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Experimental studies of adult longevity of the blowfly, Calliphora stygia (Diptera: Calliphoridae)

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"This thesis is presented as part of the requirements for the award of the Degree of Doctor of Philosophy of the University of Wollongong"

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DECLARATION

I, Megan A. Kelly, declare that this thesis, submitted in fulfillment of the requirements for the award of Doctor of Philosophy, in the School of Biological Sciences, University of Wollongong, is wholly my own work unless otherwise referenced or acknowledged. The document has not been submitted for qualifications at any other academic institution.

A. P. Zieba and D. Whittaker provided assistance in the feeding and care of fly populations and carried out parts of the phospholipid fatty acid extraction. Professor D. Griffiths of the Statistical Consulting Service, University of Wollongong, provided advice on population mortality modeling.

ABSTRACT

There are two commonly used environmental manipulations that have been shown to extend longevity across a range of animal species: temperature (in ectotherms) and dietary restriction (in endotherms and ectotherms). Using these two manipulations in model organisms, a number of correlations between levels of oxidative damage and longevity have been found, which are largely consistent with the 'oxidative stress hypothesis of ageing'. However, before mechanisms identified in a few model organisms can be accepted as being general features of the ageing process, it is essential that these manipulations are shown to extend lifespan in a broader group of animals.

Ageing is measured through both demographic measures of mortality and by determination of changes in physiological functionality at cellular and molecular levels. Rodent models are typically used, due to their physiological similarity to humans and because their body size enables thorough biochemical analysis. The lifespans of rodents, however, require experiments lasting several years to obtain demographic measurements. By contrast, invertebrate models have the demographic advantage of short lifespans and relative ease of husbandry and experimental manipulation, but their small size often limits biochemical measures, which are based on pooled samples from many individuals. The ageing process is not however, constant across tissue-types, therefore, pooling individuals can lead to both underestimating individual variation in the ageing process and missing age-related tissue specific effects.

This thesis examines longevity and membrane fatty acid composition in a new model organism, the blowfly, *Calliphora stygia.* This species has the advantage of a short lifespan but a relatively large body size among flies, that allows for individual-based measurements. This is advantageous not only for biochemical measures, but also

for implementation of treatments such as dietary restriction, where food consumption of populations can be measured over the entire lifespan. I examined the effects of temperature and diet energy content on longevity and demographic and cellular senescence. Temperature effects were examined both after exposure to constant temperatures, and after transfer to lower or higher temperatures. Diet energy content was varied by either altering the sugar content of the diet, or by varying the yeast content of the diet, to examine potential differences between diet composition and energy consumption.

Temperature has been shown to affect the longevity and life history of ectothermic invertebrates by reducing the rate of ageing at low temperatures. Exposure to temperatures over the range of 12°C to 34°C had a significant affect on the longevity of *C. stygia* increasing average and maximum longevity five to six-fold across this thermal range. As egg laying was absent at the two extreme temperatures but present for all others, it is likely these temperatures encompass the entire physiological range of *C. stygia.* Temperature effects on ectotherm longevity are often attributed to consequent changes in metabolic rate as dictated by the 'rate of living theory'. Assuming food consumption to be a valid measure of metabolic rate, my results refute this assumption as there was no difference in average daily food consumption over the moderate to high temperature range, despite a 50% difference in longevity. Mortality rates were best described by a two-phase Gompertz relation, which revealed the early phase of ageing to be the most temperature sensitive, but with relatively little influence of temperature on the rate of ageing during the later phase of ageing. In addition to the early phase of ageing having lower rates of ageing at low temperatures, this period was also significantly extended in blowflies held at low temperatures, with them showing a

delayed onset of ageing. Blowflies that were transferred from high to low temperatures showed a significant effect of thermal history, in that mortality remained higher after the transfer. A period of reduced senescence that was seen at low temperatures, was then initiated after the low-temperature transfer, despite those blowflies already having entered the late phase of ageing at the high temperature. The ability of *C. stygia* to significantly alter mortality rates during their lifespan makes them a potentially powerful model for examining the process of ageing. The accumulation of fluorescent AGE pigment, a measure of cellular oxidative damage, increased steadily over time in all blowflies, irrespective of the temporal pattern of mortality. Accumulation of pigment was found during periods of otherwise 'negligible senescence' as measured by mortality, however the rate of accumulation was significantly affected by temperature. These findings suggest that accumulation of fluorescent AGE pigment may be more a consequence of age rather than a cause of mortality.

Dietary restriction has been shown to reduce the prevalence and onset of age-related diseases and extend longevity over a range of both vertebrates (fish, rats, mice and monkeys) and invertebrates (worms and insects). Debate about whether these effects of dietary restriction result from a reduction in energy consumption or by specific reduction of certain nutrients is unresolved. Contrary to expectations, longevity increased in *C. stygia* given diets with increased sugar content and in those given diets with a reduced yeast content. The similarity between the two treatments was an increased longevity when the balance of yeast to sugar was reduced, irrespective of the calories consumed. These findings support more recent findings that diet composition is more important in affecting longevity extension than calorie consumption. Despite differences in longevity, there was no evidence of a difference between dietary

treatments in the accumulation of AGE pigments. Similar to the temperature experiment, there was little relationship between the measures of demographic senescence and cellular senescence under different dietary treatments.

A final consideration of my research was to test the 'membrane pacemaker hypothesis of ageing'. This theory states there is a functional link between the fatty acid composition of membranes, due to how this affects their susceptibility to peroxidation, and longevity. To examine this theory experimentally, blowflies were given a range of pure methyl esters or mixed oil diets and their membrane fatty acid composition and longevity were recorded. Membrane fatty acid composition of blowflies that experienced manipulation of environmental temperature or diet energy content were also examined.

Membrane fatty acid composition was very resistant to change through diet manipulation, with dietary provision of 18:0 and 18:1*n*-9 or the mixed oil diets of fish or krill oil having almost no effect. Membrane fatty acid composition was influenced by the presence of the essential polyunsaturated fatty acids (PUFA) 18:2*n*-6 and 18:3*n*-3. These two fatty acids were significantly increased in the membranes, however there was little evidence of these fatty acids being further metabolised into longer chain PUFA. Increased intake of *n*-6 PUFA resulted in high early mortality and a reduction in average longevity. The provision of the other fatty acids had a positive effect on average longevity, but this was not consistent with any specific changes in membrane peroxidisability. Therefore, this improved longevity is more likely to be a result of these fats correcting a fatty acid diet deficiency of the standard sugar/yeast food provided than from modulation of membrane composition. In support of these findings, and the relative regulation of membrane composition, there was also no evidence that

membrane fatty acid composition was responsible for the longevity extension caused by low temperature or changes to diet energy content. Blowflies exposed to low temperature treatments had a reduced rate of change and metabolic consumption of membrane fatty acids, while diet sugar or yeast content had little effect on membrane fatty acid composition.

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ABBREVIATIONS

8-OhDG	8-hydroxy-2'-deoxyguanosine
µx	age-specific mortality
AGE pigment	age-related glycation end-products pigment
ANOVA	analysis of variance
CAFE	capillary feeder
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
FAME	fatty acid methyl ester
H_2O_2	hydrogen peroxide
HPLC	high performance liquid chromatography
HHE	4-hydroxyhexanal
HNE	4-hydroxynonenal
IIS	insulin/insulin-like signalling
LEP	lifetime energy potential
MDA	malondialdehyde
MUFA	monounsaturated fatty acid
NIA	National Institute of Aging
PBS	phosphate buffered saline
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol

PI	peroxidation index
PS	phosphatidylserine
PUFA	polyunsaturated fatty acid
Q_{10}	temperature coefficient
ROS	reactive oxygen species
SEM	standard error of the mean
SFA	saturated fatty acid
SY	sugar/yeast food (standard diet)
WNPRC	Wisconsin National Primate Research Center

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1 INTRODUCTION

1.1 Ageing

Ageing is a universal process that has been identified in many organisms ranging from bacteria, to plants and animals (Stewart et al., 2005). Yet while 'age' is acknowledged to be a common risk factor for a number of diseases, what actually causes ageing or triggers its onset is mostly unknown (Kirkwood, 2011). Ageing is often described as a progressive decline in function and survival with age. Declines in physiological function that reduce the capacity to maintain homeostasis can be a result of a range of changes to cellular processes that occur with age. Due to this progressive nature of ageing, and our inability to stop time, most of our understanding of the process of ageing is only correlations between changes in function and time. Importantly, just because something is associated with age does not mean that it is necessarily a cause of ageing. Thus, experimental manipulation of factors suspected to be responsible for ageing processes is needed to identify causal mechanisms.

The best way to distinguish between causal and correlational ageing factors is to experimentally manipulate these variables to see if any are capable of positively modifying the rate or time of onset of ageing. The effects of this extended longevity on the cellular and molecular processes theorised to cause ageing can then be investigated. Population-level determination of survival and death can establish how death rates (i.e. mortality) change with age, which indicates demographic ageing or senescence. By accompanying these demographic measures of senescence with concurrent evaluation of physiological or 'functional' senescence at cellular and molecular levels, it is possible to gain a better understanding of ageing processes (Grotewiel et al., 2005). This approach can be used to address the key questions for ageing research: i) Do mortality measures

reflect physiological decline and cellular senescence? ii) Can we manipulate longevity and alter rates of physiological decline? iii) Can we manipulate the theorised causes of ageing and increase longevity?

1.2 Measuring ageing: Demographic senescence

There are a number of ways to measure the longevity and rate of ageing of a population. Following a population over time and recording the time of death for each individual gives a demographic record of that population over time, which identifies the changes in the probability of death that indicates the process of senescence (Bronikowski and Flatt, 2010). The reciprocal of average lifespan of the entire population, or average longevity, has traditionally been used as a measure of its rate of ageing. While average lifespan has sometimes been shown to correlate with changes in early mortality, it has little relationship with the rate of ageing over the lifespan and is thought to mostly reflect changes in the health of organisms (Pletcher et al., 2000). In contrast, maximum longevity, calculated as the average age of the 5-10% longest lived members of the population, inversely correlates with the rate of ageing. However, both average lifespan and maximum longevity are the result of cumulative measures and are thus unable to show age-specific patterns. Survival curves, while showing survival across all age groups, is also a cumulative measure and therefore also unable to show age-specific patterns (Bronikowski and Flatt, 2010). A greater insight into the process of ageing can be made by the examination of mortality rates over survival curves or values of average and maximum longevity (Pletcher et al., 2000; Gems et al., 2002).

Theoretical models that describe changes in age-dependent mortality of a population with time can give a simple numerical representation of mortality-related events and allow for comparison between treatments. Senescence is characterised as an increase in mortality rates over time and the rate of this increase can, in turn, be used to quantify the rate of ageing. The pattern of age-related mortality typically shows an exponential increase with time, which is often characterised using the Gompertz equation (Gompertz, 1825). Biologically, this mathematical model describes an increasing vulnerability with age, which results in an increased probability of death due to declining physiological function (Ricklefs and Scheuerlein, 2002). When mortality is expressed semi-logarithmically (Fig. 1.1), it displays a linear relationship in the form of $\ln(\mu x) = Ax + B$, where x is time and μx is mortality. The rate of ageing (A) is denoted by the slope of this line, whereas the intercept of the line (B) indicates the initial mortality of the population (Fig. 1.1).

Generally the Gompertz equation is applied to mortality data at the onset of senescence when the mortality rates are increasing (Bronikowski and Flatt, 2010). However, interpretations of the application of the Gompertz vary between studies, which can have a significant effect on the outcome of the analysis. For instance, if Gompertz is applied to all data for populations A and B, as in Figure 1.1A, then the rates of ageing would differ significantly between the populations. The slopes of these relationships show population A to age at a slower rate than population B. The intercept of the lines, which is lower for population A than population B, suggests that the initial mortality is lower in population A than B. Figure 1.1B shows the fitting of the Gompertz equation when mortality rates are increasing, i.e. at the onset of demographic senescence. This leads to a significantly different interpretation. Now it appears that

populations A and B have a similar rate of ageing, but there is a delay in the onset of ageing of population A as shown by the right shifting of the line.

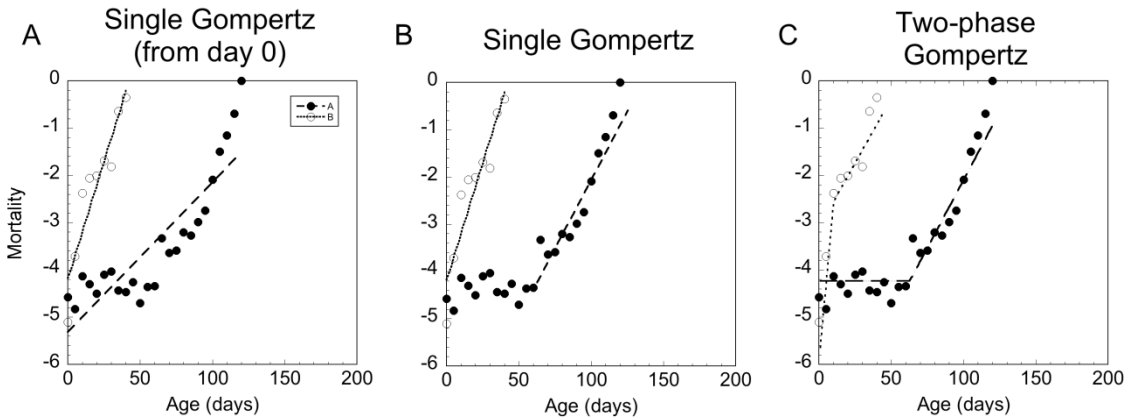

Figure 1.1 Examples of mortality rate curves fit with Gompertz models. The models are applied in different ways to two populations, A (closed circles) and B (open circles). These populations vary in longevity, with population A living longer than population B. Fig. 1.1A shows a single Gompertz applied from day 0, assuming senescence starting from day of birth or eclosion. The slopes of the line show a significant difference between populations A and B, with population A 'ageing' at a reduced rate compared to population B. Fig. 1.1B shows a single Gompertz applied from the time at which mortality rates increase with increasing age, representative of the period of senescence. Applied in this way, the differences in longevity between the two populations now indicate a delay in the onset of senescence, with the rate of ageing similar between the two populations. Fig 1.1C demonstrates the application of a two-phase Gompertz, which describes two phases of ageing which differ in their rates. For population A, the two-phase Gompertz describes the period of delay before the onset of senescence, with the horizontal line showing no increase in age-specific mortality with time and therefore 'negligible senescence' during the early phase, with the rate of ageing increased during the late phase. For population B, the early phase of ageing has high mortality indicated by the steep slope, but a reduced rate of ageing at old age. Both populations have similar rates of ageing during the second phase.

This difference in application of the Gompertz is also very sensitive to population sizes, as has been discussed by Promislow et al. (1999). Their reanalysis of the data from Orr and Sohal (1994), changed the outcome of this seminal study on overexpression of the antioxidants, superoxide dismutase and catalase, from increasing

longevity in the fruit fly, *Drosophila melanogaster*, by changing the rate of ageing (as suggested by Fig. 1.1A), to a right shifting of the Gompertz lines, resulting in similar rates of ageing, but a delay in the onset of senescence (as suggested in Fig. 1.1B).

This period of delay in the onset of senescence has previously only been inferred from survival curves and right-shifting of mortality curves (Strehler, 1961; Promislow, 1991; Garcia et al., 2010). However, it could potentially offer significant insight into the mechanisms of ageing, yet it is rarely mathematically described. A two-phase Gompertz can be used to describe an age of onset of senescence giving a more definite description of the delay in the onset of senescence. The two-phase Gompertz equation divides the mortality curve into two stages, an 'early' and a 'late' period of ageing, each with distinctly different rates of change in mortality, and the 'break-point' between the two rates of ageing, represents the age at which the change in mortality rates occurs (Shahrestani et al., 2012). This equation has been mostly utilised to describe a deceleration of ageing that occurs at old-age in a range of animals, including humans (De Reggi, 1975; Carey et al., 1992; Curtsinger et al., 1992; Fukui et al., 1993; Vaupel et al., 1998; Steinsaltz and Wachter, 2006; Shahrestani et al., 2012). Because of this decline in mortality rates at old-age, the single Gompertz equation over-estimates this decline in mortality (Vaupel et al., 2004). The two-phase Gompertz is therefore advantageous to both describe this deceleration of ageing that is seen at old-age in many species, as well as giving an age (and confidence intervals) at which this deceleration occurs, allowing for statistical comparisons between treatments and populations. Figure 1.1C shows the fitting of a two-phase Gompertz to populations A and B, with population B showing the more traditional deceleration of ageing at old-age at 10 (± 1) days of age. The delay in the onset of senescence is demonstrated in population A (Fig.

1.1C), where the initial early phase of ageing shows a very low rate of ageing until day 61 (± 4), following which, the rate of ageing increases to a rate similar to that of population B. Therefore these two populations could be considered to be 'ageing' at the same rate, but population A had a significant delay in the onset of senescence, and therefore live longer than population B.

While Gompertz is arguably the simplest model to describe senescence, there are a number of different models that can be used to describe mortality over the lifespan. Each model varies in its fit of mortality data and choosing the correct model for the shape of the data is imperative. Many of these models have been designed from an engineering perspective using failure rates, which in biological terms is a reciprocal of mortality. Although this often provides a better fit to the data, the biological relevance of many of these models can be difficult to interpret, which also needs to be considered when applying models to mortality data (Golubev, 2004).

Three other demographic models are commonly used in the analysis of mortality. The Gompertz-Makeham model introduces an additional parameter to account for mortality that is not dependent on age, and while the Makeham parameters has biological significance, it is not as widely used in interpretations of mortality changes as the Gompertz parameters (Promislow and Haselkorn, 2002; Golubev, 2004). The Weibull model, which can account for the deceleration of mortality at old age, does not assume a constant hazard rate such as in the linear models and has two parameters, a shape parameter and a scale parameter which cannot be expressed as simply in terms of the rate of ageing as the Gompertz (Ricklefs and Scheuerlein, 2002). Calculations can be made using the parameters of the Weibull to give a rate of ageing index, and as such the Weibull model is sometimes preferred over the Gompertz due to its ability to

provide a better fit during periods of decelerated ageing (Ricklefs and Scheuerlein, 2002). Finally, another common model used in geronotological research is the logistic model which also describes an asymptotic model of ageing and the decrease in mortality with old-age such as seen in humans (Carey, 1998). Similar to the Weibull, the logistic equation cannot be expressed simply in biological terms of ageing and is not used as extensively as the Weibull or Gompertz models (Arking et al., 2002).

For this thesis I have chosen the Gompertz and the two-phase Gompertz models to describe mortality patterns, due to it being the best-fit to the majority of my mortality curves as well as for its simplicity and biological relevance (Economos, 1982). After initial examination of mortality curves with temperature, I observed that the effects of temperature on the rate of demographic ageing were best analysed using a two-phase Gompertz model. This model demonstrated that temperature had a much greater influence on early ageing than late ageing. Such an insight would not be possible using the simple Gompertz or other mortality models described above. As described in Rozing and Westendorp (2008), the parallel shifting of mortality curves as seen in Figure 1.1C, has been shown for a number of treatments that were expected to alter the rate of senescence, but showed no change in the slope of this relationship (Orr and Sohal, 1994; Lin et al., 1998; Flurkey et al., 2001; Good and Tatar, 2001; Mair et al., 2003).

While it is suggested that the acceleration of mortality may give the most insight into biological effects of ageing, it may also be true that the delay in the onset of senescence described by this shift in mortality curves could hold the key to identifying important mechanisms of ageing. Wilson (1994) suggested that in addition to comparing model parameters between treatments, comparing the type of model that best-fits mortality data could also be used to infer significant insight into the ageing

process. For both simplicity and continuity throughout this thesis, only the single and two-phase Gompertz models are considered.

1.3 Theories and mechanisms of ageing

1.3.1 Development of the theories of ageing

The difference in maximum lifespan between mammalian species is generally correlated with body size, in that bigger mammals tend to live longer than smaller ones. For example, the maximum recorded lifespan of a horse is 57 years, compared to a dog which is 24 years, and compared to a mouse, which is four years (maximum longevities taken from AnAge[1] database). The basis of this body size effect on longevity was initially thought to relate to the generally slower rate of living of large mammals compared to small mammals, which prompted the initial theories of ageing to link longevity to metabolic rate (Austad, 2009). The comparative analysis of body size and metabolic rate by Rubner (1908, from Hulbert, 2008a), comparing five mammal species of varying longevities (guinea pigs, cats, dogs, cattle and horses), found a remarkable similarity between the lifetime energy potential of these mammals (LEP; calculated as total energy expenditure per kg body mass over the entire lifespan). The similarity of the LEP among these mammals that had otherwise very different longevities suggested that organisms have a similar amount of energy to expend per unit mass throughout a lifetime and that their maximum lifespan was therefore dependent on how fast they utilised that energy.

[1] http://genomics.senescence.info/species/

That organisms had a specific LEP was further supported by work on invertebrates maintained at different ambient temperatures. Both fruit flies, *Drosophila* (Loeb and Northrop, 1916, 1917) and water fleas, *Daphnia magna* (MacArthur and Baillie, 1929) kept at high temperatures, have shortened lifespans and increased metabolic rates compared to cohorts kept at low temperatures. This inverse relationship between the length of life and metabolic rate was further developed by Pearl (1928) as the 'rate of living theory'. The 'rate of living theory' suggested that the duration of life was ultimately fixed for a given species, but that the speeding up or slowing down of the pace of life could alter its duration. This association between low metabolic rate and long lifespan, i.e. a 'live-fast, die-young' relationship makes intuitive sense, and a mechanistic link between metabolic rate and ageing was provided by Harman's (1956) 'free radical theory of ageing'. The 'free radical theory of ageing' proposed that reactive oxygen species (ROS) produced as the by-product of cellular metabolism in the mitochondria, cause damage to important biomolecules. It is the accumulation of this damage that is thought to disrupt the homeostatic regulatory systems, which leads to ageing and ultimately death.

Oxidative damage occurs following the production of ROS in the mitochondria. While mitochondrial complexes I and III are the primary sites of superoxide production, several other mitochondrial sites as well as some other cellular processes have also been shown to generate ROS (Brand, 2010). Much of the superoxide produced by mitochondria is rapidly converted into hydrogen peroxide (H_2O_2) by the action of superoxide dismutases and then to H_2O and O_2 via antioxidants such as catalase (Brand, 2010). However, not all of the superoxide is detoxified and in addition to this, when H_2O_2 is in the presence of ferrous ions it can initiate a series of reactions that create a

number of highly reactive hydroxyl radicals, which can then react with almost all biological molecules, including proteins, DNA, amino acids, and membrane lipids (Halliwell and Gutteridge, 1984). In the case of the latter, reaction with lipids creates highly reactive secondary radicals causing further damage to biomolecules (Hulbert, 2005).

