifespan of adult males programmed by 0.01% or 2% larval yeast could be exchanged by co-housing. I further showed that this “social” effect could be largely reproduced by decreased the initial density of 2% flies from 30 to 15 per vial. Through lifespan experiments with 0.01% and 2% flies housed separately at different initial densities I found that increasing housing density above 15 flies per vial has dramatic detrimental effects on the lifespans of both 0.01% and 2% adults. There are several possible mechanisms that might underlie this novel "social" housing effect (discussed in detail in Chapter 7). These include the overall load or composition of gut microbiota. One possibility is that low yeast larval diet affects the adults’ ability to control intestinal flora proliferation and/or maintain gut barrier functionality. In *C. elegans*, Portal-Celhay et al. have recently shown that – although intestinal bacterial load increases as worms age (Garigan et al., 2002) – long-lived mutants can control bacterial proliferation better (Portal-Celhay et al., 2012). In *Drosophila*,

external and internal bacterial loads increase as flies age (Ren et al., 2007). Animals have developed complex mechanisms to regulate the amount and type of bacteria in their intestines, but if these fail, symbiotic bacteria can become pathogenic. Indeed, Rera et al. have shown that intestinal barrier dysfunction is linked with lifespan in *Drosophila* and is more accurate than chronological age in predicting death (Rera et al., 2012). While the authors did not link this to the possibility that it may result in intestinal bacteria becoming pathogenic, they did show that intestinal barrier dysfunction was accompanied by an increase in the expression of antimicrobial peptides. Recently, Bonnay et al. have shown that *big bang* mutants, which have loose septate junctions, display a constitutive activation of the midgut immune response and die early (Bonnay et al., 2013). Interestingly, clearing the gut microbiota with antibiotics returns the gut immune response to wild type levels and restores lifespan. In addition, ageing flies' intestinal epithelium degenerates – a phenomenon that impacts negatively on longevity because delaying it (by maintaining the gut's regenerative capacity) extends fly lifespan (Biteau et al., 2010). It is likely that a degenerating epithelium loses its barrier functionality, thus enabling the gut flora to become pathogenic, i.e. activate the gut immune response and cause chronic inflammation of the intestinal epithelium, and potentially kill the animal. From this perspective, the results of the larval diet effects, co-housing and density lifespan experiments could potentially be explained thus:

a) Larval dietary [yeast] affects both adult bacterial load and ability to cope with increased loads i.e. 0.01% adults could be less well adapted to respond to bacterial challenges, but they might also harbour fewer bacteria or a bacterial profile that is less detrimental to animal survival in the long term (such as bacteria that proliferate more slowly, or bacteria with a lower pathogenic potential). When housed separately, 0.01% animals are unchallenged and thus live long. When co-housed with 2% flies, 0.01% flies cannot cope with the change in the bacterial load/landscape brought about by the presence of 2% flies in the same vial and their survival is affected negatively.
b) 2% flies, however, benefit from the decreased bacterial load/change in the bacterial landscape brought about by co-housing with 0.01% flies and live longer.
c) In this model, decreased bacterial accumulation would also explain the increase in lifespan when adult density was decreased from 30 to 15 per vial, while the converse could be responsible for the shortened lifespan of 0.01% flies housed 60 or 90 per vial. However, decreasing density below 15 flies per vial has no further benefit as bacteria are kept below a putative threshold, at levels that do not impact on lifespan.

This model can be tested in a number of ways. First, one can assess the flies' intestinal barrier integrity by feeding animals on food supplemented with FD&C Blue Dye No. 1. In flies with uncompromised intestinal integrity, the FD&C Blue Dye No. 1 – a dye which is not absorbed in the *Drosophila* gut – will be restricted to the proboscis and intestinal tract, while disruptions in the intestinal barrier will result in the dye leaking from the gut lumen into the body cavity (Rera et al., 2011; 2012). One can further assess the inflammation status of the intestinal epithelium in both types of fly –by using Diptericin-LacZ reporter activity (Reichhart et al., 1992) – and quantify intestinal dysplasia of the adult guts at different ages, as previously described (Biteau et al., 2010). Once/if a correlation is established, causality can be experimentally tested by eliminating the gut microbiota

The possibility that changes in gut microbiota may mediate the long-term effects of nutrition during early life have received little attention in mammals to date, in part because research into the interactions between microbiota and host metabolic homeostasis is still in its infancy. However, interesting correlations between early gut colonisation and subsequent risk for metabolic complications have begun to emerge (reviewed by Kaplan and Walker, 2012). It is also interesting to note that a newly-born baby will acquire its microbiota from passing through the birth canal (if born naturally) and later from other interactions with the mother. There is now a large body of evidence demonstrating that diet influences the composition of the microbiota (reviewed by Tremaroli and Bäckhed, 2012). Maternal diet and health status would therefore have a significant impact on the microbiota a mother passes on to her baby.

8.8 Working model

The results of the work presented in this thesis demonstrate that larval dietary composition has long-lasting effects on the adult in *Drosophila melanogaster*. More specifically, low larval dietary yeast extended adult lifespan dramatically on 3 out of the 4 adult diets tested. Surprisingly, low yeast larval diet interacted with a 0.6%HG adult diet to double adult males' adiposity and abrogate the extension in lifespan. However, regardless of adult diet, low larval dietary yeast induced the accumulation of lipid droplets in the adult hindgut by 1 week of age.

Based on findings from genetic manipulations (see Chapter 5), dietary supplementation experiments (see Chapters 5, 6 & 7) and experiments investigating the effects of co-housing and housing density on lifespan (see Chapter 7), I propose a working model for the mechanism of nutritional programming in *Drosophila*. This "two component" model is

speculative, but it presents a useful framework in which to formulate future experiments (Figure 8.1). In essence, larval diet affects adult outcomes via two potentially interconnected routes. The first component of the mechanism involves larval organ sparing, translating into altered relative adult IPC sizes (at least initially, before they catch up). Altered IPC size/activity would then alter IIS signalling in peripheral organs. This would account for the premature accumulation of hindgut lipids in young 0.01% adults. In 2% adults, hindgut lipid droplets would only appear at older ages, perhaps when IPC-derived Dilps start to fall or as peripheral tissues develop insulin resistance (either would lead to lowered IIS signalling in hindgut enterocytes). This low insulin production/IIS signalling would also make 0.01% animals longer-lived. However, if fed a 0.6%HG adult diet, the low Dilp production from the undersized IPCs of 0.01% adults would be unable to compensate for the high glucose load and thus lead to premature death due to metabolic complications such as ectopic/excessive fat deposition in non-adipose tissues.

The second component of the mechanism involves diet-dependent alterations in gut content, involving the microbiota and/or the chemical composition of the faeces. Larval diet has profound consequences upon the larval microbial community and faecal composition. Therefore, following metamorphosis, the eclosing adult would inherit the gut microbiome and faecal composition of the larva. The adult gut microbiota/faeces would then be further shaped by the adult diet to determine adult physiological outcomes relevant to metabolism and lifespan.

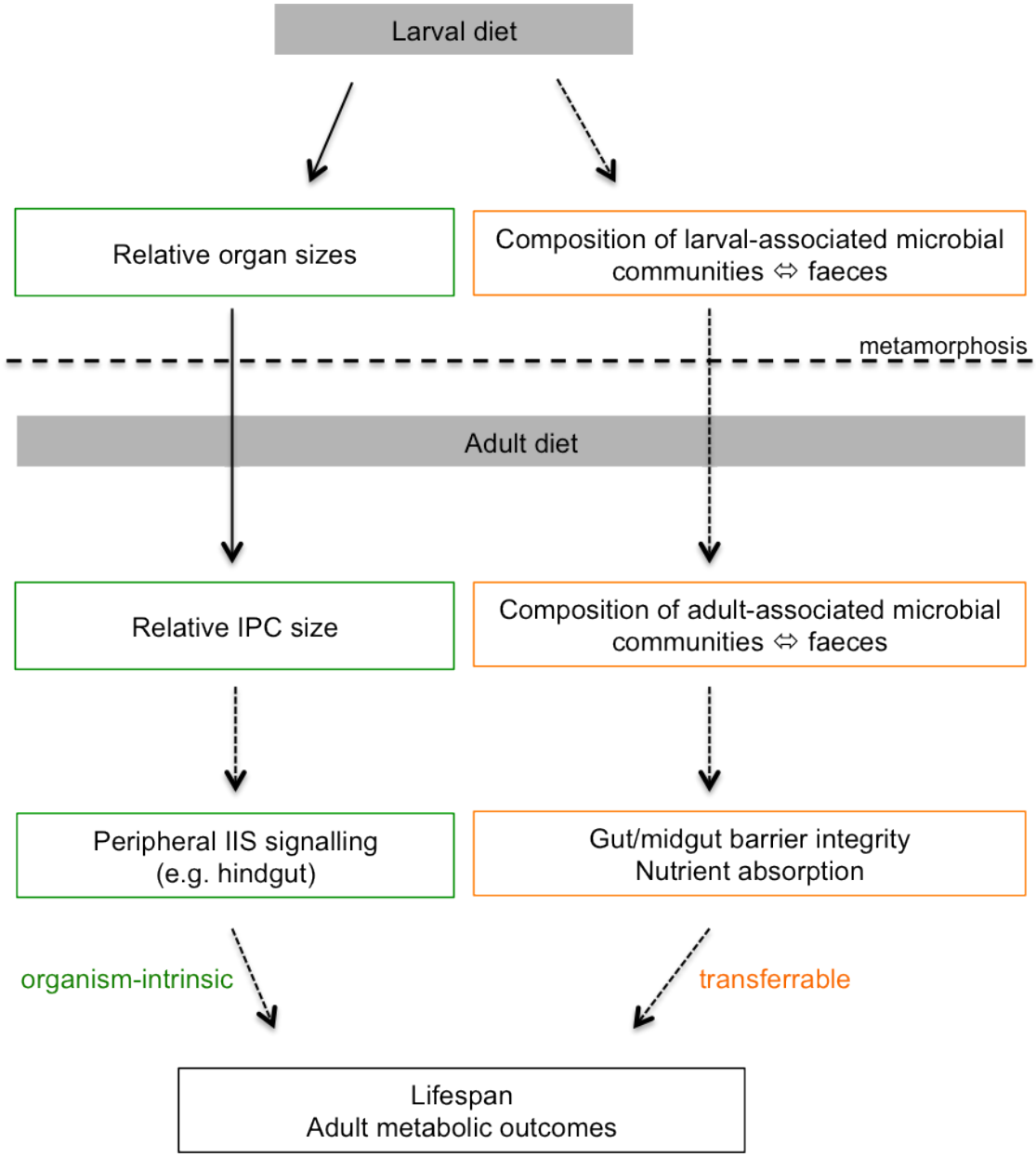

Figure 8.1: Two component working model for low-protein larval programming in *Drosophila*

9 Conclusion

The long-term impact of nutrition during early life has been extensively characterised in humans and mammalian model organisms. The molecular mechanisms underlying this medically relevant phenomenon have not been completely elucidated yet. In this thesis, I show that the programming effects of nutrition during development upon adult physiology and metabolic homeostasis are conserved between flies and mammals. I show that a low yeast larval diet induces the accumulation of lipid droplets within adult hindgut enterocytes by 1 week of age. In this work, hindgut lipid droplet accumulation emerged as a previously-uncharacterised phenomenon that also occurs in control animals with ageing. I further showed that larval dietary [yeast] interacts with adult diet to determine adult adiposity and lifespan. Intriguingly, the only adult diet on which adults from low yeast-fed larvae are not longer lived than controls is the same diet that, in combination with low larval dietary [yeast], doubles adult male adiposity and induces the accumulation of lipid droplets within adult podocytes. Finally, I propose that low larval dietary [yeast] could impact upon adult metabolism and longevity via two (potentially interconnected) "routes": an organism-intrinsic component that affects adult organ scaling and, ultimately, IIS signalling in peripheral tissue and a "transferable" component (such as gut microbiota) that impacts on the composition of faeces and may pose a challenge to gut barrier integrity. In summary, the work presented in this thesis demonstrates that nutritional programming is conserved in flies and establishes a *Drosophila* genetic model for dissecting the mechanisms underlying the developmental origins of adult health and disease

Appendix

10 Appendix

10.1 LexA/lexAop tools

Much of the beauty of *Drosophila* as a model organism comes from the availability of powerful tools for manipulating gene expression *in vivo*. The most widely used system for driving transgene expression in a precise spatiotemporal pattern in *Drosophila* is the yeast-derived GAL4-upstream activating sequence (UAS) system developed by Brand and Perrimon (1993). In recent years, the addition of GAL4/UAS-independent, binary expression systems has enabled scientists to independently express distinct transgenes in different patterns at the same time in the same organism. One such system is the bacterial LexA/lexAop binary system. Like GAL4, LexA is a DNA binding protein, which binds to an activating sequence, known as the LexA operator (lexAop), located upstream of the gene of interest. In this modified system, the bacterial LexA transcription factor is fused to the yeast GAL4 activation domain (GAD), thus rendering it suppressible by GAL80. In the lab, we already have a large collection of GAL4 driver lines specific for many of the internal larval and adult organs.

In light of this, during the early stages of this work I invested part of my efforts into generating LexA drivers for the post-embryonic CNS, midgut, oenocytes, fat body and insulin-producing cells- all organs that play important roles in metabolic homeostasis-, and lexAop reporter lines for key members of the IIS and TOR signalling pathways, the two major pathways that link nutrient availability with growth.

Detailed methodology of the approach adopted to make LexA/lexAop versions of the different lines of interest are described in Chapter 2. In short, I took two different approaches towards this goal. For the following driver lines the enhancer sequences that drive their expression are known: the oenocyte drivers pnt-GAL4 and svp-GAL4, the fat body driver Cg-GAL4 and the IPC driver dilp2-GAL4. In order to make LexA versions of these GAL4 lines and lexAop reporter lines, I used the recombinase-based Gateway system to clone the cDNAs and enhancer fragments of interest, respectively, into *Drosophila* LexA::GAD/ lexAop destination vectors, which were then sent for injection with Bestgene Inc. I tested the resulting LexA driver lines by crossing with lexAop-mCD8::GFP and comparing the pattern with the original GAL4 line.

Out of the six oenocyte-specific pnt-LexA driver lines, none drove GFP expression. This may be due to incompatibility between the LexA destination vector and the pnt insert. In a second attempt to make a LexA oenocyte driver, I cloned a 3 kb fragment of the *sevenup* (svp) enhancer upstream of LexA (Gutierrez et al., 2007) and sent it for injection. However, no GFP expression could be detected with any of the four svp-LexA lines returned by Bestgene. I also generated lexAop reporter lines for the following: $dp110^{WT}$, $dp110^{DN}$, RagA, dS6 and mouse S6.

Although incomplete, the set of LexA/lexAop tools described here can prove valuable, both as a basis to build on, or for immediate use in specific applications. For example, the dilp2-LexA, lexAop-mCD8::GFP recombinant line provides, for the first time, a means of accurately delimitating the boundaries of IPC cell bodies and their projections that is independent of GAL4. Up until now, the only GAL4-independent way of marking the IPCs has been by staining with the dilp2 antibody. However, this antibody is not commercial and, therefore, limited, and this staining does not mark the projections nor does it allow one to delineate the IPC cell body for accurate measurements. Marking the IPCs with membrane-tagged GFP in a GAL4-independent manner, allows one to use the GAL4/UAS system in order to manipulate genetically other organs and study how growth of the IPCs may be regulated systemically.