There is much experimental evidence showing no relationship between metabolic rate and longevity in a range of organisms. Long-lived *Drosophila* species did not have reduced metabolic rates in comparison to short-lived species (Promislow and Haselkorn, 2002). Furthermore, within-species comparisons of both laboratory mice and *D. melanogaster*, showed no inverse correlation between an individuals mass-specific metabolic rate and their lifespan (Speakman et al., 2000; Hulbert et al., 2004a). Similarly, voluntary exercise and its associated increase in metabolic rate does not shorten lifespan in either rats (Holloszy et al., 1985) or humans (Lee et al., 1995). As well, although caloric restriction and mutations in insulin/IGF-1 signalling (IIS) were shown to extend the lifespan of *D. melanogaster*, neither of these resulted in changes to metabolic rate (Hulbert et al., 2004a).

The ‘free radical theory of ageing’ has evolved into what is now referred to as the ‘oxidative stress theory of ageing’ (Sohal and Orr, 2012). Whilst essentially similar to the ‘free radical theory of ageing’, the ‘oxidative stress hypothesis’ proposes ageing places more emphasis on repair and antioxidant mechanisms. The ‘oxidative stress theory of ageing’ relates longevity to an imbalance between the metabolic production of ROS and the countervailing actions of both enzymatic and non-enzymatic antioxidant systems leading to the accumulation of damage to important biomolecules (Sohal and Orr, 2012). A basic schematic of the ‘oxidative stress theory of ageing’ is given in

Figure 1.2, which highlights that the susceptibility of biological structures to oxidative attack also depends on their rates of repair and antioxidant capability as well as their capacity to withstand damage.

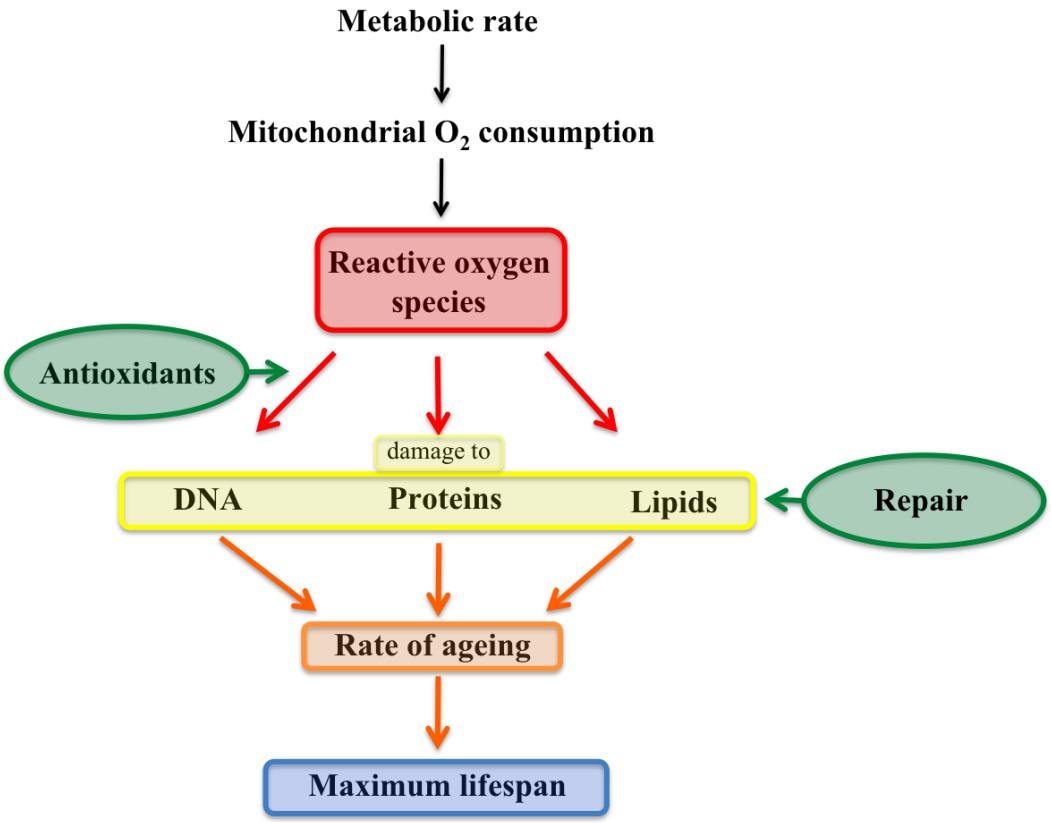

Figure 1.2 Schematic drawing of the oxidative stress theory of ageing (adapted from Hulbert et al., 2007).

The 'membrane pacemaker theory of ageing' is a modification of the 'oxidative stress theory of ageing' that incorporates the effects of membrane fatty acid composition on lipid peroxidation to longevity and the rate of ageing (Fig. 1.3). Fatty acids are highly susceptible to attack by ROS (Hulbert et al., 2007). The attack of ROS on lipids results in an autocatalytic process of formation of lipid-based ROS, which are highly

reactive and can damage a range of other biomolecules (Hulbert, 2005). In addition to the findings that body size and metabolic rate correlate with longevity, membrane fatty acid composition has also been shown to vary systematically with body size in birds and mammals (Hulbert, 2005). A number of comparative studies in mammals and birds have shown a positive relationship between peroxidation-resistant membranes and body size and longevity, with long-lived large mammals tending to have reduced polyunsaturated fatty acids (PUFA) in their membranes (Pamplona et al., 1999a; Pamplona et al., 1999b).

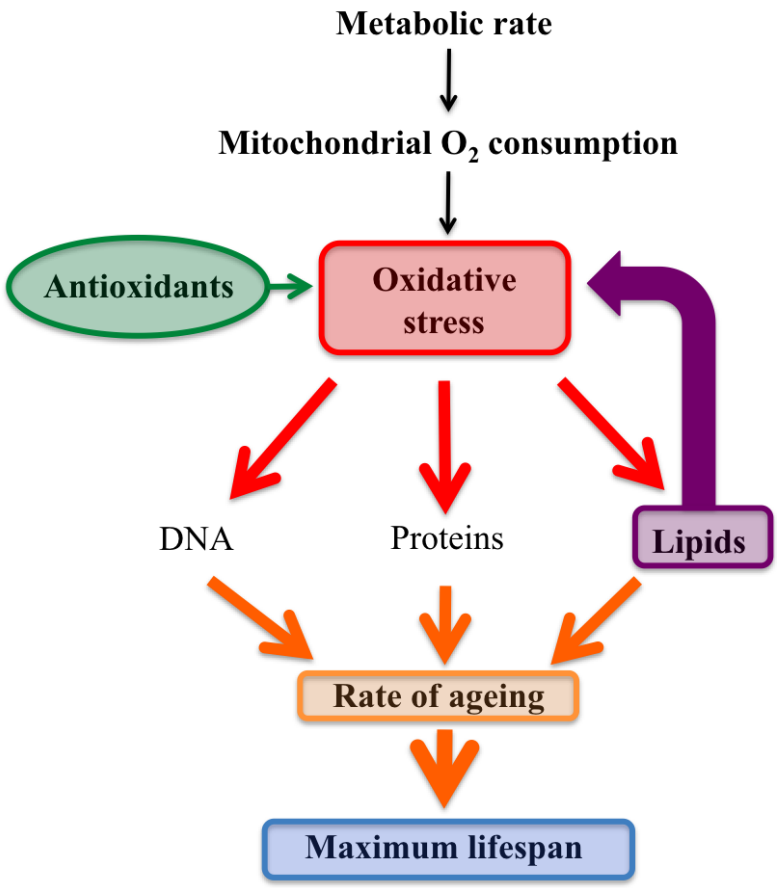

Figure 1.3 Schematic drawing of the oxidative stress theory of ageing showing the influence of lipids on oxidative damage (adapted from Hulbert et al., 2007).

The susceptibility of membrane lipids to peroxidative damage is not equal between the different fatty acid moieties (Hulbert, 2003). Fatty acids consist of a hydrocarbon chain with a carboxyl (-COOH) group and a methyl (-CH3) group at either end. Fatty acids can: i) be saturated with hydrogen and therefore have no double bonds, called saturated fatty acids (SFA); ii) contain a single double bond between carbons, called monounsaturated fatty acids (MUFA); or iii) contain multiple double-bonded carbons, called polyunsaturated fatty acids (PUFA). Fatty acids are named based on the total carbon chain length and the number of double bonds, with the positioning of the double bond also important and counted from the 'omega' carbon situated at the methyl end of the carbon chain and denoted as '*n*-' (Christie, 1985).

Within membranes fatty acids are present as phospholipids, which consist of a three-carbon glycerol backbone, with a highly charged polar head group of phosphate and alcohol attached to one of the carbons, and two fatty acyl chains attached to either of the remaining two carbons. The fatty acyl chains are denoted as being in the *sn*-1 and *sn*-2 positions, and the combination of fatty acids present in these positions has an effect on membrane structure and function (Hulbert et al., 2005). The classification of phospholipids is based on the polar head group, with the two main groups in membranes being phosphatidylcholine (PC) and phosphatidylethanolamine (PE), but phosphatidylserine (PS), phosphatidylglycerol (PG) and biphosphatidylglycerol, also known as cardiolipin, are also present (Christie, 1985). Phospholipids can be further classified by the type of linkage bond between the fatty acid and the carbon in the *sn*-1 position, with an ether bond present in some cases rather than an ester bond, such as in plasmalogens (Nagan and Zoeller, 2001).

The susceptibility of individual fatty acids to peroxidative attack is dependent upon the number of double bonds present. The carbon atom that is situated between a double bonded carbon is the most susceptible to peroxidative attack (Barja, 2004). Therefore it is the PUFA, particularly long chain highly polyunsaturated fatty acids, that are most vulnerable to attack, while SFA and MUFA are relatively peroxidation resistant (Hulbert, 2008b). The peroxidative susceptibility of a membrane can be calculated as a single value, the Peroxidation Index (PI). This calculation is based on the relative oxidation rates of isolated fatty acids, with increasing weight given to fatty acids with more double bonds. The PI is calculated as: PI = 0.025 x % monoenoics + 1 x % dienoics + 2 x % trienoics + 4 x % tetraenoics + 6 x % pentaenoics + 8 x % hexaenoics (Haddad et al., 2007). This single value does not incorporate all of the potential contributions of membrane composition to longevity. For instance, the antioxidant capacities of plasmalogens (Nagan and Zoeller, 2001), or the different potency of various lipid peroxidation products that arise from different fatty acid species (Spiteller, 2001). However, it does allow for a relatively simple comparison between species.

Longevity has been shown to negatively correlate with PI across species and phyla, accounting for the otherwise unexplainable differences in longevity seen between birds and similar-sized mammals (Hulbert et al., 2007). Comparative studies have shown that extremely long-lived species possess peroxidation-resistant fatty acids in their membranes, for example, the naked mole rat, *Heterocephalus glaber,* (maximum lifespan of 28 years, compared to the similar sized mouse of 3-4 years; Hulbert et al., 2006a), and long-lived seabirds (the Procellariiformes examined have a maximum lifespan of over 40 years compared to the similar sized Galliformes of 9 years; Buttemer

et al., 2008). Further, between species of bivalves that vary in longevity from 28 to 507 years, there was also a significant negative correlation between gill mitochondrial phospholipid PI and longevity (Munro and Blier, 2012).

Within species, the PI has been shown to correlate with longevity between different strains of mice, with long-lived wild type mice having significantly lower PI than the shorter-lived lab strains when fed the same diets (Hulbert et al., 2006b). Similarly, different strains of nematode worms, *Caenorhabditis elegans*, that have a 10-fold difference in longevity display a significant negative correlation between PI and longevity (Shmookler Reis et al., 2011). These findings from genetic mutants that have altered fatty acid metabolism capacities, and express different fatty acid membrane profiles, further emphasises the potential role of membrane fatty acid composition on ageing (Hulbert, 2011).

1.3.2 Oxidative damage and ageing

Complex antioxidant defence systems have evolved in organisms in response to the toxicity of oxygen (Halliwell, 1999). While these antioxidant defences are well developed in invertebrates and vertebrates, they are not perfect systems, and do not completely offset the adverse potential of ROS. One of the main reasons the 'oxidative stress hypothesis' has come under increased scrutiny is due to the inability of experimental manipulation of antioxidant levels to affect longevity (Gutteridge and Halliwell, 2000). For example, overexpression of superoxide dismutase and catalase delayed the onset of senescence in *D. melanogaster*, but had little effect on the rate of ageing in one strain (Orr and Sohal, 1994), and had no effect on longevity in long-lived

strains (Orr et al., 2003). Similarly, the overexpression of the same antioxidants in mice also showed no effect on longevity (Pérez et al., 2009). Furthermore, comparative studies of endogenous antioxidant capacities often show a negative relationship between antioxidants and longevity, in that, rather than long-lived animals having a greater capacity to prevent ROS-related damage, short-lived animals tend to have higher antioxidant loads to cope with their increased ROS production (Montgomery et al., 2011). However, this pattern is not consistent, and across a range of vertebrates and invertebrates there is a large variation in the relationship between antioxidant capacity and maximum lifespan (summarised in Hulbert et al., 2007).

Similarly, there is a great variation in the rates of oxidative damage accumulation and the ability of organisms to withstand oxidative damage (Buffenstein et al., 2008). The three most common markers used for measuring oxidative damage result from modifications made to DNA, proteins and lipids. Damage to DNA is commonly measured by the marker 8-hydroxy-2'-deoxyguanosine (8-OhDG). Increased DNA damage in houseflies (*Musca domestica*) with age has been linked with metabolic rate, with the amount of damage being related directly with the activity levels of these flies (Agarwal and Sohal, 1994). This link between metabolic rate and DNA damage was also shown in *D. melanogaster*, with fruit flies kept at high temperatures, and likely increased metabolic rates, having increased levels of mitochondrial DNA mutation loads compared to those maintained at lower temperatures (Garcia et al., 2010). However, naked mole rats, an exceptionally long-lived mammal for their size, exhibit extremely high loads of DNA damage when compared to mice (Andziak et al., 2006).

Protein carbonyls, formed as a result of attack from both primary and secondary ROS, have also been shown to increase with age and age-related diseases (Stadtman,

2001). Houseflies, that were maintained under conditions of low activity that are known to extend longevity, had reduced rates of protein carbonyl formation and higher protein activity levels than those under high activity conditions (Sohal et al., 1993; Yan and Sohal, 2000). Similarly, reduced protein carbonyl formation and higher protein activities were found in *D. melanogaster* maintained at low temperatures (Das et al., 2001). Lower levels of protein adducts were also found in response to low temperature exposure and dietary restriction in *D. melanogaster*, however, when examined in comparison to mortality rates, there was found to be little relationship between this marker of cell damage and demographic ageing (Jacobson et al., 2010). Furthermore, there was no significant difference in protein damage between long-lived pigeons (*Columba livia*) when compared to short-lived Wistar rats (Pamplona et al., 1996). Naked mole rats also show significantly more protein damage, even at young ages, when compared to short-lived mice (Andziak et al., 2006).

Biomarkers of lipid damage commonly incorporate measures of the secondary ROS produced by lipid peroxidation, the reactive aldehydes formed as malondialdehyde (MDA; from the *n*-6 PUFA arachidonic acid), 4-hydroxynonenal (HNE; from *n*-6 PUFA) and 4-hydroxyhexanal (HHE; from *n*-3 PUFA). These secondary ROS are very reactive and have a longer half-life than the primary ROS. As they are no longer charged molecules, they can also migrate through membranes and cause far-reaching damage (Hulbert et al., 2007). Because lipid peroxidation can cause damage to a wide variety of biomolecules, there is no single marker that accurately measures lipid damage due to these secondary ROS (Spiteller, 2001). One of the most commonly used lipid peroxidation biomarkers, MDA, has been shown to increase with age in *D. magna* (Barata et al., 2005), and to be higher in cardiac mitochondria of short-lived rats

compared to long-lived pigeons (Pamplona et al., 1999a). The rates of MDA formation decreased in the fish *Nothobranchius rachovii* when maintained at cold temperatures, a treatment that also extends its lifespan (Hsu and Chiu, 2009). Similarly, the rate of *n*-pentane production, an indicator of lipid peroxidation to *n*-6 PUFA, was reduced in houseflies kept under low temperature conditions (Sohal et al., 1985).

Although many species show increases in their levels of oxidative damage products with age, it is not clear if these are a correlate of ageing or directly contribute to the process of ageing. Recent studies of long-lived species, such as the naked mole rat, show them to have high levels of oxidative damage, which raises questions as to whether oxidative damage is responsible for ageing (Buffenstein et al., 2008). It is also important to note that mitochondrial ROS production is not directly correlated with metabolic rate as often assumed (Selman et al., 2012).

1.4 Measuring ageing: Cellular senescence

Cellular senescence has been measured as the cumulative damage of a range of markers of oxidative stress, however, there is a large variation in the correlation between longevity and damage. One of the more consistent markers with age is the accumulation of particular age-related pigments. The formation and accumulation of irreparable auto-fluorescent compounds with age are a common and universal feature of the ageing process in organisms and have been found in almost all tissue types (Szweda et al., 2003). These products are formed through a number of pathways, originating mainly from oxidative damage to lipids, proteins and DNA and include the products termed lipofuscin, age-related glycation end-products (AGE pigments), and ceroid

pigments (Yin, 1996). While these compounds are formed from many different pathways, they all share similar fluorescent characteristics, suggesting similar origins (Gerstbrein et al., 2005).

These autofluorescent compounds tend to accumulate primarily in the lysosomes within post-mitotic cells where they accumulate over the cells' lifetime and are unable to be broken down or exocytosed (Terman and Brunk, 1998). Cells therefore accumulate large quantities of age pigments which are thought to contribute to the ageing process via cumulative cellular dysfunction and eventually cell death (Terman and Brunk, 2004). This causative role in ageing is thought to occur because lysosomal enzymes are occupied in a futile attempt to degrade age pigments, instead of carrying out normal routine functions such as turning over damaged or non-functioning macromolecules and organelles (Terman and Brunk, 2004). Age pigments have also been implicated in age-related diseases such as cataracts, diabetes and Alzheimer's disease and are considered to play a significant role in the pathology of these diseases (Oudes et al., 1998).

The rate of formation of age pigment compounds, in relation to maximum lifespan, has been shown to be similar in short- and long-lived organisms (Nakano et al., 1993; Terman and Brunk, 1998). In response to low temperature or dietary restriction, a reduced rate of AGE pigment accumulation accompanies the resultant lifespan extension in *C. elegans* (Gerstbrein et al., 2005). Similarly, *D. melanogaster* maintained at low temperatures also had reduced rates of lipofuscin accumulation compared to fruit flies kept at higher temperature (Sheldahl and Tappel, 1974).

Recent investigations of auto-fluorescent AGE pigments in *D. melanogaster* support both its use as an ageing marker and its potential role in the ageing process

(Jacobson et al., 2010). It was found to be the only true marker of age out of a range of other molecular biomarkers of oxidative damage to proteins (glutamic semialdehdye and aminoadipic aldehyde), carbohydrates (*N*-(carboxyethyl)-lysine and *N*-(caboxymethyl)-lysine) and lipids (*N*-malondialdehyde-lysine), correlating with mortality rates when *D. melanogaster* were under low temperature or dietary restriction treatments.

1.5 Examining ageing: treatments that extend longevity

The best methods for examining the ageing process are to use experimental treatments that are known to extend longevity. In the last 20 years, there have been a number of genetic mutations and single gene knock-outs that result in increased longevity in some model organisms (Finch and Ruvkun, 2001; Christensen et al., 2006). There are also two environmental manipulations that have been used to successfully extend longevity across a range of species, these are temperature treatments and manipulation of diet energy content (dietary restriction). However, one treatment that has received minimal attention is the potential for manipulation of membrane fatty acid profiles to affect longevity. If variation in dietary fatty acid profile permits modification of membrane fatty acid composition, then the effects of membrane fatty acid characteristics would permit an experimental test of the 'membrane pacemaker theory of ageing'.

1.5.1 The effects of temperature on longevity, ageing and metabolism

Temperature affects many aspects of animal physiology, particularly in ectotherms that do not metabolically regulate their body temperature at a stable level like endotherms (Clarke and Pörtner, 2010). Ectotherms are known to reduce activity at low temperatures, with houseflies having 10- to 15-fold decrease in flying and walking activity when the ambient temperature is reduced from 26°C to 17°C (Buchan and Sohal, 1981). Measures of resting metabolic rate of ectotherms typically show exponential decreases in metabolic rate with decreasing temperature (Gillooly et al., 2001; Clarke and Fraser, 2004). These relationships between metabolic processes and temperatures are often described by the temperature coefficient, Q_{10}, which expresses a ratio of the rates of processes measured over a thermal differential of 10°C. Many metabolic processes measured have a Q_{10} around two to three (i.e. a doubling to tripling of rate with a 10°C increase in temperature), which is thought to reflect the effect of temperature on simple chemical and enzymatic reactions. Values closer to one show little sensitivity to temperature and can indicate either a temperature-independence of the process or a physiological regulatory or compensatory mechanism, such that temperature fluctuations cause no change in the rate of the process. Conversely, large Q_{10} values (>4) show a high sensitivity to temperature and therefore large changes in rates, but can also indicate a physiological regulatory or compensatory mechanism that acts to significantly alter the rate of the process more than would be expected by temperature effects alone.

While metabolic processes typically exhibit a Q_{10} of two to three, longevity is also altered to a similar magnitude in response to temperature. A number of studies on longevity in *Drosophila* have given Q_{10}'s between two and three for the inverse

relationship between temperature and longevity (Table 1.1). Loeb and Northrop (1916, 1917) were the first to experimentally increase longevity in an animal species and did so by showing that low temperature extended the lifespan of *Drosophila.* Indeed, it was the observation that the quantitative effects of temperature on metabolic rate was the same as those on the rate of ageing that formed the basis of the 'rate of living theory' (Pearl, 1928).

Although the 'rate of living theory' of ageing has an intuitive appeal, closer scrutiny of its assumptions, challenge its robustness in explaining longevity. As already discussed, just because temperature affects the rate of many processes and influences both longevity and metabolic rate does not necessarily mean there is a cause-and-effect relationship between them. For example, cockroaches (*Periplaneta americana*) transferred from 10°C to 20°C have a higher metabolic rate than those transferred from 26°C to 20°C (Dehnel and Segal, 1956). Similarly, milkweed bugs, *Oncopeltus fasciatus*, maintained at 25°C have higher metabolic rates at old age than those maintained at 30°C (McArthur and Sohal, 1982). In addition, many of the earlier studies on temperature-affected longevity have assumed that metabolic rate tracked temperature, as predicted from measurements taken while animals were inactive, and that rates of oxidative damage were similarly affected. It is important to recognize that resting metabolic rate is not the only contributor to the production of metabolically-derived ROS. Metabolic rate is also determined by activity, which is, in turn, significantly affected by temperature (Buchan and Sohal, 1981). Activity levels have been shown to affect longevity, with *D. melanogaster* prevented from flying having increased longevity and reduced rates of ageing than flying controls (Magwere et al., 2006). However, similar to the observations on metabolic rate, temperature does not

necessarily affect activity in a predictably exponential fashion. Houseflies show a 10- to 15-fold increase in flying and walking activity within a less than 10°C increase in temperature (Buchan and Sohal, 1981), which is much more dramatic than a doubling of rate which would be expected from a simple temperature relationship. Also, over the temperature range 20°C to 30°C, six species of colubrid snakes showed very little effect of temperature on either burst speed or sprint speed activity (Robert et al., 2007).

Table 1.1. Temperature coefficient (Q_{10}) values for longevity and life history parameters of *Drosophila*

Species	Q_{10}	Temperature range (°C)	Number of temperatures	Source
Rate of ageing & senescence				
Rate of ageing (average longevity^{-1})				
Drosophila	2.1	9 – 34 °C	6	Loeb and Northrop (1916)
Drosophila	3.0	10 – 30 °C	5	Loeb and Northrop (1917)
D. melanogaster	2.1	18 – 28 °C	3	Alpatov and Pearl (1929)
Drosophila	2.1	16 – 30 °C	3	Northrop (1926)
D. suboscura (♂)	3.3	3 – 30 °C	5	Clarke and Smith (1961)
D. melanogaster (♂)	4.4	18 – 30 °C	4	Miquel et al., (1976)
D. melanogaster (♂)	3.8	19 – 29 °C	3	Sestini et al., (1991)
D. melanogaster (♀)	3.1			
D. melanogaster (♂)	2.5	18 – 29 °C	3	Zheng et al., (2005)
D. melanogaster (♀)	2.8			
Metabolic Rate				
O_2 consumption rate[a]				
D. melanogaster (♂)	2.6	18 – 27 °C	3	Miquel et al., (1976)
D. melanogaster (♂)	2.3	18 & 25 °C	2	Berrigan and Partridge (1997)
CO_2 production rate[b]				
Drosophila	1.5	16 – 30 °C	3	Northrop (1926)

[a] = calculated as average µl O_2. fly^{-1}. day^{-1} and ml O_2. g^{-1}.hr^{-1}
[b] = measured as mg CO_2 per 100 flies per day

It is also important to note that while temperature effects on longevity have been examined mainly in ectothermic animals due to the pronounced influence of temperature on the metabolic processes of these animals, there is also evidence of temperature effects on longevity in mammals (Carrillo and Flouris, 2011). Dietary restriction in rats, mice, primates and humans has been shown to induce a reduction in core body temperature which is thought to contribute to lifespan extension under dietary restriction (Weindruch et al., 1979; Duffy et al., 1990; Lane et al., 1996; Conti, 2008). Genetic variation is known to affect the extent to which dietary restriction affects longevity. In a study of six mouse strains, all showed a reduced core body temperature in response to dietary restriction, but the degree of the temperature reduction varied between strains (Rikke et al., 2003). These findings that lowered core body temperature may influence longevity effects were further confirmed in a transgenic mouse model in the absence of dietary restriction (Conti et al., 2006). Mice that overexpressed uncoupling protein 2 in their hypocretin neurons had a resetting of their central thermostat and a subsequent sustained reduced core body temperature. This slight reduction, of up to 0.5°C in core body temperature, increased the average longevity of the transgenic mice due to an apparent delay in the onset of senescence. Signalling mechanisms for both nutrient and thermal regulation occur in the hypothalamus, although in different nuclei, and the investigation of interactions between the signalling mechanisms of body temperature and nutrient sensing could prove important in the understanding of the ageing process (Tabarean et al., 2010).

Despite inconsistencies in temperature effects on metabolism and activity, experimentally using temperature as a means to ‘speed up’ living in ectotherms has been utilised to measure a number of changes in oxidative damage accumulation in

accordance with the ‘oxidative stress theory of ageing’. Protein carbonyl formation rate is increased with increasing ambient temperature in fish *N. rachovii* (Hsu and Chiu, 2009) and fruit flies *D. melanogaster* (Das et al., 2001; Rebrin et al., 2004; Jacobson et al., 2010). The rate of mitochondrial DNA mutation has also been shown to increase with increasing temperature in fruit flies (Garcia et al., 2010). Measures of various products of lipid peroxidation also show an increase in accumulation with increased ambient temperature in various fly species (Miquel et al., 1976; Sohal et al., 1981; Sohal et al., 1985; Sestini et al., 1991; Zheng et al., 2005). Similarly, fluorescent AGE pigments accumulate more rapidly at high temperatures compared to low temperatures in age-matched fish, flies and worms (Gerstbrein et al., 2005; Valenzano et al., 2006; Hsu and Chiu, 2009; Jacobson et al., 2010).

While temperature manipulation has resulted in many correlations between markers of oxidative damage and longevity, temperature is also known to induce compensatory mechanisms in metabolic rate, gene expression and membrane composition. Isolated mouse brain mitochondria exposed to temperatures ranging between 32°C and 37°C had decreased rates of resting and phosphorylating respiration, but significantly increased rates of ROS production and H_2O_2 release at low temperature (Ali et al., 2010). Although examined *in vitro* and therefore not necessarily representative of processes occurring *in vivo* under the same temperature conditions, increased H_2O_2 production rates have also been shown in houseflies maintained at 20°C compared to those kept at 28°C (Farmer and Sohal, 1987). Increased proton leak and increased ROS production at low temperature may reflect functional adjustments related to the use of ROS signalling mechanisms in maintaining cellular homeostasis (reviewed

in D'Autréaux and Toledano, 2007; Ristow and Zarse, 2010; Ristow and Schmeisser, 2011).

Low ambient temperature has been associated with the upregulation of antioxidant enzymes and transcription factors related to protection against oxidative stress and also increased mitochondrial energy production in both the zebrafish *Danio rerio*, and the annual fish *N. rachovii* (Malek et al., 2004; Hsu and Chiu, 2009). This is supported by a study showing that a 10°C decrease in temperature had no effect on measures of DNA or lipid peroxidation in *D. rerio* while protein carbonyl formation actually increased with decreasing temperature (Malek et al., 2004).

Membrane composition is often altered in response to ambient temperature in a process termed homeoviscous adaptation (Hazel, 1995). Low temperatures result in the increased polyunsaturation of membrane fatty acids, theorised to increase fluidity and maintain cell function at normal rates (Crockett, 2008). However, these changes also increase the potential for lipid peroxidation, which is at odds with the longevity extension commonly seen at low temperatures (Hulbert et al., 2007). The formation of plasmalogens, which are effective antioxidants, has been shown to increase with increased temperature, suggestive of a mechanism to reduce peroxidative damage (Kraffe et al., 2007). Temperature-induced changes to membranes can also involve shuffling the fatty acids to make new molecular species without changing the overall fatty acid composition, or changing the class of phospholipids (phospholipid head group), with changes in the ratios of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) the most common head groups affected (Käkelä et al., 2008).

Evidence of lipid restructuring in response to temperature change has been determined for many taxonomic groups, revealing a wide variation in the degree and types of responses seen between species and even between tissue types (Crockett, 2008). Small but directional changes in membrane fatty acid composition in response to changes in ambient temperature have been reported in *D. melanogaster* (Overgaard et al., 2005; Overgaard et al., 2008). Membrane fatty acid composition of muscle mitochondria in the trout, *Onchorhyncus mykiss*, was affected by cold-temperature acclimation, with most changes involving specific phospholipid classes rather than an overall unsaturation of the membrane (Kraffe et al., 2007). Kraffe et al. (2007) also found increased plasmalogen formation following acclimation to warm temperatures, and a similar result was found under warm-acclimation in the brain tissue of another fish species, *Carassius carassius* (Käkelä et al., 2008).

The majority of studies examining the effects of temperature on biomarkers of ageing are generally limited to comparing 'young' and 'old' animals within the different temperature treatments. While this has demonstrated damage accrual with chronological time, it gives little insight into changes with physiological age. Teaming measures of oxidative damage with mortality measures over a wide range of ages throughout the lifespan is needed to identify functional senescence and therefore, detect markers that not only increase with chronological time, but also contribute to physiological ageing. Mair et al., (2003) concluded that temperature and dietary restriction acted under potentially different mechanisms after examination of *D. melanogaster* mortality curves and found temperature to act more on the rate of ageing, while dietary restriction tended to delay the onset of ageing without affecting the rate. By utilising a cross-over study design, they found the mechanisms that reduce longevity at high temperatures to be

irreversible. This does not seem to be the case for dietary effects on longevity, in which diet history appears to have no permanent effect on mortality (Mair et al., 2003).

1.5.2 The effects of dietary restriction on longevity and ageing

Dietary restriction is defined as 'undernutrition without malnutrition', and is often described as a reduction of energy intake. It is known to benefit both the lifespan and 'health span' of many different species, from yeast and flies to rodents and primates (Mair and Dillin, 2008). The original studies on dietary restriction by McCay et al. (1935) described the effects of undernutrition on slowed growth and longevity in line with the 'rate of living theory'. Over 40 years later, dietary restriction became a powerful ageing experimental model, after studies in mice given restricted diets in adulthood revealed both an extended lifespan and a delayed onset of age-related disease (Weindruch and Walford, 1982; Maeda et al., 1985; Yu et al., 1985; Weindruch et al., 1986).

In rodent models, dietary restriction both reduces the incidence of, and delays the onset of age-related diseases such as cancer, obesity, diabetes and neuro-degeneration, while increasing longevity (Weindruch et al., 1986; Masoro, 2002; Anderson et al., 2009). Studies on humans and other non-human primates are obviously difficult to undertake due to their long lifespans, however a 20-year ongoing study of rhesus monkeys (*Macaca mulatta*) by the Wisconsin National Primate Research Center (WNPRC) also reported increased longevity, and reduced incidences of age-related disease such as diabetes, cancer, and cardiovascular disease as well as reduced muscle and neuro-degeneration to result from dietary restriction (Colman et al., 2009). Short-

term human trials are also showing promising similarities in health benefits to those of rodents and other primates (Anderson et al., 2009). Evidence from human populations, such as the Okinawan population of Japan, who have a cultural tendency to have a low energy intake, show reduced rates of cardiovascular disease and cancer and lower mortality rates than the Japanese average (Willcox et al., 2006).

In contrast, a recent study of dietary restriction in rhesus monkeys by the National Institute on Aging (NIA), one similar to that performed by the WNPRC, contradicts some of the previous observations on this mammalian model (Colman et al., 2009; Mattison et al., 2012). In these later findings, there is no evidence of longevity benefits of dietary restriction and relatively few health benefits as compared to previous reports from the WNPRC study (Mattison et al., 2012). The NIA monkeys (both control and dietary restriction treatments) showed increased longevity compared to the monkeys in the WNPRC study (Mattison et al., 2012). The major point of difference between these studies was the diet composition. Although diets used in both studies had similar overall compositions of protein, fat and carbohydrates, the source of these components was very different. For example, both diets were ~60% carbohydrates, but this was derived equally from corn starch and sucrose in WNPRC diets resulting in a diet containing 29% sucrose, as compared to only 4% sucrose in the NIA study.

This difference in diet could be highly significant, as high sucrose content could increase the incidence of type II diabetes. It is likely that the WNPRC study, rather than increasing the longevity of dietary restriction subjects, the control diet instead both reduced the lifespan of, and increased the incidence of disease (such as diabetes) in control subjects. Reports of reduced incidence of disease and extended longevity as a result of dietary restriction in long-lived mammals therefore remains inconclusive

(Austad, 2012). These results highlight the complexity of examining ageing in long-lived cohorts, as there was little knowledge 20 years ago about how the types of components comprising the calories provided might affect longevity independently of their caloric content. It also shows the importance of understanding how diet composition, rather than just energy consumption, influences longevity.

These more recent findings aside, the reported health benefits from dietary restriction has led to an increased desire to understand the mechanisms by which it modulates longevity and ageing. The use of invertebrate models is proving indispensible for this outcome. The health and longevity benefits of dietary restriction have been demonstrated in yeast, worms and flies (see review in Fontana et al., 2010), and their use as models for investigation of the genetics and mechanisms for the dietary restriction effects on ageing, has led to important discoveries in this area (Partridge, 2009, 2011).

Dietary restriction in mammalian models is often applied through reduced provision of calories by controlling the total intake of food. However, in invertebrate models, dietary restriction is harder to implement in this way. While there has been some success in using invertebrate models such as the grasshopper, *Romalea microptera* (Drewry et al., 2011) and rotifer, *Asplanchna brightwelli* (Verdone-Smith and Enesco, 1982), attempts to give reduced quantities of food to fruit flies *D. melanogaster* (Le Bourg and Minois, 1996), medflies, *Ceratitis capitata* (Carey et al., 2002) or houseflies, *M. domestica* (Cooper et al., 2004) has failed to identify dietary restriction effects on longevity. In *C. elegans*, eight different dietary restriction protocols have been used, ranging from reducing the density of bacterial food provided, to using worms with a mutation that limits the amount of food these worms can

consume. An analysis of genetic pathways in *C. elegans* suggests that these different protocols act to extend longevity through different mechanisms (Greer and Brunet, 2009). There is therefore a need to standardise how dietary restriction is implemented in ageing studies involving invertebrate models.

The most common way to achieve dietary restriction treatments for flies is through simple dilution of their diet, with the diluted diet provided in excess. In this way, the total amount of food animals eat is not directly controlled, but they are given a diet with reduced energy and nutrient density. This method of dietary restriction has been successful in extending longevity, and analysis of mortality rate suggests that dietary restriction delays the onset of ageing rather than altering the rate of ageing in these animals, as found in mammals (Chapman and Partridge, 1996; Mair et al., 2003; Magwere et al., 2004; Zheng et al., 2005; Jacobson et al., 2010). Dietary restriction studies in *D. melanogaster* suggest that diet-induced benefits to lifespan can be implemented at any stage in their life with little carry-on of negative effects due to previous dietary history (Mair et al., 2003).

However, a major flaw in a number of these studies is that flies given these diluted diets are only assumed to be dietary restricted, as surprisingly food intake is not routinely measured. There is the likelihood that flies will compensate for these diluted diets by increasing the amount of food they eat, negating the dietary restriction regime and calling into question the contribution of dietary restriction in these findings.

Compensatory feeding in flies is not unexpected. Blowflies (*Phormia regina*, *Protophormia terraenovae* and *Lucilia cuprina*) are known to sense the nutritional content of their food source and alter their feeding behaviour accordingly (Dethier, 1961; Roberts and Kitching, 1974; Greenberg and Stoffolano Jr., 1977). Studies that

have measured food intake show that flies adjust their feeding rates when given the diluted diets. Carvalho et al. (2005) showed that *D. melanogaster* fruit flies fed a range of diluted diets sufficiently compensated for the diluted diets by consuming more. Ja et al. (2009) and Lee et al. (2008) also showed that fruit flies not only compensated for diluted diets by consuming more, they also adjusted their degree of compensatory feeding in relation to the nutritional content of the diet. *Drosophila melanogaster* tend to increase food consumption when the sugar content of the diet is increased, while increasing dietary protein levels tends to suppress feeding (Skorupa et al., 2008). Queensland fruit flies, *Bactrocera tryoni,* increased their consumption of foods that had an imbalance between the yeast and sugar content, which overcame any subsequent reduction in energy content (Fanson et al., 2009).

To attempt to decipher compensatory feeding in flies, a variety of methods have been employed to measure food consumption. Methods for examining food consumption in *D. melanogaster* have included calorimetry (Min et al., 2007), spectrophotometric measures of gut or faecal dye content when feeding on a dye-laden food (Min and Tatar, 2006; Wong et al., 2009), radiolabelling of food (Carvalho et al., 2005), measuring the volume of food consumed (Ja et al., 2009), or recording the time spent feeding by observing the number of proboscis extensions (Mair et al., 2005). Calorimetry, radiolabelling of food or spectrophotometric analysis of dye-labelled food can represent the direct measurement of calorie or food intake, yet these are short-term measures, because flies are exposed to these treatments for a relatively small time period. Furthermore, they require individuals to be killed before sampling and cannot, therefore, be used for long-term measurement of individual food consumption (Carvalho et al., 2005; Min et al., 2007; Wong et al., 2009).

Two non-harmful methods have been suggested, also with their detractors. Examining proboscis lowering and the time spent feeding have been utilised, with further spectrophotometric analysis suggesting they reflect feeding rates (Mair et al., 2005; Wong et al., 2009). However, they are also only measured over a small time period and may not accurately reflect lifetime feeding behaviour. Furthermore, proboscis extension is associated with a number of different behaviours, such as cleaning and in response to odours, not just feeding, and so may be an unreliable method for inferring food consumption (Dethier, 1976).

Arguably the simplest method for measuring food consumption is through directly measuring the difference by weight or volume of food provided. Capillary feeders (CAFE) have been used to feed flies a liquid food-type which can accurately measure the volume of food consumed (Carvalho et al., 2005; Lee et al., 2008; Ja et al., 2009). However, there is evidence that fruit flies have reduced reproductive output and lifespan on these liquid-diets, which may impact on longevity effects (Wong et al., 2009).

There is therefore a great benefit in using a sufficiently large insect model that permits direct measurement of food consumption over its entire lifespan. Large flies, such as blowflies are suitable for such studies, as they are known to consume about 40-fold more food than *D. melanogaster* (Carvalho et al., 2005; Min and Tatar, 2006; Piper and Partridge, 2007). This allows food consumption to be determined by simply measuring changes in weight of food provided over time. This non-harmful method can therefore track food consumption of cage populations or individuals throughout their lifetime.

The focus of dietary restriction in flies and other invertebrates is currently moving away from considering only the influence of energy consumption and more towards examining diet composition. This change in focus has been provoked by findings that altering the yeast and sugar components of the diet separately affect the degree of longevity extension (Mair et al., 2005). Fruit flies on a low-yeast, high-sugar diet lived longer than flies on an iso-caloric diet containing high-yeast and low-sugar (Mair et al., 2005). Similarly, Lee et al. (2008) found that, rather than any effect of energy consumption affecting longevity, lifespan was maximised in *D. melanogaster* given a diet with a low yeast to sugar ratio. These findings have been replicated in another fruit fly species, *B. tryoni* (Fanson et al., 2009; Fanson and Taylor, 2011) and the lower extremes of this relationship have been examined in *D. melanogaster* (Lushchak et al., 2012).

These studies have calculated the composition of their diets based on a protein to carbohydrate basis, considering yeast to be a protein and carbohydrate source, and sugar to be purely carbohydrate. However, it is important to remember that yeast is more than just protein and carbohydrates, as it also contains an array of micro and macro nutrients that may also influence longevity (Fanson and Taylor, 2012). The use of chemically defined diets in crickets, *Teleogryllus commodus*, to compare protein content to energy content as the driver of longevity changes, are supportive of protein being responsible (Maklakov et al., 2008; Maklakov et al., 2009). However, a more detailed examination of chemical-based diets in *B. tryoni*, have suggested a more complex relationship, with interactions evident between protein and specific essential amino acids (Fanson and Taylor, 2012).

While mammalian dietary restriction is often still considered to be primarily an effect of reduced energy consumption (McDonald and Ramsey, 2010), there are suggestions that energy consumption alone may not be the driver of dietary restriction effects in these models. Increasing evidence suggests that proteins and even single amino acids can have significant effects on longevity (Pamplona and Barja, 2006). Protein restriction in mammals, in particular methionine, has been shown to extend longevity in rodents (Orentreich et al., 1993; Richie Jr et al., 1994; Zimmerman et al., 2003; Miller et al., 2005). The use of amino acid supplementation in *D. melanogaster* has also demonstrated that dietary restriction of methionine can extend longevity of the fruit fly (Grandison et al., 2009). Studies are required in mammals that incorporate a greater range of diets than a control versus dietary restriction treatment to determine whether composition or quantity affects mammalian longevity (Simpson and Raubenheimer, 2009). As evident in the discrepancies between the WNPRC and NIA rhesus monkey studies (Colman et al., 2009; Mattison et al., 2012), diet composition clearly plays an important role in health and longevity.

There are currently a number of nutrient sensing pathways that are implicated in the regulation of lifespan (see reviews by Mair and Dillon, 2008, Fontana et al., 2010). The prevailing theory about why dietary restriction is such a universal and conserved mechanism across species is that it likely serves as a survival mechanism during periods of famine (Mair and Dillin, 2008). With resources being directed towards somatic maintenance, and away from reproduction, during periods of food deprivation, which is thought to favour longevity (Grandison et al., 2009). There is a well demonstrated relationship of reduced fecundity and extended longevity under dietary restriction in *D. melanogaster*, (Chapman and Partridge, 1996). Interestingly, Grandison et al., (2009)

found that the provision of a single amino acid, methionine, to *D. melanogaster*, was able to restore fecundity to levels observed under full feeding conditions, without negatively affecting the longevity benefits of dietary restriction. Methionine is one of the more likely amino acids to be attacked by ROS due to the presence of a sulfhydryl group (Stadtman et al., 2005). While the products of methionine oxidation are reversible the accumulation of oxidative damage to methionine has been associated with age (Stadtman et al., 2005).

To date there has been conflicting evidence regarding changes to oxidative damage measures in response to dietary restriction. Dietary restriction has shown no effect on the activity of the antioxidants superoxide dismutase in studies of both *C. elegans* (Yen et al., 2009) and *D. melanogaster* (Kabil et al., 2007). Examination of somatic DNA damage in *D. melanogaster*, showed no difference in DNA mutation accumulation between dietary restriction and *ad libitum* feeding conditions (Edman et al., 2009). However, the accumulation of lipid peroxidative damage (HNE) was delayed in *D. melanogaster* under dietary restriction conditions (Zheng et al., 2005). Likewise, the accumulation of fluorescent AGE pigments was lower in both *C. elegans* (Gerstbrein et al., 2005) and *D. melanogaster* (Jacobson et al., 2010) under dietary restriction compared to control conditions.

Currently the most promising mechanisms by which dietary restriction effects are actioned, is through the target of rapamycin (TOR) signalling pathway (reviewed in Kenyon, 2005; Evans et al., 2011), and the insulin/insulin-like growth factor-like signalling (IIS) pathways (reviewed in Bartke, 2005; Kenyon, 2005; Giannakou and Partridge, 2007; Taguchi and White, 2008). These nutrient-sensing pathways are thought to act on various transcription factors that induce metabolic changes and

protective enhancements that reduce the accumulation of age-related damage (Fontana et al., 2010).

1.5.3 The effect of dietary fatty acid profile on membrane fatty acid composition and longevity

The ‘membrane pacemaker theory of ageing’ suggests that longevity may be a consequence of the relative peroxidative susceptibility of organism’s membranes. Membrane composition has been shown to vary systematically with size, such that large animals (that are generally long-lived) have more peroxidation-resistant membranes than smaller short-lived species (Hulbert, 2003). Furthermore, animals that are extremely long-lived compared to similar-sized animals within the same taxa, also show more peroxidation resistant membranes (Pamplona et al., 2002; Buttemer et al., 2008). However, little is known about what mechanisms are responsible for differences in membrane composition between species or even how membrane composition is regulated within species (Hulbert et al., 2006b). A test of the ‘membrane pacemaker theory’ would require experimental manipulation of membrane fatty acid composition to see how this affects longevity.