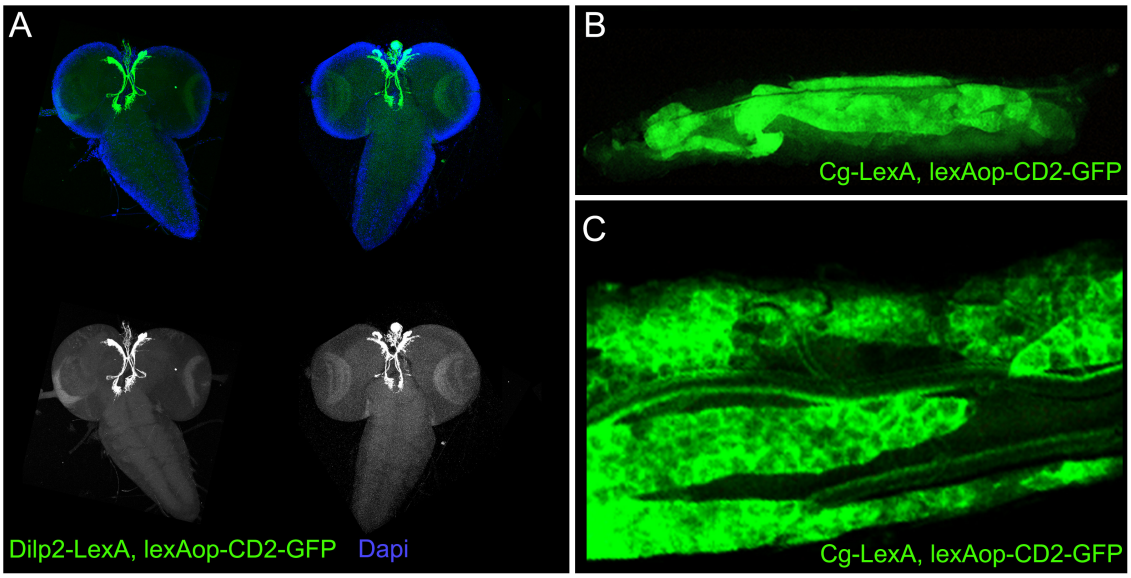

Figure 10.1: LexA versions of fat body- and IPC-specific drivers. (A) dilp2-LexA drives lexAop-mCD8::GFP expression in the IPCs. Shown are brains of wL3 larvae. **(B)** Cg-LexA drives expression of lexAop-mCD8::GFP specifically in the fat body. **(C)** Magnification of (B).

10.2 Wolbachia

During the course of the lifespan studies, it became clear that w^{1118}iso is infected with *Wolbachia*. *Wolbachia* is a maternally transmitted endosymbiotic bacterium that infects a wide range of arthropods, including *Drosophila* (Werren, 1997). It is estimated that more than >30% of laboratory stocks carry *Wolbachia* (Clark et al., 2005). The presence of *Wolbachia* has been shown to increase insulin signalling in *Drosophila* and, thus, partially mask the effects of genetic manipulations that lower signalling via the IIS pathway (Ikeya et al., 2009). However, the fitness benefit conferred by *Wolbachia* may depend on genetic background (Dean, 2006). Some *Wolbachia* strains can also become virulent and affect lifespan (Min and Benzer, 1997). In addition, it also appears to protect insects from a number of pathogens, including viruses and bacteria - even though the *Wolbachia*-mediated antibacterial protection could not be reproduced in *Drosophila* (Teixeira et al., 2008; Wong et al., 2011b). PCR diagnosis with primers specific for the *wsp* (*Wolbachia* surface protein) gene revealed that the wild-type w^{1118}iso line is positive for *Wolbachia*, but not the IPC and fat body drivers from which females were collected for genetic manipulations (data not shown). This indicates that the ectopic hindgut lipids found in 0.01% adults are not attributable to *Wolbachia*, as these genetically-manipulated adults also showed 0.01%-induced hindgut lipid accumulation. To clear the w^{1118}iso of *Wolbachia*, flies were reared on 2% yeast medium supplemented with 50 µg/mL tetracycline for four generations, at the end of which they were confirmed to be *Wolbachia*-free by PCR (data not shown). As the antibiotic treatment will have also removed the animals' resident microbiota (Ridley et al., 2012), the stock was then amplified and allowed to be recolonised by microbiota for several generations. Allowing the stock to recover is also important because tetracycline impacts negatively on mitochondrial efficiency and density for at least two generations after treatment (Ballard and Melvin, 2007). Preliminary lifespan experiments with w^{1118}iso *Wolbachia*-free flies, however, suggest that *Wolbachia* does not play a role in mediating the long-term effects of larval dietary [yeast] upon lifespan (data not shown).

Table 10.1: Composition of the *Drosophila* diets used by different labs. The "sugar:yeast ratio" was calculated based on the amount of "pure" sugar added to the diet, not taking into account the extra sugar that cornmeal or yeast might contribute with.

Lab	% yeast	% sugar	% cornmeal	% agar	Sugar:yeast ratio	Reference
Gould[1] (yeast=yeast extract)	9.36% in 9% food 2.34% in 2% food 0.585% in 0.6% food 0.0117% in 0.01% food	Normal glucose= 5.85% High glucose= 40%	6.63%	0.7%	0.625:1 in 9% 17:1 in 2%HG 2.5:1 in 2% 68.4:1 in 0.6%HG 4.3:1 in 9%HG 487.5:1 in 0.01% 10:1 in 0.6%	See Chapter 2
Partridge	10% for DR 20% for fully-fed	5% sucrose	No cornmeal	1.5%	0.5:1 for DR medium 0.25:1 for control food	Grandison et al. (2009a)
Pletcher (treatment	2.5% to 40%	2.5% to 40% sucrose	No cornmeal	1.5%	16:1 2.5:1 0.25:1 8:1 1:1 0.125:1	Skorupa et al. (2008)
Pletcher[3] (larval diet)	2.5%	5.5% glucose + 3% sucrose	6%	1%	3.4:1	Skorupa et al. (2008)
Pletcher[3] (mating diet)	10%	10% sucrose	No cornmeal	2%	1:1	Skorupa et al. (2008)
Baranski and Cagan[2]	8% Brewer's yeast + 2% yeast extract => 10% + 2%	5.1% sucrose	No cornmeal	1%	0.425:1 (control)	Na et al. (2013)
	"	34.2% sucrose	No cornmeal	1%	2.85:1 (high sucrose)	
	" + 15% soy protein	5.1% sucrose	No cornmeal, but 6.6% Crisco	1%	0.19:1 (high protein)	
	"	5.1% sucrose	" w/ 15.1% Crisco	1%	0.425:1 (high fat)	
Tatar	16% for HY diet 4% for LY diet	16% sucrose for HS diet 4% sucrose for LS diet	5.2%		0.25:1 for HY/LS 1:1 for LY/LS and HY/HS	Min et al. (2007)
Kapahi[3] (larval diet)	1.6% yeast	5% sucrose + 10% dextrose	8.6% cornmeal	0.46%	9.375:1	Katewa et al. (2012) Zid et al. (2009)
Kapahi [4] (yeast extract adult	0.5% yeast extract for DR 5% yeast extract for ctrl	5% sucrose	8.6% cornmeal	0.46%	10:1 for DR 1:1 for ctrl	Katewa et al. (2012)
Leulier[5]	0%, 0.4%, 0.8%, 1% or 8% yeast extract	No sugar added	8% cornmeal	0.82%		Storelli et al. (2011)
Lee[6]	0.1% to 2% yeast 0.5% yeast for sugar-rich	9.6% sucrose 9.6% sucrose or glucose	6.97% cornmeal	1.5%	96:1 down to 4.8:1 depending on [yeast]	Shin et al. (2011)