Queen bees, which are genetically identical to worker bees, yet live an order-of-magnitude longer, have membrane fatty acids with a much lower PI than those of worker bees (Haddad et al., 2007). Newly emerged worker and queen bees have very similar membrane phospholipid composition, but these start to diverge within the first week of emergence. This difference in their membrane composition appears to be a result of the different diets fed to workers and queens, as queen bees are fed only royal

jelly, which is significantly lower in PUFA content than the pollen fed to the worker bees (Haddad et al., 2007). Food intake rate is also known to influence phospholipid composition in vertebrates. Mice given diets of varying caloric content had a negative correlation between caloric content of the diet and longevity (Faulks et al., 2006). While the diet treatments had no effect on metabolic rate, within one month of starting the diet, changes in the fatty acid composition of skeletal muscle, liver, kidney, heart and brain were observed, such that the calorie restricted mice with extended longevity had more peroxidation resistant membranes (Faulks et al., 2006). Similar results were shown in the liver tissue of protein-restricted rats (Ayala et al., 2007).

Fatty acids are fundamental for life, being an essential component of membranes (Hulbert and Abbott, 2011). Most of our knowledge of membrane fatty acids comes from vertebrates especially mammals. Relatively little is known about the membrane fatty acids of insects and their synthesis and metabolism (Stanley-Samuelson et al., 1988). Until recently, many studies of insect fatty acid content did not examine fatty acids above 18 carbon chain length as it was thought that insects did not have long chain PUFA (Blomquist et al., 1991). Mammals can synthesise palmitic acid (16:0) *de novo* through lipogenesis, and this basic building block can be elongated and desaturated into a number of other fatty acid species (Fig. 1.4). However, mammals cannot synthesise the essential PUFA, and they need to obtain both *n*-6 and *n*-3 fatty acids from the diet (Hulbert et al., 2005). These *n*-6 and *n*-3 PUFA are essential components of membranes and play significant roles as hormone precursors (Tapiero et al., 2002). As well as being essential nutrients, the inability of animals to interconvert *n*-6 and *n*-3 PUFA means that the balance of these two fatty acids in the diet can significantly influence membrane composition (Hulbert and Abbott, 2011).

These dietary fatty acids appear to be essential for almost all vertebrates, but this does not seem to be the case for a number of invertebrates. The nematode, *C. elegans*, is capable of synthesising almost all fatty acids (Pereira et al., 2003). A number of insect species, including the cockroach, *P. americana* and the house cricket, *Acheta domestica*, are capable of synthesising 18:2*n*-6 *de novo* (Blomquist et al., 1991), while mosquitoes are unable to elongate or desaturate 18-carbon fatty acids and require >20 carbon fatty acids in the diet (Blomquist et al., 1991). Fruit flies, *D. melanogaster*, are different again, in that they seem to not require fatty acids of longer than 18 carbons, and utilise a different fatty acid for controlling membrane lipid organisation (Dobrosotskaya et al., 2002).

Membrane composition is generally tightly regulated both between species and within different strains of the same species. For example, wild type and lab-bred mice fed the same diets showed significant differences in their membrane fatty acid composition, with the wild mice having reduced levels of 22:6*n*-3 in liver and skeletal muscle membranes (Hulbert et al., 2006b). While the precise mechanisms for this regulation of membrane composition is unknown, it is likely to involve the enzymes involved in membrane remodelling, the acyltransferases (Abbott et al., 2012). These acyltransferases preferentially incorporate PUFA into the s*n*-2 position, but do not distinguish between *n*-6 or *n*-3 fatty acids (Lands et al., 1982). Potentially, as a result of this enzymatic remodelling of membranes, the majority of changes to membrane composition as a result of dietary fats is generally observed in the *n*-6 and *n*-3 PUFA (Hulbert et al., 2005).

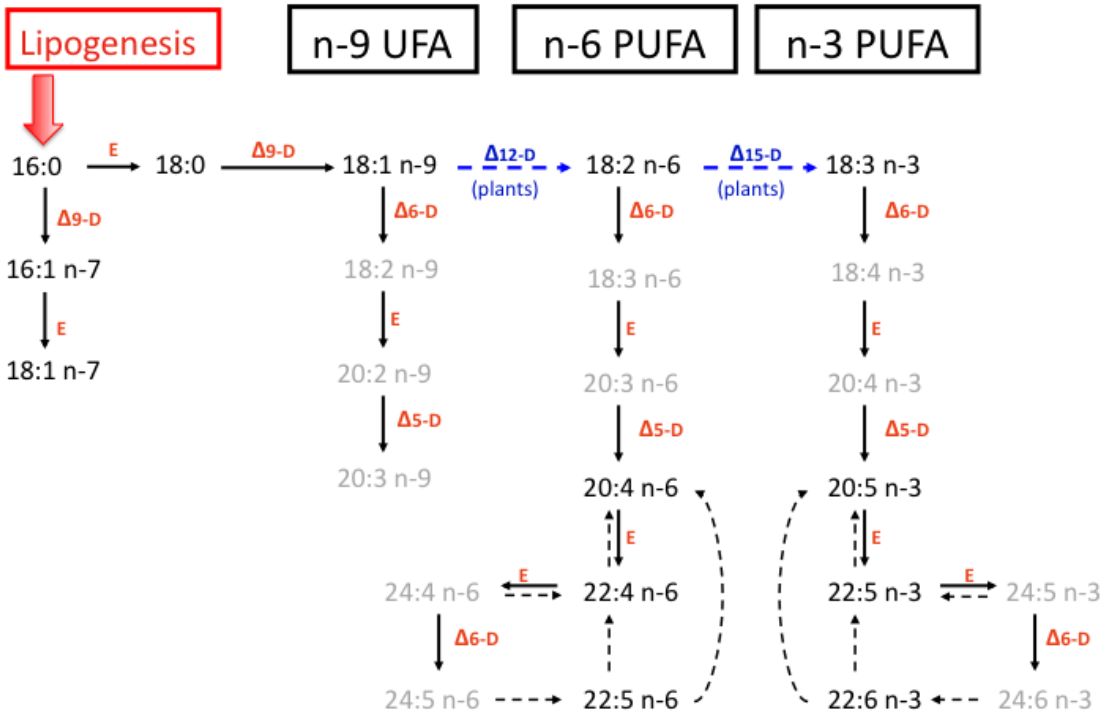

Figure 1.4. Mammalian pathway for the biosynthesis of *n*-9, *n*-6 and *n*-3 polyunsaturated fatty acids (figure modified from Pereira et al., 2003). Enzymes include elongase (E) and the desaturases (Δ5, Δ6, Δ9, Δ12, and Δ15). The blue dotted lines represent steps that are unable to be performed in higher animals that are lacking these desaturases (Δ12 and Δ15), requiring these fatty acids from plants. Solid lines represent reactions occurring in the endoplasmic reticulum, dashed lines represent partial ß-oxidation occurring in the peroxisomes.

An extensive experiment on *Rattus rattus*, where animals were fed one of 12 diets that varied only in fatty acid composition, demonstrated the strong regulation of membrane fatty acid composition (Abbott et al., 2010; Abbott et al., 2012). They showed that some tissues, for example the brain, had a stronger regulation of fatty acid composition than other tissues (Abbott et al., 2010; Abbott et al., 2012). The most variation in the membrane fatty acid composition was seen in the relative amounts of the *n*-3 and *n*-6 PUFA, with the PUFA balance (% *n*-3 as % total PUFA) being most responsive when the dietary levels of *n*-3 PUFA were extremely low (<10%).

Membrane fatty acid composition of gill phospholipids of three species of bivalves also respond to variation in dietary fatty acids, with most of these changes being seen in the long chain PUFA (Delaporte et al., 2005). Further, the trout *O. mykiss*, fed diets of varying fatty acid profiles showed significant differences in a range of fatty acids of muscle mitochondria phospholipids (Guderley et al., 2008). However there was also evidence of extensive regulation of phospholipids, with membrane composition being consistently distinct from the diet fatty acid composition (Guderley et al., 2008).

High-fat diets have been shown to induce the metabolic syndrome and cardiac dysfunction in *D. melanogaster* in a similar manner to mammals (Birse et al., 2010). Previous work on *Calliphora stygia* has shown that the addition of three grams of fat (olive oil) had no negative effect on lifespan, while any further amounts of fat were detrimental (Ujvari et al., 2009). This study of *C. stygia* also showed that after 10 days of adult life, membrane fatty acid composition was different to that seen on the day of eclosion, suggesting remodelling of membrane composition with time. They also found that the dramatic decreases in longevity associated with the five grams or more of fat added to diet were not associated with membrane fatty acid changes, specifically variations in peroxidation index (Ujvari et al., 2009).

1.6 Invertebrates as models in ageing research

Invertebrates as ageing models confer a number of advantages over vertebrate models. Their relatively short lifespans and ease of husbandry makes them ideal for examining age-specific mortality changes in response to treatment manipulations. Demographic studies require the use of large populations sizes to avoid bias, and this is

more difficult to achieve in mammalian studies (Pletcher, 1999). For instance, significant insights into the deceleration of ageing at old-age in humans was initially suggested after the examination of the mortality curves of 1.2 million medflies (Carey et al., 1992). These findings were controversial at the time of publication due to the expectation that the probability of death continually increased with increasing age and a lack of convincing large scale long-term data to suggest otherwise (Vaupel et al., 1998).

The most common invertebrate models, the fruit fly, *D. melanogaster* and the nematode worm, *C. elegans*, have had their entire genome sequenced, making them amenable to exploring genetic manipulation (Grotewiel et al., 2005). In conjunction with their short lifespan, this allows rapid analysis of potential 'ageing genes' that can then be further investigated in rodent models. Indeed, numerous genes having a significant role in influencing longevity have first been identified in invertebrate models and then determined to have a corresponding analogous function in vertebrates (see reviews by Finch and Tanzi, 1997; Finch and Ruvkun, 2001; Tissenbaum and Guarente, 2002; Kenyon, 2005; Vijg and Suh, 2005; Christensen et al., 2006; Partridge, 2009, 2011).

The universality of the ageing process suggests the possibility that the mechanisms controlling ageing are highly conserved across taxa. It is therefore important that lifespan extension pathways are also confirmed across a range of species (Mair and Dillin, 2008). For example, while dietary restriction is considered a mechanism to increase lifespan in all species, it has failed to extend longevity in a number of species of flies (Shanley and Kirkwood, 2006). Attempts to administer dietary restriction conditions to medflies (Curtsinger et al., 1992) and houseflies (Cooper et al., 2004) failed to extend longevity.

The ageing process is not uniform within an individual, with great variation in the susceptibility of tissues types to ageing (Simm and Johnson, 2010). Exploring differential ageing rates among tissues or organs is difficult in most invertebrate models. Their small size dictates that biochemical analyses be performed on whole body homogenates of pooled individuals. This removes the individual variation that may be present and can mask the role of certain tissue-specific mechanisms when diluted by whole-body measures. There is therefore a great advantage in using an invertebrate model with a larger body size than those typically studied, which permits both individual and tissue-specific examination of the biochemistry of ageing.

1.6.1 Life history of a novel model organism, the blowfly, *Calliphora stygia* (Fabricius, 1781)

The blowfly, *C. stygia* (Diptera: Calliphoridae), is a common carrion-breeding fly species native to Australia. It is found across Australia and is well adapted to cooler climates (Department of Primary Industries, 2007). *Calliphora stygia* is significantly larger than the common insect model, the fruit fly *D. melanogaster*, with a body mass roughly 80 times larger (~80 mg compared to ~1 mg) and a body length roughly four times larger (~10 mm compared to 2.5 mm; Bross et al., 2005).

These blowflies lay their eggs on decomposing animals on which the larvae feed. However as adults, *C. stygia* consume nectar and sugary liquids as well as products of organic decomposition much like the *Drosophilids* (Colless and McAlpine, 1991). Adults can survive well on a standard *Drosophila*-type diet of sugar and yeast set

in agar (Ujvari et al., 2009). The average longevity of the adult stage under these conditions at 25°C is approximately 30-40 days (own observations).

Therefore this species has numerous advantages in ageing research over other invertebrate models. While having a similarly short lifespan and ease of husbandry, their larger body size allows for the biochemical measurement of individuals, as well as more tissue-specific analysis. The similarity with the *Drosophilids* is also an advantage in comparing treatments that affect ageing. Further, while measurement of food consumption is relatively difficult in the smaller *Drosophila,* it is estimated that *C. stygia* consume approximately 40 times more food (Carvalho et al., 2005; Min and Tatar, 2006; Piper and Partridge, 2007), making measurement of food consumption significantly easier.

1.7 Aims of this thesis

The aims of this study are to examine the effects of three environmental treatments on the demographic and cellular senescence of the novel model organism *C. stygia.* The three treatments are temperature, dietary energy content and dietary fatty acid profile.

This thesis is divided into three main sections addressing these aims:

Part A presents the effects of different constant temperatures (Chapter 3) as well as a temperature crossover experiment (Chapter 4). The range of temperatures used (12°C to 34°C) covered the entire physiological range of this species.

Part B presents the effects of different diet energy contents through manipulation of either sugar (Chapter 5) or yeast (Chapter 6).

Part C presents the effects of varying dietary fatty acid profiles on membrane fatty acid composition. Membrane fatty acid composition of flies undergoing temperature or diet energy content treatments was also examined, as an experimental test of the 'membrane pacemaker theory of ageing'.

2 METHODS

2.1 Animals and animal maintenance

To examine adult lifespan, pupae of the eastern golden-haired blowfly (*Calliphora stygia*) were purchased from a commercial supplier (Sheldon's Bait, Parawa, SA, Australia) during 2009 and 2010. Within each experiment equal amounts (by weight) of pupae were added to replicate cages prior to eclosion, to ensure similar population sizes (Part A and B ~350 per cage, Part C ~120 flies per cage). Blowflies were allowed to hatch over a two-day period, following which remaining pupae were removed from each cage. This meant that each blowfly present in the cage was of the same age (± one day), and throughout all calculations it was assumed that all blowflies hatched in the middle of this 48-hour period. Consequently the overall population size per cage was not known until all the blowflies in that cage had died.

Fly cages were constructed of 350 mm x 400 mm x 350 mm aluminium frames covered with insect-screen. One end of each cage was covered with pantyhose (with the feet cut off) allowing hand access for changing food and water and removal of dead blowflies while preventing the escape of living blowflies. Temperature and humidity were monitored continuously throughout the housing period using Gemini Tinyview Dataloggers (Chichester, UK). Blowflies were provided water *ad libitum*, and were fed daily with continual access to a standard sugar-yeast (SY) food mixture set in petri dishes as previously described in Ujvari et al. (2009).

2.2 Food preparation and measurement of food consumption

The SY diet consisted of 200 g of sugar and 200 g of yeast (Enoferm M1 general wine yeast, Laffort Oenologie, Lallemand, Underdale, SA, Australia), 7.2 g of agar (technical No.3, Oxoid, Adelaide, SA, Australia) and 0.5 g of nipagin (Sigma-Aldrich, Sydney, NSW, Australia) to inhibit any bacterial or fungal growth. This food mixture was then dissolved in 400 ml of water and dispersed in 15 ml portions into 100 mm diameter petri dishes. In Part B, sugar and yeast content per gram of food were calculated from the final weight of food to account for water loss during preparation. Energy content of food was calculated based on estimations from Bass et al. (2007) and the USDA database[2] based on sugar = 16 kJ/g and yeast = 13.6 kJ/g. An approximation of metabolic rate was made by converting the calculated daily energy consumption per fly, firstly to a measure of energy consumption per mg per hr (based on flies having an average body mass of 80 mg and assuming a constant feeding over a 24 hour period), then using the conversion of 20 kJ/L O_2 (Burton, 2000). By using food consumption in this context it is assumed that it is related to metabolic needs. If food consumption does not match metabolic rate then there should be changes in body mass. A previous study using this species measuring food consumption found individuals to maintain body mass throughout their lifespan (Hulbert et al., 2004b) and showed similar food consumption changes, and I have assumed this is the case in this study.

Each cage was provided with a number of f.ood dishes, with food always available in excess. To measure daily food consumption per cage, food plates were weighed (± 0.1 mg) using a Mettler Toledo AB204-5 Balance, before being placed in

[2] http://ndb.nal.usda.gov/ndb/foods

the cage and then weighed again upon removal. All food consumption values were corrected for evaporative water loss from control food petri dishes placed under the same temperature and humidity conditions. Daily food consumption per cage was recorded and divided by the number of blowflies in each cage present at that day. This standard SY diet was provided to all cages, excepting during experiments described in Part B, where the sugar and yeast components of the food were varied, and Part C where an additional three grams of various methyl esters or oil mixtures were added to each 100 ml of standard SY mixture.

During one experiment blowflies were housed individually and the food consumption and longevity determined. In this experiment, nine males and ten females were placed into individual specimen jars (100 ml) and maintained at 25°C and ~60% relative humidity. Specimen jars were plugged with foam stoppers to allow for gas exchange (Hulbert et al., 2004b). They were provided with water and standard SY food in separate small containers (1.5 ml eppendorf tube caps). Food containers were replaced daily and weighed to determine food consumption. Five control containers devoid of blowflies were used to measure, and correct for, evaporative mass loss of food.

2.3 Measurement of egg laying

Egg laying in *C. stygia* often occurred on the food dishes within the cages. Cages and food plates were checked daily for evidence of female oviposition, and the presence or absence of eggs each day was recorded. This was used to determine the

reproductive period of the blowflies as an indication of the effect of treatments on normal function of the blowflies.

2.4 Measurement of longevity

Dead blowflies were removed and counted daily and the gender of each dead blowfly was recorded. Daily deaths were used to create life tables and survivorship curves using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). Differences in survivorship curves between treatments were examined for significance using the Log-Rank (Mantel-Cox) test. For all treatments replicate cages were compared and no significant differences determined in survival and as a result all data was examined from the pooled replicate cages. There were no consistent gender differences for many of the parameters measured. Genders are combined for all data presented within the thesis, however the few significant gender differences are tabulated and presented in Appendix A. Average longevity was calculated as the mean longevity of the population (from pooled replicate cages). The maximum longevity values presented in this thesis are the calculated mean longevity of the 5% longest-lived blowflies for each treatment of the pooled replicate cages.

2.5 Measurement of demographic senescence

To measure demographic senescence (the rate of ageing) I have used the Gompertz analysis of mortality curves derived from the constructed life tables. The Gompertz model, which describes an exponential increase in the rate of ageing with

time, is expressed as $\mu_x = A.exp^{Bx}$, where A is the intercept and B is the slope or the change in mortality rate with time (i.e. the rate of ageing). Age-specific mortality (μ_x) was calculated as: $\mu_x = -\ln(q_x)$, where $q_x = 1-(l_{x+1}/l_x)$, and l_x is the fraction of animals surviving at day x. When the mortality data at lower temperatures was examined with this method, it was obvious that a single phase Gompertz was inadequate to describe the mortality curve. There was a significant period before the onset of the exponential increase in daily mortality and in order to quantify this early period of low senescence, I used a segmental linear regression technique (GraphPad Prism 5 Software, Inc, La Jolla, CA, USA) to fit a two-phase Gompertz curve as described by Carey (1998).

The two-phase Gompertz is described as $\mu_x = A_1.exp^{B_1 x}$ if $x \leq bp$ / $A_2.exp^{B_2(x-bp)}$ if $x > bp$, where bp represents the day of the break point in the lines and describes the length of the period of early ageing (B_1) often used to explain the decreased rate of ageing in late life. As with a regular Gompertz, the slope of the line is the rate of senescence (B) as described by $\ln(\mu_x) = A + Bx$. The two-phase Gompertz shows two distinct phases of ageing, which I refer to herein as the early phase of ageing and the late phase of ageing. To ensure the significance of the segmental linear regression relationship for each temperature, I performed an F-test to compare the model with a simple Gompertz linear regression. In some instances a two-phase Gompertz equation could not be fit to the mortality curves, in these circumstances a single Gompertz equation was applied.

2.6 Measurement of cellular senescence

To measure cellular senescence, I have measured a commonly used marker of ageing, the accumulation of fluorescent AGE pigment. Collection of live blowflies for measurement of their AGE pigment concentration was performed over the time of the experiment for the temperature and diet treatments only (Part A and Part B). Where population numbers were sufficient, six blowflies were collected from each treatment for assaying at various time points. Blowflies maintained at high temperatures or on low quality diets, had shortened lifespans and so were not collected at all time points. Initially, AGE pigment concentration was measured in whole blowflies (Part A only). Later, individual blowflies were dissected into head, thorax and abdomen and the AGE pigment content of these body parts was measured and summed together to determine the whole blowfly concentration. All blowflies removed from the populations as live blowflies were not included in the calculation of life tables and survival curves.

Fluorescent AGE pigment determination was assayed using the methods of Oudes et al. (1998) and Jacobson et al. (2010). Briefly, individual blowflies (or fly parts) were placed in a 1.5 ml eppendorf tube, containing 900 µl of phosphate buffered saline (PBS) with 10 mM Na_2 ethylenediaminetetraacetic acid (EDTA) and homogenized using a hand-held homogeniser. Following homogenisation, 100 µl of trypsin (0.1 g of trypsin dissolved in 100 µl of PBS/10 mM EDTA) was added to the homogenate and tubes incubated for 24 hours at 37°C. The digested homogenate was centrifuged at 11000 *g* for five minutes, the supernatant removed and spin-filtered through a 0.22 µm cellulose acetate membrane (Costar spin-X, Corning, NY, USA) at 11000 *g* for five minutes. The filtrate was added to 96-well plates in 50 µl aliquots,

which were made up to 200 µl by addition of 150 µl of PBS/10 mM EDTA. Fluorescence was measured using a FLUOstar Optima (BMG Labtech, VIC, Australia) at excitation and emission wavelengths of 360 ± 10 nm and 440 ± 10 nm respectively. The dilution of samples ensured they remained within the linear range of fluorescence, as determined by preliminary experiments.

Each fluorescence measurement was corrected for blanks and performed in triplicate. Individual AGE pigment concentration was taken as the mean fluorescence from triplicate wells, and where triplicates varied by >5%, averages were calculated from the two closest values. Inter-assay variation was determined using a standard sample and averaged 1.4%. Data of whole blowflies in Part A are expressed relative to fluorescence values measured for blowflies post-eclosion. All remaining data are expressed in fluorescence units.

2.7 Measurement of fatty acid composition

Fatty acid composition was measured on total lipids from the diet, and phospholipids from whole blowflies, and in one experiment (Part C), on isolated thoracic mitochondria phospholipids. Whole blowflies were collected live at various time points throughout the experiments and then stored at -80°C until measurement. All blowflies collected from the population cages as live blowflies were not included in the measurement of survival curves or construction of life tables.

Mitochondria were isolated from frozen blowflies by homogenising separated thorax in cold media (100 mM KCl, 50 mM Tris-HCl and 2 mM ethylene glycol tetraacetic acid (EGTA); pH 7.4 at 4°C; Sigma-Aldrich, Sydney, NSW, Australia)

containing protease subtilisin A (Sigma-Aldrich, Sydney, NSW, Australia) and digested on ice for 10 minutes. The homogenate was centrifuged at 1000 *g* at 4°C for 10 minutes and the supernatant removed and then spun at 4000 *g* at 4°C for 20 minutes. The resulting mitochondrial pellet was then used for fatty acid extraction. Separate experiments on live blowflies demonstrated that this pellet contained functional mitochondria that consumed oxygen and responded to appropriate mitochondrial substrates and inhibitors (Hulbert, personal communication).

For fatty acid composition analysis, all solvents used were of HPLC grade (Crown Scientific, Moorebank, NSW, Australia) and contained 0.01% (w/v) butylated hydroxytoluene (Sigma-Aldrich, Sydney, NSW, Australia) as an antioxidant. Diet samples, whole blowflies and isolated thoracic mitochondria were homogenised using glass-glass homogenisers containing a 2:1 (v/v) chloroform:methanol solution and the resulting homogenate rotated overnight (at least 12 hours). Sulphuric acid solution (1 M) was added to the homogenate and centrifuged to ensure phase separation. The bottom phase (of chloroform containing lipids) was collected and filtered through silane treated glass wool. Phospholipids were separated from neutral lipids using solid phase extraction on Sep-Pak® classic silica cartridges (Waters Corp, Rydalmere, NSW, Australia).

Phospholipids (and food total lipids) were transmethylated using the methods of Lepage and Roy (1986). Fatty acid methyl esters were separated and measured by gas-liquid chromatography on a Shimadzu GC-17A gas chromatograph (Shimadzu, Rydalmere, NSW, Australia), using a fused silica capillary column (Varian WCOT 50 m x 0.25 mm internal diameter, CP7419, Sydney, NSW, Australia). The temperature program used was as follows: 150°C initial temperature, 17.5°C. min^{-1} to 170°C; 0.5°C.

min^{-1} to 178°C; 15°C. min^{-1} to 222°C; 2°C. min^{-1} to 232°C. Individual fatty acids were identified by comparison to an external standard (FAME mix C4 - C24; Sigma-Aldrich, Sydney, NSW, Australia), and expressed as mole percent of total fatty acids.

From the fatty acid composition I calculated the Peroxidation Index (PI), which is an indicator of the peroxidisability of membranes, and the PUFA balance, representing the balance between *n*-6 and *n*-3 PUFA. These parameters were calculated as from Haddad et al. (2007) and Abbott et al. (2010): PI = (0.025 x % monoenoics) + (1 x % dienoics) + (2 x % trienoics) + (4 x % tetraenoics) + (6 x % pentaenoics) + (8 x % hexaenoics); PUFA balance = *n*-3 PUFA as a percent of total PUFA.

2.8 Experimental treatments

2.8.1 Part A: Effect of temperature

In order to examine temperature effects on longevity of adults independent of temperature effects on eclosion, during the initial two-day eclosion period, all blowflies were kept at 25°C, and excess pupae were then removed. At this time, replicate cages of blowflies were moved to one of six different temperature- and humidity-controlled rooms (at 12°C, 15°C, 20°C, 25°C, 29°C or 34°C) and maintained on a 12 hour day: 12 hour night light cycle, at approximately 60% humidity for the remainder of each population's lifespan. Approximately 30 g of pupae were added per cage, with average ± SEM cage density of 370 ± 18 blowflies (range 334 to 395).

In a temperature cross-over experiment, four additional cages (of similar blowfly density) were maintained at 15°C and 29°C. Following a period of either 14- or 28-

days, replicate cages were switched between these two temperature controlled rooms and maintained at the new temperature for the remainder of their lifespan.

2.8.2 Part B: Effect of dietary composition

Two separate experiments were performed on different batches of pupae, with blowflies maintained at 25°C and 60% relative humidity over both experimental periods. The standard SY diet contains only sugar or yeast for energy and nutrient availability and under traditional dietary restriction regimes a dilution of all elements of the food would represent the 'restricted' diets. However I altered the sugar and yeast contents separately and also increased the content of both to ensure I encompassed a range of dietary compositions rather than just a restriction of the assumed 'standard' food, which may inadvertently be already a 'restriction' of the dietary requirements for *C. stygia*. Firstly (Chapter 5, April 2009), under a sugar dietary treatment, blowflies were given diets where the yeast component was kept constant while the sugar content was varied from 0.25*x* standard SY diet to 2.0*x* standard SY diet levels. The second dietary treatment (Chapter 6, July 2009) varied the yeast component while maintaining sugar content, with yeast varied from 0.0*x* standard SY diet to 1.5*x* standard SY diet levels.

2.8.3 Part C: Effect of membrane fatty acid composition

In order to examine the effect of membrane fatty acid composition on longevity, the fatty acid composition of the diet was altered (Chapter 7, September 2009). Five

populations (~120 blowflies per population) were fed different diets for their entire population lifetime while maintained at 25°C and 60% relative humidity. One population was fed the standard SY diet, while four populations were fed the standard SY diet with a specific fatty acid methyl ester added (3 g per 100 ml of food). The pure methyl esters of 18:0, 18:1*n*-9, 18:2*n*-6 and 18:3*n*-3 were obtained from Sigma-Aldrich (Sigma-Aldrich, Sydney, NSW, Australia). No antioxidants were added to the food mixtures, however food was stored frozen and only removed to briefly defrost before being placed into the blowfly cages. Live blowflies (n = 12) were removed from cages at days 0, 7, 14, 21, 28 and 42 and frozen at -80°C for later phospholipid fatty acid analysis of whole blowflies and isolated thoracic mitochondria. The remaining blowflies were used for survival and longevity measures. Live blowflies removed from the cages were not included in the life table calculations.

A second experiment was performed on a separate small cohort of blowflies, given one of two diets that were supplemented with three grams of either fish oil or krill oil (per 100 ml of standard SY food). Live blowflies were removed at days 0, 7, 14, 21 and 28 for phospholipid fatty acid analysis of whole blowflies. No longevity measurements were performed for these treatments.

2.9 Statistical analysis

Unless otherwise indicated, all results are expressed as means ± standard error of the mean (SEM), with N indicating the number of animals used for each treatment or assay. Average longevity was invariant between replicate cages (P >0.05) and so all analyses were performed on data pooled from replicate cages with genders combined.

As a partial control for situations of repeated statistical comparisons, Bonferroni corrections were used and reported where appropriate. Where data could not be transformed to achieve normality, non-parametric tests were used.

Linear regressions and segmental linear regressions for mortality, food consumption and fluorescent AGE pigment accumulation over time were performed using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). Comparisons of models or parameters obtained from regressions were made using an F-test provided by GraphPad Prism. Where this was not appropriate, comparisons were made between treatments by comparison of 95% confidence intervals.

Comparison of means was determined by ANOVA and Tukey HSD post-hoc test, where values could not achieve normality non-parametric Kruskal-Wallis analysis was performed with Dunns multiple comparison post-hoc test used to determine differences between treatments.

PART A

EFFECTS OF TEMPERATURE ON LONGEVITY

3 EFFECT OF CONSTANT TEMPERATURE

3.1 Preface

This chapter examines the effects of constant temperature treatments on i) adult longevity, ii) egg laying (as a measure of reproduction), iii) food consumption, iv) demographic senescence, and v) cellular senescence of the blowfly. In addition to the temperature treatments, a small cohort of blowflies were maintained individually at 25°C, as a test of the 'rate of living theory'.

To account for the large number of statistical comparisons made between temperature treatments, Bonferroni adjustment resulted in statistical significance being set to $P < 0.001$ for ANOVA and *F*-test analyses. For analyses comparing individuals or within temperatures, statistical significance was kept at $P < 0.05$.

3.2 Results

3.2.1 Temperature effects on longevity

Ambient temperature had a significant effect on survivorship in adult *C. stygia* ($\chi^2 = 2413$, $df = 5$, $P < 0.0001$). Both average and maximum longevity were significantly extended when blowflies were maintained at lower temperatures (Table 3.1). Average longevity increased six-fold, from 15 days at 34°C to 91 days at 12°C, while maximum longevity was extended almost five-fold over this temperature range.

Table 3.1. Longevity of adult *Calliphora stygia* maintained at different ambient temperatures from 12°C to 34°C.

Temperature	**Average longevity (days)**	**Maximum longevity (days)**	***N***
12°C	90.8 ± 1.6^{a}	155.8 ± 1.5^{a}	685
15°C	87.9 ± 1.5^{a}	152.2 ± 1.4^{a}	733
20°C	45.1 ± 1.2^{b}	105.6 ± 0.7^{b}	742
25°C	27.7 ± 0.7^{c}	66.9 ± 0.7^{bc}	747
29°C	23.2 ± 0.5^{c}	55.4 ± 1.5^{cd}	757
34°C	15.2 ± 0.2^{d}	32.0 ± 0.6^{d}	777

All values are means ± SEM. Maximum longevity is calculated as the average longevity of the 5% longest-lived animals for each group. Average and maximum longevities are compared between temperatures by Kruskal-Wallis ANOVA with temperature pairs compared using Dunn's multiple comparison post-hoc test (Average longevity: $K\text{-}W = 1984$, $df = 5$, $P < 0.0001$; Maximum longevity: $K\text{-}W = 200.8$, $df = 5$, $P < 0.0001$). Values sharing the same letter are not significantly different ($P > 0.001$).

3.2.2 Temperature effects on food consumption

Total food consumption was measured for each population cage and then divided by the number of blowflies present to calculate the average daily food consumption per individual blowfly (Fig. 3.1). There was a similar pattern in daily food consumption over the lifespan of the six different populations, with daily food consumption decreasing over time after an initial increase. An ANOVA (food consumption as a dependent variable, days as a covariate and temperature as factor; $F_{11, 388} = 54.1$, $P < 0.0001$) found significant effects of time ($F_{1, 1} = 25.3$, $P < 0.0001$) and temperature ($F_{5, 5} = 24.8$, $P < 0.0001$) on food consumption, as well as a significant interaction between time and temperature (days*temperature: $F_{5, 5} = 8.7$, $P < 0.0001$). Because the food consumption rate was measured at a population level and not an individual level, there are two alternate explanations for this decrease in food

consumption with time. Firstly, the most obvious explanation is that individual blowflies reduce their food consumption with age. Alternately, if there is a large variation between individual blowflies in food consumption and high food consumers have a shorter lifespan than low food consumers, a consequence of this will be that over time the calculated average food consumption per blowfly decreases. This I will call a 'rate of living' effect, assuming that food consumption per individual is a correlate of its metabolic rate (i.e. its 'rate of living'). Of course, the explanation could also be a combination of these two. In order to differentiate between these two explanations, the food consumption of individually housed blowflies was measured over time at 25°C.

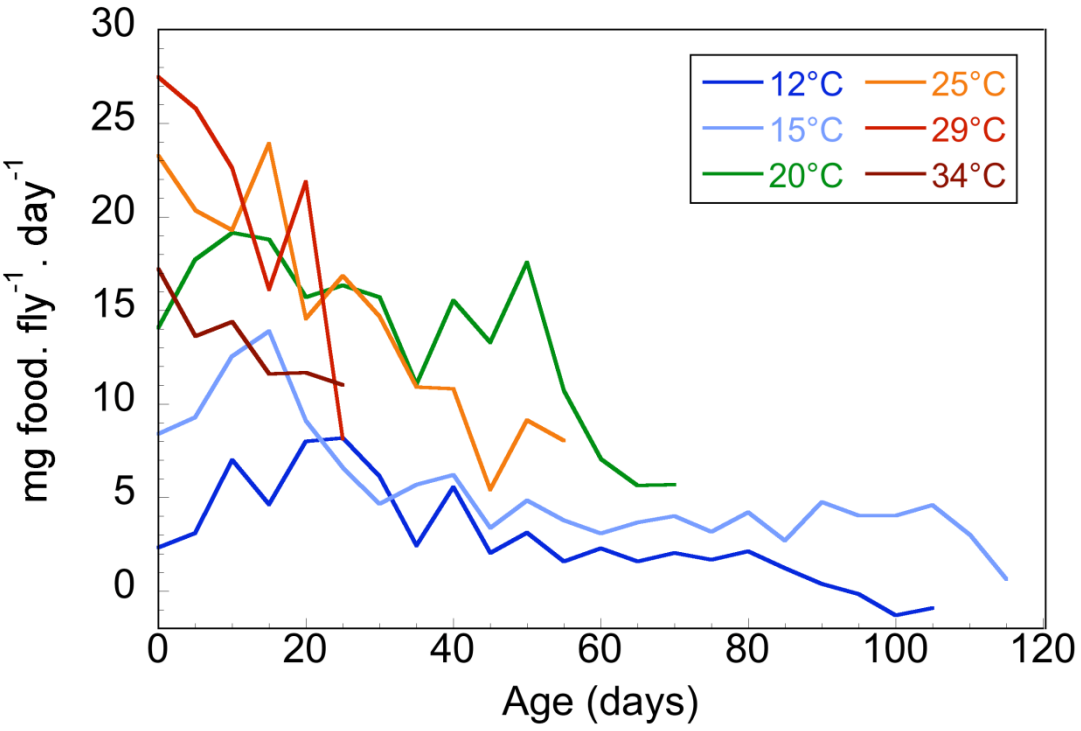

Figure 3.1. Food consumption over time in adult *Calliphora stygia* maintained at different constant temperatures. Data is from pooled replicate cages and averaged over a 5-day period. Error bars are omitted for clarity (see Appendix B for means ± SEM).

3.2.3 A test of the 'rate of living' effect: food consumption and longevity of individually housed flies.

The daily food consumption and longevity of individual blowflies maintained at 25°C is shown in Figure 3.2. These results provide support for the hypothesis that individual flies reduce their food consumption with age. Individually housed blowflies showed a similar decrease in daily food consumption with time as when food consumption was measured at the population level at 25°C (Fig. 3.2A compared to Fig. 3.1). Further, with respect to the second explanation, there was little variation in food consumption between individuals (see error bars in Fig. 3.2A).

The 'rate of living theory' predicts a 'live-fast die-young' relationship. If a high metabolic rate necessitates high food consumption it predicts a negative relationship between food consumption and individual longevity. However, average daily food consumption was not inversely related to individual longevity when examined collectively ($F_{1,17} = 4.115$, $P > 0.05$; Fig. 3.2B). Similarly, the 'rate of living theory' also predicts constant lifetime energy consumption among blowflies, i.e. flies have the same lifetime food consumption irrespective of longevity. However, contrary to this prediction of no relationship, there was a strong positive relationship between total lifetime food consumption and total lifespan for individuals ($F_{1,17} = 13.70$, $P < 0.01$; Fig. 3.2C).

When individually measured food consumption at 25°C was compared to that measured at the population level (also held at 25°C), I found no significant difference in the daily food consumption between the two populations (Welch-corrected $t = 0.62$, $df = 241$, $P > 0.05$). Individually housed blowflies had an overall average daily food

consumption of 14.5 ± 1.0 mg per fly, and blowflies in the population cages held at 25°C consumed an average of 15.3 ± 0.9 mg per fly.

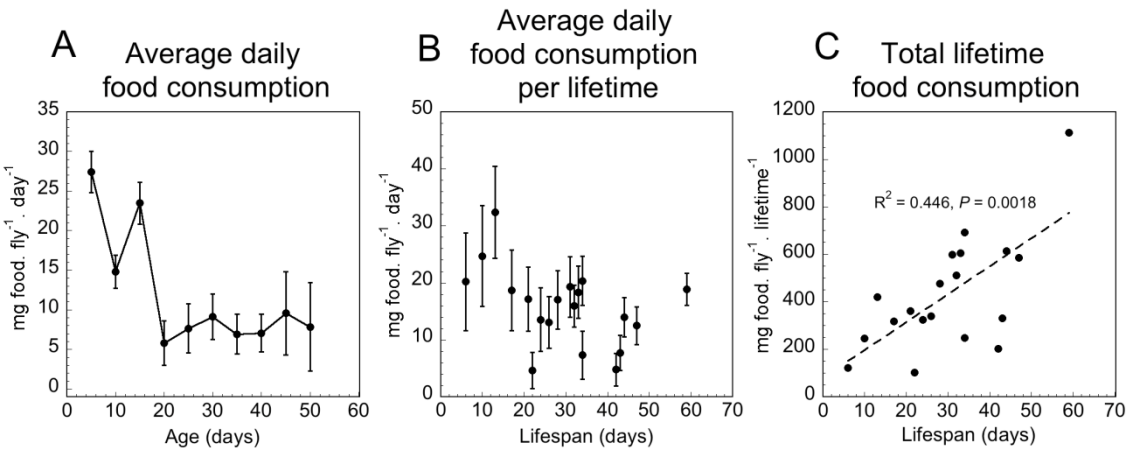

Figure 3.2. Food consumption of *Calliphora stygia* kept individually at 25°C (*N* = 10 females and 9 males). Figure 3.2A. Average daily food consumption of adult blowflies fed *ad libitum*. Data points are averaged over all blowflies for a 5-day period, error bars are ± one SEM. Figure 3.2B. There was no negative relationship between average daily food consumption and lifespan as would be predicted by the rate of living theory ($F_{1, 17}$ = 4.115, *P* >0.05). Values are means ± SEM. Figure 3.2C. Total lifetime food consumption was calculated per individual by multiplying average food consumption by total longevity. There was a significant positive relationship between lifetime food consumption and lifespan ($F_{1, 17}$ = 13.7, *P* <0.01).

3.2.4 Temperature effects on average and lifetime food consumption

For each temperature treatment, daily food consumption was averaged over the period of measurement (Fig. 3.3A) and this average daily food consumption was combined with the average longevity (Table 3.1) to calculate an 'average lifetime food consumption' per individual blowfly at the six different temperatures (Fig. 3.3B). Average daily food consumption was significantly influenced by temperature (Kruskal-Wallis test = 202.4, *df* = 5, *P* <0.0001), with food consumption significantly lower at 12°C and 15°C compared to all other temperatures (Dunns multiple comparison post-hoc test *P* <0.0001, Fig. 3.3A). Food consumption was highest at 29°C with 17.9 ± 2.1

mg food consumed per fly per day, and lowest at 12°C with only 2.8 ± 0.4 mg food consumed per fly per day. While there was a general increase in average daily food consumption with increasing temperature, there was no significant difference in food consumption between populations held at temperatures at or above 20°C (Dunn's multiple comparison post-hoc test $P > 0.05$).

Average lifetime food consumption also showed relatively little difference between blowflies maintained at 15°C and 29°C (Fig. 3.3B). There was a large decrease in average lifetime food consumption in blowflies held at the two extremes of 34°C and 12°C (204 -254 mg food per fly per lifetime), being almost half that of the more moderate temperatures (413 -608 mg food per fly per lifetime).

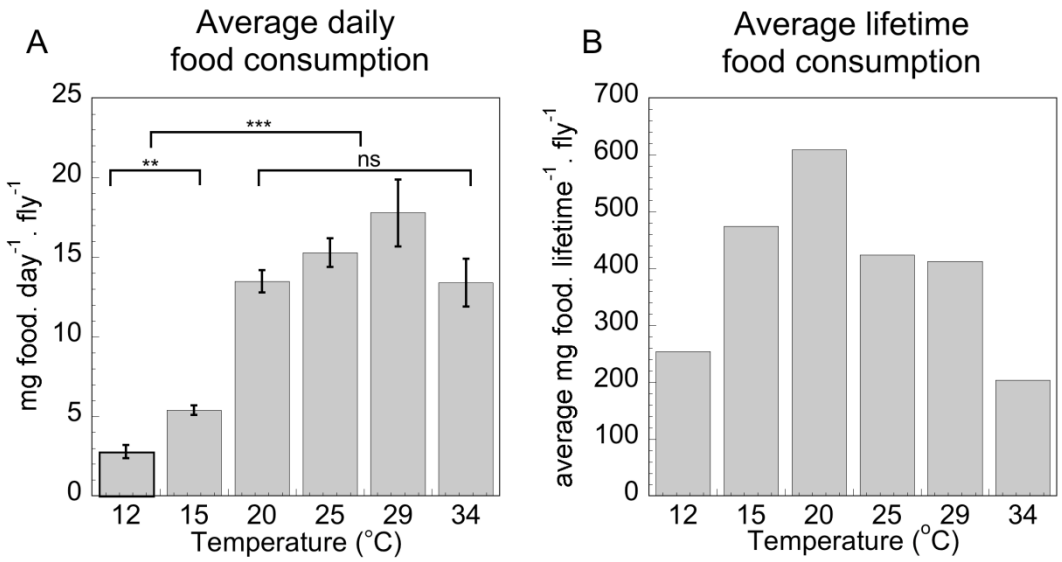

Figure 3.3. Average and lifetime food consumption of *Calliphora stygia* maintained at different constant temperatures. Fig 3.3A. Average daily food consumption of blowflies was significantly less at very low temperatures (12°C and 15°C), however there were no significant differences in average food consumption between the moderate to high temperatures (20°C to 34°C). Values are means ± SEM. ** is P <0.001; *** is P <0.0001, 'ns' represents no significant difference (P >0.001). Figure 3.3B. Average lifetime food consumption per fly was approximated by multiplying the average daily food consumption per fly by average lifespan (in days).

3.2.5 Temperature effects on egg laying

Egg laying was used as an indicator that conditions of housing and food allowed normal physiological function. The data for the period of egg laying relative to female maximum lifespan for flies maintained at the different temperatures is presented in Figure 3.4. There were no eggs layed at either the highest temperature of 34°C or the lowest temperature of 12°C. This indicates that the temperature range examined incorporates the entire physiological range of *C. stygia.*

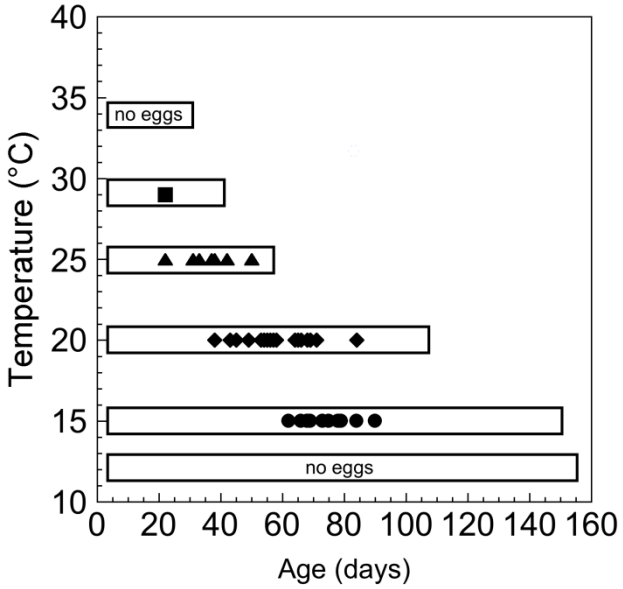

Figure 3.4. Frequency and duration of egg laying of female *Calliphora stygia* kept at different ambient temperatures. Data points represent the presence of eggs on food dishes, with the box around the data representing the female maximum lifespan for each temperature treatment (n = 351; 388; 379; 402; 399; and 401 from 12°C to 34°C respectively).