[1] Additional ingredients: methylparaben and nipagin dissolved in 1% (v/v) EtOH
[2] Larval food: Bloomington Std. Cornmeal Food. Adult foods based on: Bloomington semi-defined medium. Additional ingredients: 0.2% 1M $MgSO_4$ 0.34% 1M $CaCl_2$, 0.6% propionic acid, 1% mold inhibitor
[3] Standard Caltech Fly Food based on Lewis (1960). Additional ingredients: 1% acid mix.
[4] Additional ingredients: 1% acid mix. Yeast extract: #212750 Bacto™ Yeast Extract, B.D. Diagnostic Systems, Sparks, MD
[5] Additional ingredients: 0.52% (w/v) methylparaben sodium salt and 0.4% (v/v) 99% propionic acid. Yeast extract: VWR, Cat. No. #24979.413. Cornmeal: Westhove, Farigel maize H1.
[6] For stock keeping only, 0.03% bokinin and 0.5% propionic acid were added to the standard cornmeal-yeast-sucrose-agar medium. Yeast source: Lessafre, France. Cornmeal source: Sunglim Co., South Korea

Table 10.2: Dietary manipulations that increase lifespan in *Drosophila*. n.a. = not applicable; n.d. = not determined

Manipulation	Increase in median male lifespan	Increase in median female lifespan	Reference
Low [yeast] larval diet	90-150% on 9%HG ~90% on 9% 20-33% on 0.6% no effect on 0.6%HG	50% on 9%HG no effect on 0.6%HG	n.a.
Diet supplemented with 10 mg/mL of apple polyphenols	10%	n.d.	Peng et al. (2011)
Diet supplemented with 10 mg/mL of black tea theaflavins	12%	n.d.	Peng et al. (2009)
Diet supplemented with 5 mg/mL of blueberry extract	12.8%	n.d.	Peng et al. (2012)
Adults fed on food with dietary yeast deficient in CoenzymeQ	<10%	>35%	Palmer and Sackton (2003)
PDTC (NF-κB inhibitor; supplemented to adult food in yeast paste at 20 mg/L)	20%	13%	Moskalev and Shaposhnikov (2011)
+ 200 μM Rapamycin	6%	10-13%	Bjedov et al. (2010)
Dietary restriction in the adult	50%	66.7%	Min and Tatar (2006)
Yeast deprivation during L3	No effect Transiently elevated mortality at young ages		Tu and Tatar (2003)

Table 10.3: Genetic manipulations reported to increase lifespan in *Drosophila*. n.a. = not applicable; n.d. = not determined

Manipulation	Increase in median male lifespan	Increase in median female lifespan	Reference
Low [yeast] larval diet	Up to 150%	Up to 50%	n.a.
Adult IPC ablation	10.5%	33.5%	Broughton et al. (2005)
Imp-L2 overexpression	No effect	16.4%	Alic et al. (2011)
FOXO overexpression in abdominal FB ($S_1$106 driver)	No effect	38.5%	Giannakou et al. (2004)
”	No effect	No effect	Hwangbo et al. (2004)
PTEN overexpression in head FB ($S_1$32 driver)	20%	17.4%	
FOXO overexpression in head FB ($S_1$32 driver)	15.5%	19.4%	
Overexpression of protein carboxyl methyltransferase (PCMT)	n.d.	32-32% @ 29° C No effect @ 25° C	
Altered expression of TOR pathway members ubiquitously	11.5% for da-G4>UAS-TSC1 or >UAS-TSC2 15% for da-G4>UAS-TOR^{FRB} or >UAS-S6K^{KQ}	n.d.	Kapahi et al. (2004)
TSC2 overexpression ubiquitously	5% @ 0.3% dietary yeast 13% @ 1% dietary yeast 23% @ 3% dietary yeast 95% @ 9% dietary yeast	n.d.	
Overexpression of activated 4E-BP ubiquitously (strong allele)	11% @ 5% dietary yeast No effect @ 0.25% yeast	22% @ 5% dietary yeast No effect @ 0.25% yeast	Zid et al. (2009)
Overexpression of dilp6 in abdominal FB ($S_1$106 driver) *appears to mediate the effects of dFOXO overexpression	No effect	15% @ 2% dietary yeast no effect @ 8% dietary yeast	Bai et al. (2012)
Overexpression of dilp6 in head FB ($S_1$32 driver)	No effect	No effect @ 2% dietary yeast 8.7% @ 8% dietary yeast	Bai et al. (2012)
Overexpression of Parkin ubiquitously in adult tissues	No effect	~20%	Rana et al. (2013)
Overexpression of Parkin in adult neurons	No effect	<15%	Rana et al. (2013)
Overexpression of UCP in adult IPCs	10%	19%	Fridell et al. (2009)
Overexpression of *Drosophila* or human insulin degrading enzyme (IDE) in the IPCs (late L3 onset)	n.d.	14-18%	Hyun and Hashimoto (2011)

Table 10.4: Mutations reported to increase lifespan in *Drosophila*. n.a. = not applicable; n.d. = not determined

Mutation	Increase in median male lifespan	Increase in median female lifespan	Reference
Low [yeast] larval diet	Up to 150%	Up to 50%	n.a.
chico$^{1/+}$	8.8%	36.4%	Clancy et al. (2001)
chico^{1}	Slight decrease	48%	
InR$^{p5545/E19}$	No effect, but reduced mortality at late ages	85%	Tatar et al. (2001)
methuselah$^{-/-}$	35%	n.d.	Lin et al. (1998)
Dilp1$^{-/-}$	No effect	No effect	Gronke et al. (2010)[7]
Dilp2$^{-/-}$	9%	8-13%	
Dilp3$^{-/-}$	No effect	No effect	
Dilp4$^{-/-}$	No effect	No effect	
Dilp5$^{-/-}$	No effect	No effect	
Dilp6$^{-/-}$	No effect	No effect	
Dilp7$^{-/-}$	No effect	No effect	
Dilp2-3$^{-/-}$	n.d.	12%	
Dilp2-3,5$^{-/-}$	No effect	No effect in Wol- $w^{Dahomey}$ 22-29% in Wol+ $w^{Dahomey}$	
Dilp1-4$^{-/-}$	No effect	No effect	
Lnk$^{d07478/d07478}$	17.5%	14%	Slack et al. (2010)
Lnk$^{Del29/Del29}$	12.7%	11.5%	

[7] Data for *Wolbachia*-free flies, unless otherwise noted. Lifespan was recorded on the 1x Sucrose-Yeast-Agar Partridge food (see Table with food compositions)

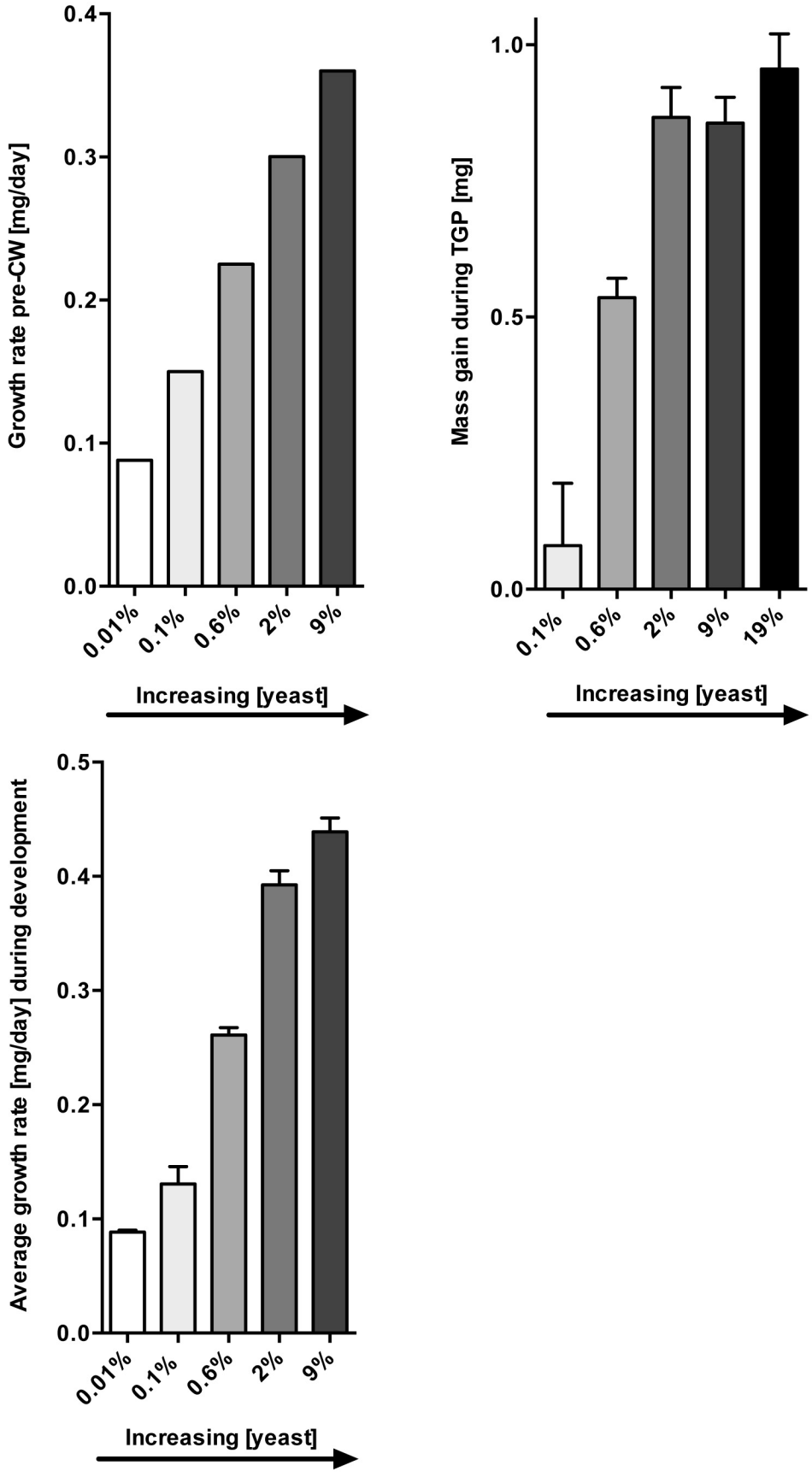

Figure 10.2: Effect of dietary yeast on growth rate pre-CW, during TGP and throughout development

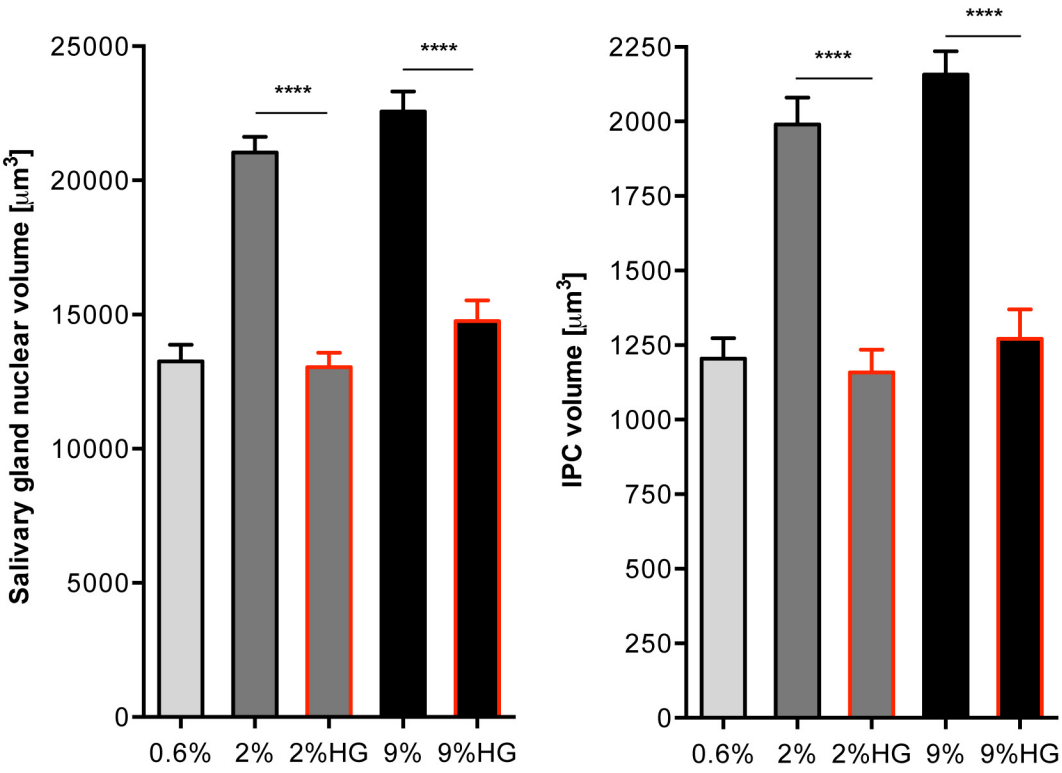

Figure 10.3: Salivary gland and IPC soma volumes for WPP raised on high-glucose diets compared to "standard" glucose diets

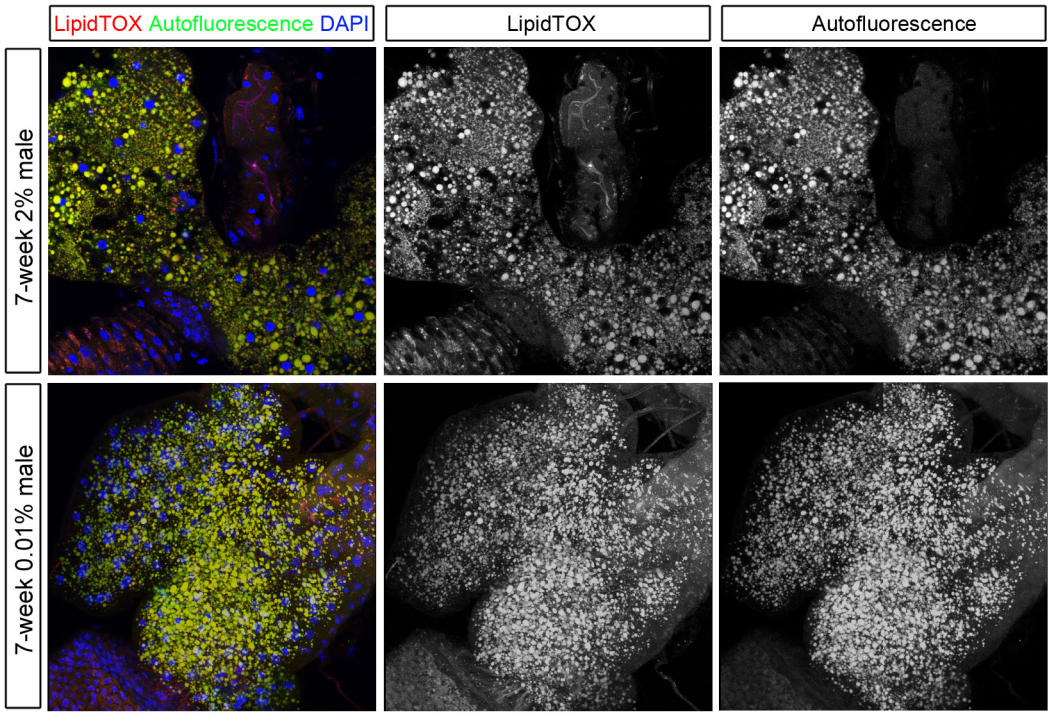

Figure 10.4: Fat body lipid droplets become autofluorescent with age.