The age at first egg laying decreased with increasing temperature, with blowflies at 25°C beginning egg laying at 22 days of adult life, while blowflies maintained at 15°C began 40 days later at 62 days post-eclosion (Fig. 3.4). However, when expressed as a percent of the female maximum longevity, the reproductive period was similar for all temperatures, with the median egg-laying day occurring at 49%, 56%, 64% and 54% of the maximum longevity at 15°C, 20°C, 25°C and 29°C, respectively. When scaled to maximum longevity, the length of time over which females were reproductive was shortened at 15°C and 29°C, with only one laying day observed at 29°C. Females at 15°C layed for only 19% of their maximum longevity, while in comparison females kept at 20°C and 25°C were reproductive for up to 50% of their maximum longevity.

3.2.6 Temperature effects on demographic senescence

Initial mortality rates of populations were not significantly different between temperatures (Table 3.2). However, the changes in age-specific mortality with time were significantly affected by temperature (Fig. 3.5). All mortality curves were best fit by a two-phase Gompertz over a single Gompertz (P <0.0001 for all temperatures; Table 3.2). For temperatures ≤20°C, the rate of ageing (i.e. the slope of the line) during the early phase was not significantly different from zero, which indicates there was negligible demographic senescence occurring during this phase at these low temperatures. While there was no statistically significant increase in mortality rates during this early phase at these low temperatures, there was a significant difference between temperatures in the level of mortality during this period. Populations kept at 20°C had a higher level of mortality during this early period than those kept at the two lower temperatures of 12°C and 15°C.

Table 3.2. Mortality parameter estimates from a two-phase Gompertz fit to daily mortality of adult *Calliphora stygia* maintained at different ambient temperatures.

Temperature	**Initial mortality (% mortality.day^{-1})**	**Duration of early ageing (days)**	**Early rate of ageing (% Δ in mortality rate.day^{-1})**	**Late rate of ageing (% Δ in mortality rate.day^{-1})**	**Two phase vs. single Gompertz (R^2)**
12°C	0.6 ± 0.4 [a]	83 ± 6[a]	0.76 ± 0.27[a#]	4.01 ± 0.28[a]	**0.81** vs. 0.72
15°C	0.6 ± 0.1 [a]	48 ± 5[b]	-0.58 ± 0.61[a#]	3.48 ± 0.16[b]	**0.84** vs. 0.78
20°C	0.9 ± 0.7 [a]	61 ± 4[ab]	-0.35 ± 0.49[a#]	5.68 ± 0.57[c]	**0.68** vs. 0.49
25°C	1.1 ± 0.2 [a]	55 ± 4[b]	2.35 ± 0.43[b]	9.29 ± 1.76[d]	**0.74** vs. 0.66
29°C	0.6 ± 0.3[a]	17 ± 3[c]	16.90 ± 3.38[c]	2.97 ± 0.57[e]	**0.77** vs. 0.64
34°C	0.6 ± 0.1 [a]	10 ± 1[c]	33.32 ± 5.42[d]	5.46 ± 1.06[abc]	**0.88** vs. 0.72

Initial mortality, calculated as the average mortality over the first 5-days, was invariant between temperatures as compared by Kruskal- Wallis ANOVA (K-W = 0.65, df = 5, P >0.05). Daily mortality data was fitted by segmental linear regression, giving two phases of ageing (early and late) and a time point at which the two phases intersect (length of early ageing period). For each mortality parameter, temperatures are compared between pairs by an F-test (P <0.001). Values that share the same letter are not significantly different. [#]The early phase of ageing was also tested to determine whether the rate of ageing (i.e. the slope of the line) was significantly different from 0. Slopes that were not significantly different from 0 are denoted by [#].

For temperatures ≥25°C, there was significant demographic senescence (i.e. the slope was significantly greater than zero) during this early phase, and this rate increased with increasing temperature. As well as the rate of early ageing being temperature dependent, the duration of this early phase was also influenced by temperature (Table 3.2). However, unlike the rate of ageing which increased with increasing temperature, the duration of the early phase decreased with increasing temperature (Fig. 3.5).

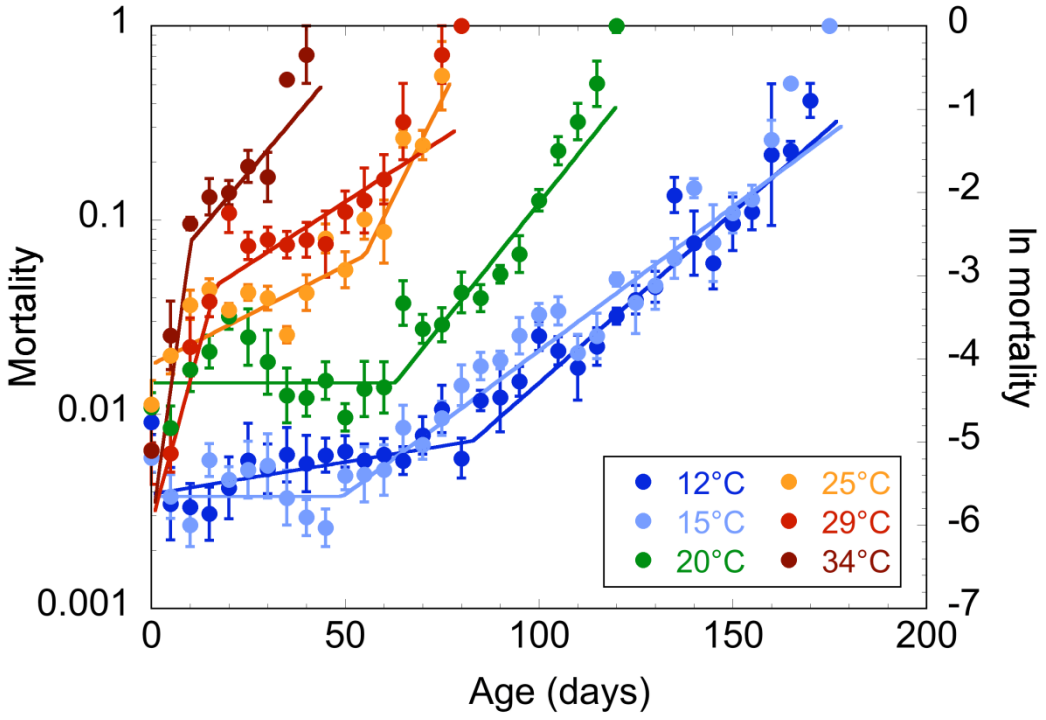

Figure 3.5. Mortality rates of adult *Calliphora stygia* maintained at different ambient temperatures ranging from 12°C to 34°C. A two-phase Gompertz was fitted to daily ln mortality by segmental regression and is represented by the solid line for each temperature (see Table 3.2 for parameters). Data is pooled from replicate cages, with plotted points being the average of a 5-day period, with error bars ± one SEM (*N* = 12°C = 685, 15°C = 733, 20°C = 742, 25°C = 746, 29°C = 618, and 34°C = 778).

The later phase of demographic senescence showed much less temperature influence than the early phase (Fig. 3.5 and Table 3.2). For example, in the early phase, the change in mortality rate varied from 0% to 33% per day, while during the late phase, the change in mortality rate varied from 3% to 9% per day. At temperatures at or below 25°C, the rate of ageing during the late phase was faster than that during the early phase, however at temperatures above 25°C this relationship was reversed (i.e. the late rate of ageing was slower than the early rate of ageing).

The relationship between temperature and the rate of a physiological process can be expressed as a Q_{10} (see introduction). On examination of the relationship between temperature and the rate of ageing (Fig. 3.6), a strong effect of temperature is evident on the early rate of ageing, but almost no temperature effect on the late rate of ageing. The early phase of ageing exhibited a Q_{10} of 5.7. This is exceptionally high compared to other biological processes (such as metabolism or food consumption), which generally have a Q_{10} of between 2-3. In contrast, the late rate of ageing had a Q_{10} of 1.1, which indicates almost no temperature sensitivity.

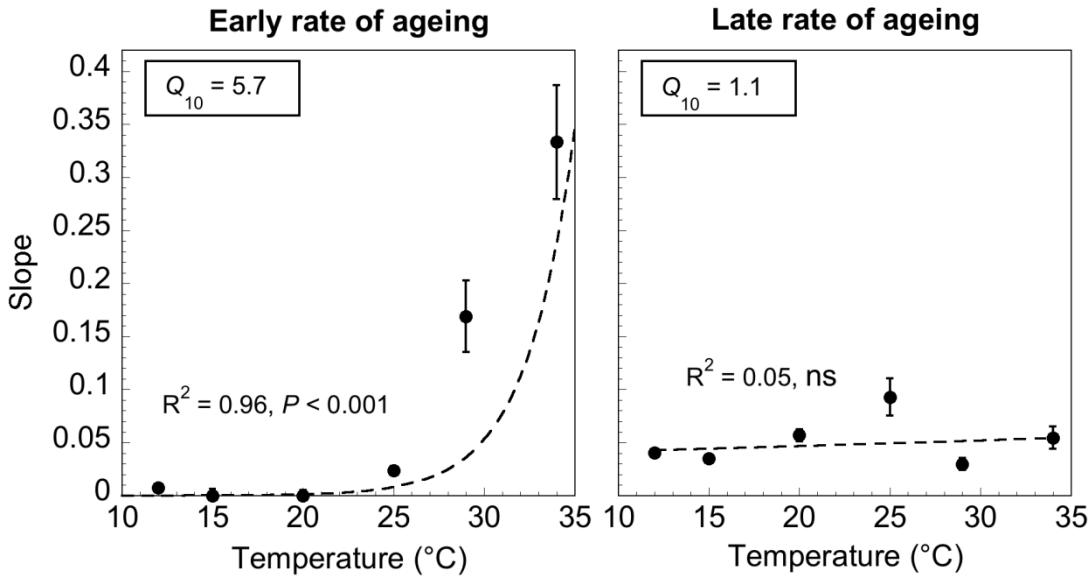

Figure 3.6. The exponential relationship between early and late-phase rate of ageing in *Calliphora stygia* and temperature. The estimates of Q_{10} were calculated from the slope of the regression line (values are means ± SEM, where error bars are absent, error is less than the size of the marker).

3.2.7 Temperature effects on cellular senescence

Cellular senescence was measured as the accumulation of fluorescent AGE pigment in whole body and body segments. Initially, fluorescent AGE pigment was measured in homogenised whole blowflies and the change in fluorescence expressed relative to the level found in newly emerged adults (Fig. 3.7). Later, fluorescent AGE pigment accumulation was measured separately in body segments (head, thorax, and abdomen) and expressed in fluorescence units (Fig. 3.8). In all cases newly emerged blowflies had measurable levels of fluorescent AGE pigment present that was presumably accumulated during larval and pupal life stages. At temperatures above 15°C, there was a rapid increase in fluorescent AGE pigment (which indicates cellular senescence; $F_{6, 35}$ = 14.2, P <0.0001), over the first eight days of adult life. Fluorescent

AGE pigment was accumulated throughout the periods of 'negligible demographic senescence' observed during the early phase of ageing at low temperatures (see previous section).

Temperature affected the accumulation rate of fluorescent AGE pigment, occurring more rapidly at higher temperatures (Fig. 3.7A). When the rate of accumulation in whole blowflies is averaged over the total experimental period, this relationship between temperature and accumulation rate was calculated to have a Q_{10} of 2.5 (Fig. 3.7B). This value is within the range expected for normal metabolic processes.

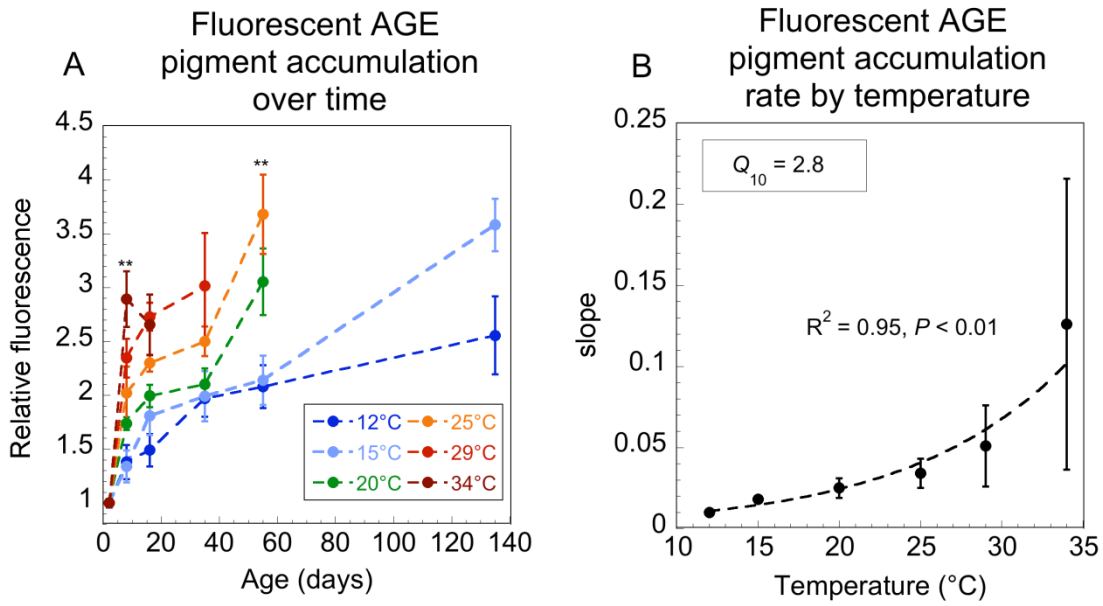

Figure 3.7. Fluorescent AGE pigment accumulation at different ambient temperatures in *Calliphora stygia*. Figure 3.7A. Fluorescent AGE pigment accumulation with chronological age for each temperature treatment. Values are means ± SEM (N = 6). ** represents a significant difference between temperatures at that age (Tukey post-hoc test, P <0.001), no significant differences were seen at other days. Figure 3.7B. Temperature sensitivity of AGE pigment as described by the rate of accumulation (calculated from the linear slopes derived from data given in Fig. 3.7A). The Q_{10} estimate was calculated from the slope of the exponential relationship between temperature and rate of accumulation. Values are means ± SEM.

When fluorescent AGE pigment was examined in the body segments separately, cellular senescence was highest in the heads of individual flies, with little fluorescence observed in the abdomen, while levels in the thorax were below our detection limits (Fig. 3.8). This may indicate cellular senescence does not occur evenly throughout the blowfly body. Within the head, the rate of accumulation of AGE pigment was faster at higher temperatures (a calculated Q_{10} = 2.6, figure not shown) in a manner similar to that seen in whole blowflies (Fig. 3.7).

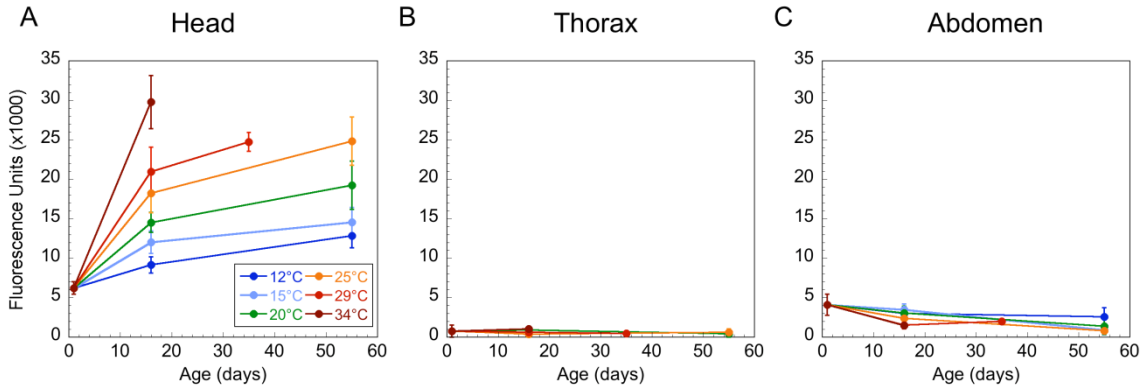

Figure 3.8. Fluorescent AGE pigment accumulation in separated body segments of head (A), thorax (B) and abdomen (C) of *Calliphora stygia* kept at one of 6 ambient temperatures. All values are means ± SEM (N = 6).

3.3 Discussion

Temperature had a significant effect on the adult life history of *C. stygia*. The fact that egg laying occurred at all but the two extreme temperatures suggests the entire physiological temperature range was examined in the current study. Reducing the environmental temperature delayed the start of reproduction, while increasing the

temperature shortened the reproductive period. Shorter breeding periods can result in either reduced lifetime fecundity as demonstrated in the rotifer, *A. brightwelli* (Verdone-Smith and Enesco, 1982), or can be associated with increased daily fecundity (Huey et al., 1995) with a consequent increase in total lifetime reproductive success. The latter has been observed in both *D. melanogaster* (Partridge et al., 1995; Sisodia and Singh, 2002) and the butterfly *Bicyclus anynana* (Steigenga and Fischer, 2007). In the current study only the frequency of egg laying was measured, not fecundity *per se*, and so the interactive effects of temperature and fecundity on longevity cannot be determined. However, a negative relationship between lifetime fecundity and longevity has been often demonstrated in other insects (Chippindale et al., 1993; Djawdan et al., 1996; Harshman and Zera, 2006).

The assumption that low temperature extends longevity through a reduction in metabolic rate (the 'rate of living') was not supported in the current study as evidenced by food consumption rates of populations or individuals. Food consumption values can infer total metabolic energy requirements (Hulbert et al., 2004b). In this study however, there was no significant difference in the calculated average food consumption of blowflies over the range of 20°C to 34°C. This may indicate there was no effect of temperature on metabolic rate over this temperature range, despite a three-fold increase in average and maximum longevity between 20°C and 34°C. Further, examination of individual blowflies maintained at 25°C showed no relationship between individual longevity and food consumption. An earlier study on the same species where blowflies were provided a sucrose solution, found no relationship between an individuals sucrose consumption and longevity (Hulbert et al., 2004b), which confirms the findings of the present study. While there is little doubt that temperature has a substantial effect on both

activity and many physiological processes, the magnitude of difference in longevity in relation to temperature that was observed in the current study, was not mirrored by a similar change in food consumption, suggesting the influence of mechanisms other than the 'rate of living'.

Temperature affected both demographic and cellular senescence of adult *C. stygia*. Demographic ageing was best described by a two-phase Gompertz, which has been used extensively in the examination of mortality in flies (Carey et al., 1992; Curtsinger et al., 1992; Fukui et al., 1993; Shahrestani et al., 2012). The two-phase Gompertz was preferred at all temperatures, with the early phase of ageing being far more temperature sensitive than the late phase of ageing. Possibly the strongest influence of temperature observed in the current study was the fact that low temperature delayed the onset of 'normal' demographic senescence and resulted in an extended period of 'negligible' demographic senescence. Such a 'delay' in the onset of senescence at low temperatures has previously been inferred from survival curves in other species including *D. melanogaster* (Miquel et al., 1976; Garcia et al., 2010) and mice (Conti et al., 2006), however, this is one of the first instances of this period being quantified.

An increase in mortality rate with time is generally considered to be a marker of senescence. Interestingly, once *C. stygia* reached this period of their adult life, temperature had little influence on their rate of ageing. Therefore, while a decrease in temperature had a dramatic effect on the early rate of ageing and delayed the onset of 'normal' senescence, it appeared that once senescence began, ageing occurred at a similar rate at all temperatures. A study in the milkweed bug, *O. fasciatus*, showed that while temperature greatly affected metabolic rate (measured as oxygen consumption)

early in adult life, it had less influence on the metabolic rate during late adult life (McArthur and Sohal, 1982). Thus, the influence of temperature is not constant across the adult lifespan of some insects. That some species may experience an extended delay before the onset of senescence when kept at low temperatures, is an important factor to consider when examining ageing processes.

The process of ageing (i.e. senescence) can be measured both demographically and at a cellular level. When determined demographically, mortality (i.e. lifespan) is the parameter measured. However, when cellular senescence is examined, the parameter measured may increase with age but this increase may not necessarily be associated with a decline in function leading to death (Grotewiel et al., 2005). At all temperatures measured, there was an initial rapid increase in accumulation rate of fluorescent AGE pigment, which was faster at high temperatures. Sheldahl and Tappel (1974) also found faster accumulation of lipofuscin with increasing temperature in fruit flies, with a more rapid accumulation early in adult life. In *C. stygia* this rapid initial increase in accumulation occurred even during the delayed 'negligible' demographic senescence of blowflies at low temperatures. Fluorescent AGE pigments are used as a marker of cellular senescence that accumulate with age and are correlated with the rate of ageing (Clancy and Birdsall, 2012). However, the lack of correlation between cellular senescence and demographic senescence in my study, may indicate that AGE pigments do not directly contribute to mortality, but rather be a consequence of increasing time. Conversely, Jacobson et al. (2010) reported that fluorescent AGE pigment was the only marker of irreversible cellular damage that correlated with mortality rates in *D. melanogaster*, however, they did not evaluate this process at very low temperatures.

Many studies examining biochemical markers of cellular senescence fail to encompass the entire lifespan, indeed most use only a single time point comparison (e.g. (Sohal et al., 1985; Farmer and Sohal, 1987; Malek et al., 2004; Rebrin et al., 2004; Hsu and Chiu, 2009). Such measures would most likely miss the onset of senescence in the long-lived cohorts and over-emphasise the temperature effects that are seen in the early temperature-sensitive phase of ageing. Increased rates of mitochondrial DNA damage (Garcia et al., 2010), and higher rates of protein carbonylation (Das et al., 2001) in *D. melanogaster* and AGE pigment accumulation in insects and fish (McArthur and Sohal, 1982; Valenzano et al., 2006; Jacobson et al., 2010) have been shown with high temperature exposure, yet in all studies the period over which the measures were made were at most equal to the average longevity of the long-lived cohort. In contrast, measures of lipid peroxidation products in *D. melanogaster* kept at different temperatures taken over the entire lifespan, were shown to be similar when longevity was normalised to maximum longevity (Zheng et al., 2005). In the current study AGE pigment was measured up to day 135 for the long-lived cohorts, roughly 85% of their maximum lifespan. Between 55 days and 135 days there was a dramatic increase in AGE pigment seen for 15°C, but less so in flies kept at 12°C, despite similar demographic senescence between these two temperatures.

There is a potential problem with sampling a live population to measure the accumulation of AGE pigment, in that the sample may include unknown proportions of physiologically young and physiologically old flies (i.e. close to death), and these may differ in their AGE pigment levels. To attempt to examine this question I measured AGE pigment in three groups of flies (data not shown, see Appendix C). These groups were defined as: alive, dead and almost dead (lying on their back and unable to turn

over), at two different times (16 days and 35 days), while held at 25°C. The results were somewhat inconclusive, as while there was a significant difference between alive and dead flies at day 16, there was no difference by day 35. Similarly, there was also no difference in AGE accumulation between dying and dead flies at either day 16 or day 35. Gerstbrein et al. (2005) performed a similar examination of fluorescent AGE pigment in *C. elegans* that were age-matched but differing in physiological health, finding animals that were deemed less physiologically healthy and therefore closer to death had higher levels of pigment accumulation.

Accumulation of AGE pigment in *C. stygia* was shown to be limited to the head region, which has also been reported for the flesh-fly, *Sarcophaga bullata* (Ettershank et al., 1993) and housefly, *M. domestica* (Donato and Sohal, 1978). No accumulation of AGE pigment with age was observed in the abdominal segment or thorax. Ettershank et al. (1993) also found no change in pigment accumulation in the abdominal region with age. In addition they measured lipofuscin content of the larval phase and found the accumulation of lipofuscin with age in the larvae similar to that in the adult, with a loss of lipofuscin between the larval and pupal phases and again between the pupal and adult phases, suggesting some elimination of this pigment during transition between life history phases.

The separate measurement of fluorescent AGE pigment in different body segments permits consideration of how AGE pigment accumulation may differ among tissue types. This is because the thorax consists of mainly muscle, the abdomen being primarily digestive and reproductive tissues, and the head containing predominantly neural tissue. Mitochondrial DNA damage was found to be highest in the thorax of *D. melanogaster* (Garcia et al., 2010), and muscles also appear to be highly sensitive to

ageing (Girardot et al., 2006). Yet, changes in physiological age, both chronologically and through exposure to heat stress, were best described by changes in lipid profiles in the head of medflies as compared to the other body segments (Pujol-Lereis et al., 2012). Examination of fatty acid composition of the phospholipids of the head, thorax and abdomen of bees, showed that the peroxidation susceptibility of the heads of worker bees was double that of the other two segments (Haddad et al., 2007). These differences between tissue types in their response to ageing and their susceptibility to various ageing-related stressors highlight the complexity of the ageing process (Simm and Johnson, 2010).

4 A TEMPERATURE CROSS-OVER EXPERIMENT

4.1 Preface

In the previous chapter it was demonstrated that constant ambient temperature exposure during adult life had a dramatic effect on both demographic and cellular senescence. Another approach is to change temperature during the adult life of the blowfly and examine the effects on demographic and cellular senescence. This chapter reports the results of such an experiment. Some cages of blowflies, from the same cohort as the previous chapter, were transferred between 15°C or 29°C after a period of 14- or 28-days. The data for constant 15°C and 29°C treatments are the same as those presented in the previous chapter as the experiments were performed on the same batch of pupae at the same time and under the same conditions.

4.2 Results

The results of this experiment are consistent with the findings from the previous chapter, in that low temperature extended lifespan while high temperature shortened lifespan. In all cases of temperature transfer, average and maximum longevity were altered to a value intermediate between the longevity of the two constant temperature groups (15°C and 29°C), with the degree of change in longevity dependent on the time of transfer (Table 4.1).

Table 4.1 Average and maximum longevity of *Calliphora stygia* transferred between ambient temperatures after 14- or 28-days.

Temperature	**Average longevity (days)**	**Maximum longevity (days)**	***N***
15°C	87.9 ± 1.5^{a}	152.2 ± 1.4^{a}	733
transfer from 29°C to 15°C			
at 14 days	47.0 ± 1.5^{b}	143.1 ± 1.5^{ab}	782
at 28 days	25.6 ± 0.8^{c}	99.6 ± 2.8^{bc}	790
transfer from 15°C to 29°C			
at 14 days	32.2 ± 0.5^{b}	61.8 ± 1.2^{de}	709
at 28 days	45.4 ± 0.5^{d}	71.0 ± 0.8^{cd}	785
29°C	23.2 ± 0.5^{c}	55.4 ± 1.5^{e}	757

All values are means ± SEM. Maximum longevity is calculated as the average longevity of the 5% longest-lived animals for each group. Average and maximum longevities are compared between temperatures by Kruskal-Wallis ANOVA with temperature pairs compared using Dunn's multiple comparison post-hoc test (Average longevity: $K\text{-}W = 1984$, $df = 5$, $P < 0.0001$, Maximum longevity: $K\text{-}W = 200.0$, $df = 5$, $P < 0.0001$). Values that share the same letter are not significantly different. Values for 15°C and 29°C are taken from the previous chapter.

The effect of temperature transfer on mortality rates is presented in Figure 4.1, where two-phase Gompertz equations were fitted to daily mortality rates from the day of transfer. Temperature had a very rapid effect on mortality rate at both low and high temperature, with changes to mortality patterns evident immediately following temperature transfer. The transfer of blowflies to the low temperature at both 14 days and 28 days resulted in a period of 'suspended' demographic senescence, which was at a level of mortality unchanged from the previous high mortality experienced prior to transfer from high temperature (Fig. 4.1).

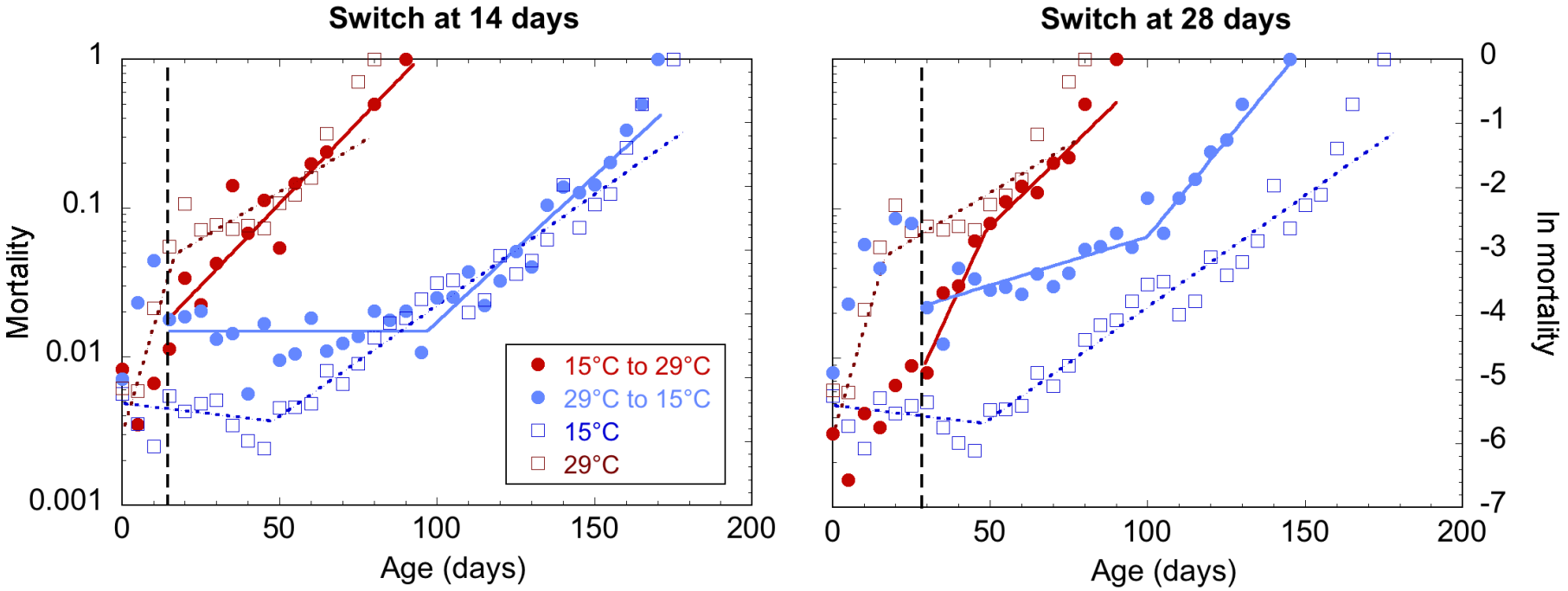

Figure 4.1. Age-specific mortality of adult *Calliphora stygia* transferred between high (29°C) and low (15°C) temperatures after 14- or 28-days are presented with points calculated as an average of a 5-day period (error bars are omitted for clarity). See Table 4.2 for parameters.

The time of transfer had an effect on the late rate of ageing, with blowflies transferred to low temperature at 28 days having a faster rate of ageing than those transferred at 14 days, and those transferred at 14 days having a faster rate of ageing than those maintained constantly at 15°C (Table 4.2). The time of transfer also affected blowflies transferred from low to high temperature (15°C to 29°C). While both populations experienced a rapid increase in mortality rates with exposure to the new temperature, the blowflies transferred at 14 days showed no slowing of the rate of ageing with old-age, while blowflies transferred after 28 days and those held constantly at 29°C did exhibit a slower rate of late ageing (Table 4.2).

Overall, the rate of ageing during the late phase was similar for all transferred blowflies (5-6% change per day in mortality rate), but the greatest contribution to the large differences in maximum longevity (up to a two-fold increase) was due to the duration of 'negligible' senescence experienced by flies transferred to low temperature. Blowflies that experienced a cross-over all showed a significantly faster rate of late ageing compared to the blowflies that did not experience a temperature cross-over, which had a 3% change in daily mortality rate (Table 4.2).

Table 4.2 Mortality parameter estimates from a two-phase Gompertz fit to daily mortality of adult *Calliphora stygia* following transfer between 15°C and 29°C after 14- or 28-days.

Temperature	**Initial mortality**	**Immediately post-transfer**		**2nd phase post-transfer ageing (% Δ in mortality rate.day^{-1})**	**Two-phase vs. single phase Gompertz (R^2)**
	(% mortality.day^{-1})	**Duration (days)**	**Rate of ageing (% Δ in mortality rate.day^{-1})**		
15°C	0.56 ± 0.13^{a}	48 ± 5^{a}	-0.58 ± 0.61$^{\#a}$	3.48 ± 0.16^{a}	**0.84** vs. 0.78
transfer 29°C to 15°C					
at 14 days	0.74 ± 0.34^{a}	83 ± 4^{c}	0.01 ± 0.03$^{\#b}$	4.59 ± 0.41^{b}	**0.71** vs. 0.50
at 28 days	0.85 ± 0.22^{a}	72 ± 8^{ac}	1.51 ± 0.41$^{\#c}$	5.99 ± 1.38^{c}	**0.63** vs. 0.54
transfer 15°C to 29°C					
at 14 days	0.75 ± 0.35^{a}	na	na	5.15 ± 0.53^{bc}	na vs. 0.64
at 28 days	0.31 ± 0.31^{a}	21 ± 5^{b}	10.75 ± 2.00^{d}	4.81 ± 0.93^{bc}	**0.82** vs. 0.79
29°C	0.55 ± 0.31^{a}	17 ± 3^{b}	16.90 ± 3.38^{e}	2.97 ± 0.57^{d}	**0.77** vs. 0.64

Initial mortality, calculated as the average mortality over the first 5-days, was invariant between temperatures as compared by Kruskal- Wallis ANOVA (K-W = 6.67, df = 5, P >0.05). Daily mortality data was fitted by segmental linear regression, giving two phases of ageing (early and late) and a time point at which the two phases intersect (length of early ageing period). Parameters noting 'na' represent where a two-phase Gompertz could not be fit and only the corresponding single phase Gompertz parameters are given. For each mortality parameter, temperatures are compared between pairs by an F-test (P <0.01). Values that share the same letter are not significantly different. $^{\#}$The early phase of ageing was also tested to determine whether the rate of ageing (i.e. the slope of the line) was significantly different from 0 (P <0.05). Slopes that were not significantly different to 0 are denoted by $^{\#}$

Egg laying showed a variable response to a change in temperature based on the day of transfer (Fig. 4.2). For the blowflies transferred at 14 days, their subsequent egg laying resembled that of the blowflies kept constantly at their transfer temperature. Blowflies transferred to 15°C layed eggs for the same amount of time as those kept constantly at 15°C, although had fewer laying days. Blowflies transferred to 29°C had only one egg laying event.

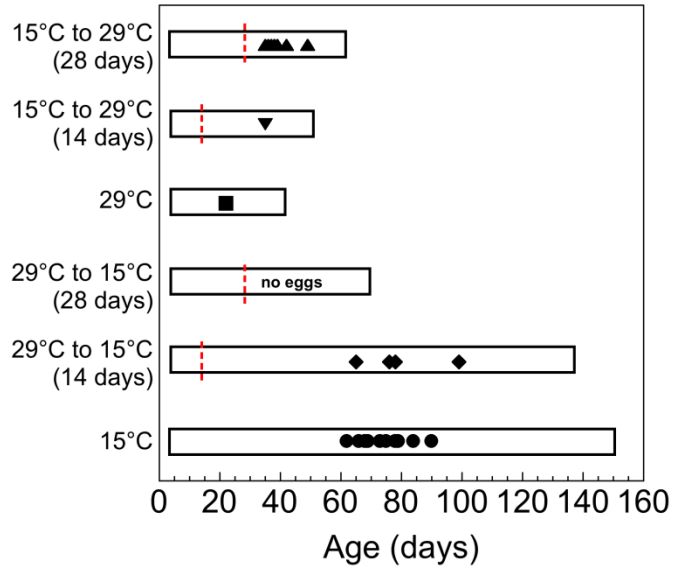

Figure 4.2. Egg laying behaviour of *Calliphora stygia* transferred between 15°C and 29°C after 14- or 28-days. The box around the data indicates the length of female maximum longevity for that treatment. The red dotted line shows the time of the temperature transfer (*n* =389, 397, 399, 399, 376, and 388, listed from the top to bottom categories respectively).

Blowflies transferred to 15°C after 28 days did not lay eggs. This may be a result of the blowflies maintained constantly at 29°C having only one egg laying day that occurred prior to 28 days. Thus, the period of egg laying may have already passed before the blowflies were transferred to 15°C, with no reversal of this upon exposure to the lower temperature. Although it is unknown as to why they did not lay prior to their

transfer. In contrast, blowflies that were transferred to high temperature at 28 days had increased frequency and duration of egg laying than those maintained constantly at 29°C. This was in contrast to blowflies kept constantly at 15°C that laid eggs earlier and for a shortened period of time.

Due to limited sampling, the effects of temperature transfer on the accumulation of fluorescent AGE pigments showed few differences between treatments (Fig 4.3). The most obvious change in accumulation occurred in blowflies transferred to high temperature after 28 days, with a 50% increase in AGE pigment compared to those kept chronically at 15°C. There was no corresponding increase seen for blowflies transferred at 14 days. Blowflies transferred to low temperature after 14 days showed a small decrease in AGE pigment, yet this was not evident in the blowflies transferred after 28 days.

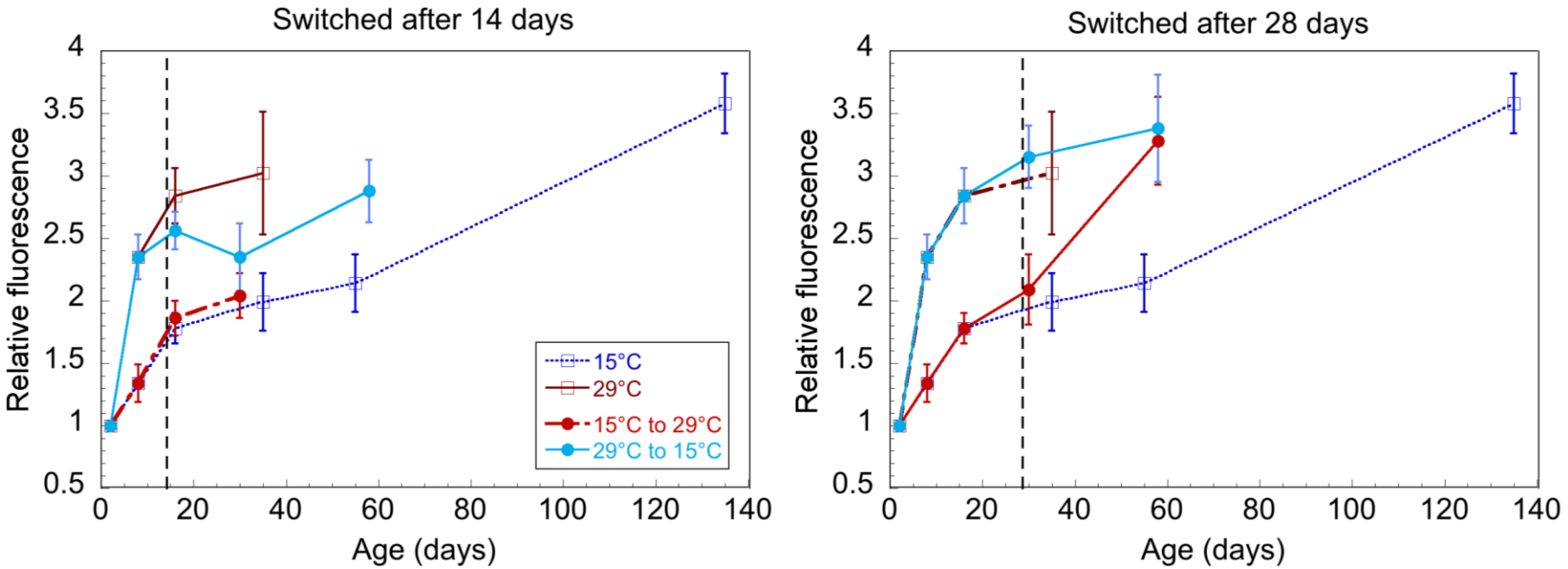

Figure 4.3. Fluorescent AGE pigment accumulation in *Calliphora stygia* transferred between high and low temperatures after a period of 14- or 28-days (represented by the vertical dotted line). Values are means ± SEM (N = 6).

4.3 Discussion

The examination of temperature effects on ageing and longevity may have been previously undervalued because of the assumptions of temperature-induced 'rate of living' type effects (Yen and Mobbs, 2010). Nevertheless, thermal perturbation studies of ectotherms provide a potentially powerful tool to examine the processes of ageing (Jacobson et al., 2010). Thermal history had a strong impact on the level of mortality of transferred flies, however their rate of ageing reflected the new temperature conditions. Flies transferred from high to low temperature showed a mid-life 'suspension' of senescence that was similar to the 'negligible' senescence of low temperature flies in the constant temperature experiment. The ability to induce this reduced senescence after previous 'fast' ageing, has the potential to present significant insights into the processes involved in ageing and longevity.

Mortality patterns of *D. melanogaster* under temperature or dietary restriction regimes were examined in a transfer experiment by Mair et al. (2003). The two environmental manipulations differed in how they affected demographic ageing responses, with thermal history playing an important role in determining mortality rates post-switch, whereas the dietary restriction conditions that followed the switch were the only nutritional factor that contributed to post-switch mortality. These results are similar to the current study with the mortality level of flies being dependent on thermal history, and the rate of senescence following temperature-transfer reflecting that of the new temperature. However, in contrast to the slowed rates of ageing at low temperature observed in *D. melanogaster* (Mair et al., 2003), *C. stygia* demonstrated similar rates of ageing at old age regardless of temperature conditions.

The ‘suspension’ of senescence observed after transfer to low temperature likely reflects a temperature-sensitive mechanism that either decreases the rate of damage or increases the rate of repair, or perhaps a combination of both. To begin to examine this, the accumulation of fluorescent AGE pigment, as a marker of cellular senescence and age-related damage, was measured over the lifespan of the blowflies. Accumulation of endogenous fluorescent compounds with age has been demonstrated in a range of both vertebrate and invertebrate animals (Terman and Brunk, 1998). Further, increased rates of accumulation with increasing temperature have been found in annual fish, *Nothobranchius spp.* (Valenzano et al., 2006; Hsu and Chiu, 2009), milkweed bugs, *O. fasciatus* (McArthur and Sohal, 1982), nematodes, *C. elegans* (Gerstbrein et al., 2005) and the fruit fly *D. melanogaster* (Sheldahl and Tappel, 1974; Miquel et al., 1976; Jacobson et al., 2010). Jacobson et al. (2010) who investigated six markers of oxidative damage in *D. melanogaster*, found a strong correlation between the rate of mortality and fluorescent AGE pigment, but not in the other five markers. Similarly, in the current study AGE pigment accumulated with age, with faster rates of accumulation at high temperature. However, the results are less clear following temperature transfer, possibly due to limited times of sampling. There was a large increase in AGE accumulation rate upon transfer to high temperature after 28 days, and a slight decrease after transfer to low temperature after 14 days, however, the other two transfers showed no obvious temperature effect. These relatively small changes in AGE pigment accumulation did not reflect the dramatic changes seen in demographic senescence and seems to indicate a partial decoupling between cellular senescence and demographic senescence in *C. stygia*, thereby supporting the findings of the previous chapter where AGE pigment was accumulated during periods of negligible senescence.

PART B

EFFECTS OF DIETARY COMPOSITION ON LONGEVITY

5 EFFECT OF DIET SUGAR CONTENT

5.1 Preface

The standard fly diet used throughout this thesis consisted of equal amounts of sugar and yeast (see Methods). This chapter reports on the effects of altering the dietary sugar content (with the yeast content kept constant) on longevity, demographic senescence and cellular senescence. The effects of four other diets were examined and compared to the standard diet, with statistical analyses being limited to comparisons between these different diets and the standard diet, except where otherwise stated. Statistical significance is set at $P < 0.05$.

It must be noted that the blowflies on the standard diet in this chapter are under the same conditions as those held at 25°C in the previous constant temperature experiment (Chapter 3). These two experiments were conducted at different times of the year, and on a different batch of pupae, and while they show similarities in their mortality, longevity and food consumption, there are small significant differences between the two 'control' groups and therefore the experiments cannot be directly compared (see Appendix D for a comparison of 'control' cohorts between experiments).

5.2 Results

5.2.1 Effects of diet sugar content on food consumption

The proportions of sugar added to the diet varied from a low of 0.25 times, to a maximum of 2.0 times the standard level of sugar (1.0), while the amount of yeast was the same for all diets (Table 5.1). Those different proportions influenced the density of

the food, as well as the energy content per gram. Standard fly food has an energy content of 7.7 kJ/g and the four other diet treatments that were examined ranged in energy content from a reduction of 25% to an increase of 20% compared to the standard diet (Table 5.1).

Food consumption decreased over time for all diet treatments (Fig. 5.1), a pattern that has been shown consistently in this species (see Chapter 3, Ujvari et al., 2009). Food consumption increased as relative sugar content increased, however, these differences were not significantly different from the consumption of standard food when averaged over the measurement period ($F_{4,\ 101} = 1.62$, $P > 0.05$). When food consumption was estimated as volume of food consumed, the pattern of blowflies consuming increased volumes of food with increasing food sugar content was still evident, however, there was less variation in the volume of food consumed between diet treatments.

Table 5.1. Composition and energy content of diets and respective food consumption of *Calliphora stygia*.