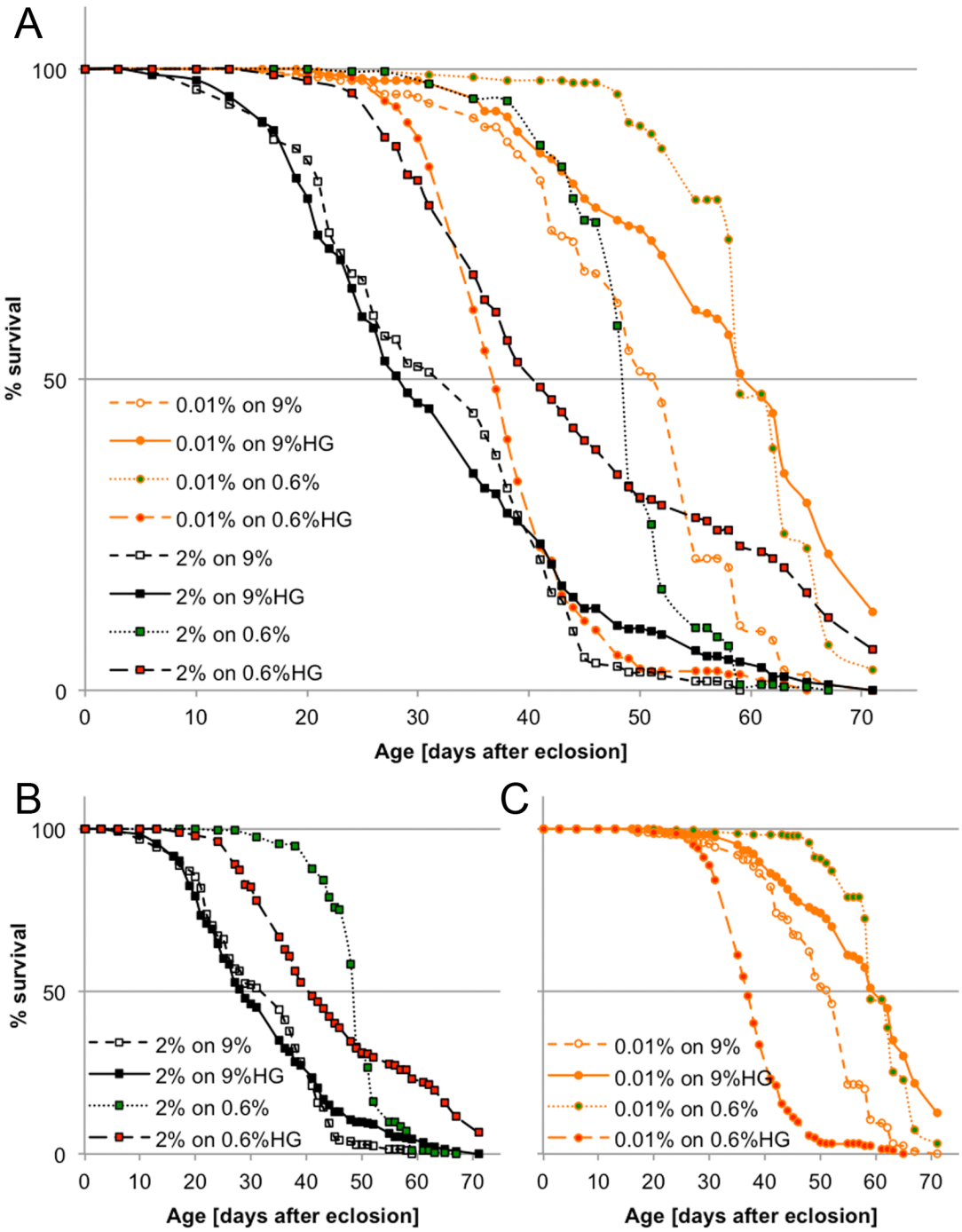

Figure 10.5: Effect of adult diet on the lifespan of males fed on either a 2%- or on a 0.01%-yeast diet as larvae

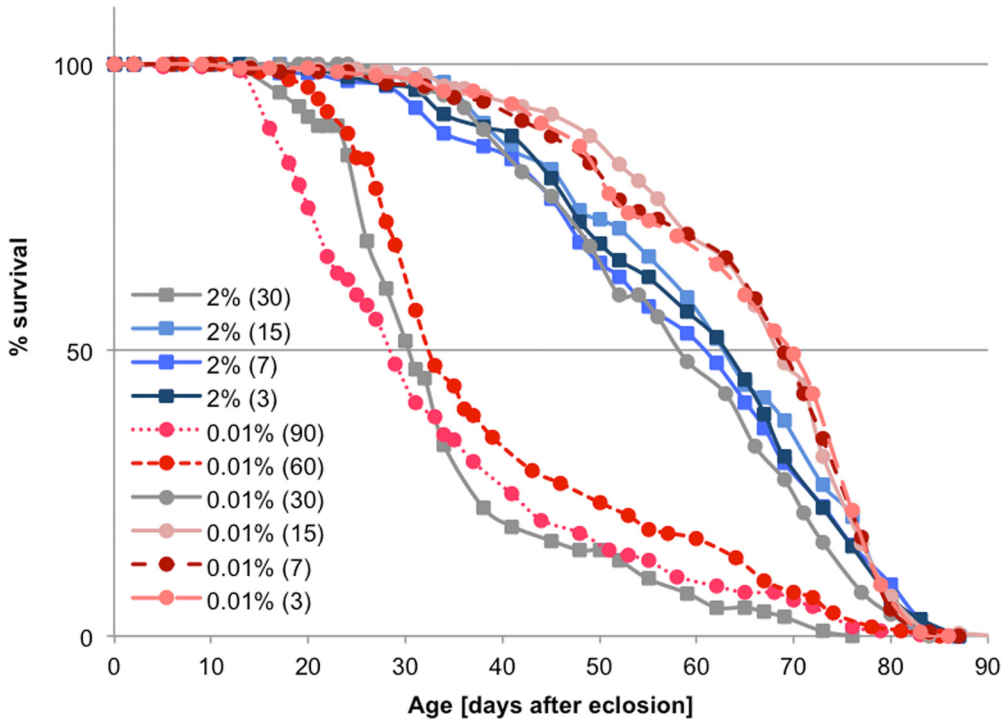

Figure 10.6: Effects of housing density on the lifespan of males fed on either a 2%- or on a 0.01%-yeast diet as larvae

Table 10.5: Mean and median lifespan data for Figure 6.1

Panel A	No. of subjects	Restricted mean			Age in days at % mortality					
		Days	Std. error	95% C.I.	25%	50%	75%	90%	100%	95% Median C.I.
0.01% on 9%HG	214	57.58	0.83	55.95 ~ 59.21	49	61	67	-	-	59 ~ 62
0.1% on 9%HG	211	54.56	0.88	52.83 ~ 56.29	43	54	69	-	-	53 ~ 59
0.6% on 9%HG	193	42.85	0.94	41.01 ~ 44.69	36	42	50	62	-	40 ~ 42
2% on 9%HG	224	31.98	0.89	30.23 ~ 33.73	21	29	41	49	71	27 ~ 31

Panel B	No. of subjects	Restricted mean			Age in days at % mortality					
		Days	Std. error	95% C.I.	25%	50%	75%	90%	100%	95% Median C.I.
0.01% on 9%	218	49.80	0.67	48.48 ~ 51.12	42	52	55	61	71	49 ~ 52
0.1% on 9%	217	50.39	0.64	49.15 ~ 51.64	45	54	58	60	64	- ~ -
0.6% on 9%	186	38.39	0.87	36.68 ~ 40.10	30	40	46	53	64	38 ~ 40
2% on 9%	213	31.70	0.76	30.21 ~ 33.18	22	35	41	44	59	28 ~ 35

Panel C	No. of subjects	Restricted mean			Age in days at % mortality					
		Days	Std. error	95% C.I.	25%	50%	75%	90%	100%	95% Median C.I.
0.01% on 0.6%	223	60.15	0.47	59.22 ~ 61.07	58	59	65	67	-	59 ~ 61
0.1% on 0.6%	220	58.63	0.44	57.77 ~ 59.49	57	58	62	66	-	58 ~ 60
0.6% on 0.6%	162	51.60	0.52	50.58 ~ 52.63	49	52	56	60	68	50 ~ 52
2% on 0.6%	216	48.62	0.41	47.81 ~ 49.43	48	49	52	57	67	- ~ -

Panel D	No. of subjects	Restricted mean			Age in days at % mortality					
		Days	Std. error	95% C.I.	25%	50%	75%	90%	100%	95% Median C.I.
0.01% on 0.6%HG	206	38.19	0.50	37.21 ~ 39.18	35	37	41	46	65	36 ~ 37
0.1% on 0.6%HG	193	39.44	0.68	38.10 ~ 40.78	35	38	42	48	-	36 ~ 38
0.6% on 0.6%HG	178	42.93	1.13	40.71 ~ 45.16	31	38	53	68	-	37 ~ 40
2% on 0.6%HG	211	45.23	1.07	43.13 ~ 47.33	35	41	59	71	-	38 ~ 43

Table 10.6: Mean and median lifespan data for Figure 6.2

Panel A	No. of subjects	Restricted mean			Age in days at % mortality					
		Days	Std. error	95% C.I.	25%	50%	75%	90%	100%	95% Median C.I.
0.01% on 9%HG	198	51.04	0.82	49.44 ~ 52.65	45	56	59	62	69	52 ~ 52
0.1% on 9%HG	242	51.53	0.62	50.31 ~ 52.74	45	52	59	62	73	52 ~ 52
0.6% on 9%HG	253	31.18	0.79	29.63 ~ 32.72	21	27	42	52	62	27 ~ 27
2% on 9%HG	250	27.77	0.77	26.27 ~ 29.27	17	24	34	45	62	24 ~ 24

Panel B	No. of subjects	Restricted mean			Age in days at % mortality					
		Days	Std. error	95% C.I.	25%	50%	75%	90%	100%	95% Median C.I.
0.01% on 0.6%	248	66.29	0.69	64.95 ~ 67.64	66	69	73	76	83	- ~ -
0.1% on 0.6%	245	68.02	0.45	67.13 ~ 68.90	66	69	73	76	80	- ~ -
0.6% on 0.6%	235	66.18	0.67	64.88 ~ 67.49	62	69	73	76	80	- ~ -
2% on 0.6%	247	54.10	0.53	53.06 ~ 55.14	48	52	56	66	79	- ~ -

Panel C	No. of subjects	Restricted mean			Age in days at % mortality					
		Days	Std. error	95% C.I.	25%	50%	75%	90%	100%	95% Median C.I.
0.01% on 0.6%HG	91	40.71	1.14	38.48 ~ 42.94	34	38	45	56	69	34 ~ 38
0.1% on 0.6%HG	220	49.28	0.86	47.59 ~ 50.96	38	48	59	66	85	45 ~ 45
0.6% on 0.6%HG	239	51.65	0.95	49.78 ~ 53.51	38	52	66	69	76	48 ~ 52
2% on 0.6%HG	245	47.66	1.13	45.44 ~ 49.88	34	42	66	73	79	38 ~ 45

Table 10.7: Mean and median lifespan data for Figure 7.5

Panel A	No. of subjects	Restricted mean			Age in days at % mortality					
		Days	Std. error	95% C.I.	25%	50%	75%	90%	100%	95% Median C.I.
0.01% on 9%HG	85	58.82	1.43	56.02 ~ 61.61	48	61	69	76	-	58 ~ 61
2% on 9%HG	121	32.31	1.28	29.80 ~ 34.83	23	28	36	58	73	26 ~ 29
co-housed 0.01% on 9%HG	114	37.06	1.45	34.22 ~ 39.90	28	31	43	65	76	31 ~ 31
co-housed 2% on 9%HG	119	47.28	1.58	44.19 ~ 50.38	35	44	62	69	82	39 ~ 47

Panel B	No. of subjects	Restricted mean			Age in days at % mortality					
		Days	Std. error	95% C.I.	25%	50%	75%	90%	100%	95% Median C.I.
0.01% on 9%HG	146	66.07	1.09	63.94 ~ 68.20	55	70	77	80	87	66 ~ 70
2% on 9%HG	180	32.54	1.01	30.55 ~ 34.52	23	29	37	55	76	26 ~ 27
co-housed 0.01% on 9%HG	229	38.45	0.81	36.87 ~ 40.03	30	39	43	53	79	37 ~ 39
co-housed 2% on 9%HG	239	61.31	0.86	59.62 ~ 62.99	50	62	72	79	86	59 ~ 63
2% (15) on 9%HG	106	61.76	1.42	58.98 ~ 64.54	55	63	72	79	81	61 ~ 65

Table 10.8: Mean and median lifespan data for Figure 7.6

Panel A	No. of subjects	Restricted mean			Age in days at % mortality					
		Days	Std. error	95% C.I.	25%	50%	75%	90%	100%	95% Median C.I.
2% (30) on 9%HG	120	34.93	1.24	32.51 ~ 37.35	26	31	38	55	76	30 ~ 32
2% (15) on 9%HG	128	62.35	1.36	59.68 ~ 65.01	48	65	76	80	87	62 ~ 67
2% (7) on 9%HG	133	59.52	1.48	56.61 ~ 62.42	48	62	73	80	87	55 ~ 62
2% (3) on 9%HG	139	61.02	1.37	58.34 ~ 63.70	48	65	73	80	87	59 ~ 65

Panel B	No. of subjects	Restricted mean			Age in days at % mortality					
		Days	Std. error	95% C.I.	25%	50%	75%	90%	100%	95% Median C.I.
0.01% (90) on 9%HG	355	33.98	0.91	32.19 ~ 35.77	20	29	41	62	86	29 ~ 29
0.01% (60) on 9%HG	300	40.03	0.97	38.12 ~ 41.94	28	33	50	67	85	33 ~ 33
0.01% (30) on 9%HG	208	58.91	1.02	56.91 ~ 60.90	49	59	71	77	84	56 ~ 59
0.01% (15) on 9%HG	217	66.39	0.93	64.57 ~ 68.22	59	69	77	80	91	69 ~ 71
0.01% (7) on 9%HG	153	65.56	1.21	63.18 ~ 67.94	54	69	77	80	87	69 ~ 71
0.01% (3) on 9%HG	146	65.86	1.21	63.48 ~ 68.24	53	70	76	79	86	68 ~ 72

Table 10.9: Lifespan statistical analyses for Figures 6.1 and 6.2 in Chapter 6.

p-values are derived from non-parametric Log-rank test and were calculated in OASIS (Yang et al., 2011)

		p-value
Fig. 6.1.A	**0.01% on 9%HG** *v.s.* **0.1% on 9%HG**	0.1333
	0.01% on 9%HG *v.s.* **0.6% on 9%HG**	0.00E+00
	0.01% on 9%HG *v.s.* **2% on 9%HG**	0.00E+00
	0.1% on 9%HG *v.s.* **0.6% on 9%HG**	0.00E+00
	0.1% on 9%HG *v.s.* **2% on 9%HG**	0.00E+00
	0.6% on 9%HG *v.s.* **2% on 9%HG**	0.00E+00
Fig. 6.1.B	**0.01% on 9%** *v.s.* **0.1% on 9%**	0.2699
	0.01% on 9% *v.s.* **0.6% on 9%**	0.00E+00
	0.01% on 9% *v.s.* **2% on 9%**	0.00E+00
	0.1% on 9% *v.s.* **0.6% on 9%**	0.00E+00
	0.1% on 9% *v.s.* **2% on 9%**	0.00E+00
	0.6% on 9% *v.s.* **2% on 9%**	0.00E+00
Fig. 6.1.C	**0.01% on 0.6%** *v.s.* **0.1% on 0.6%**	1.90E-08
	0.01% on 0.6% *v.s.* **0.6% on 0.6%**	0.00E+00
	0.01% on 0.6% *v.s.* **2% on 0.6%**	0.00E+00
	0.1% on 0.6% *v.s.* **0.6% on 0.6%**	0.00E+00
	0.1% on 0.6% *v.s.* **2% on 0.6%**	0.00E+00
	0.6% on 0.6% *v.s.* **2% on 0.6%**	1.10E-08
Fig. 6.1.D	**0.01% on 0.6%HG** *v.s.* **0.1% on 0.6%HG**	0.1365
	0.01% on 0.6%HG *v.s.* **0.6% on 0.6%HG**	3.20E-06
	0.01% on 0.6%HG *v.s.* **2% on 0.6%HG**	0.00E+00
	0.1% on 0.6%HG *v.s.* **0.6% on 0.6%HG**	0.001
	0.1% on 0.6%HG *v.s.* **2% on 0.6%HG**	1.30E-07
	0.6% on 0.6%HG *v.s.* **2% on 0.6%HG**	0.3813
Fig. 6.2.A	**0.01% on 9%HG** *v.s.* **0.1% on 9%HG**	0.6178
	0.01% on 9%HG *v.s.* **0.6% on 9%HG**	0.00E+00
	0.01% on 9%HG *v.s.* **2% on 9%HG**	0.00E+00
	0.1% on 9%HG *v.s.* **0.6% on 9%HG**	0.00E+00
	0.1% on 9%HG *v.s.* **2% on 9%HG**	0.00E+00
	0.6% on 9%HG *v.s.* **2% on 9%HG**	0.0042
Fig. 6.2.B	**0.01% on 0.6%HG v.s. 0.1% on 0.6%HG**	1.00E-08
	0.01% on 0.6%HG v.s. 0.6% on 0.6%HG	0.00E+00
	0.01% on 0.6%HG v.s. 2% on 0.6%HG	5.50E-06
	0.1% on 0.6%HG v.s. 0.6% on 0.6%HG	0.0069
	0.1% on 0.6%HG v.s. 2% on 0.6%HG	0.3719
	0.6% on 0.6%HG v.s. 2% on 0.6%HG	0.2395
Fig. 6.2.C	**0.01% on 0.6%HG** *v.s.* **0.1% on 0.6%HG**	1.00E-08
	0.01% on 0.6%HG *v.s.* **0.6% on 0.6%HG**	0.00E+00
	0.01% on 0.6%HG *v.s.* **2% on 0.6%HG**	5.50E-06
	0.1% on 0.6%HG *v.s.* **0.6% on 0.6%HG**	0.0069
	0.1% on 0.6%HG *v.s.* **2% on 0.6%HG**	0.3719
	0.6% on 0.6%HG *v.s.* **2% on 0.6%HG**	0.2395

Table 10.10: Lifespan statistical analyses for Figures 7.5 and 7.6 in Chapter 7.

p-values are derived from non-parametric Log-rank test and were calculated in OASIS (Yang et al., 2011)

		p-value
Fig. 7.5.A	**0.01% on 9%HG v.s. 2% on 9%HG**	0.00E+00
	0.01% on 9%HG v.s. co-housed 0.01% on 9%HG	0.00E+00
	0.01% on 9%HG v.s. co-housed 2% on 9%HG	0.0004
	2% on 9%HG v.s. co-housed 0.01% on 9%HG	0.0055
	2% on 9%HG v.s. co-housed 2% on 9%HG	0.00E+00
	co-housed 0.01% on 9%HG v.s. co-housed 2% on 9%HG	6.30E-06
Fig. 7.5.B	**0.01% on 9%HG** *v.s.* **2% on 9%HG**	0.00E+00
	0.01% on 9%HG *v.s.* **co-housed 0.01% on 9%HG**	0.00E+00
	0.01% on 9%HG *v.s.* **co-housed 2% on 9%HG**	0.0001
	0.01% on 9%HG *v.s.* **2% (15) on 9%HG**	0.0001
	2% on 9%HG *v.s.* **co-housed 0.01% on 9%HG**	0.0001
	2% on 9%HG *v.s.* **co-housed 2% on 9%HG**	0.00E+00
	2% on 9%HG *v.s.* **2% (15) on 9%HG**	0.00E+00
	co-housed 0.01% on 9%HG *v.s.* **co-housed 2% on 9%HG**	0.00E+00
	co-housed 0.01% on 9%HG *v.s.* **2% (15) on 9%HG**	0.00E+00
	co-housed 2% on 9%HG *v.s.* **2% (15) on 9%HG**	0.9427
Fig. 7.6.A	**2% (30) on 9%HG v.s. 2% (15) on 9%HG**	0.00E+00
	2% (30) on 9%HG v.s. 2% (7) on 9%HG	0.00E+00
	2% (30) on 9%HG v.s. 2% (3) on 9%HG	0.00E+00
	2% (15) on 9%HG v.s. 2% (7) on 9%HG	0.5639
	2% (15) on 9%HG v.s. 2% (3) on 9%HG	0.6741
	2% (7) on 9%HG v.s. 2% (3) on 9%HG	0.8073
Fig. 7.6.B	**0.01% (90) on 9%HG v.s. 0.01% (60) on 9%HG**	0.0002
	0.01% (90) on 9%HG v.s. 0.01% (30) on 9%HG	0.00E+00
	0.01% (90) on 9%HG v.s. 0.01% (15) on 9%HG	0.00E+00
	0.01% (90) on 9%HG v.s. 0.01% (7) on 9%HG	0.00E+00
	0.01% (90) on 9%HG v.s. 0.01% (3) on 9%HG	0.00E+00
	0.01% (60) on 9%HG v.s. 0.01% (30) on 9%HG	0.00E+00
	0.01% (60) on 9%HG v.s. 0.01% (15) on 9%HG	0.00E+00
	0.01% (60) on 9%HG v.s. 0.01% (7) on 9%HG	0.00E+00
	0.01% (60) on 9%HG v.s. 0.01% (3) on 9%HG	0.00E+00
	0.01% (30) on 9%HG v.s. 0.01% (15) on 9%HG	6.30E-07
	0.01% (30) on 9%HG v.s. 0.01% (7) on 9%HG	1.70E-05
	0.01% (30) on 9%HG v.s. 0.01% (3) on 9%HG	0.0002
	0.01% (15) on 9%HG v.s. 0.01% (7) on 9%HG	0.8754
	0.01% (15) on 9%HG v.s. 0.01% (3) on 9%HG	0.3337
	0.01% (7) on 9%HG v.s. 0.01% (3) on 9%HG	0.5722

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