Treatment (*x* sugar of standard diet)	Sugar content (g/100g food)	Yeast content (g/100g food)	Energy content (kJ/g)	Density of food (g/ml)	Average daily food consumption ($mg.fly^{-1}$)	($kJ.fly^{-1}$)	($\mu l.fly^{-1}$)[$]	% Δ in daily kJ consumed from standard
0.25	8.3	33.4	5.88	0.45	16.3 ± 3.2^{ns}	0.10 ± 0.02^{ns}	15.2	-44%
0.50	15.2	30.4	6.57	0.52	18.4 ± 3.2^{ns}	0.12 ± 0.02^{ns}	16.1	-33%
0.75	20.8	27.7	7.10	0.55	21.3 ± 3.2^{ns}	0.15 ± 0.02^{ns}	18.8	-17%
1.0	**26.1**	**26.1**	**7.73**	**0.61**	**23.7 ± 3.3**	**0.18 ± 0.03**	**20.1**	
2.0	40.8	20.4	9.31	0.76	26.8 ± 2.5^{ns}	0.25 ± 0.02^{ns}	21.6	+39%

Standard diet is in bold. Calculation of energy content is based on final food weight and the relative weight contributions of both sugar and yeast. Kilojoule content of yeast (*Saccharomyces cerevisiae*, Brewers yeast, Enoferm M1, Lallemand, SA) and sugar was based on estimations from Bass et al., (2007) and USDA database (sugar = 16 kJ/100g, yeast = 13.6 kJ/100g). Average daily food consumption (mg and kJ per fly) is calculated as the average over the measurement period from replicate cages. [$]Average volume of food consumed was estimated from the average daily food consumption in mg and therefore no statistical analysis was performed. Sample sizes for each diet population (combined replicate cages) are 0.25*x* N = 498; 0.5*x* N = 464; 0.75*x* N = 492; 1.0*x* N = 491 and 2.0*x* N = 487 flies respectively. 'ns' indicates no significant difference between treatments.

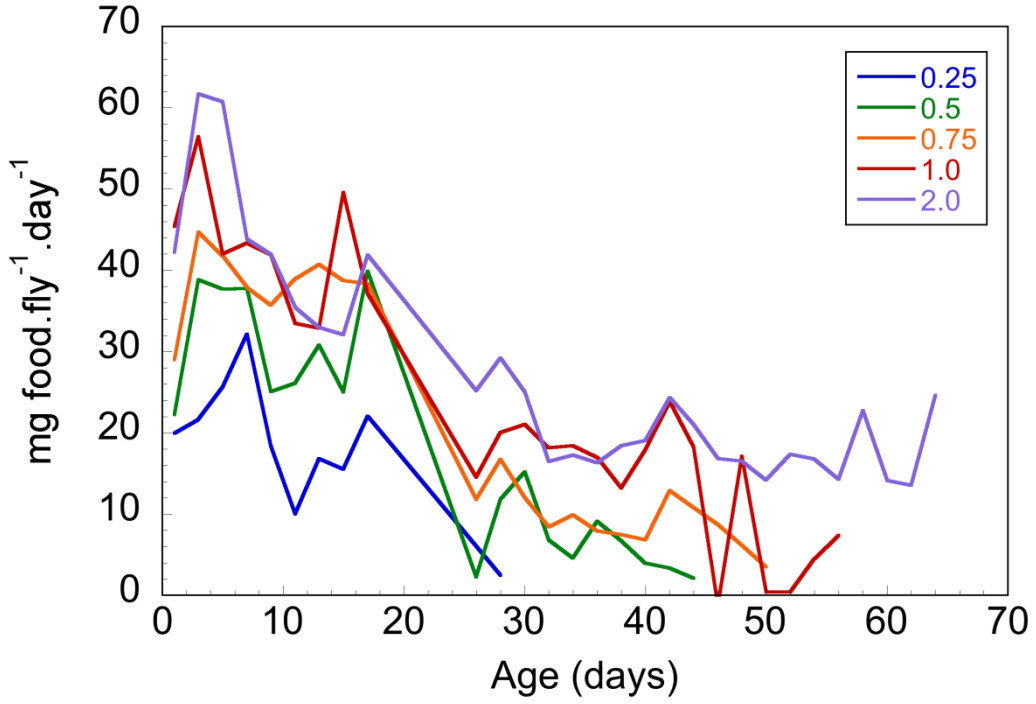

Figure 5.1. Daily food consumption of *Calliphora stygia* fed diets varying in sugar content. Points are plotted as an average from replicate cages (with food consumption measured every 2nd day). Error bars are omitted for clarity (averages ± SEM are presented in Table 5.1; see Appendix B for full data).

The increased consumption of high sugar diets showed a strong influence on the amount of food energy consumed and there was a significant positive correlation between the total kJ content of the diet and both mg of food consumed and kJ of food consumed per fly (Fig. 5.2). Blowflies on the highest energy diet had increased food energy consumption compared to the all the reduced sugar diets ($F_{4, 101}$ = 6.03, P <0.01, Tukey HSD post-hoc test). For example, blowflies fed the highest sugar diets consumed 150% more energy per day than blowflies fed the lowest sugar diet. This was a combination of the facts that they ate 64% greater mass of food per day and this food contained 69% more energy per gram. However, as with mg of food consumed, there

was no significant difference in average daily kJ consumption per fly between diets when compared to the standard diet (Table 5.1).

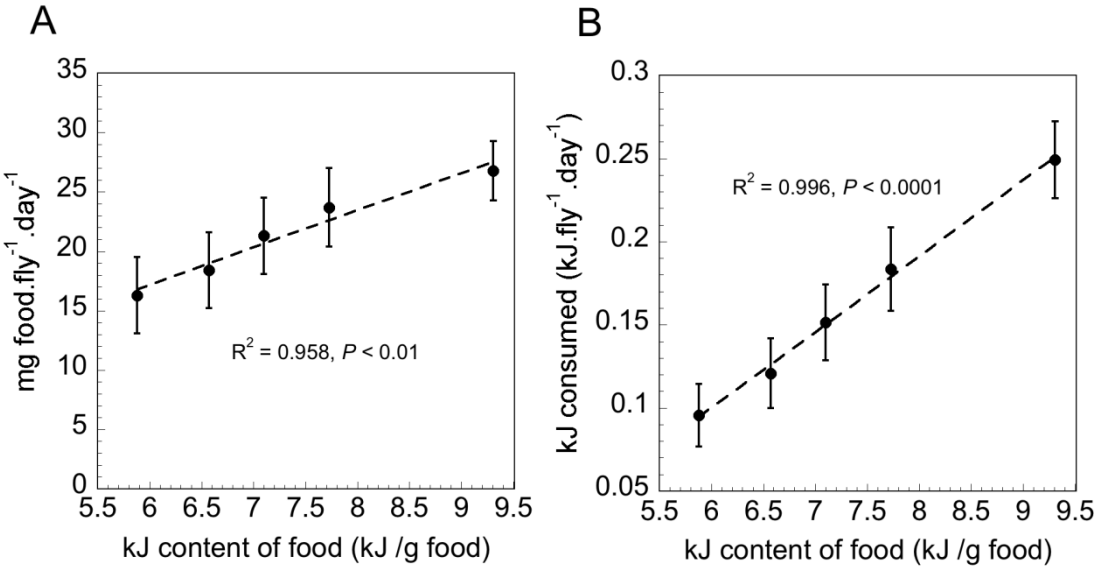

Figure 5.2. Relationship between kJ content of food and the average daily mg and kJ of food consumed per fly in *Calliphora stygia*. Fig. 5.2A, shows a positive correlation with increasing average mg of food consumed with increasing kJ content. The average kJ consumed per fly (Fig. 5.2B) also shows a strong positive correlation with the energy content of the diet. Values are means ± SEM.

The proportions of sugar and yeast consumed were significantly influenced by the sugar content of the diet (Fig. 5.3). Blowflies consumed equal kJ from yeast on all diets ($F_{4, 101}$ = 0.19, P >0.05), while those on the high sugar diet obtained significantly more kJ from sugar compared to blowflies on the standard diet, and blowflies on the lower-energy diets consumed significantly less kJ obtained from sugar ($F_{4, 101}$ = 22.78, P <0.0001, Tukey HSD post-hoc test). As such, blowflies on the 0.5*x* and 0.25*x* sugar-restricted diets have reduced energy consumption compared to the standard diet, while

those on the highest sugar diet have increased energy consumption compared to the standard diet.

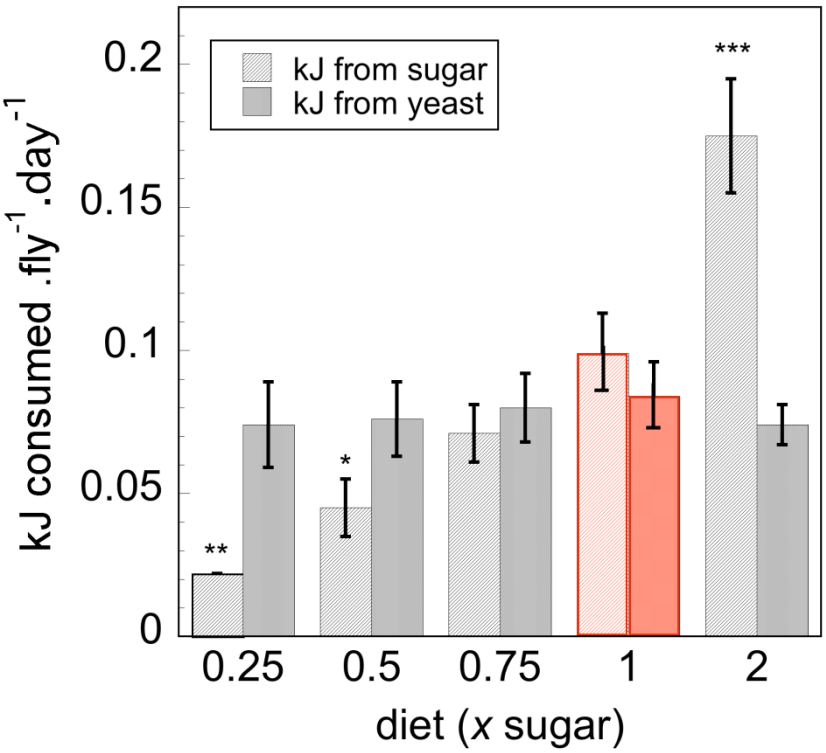

Figure 5.3. Average daily consumption of yeast or sugar (kJ per fly) in *Calliphora stygia* when fed diets varying in sugar content. * represents a significant difference between that treatment and the standard diet (1.0*x*); * P <0.05, ** P <0.01, *** P <0.0001. Values are means ± SEM. Red bars represent the standard diet treatment.

5.2.2 Effects of diet sugar content on egg laying

Sugar content of the diet had no effect on the frequency or duration of egg laying of adult *C. stygia* (Fig. 5.4), suggesting all diets examined were of sufficient energy and nutrient content to prevent malnutrition. Blowflies began to lay eggs after 18

days and egg laying continued to day 45 on average. A few egg laying episodes occurred beyond this time.

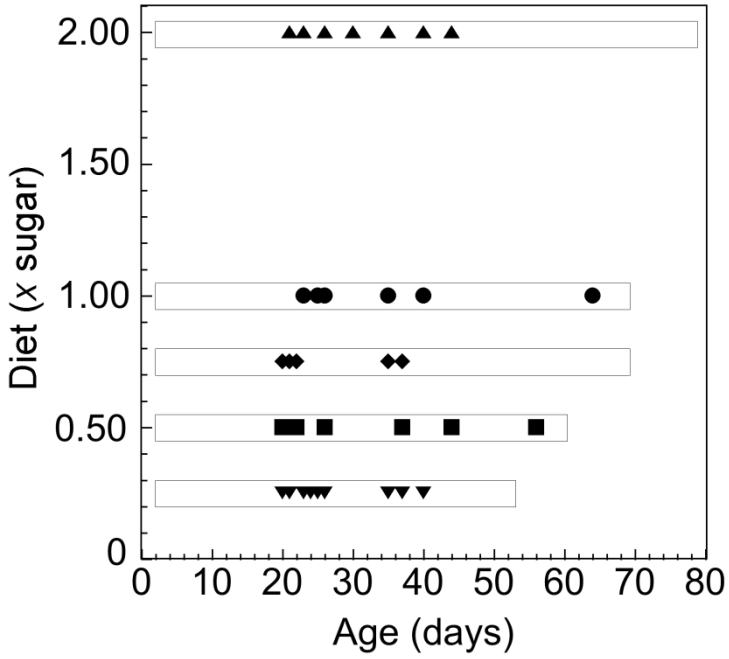

Figure 5.4. Egg laying behaviour of female adult *Calliphora stygia* fed on diets of varying sugar content. Boxes surrounding data represent the maximum lifespan of female blowflies for each diet treatment (n =240, 231, 250, 230 and 223 from diets 0.25x to 2.0x, respectively).

5.2.3 Effects of diet sugar content on longevity

Longevity of blowflies was significantly affected by the sugar content of the diet (Table 5.2). In contrast to energy restriction predictions, it was the blowflies on the highest sugar diet that demonstrated a significant increase in both average longevity (+17%) and maximum longevity (+21%). Blowflies given the moderately sugar-restricted diets (0.75x and 0.5x) showed no change in average longevity, but there was a small significant decrease in maximum longevity on the 0.5x diet. Blowflies on the

lowest sugar content (0.25*x*) diet had significant reductions in both average and maximum longevity.

In addition to longevity increasing with greater sugar content of the diet, both average and maximum longevity were positively correlated with blowfly average daily kJ consumption, which was attributable to their daily sugar consumption as opposed to their daily yeast consumption (Fig. 5.6).

Table 5.2 Comparison of average and maximum longevity of adult *Calliphora stygia* given diets varying in sugar content.

Diet (*x* sugar)	***N***	**Average longevity (days)**	**% Δ from standard**	**Maximum longevity (days)**	**% Δ from standard**
0.25	498	$25.8 \pm 0.8^{***}$	- 28%	$58.7 \pm 1.1^{***}$	- 26%
0.5	464	33.4 ± 0.9^{ns}	- 6%	$69.5 \pm 1.2^{*}$	- 12%
0.75	492	33.6 ± 0.9^{ns}	- 6%	75.6 ± 1.5^{ns}	- 5%
1.0	**491**	**35.6 ± 1.0**		**79.4 ± 1.8**	
2.0	487	$41.6 \pm 1.2^{*}$	+17%	$95.9 \pm 2.5^{*}$	+21%

All values are means (± SEM). Maximum longevity is calculated as the average longevity of the 5% longest-lived animals for each group. Average and maximum longevities are compared to the standard diet (1.0*x*) by Kruskal-Wallis ANOVA with pairs selected using Dunn's multiple comparison post-hoc test. * is * P = <0.05, ** P <0.001 and *** P <0.0001.

5.2.4 Effects of diet sugar content on demographic senescence

Initial mortality was similar for all diet treatments (Table 5.3). Mortality curves were better fit by a two-phase Gompertz than a single Gompertz equation. This revealed the long-lived blowflies on the highest sugar diet to have a significantly longer period of slowed senescence (Fig. 5.5; Table 5.3). There was no effect of high sugar content on the rate of ageing of either the early or late phases. The shortening of longevity when diet sugar is reduced is due to a mixture of effects both on the duration of the early

phase and the rate of ageing compared to the standard diet. In blowflies given the 0.5*x* diet, the effect of the shortened early phase was dominant over the reduced rate of early ageing. There was no significant effect on the rate of late ageing. In contrast, the blowflies on the 0.25*x* diet showed no significant effect on early ageing (either duration or rate). However the large reduction in longevity of these blowflies was associated with a significantly faster rate of late ageing (Table 5.3).

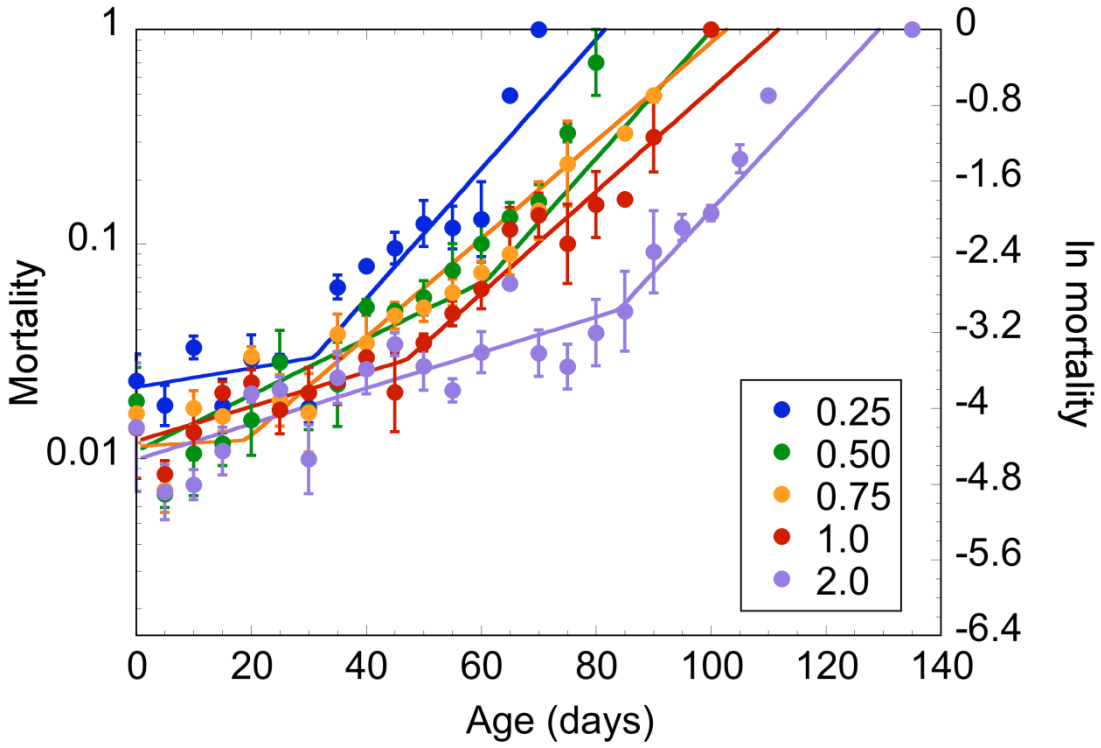

Figure 5.5. Mortality curves of *Calliphora stygia* fed diets of varying sugar content. Two-phase Gompertz equations were fit to the ln(μx) of each diet treatment (see Table 5.3 for parameter estimates and correlation coefficients). Values are averaged over a 5-day period, error bars represent ± one SEM.

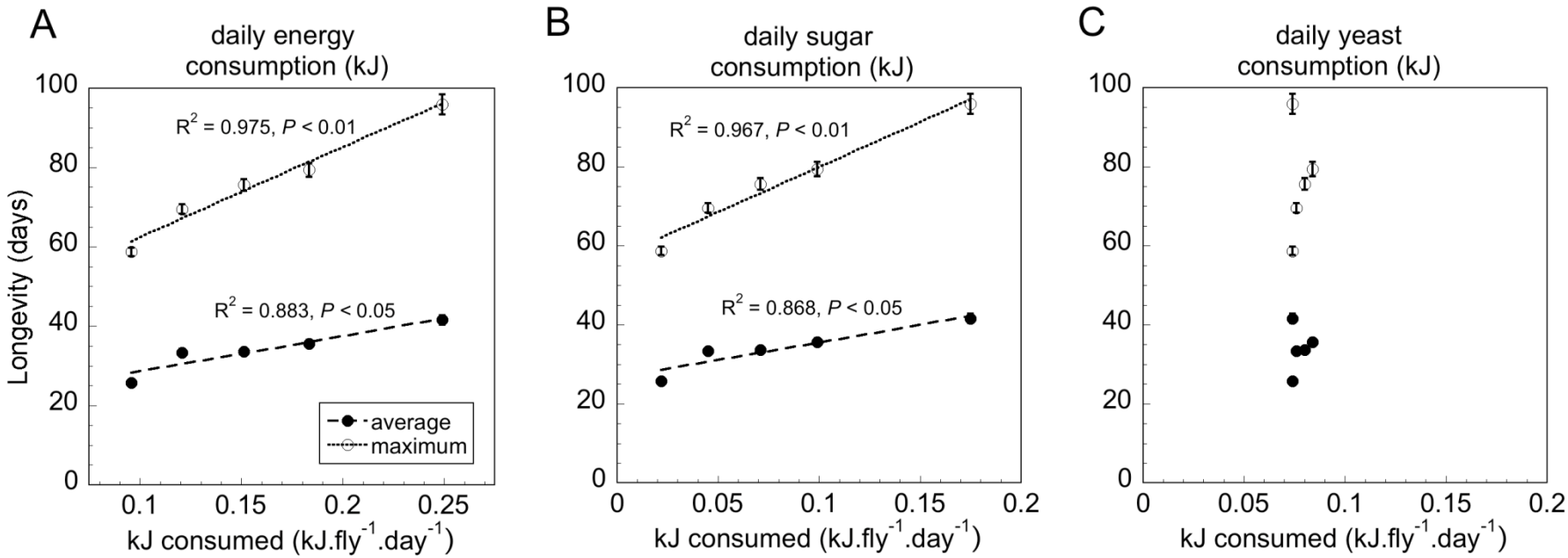

Figure 5.6. Correlation between average and maximum longevity and the daily energy consumption (kJ/g) of *Calliphora stygia* on diets of varying sugar content (Fig. 5.5A). Fig. 5.5B shows daily sugar consumption is responsible for this relationship, while there was no correlation between daily yeast consumption and longevity (Fig. 5.5C). Values are means ± SEM (where error bars are absent, error is less than the marker size).

Table 5.3. Mortality parameter estimates of adult *Calliphora stygia* maintained on diets varying in sugar content.

Diet (*x* Sugar)	**Initial mortality (% per day)**	**Duration of early ageing (days)**	**Early rate of ageing (% Δ in mortality rate.day^{-1})**	**Late rate of ageing (% Δ in mortality rate.day^{-1})**	**Two-phase vs. single Gompertz (R^2)**
0.25	2.4 ± 0.2^{ns}	31 ± 4^{ns}	$1.0 \pm 1.1^{ns\#}$	$7.0 \pm 0.8^{***}$	0.75 vs. 0.67
0.50	1.8 ± 0.3^{ns}	$19 \pm 7^{**}$	$0.3 \pm 2.7^{*\#}$	5.3 ± 0.5^{ns}	0.76 vs. 0.74
0.75	1.3 ± 0.4^{ns}	61 ± 6^{ns}	3.0 ± 0.4^{ns}	6.9 ± 1.2^{ns}	0.77 vs. 0.74
1.0	**1.3 ± 0.4^{ns}**	**47 ± 6**	**1.9 ± 0.6**	**5.5 ± 0.6**	**0.76 vs. 0.71**
2.0	1.2 ± 0.6^{ns}	$84 \pm 5^{***}$	1.9 ± 0.3^{ns}	6.6 ± 1.2^{ns}	0.69 vs. 0.62

Initial mortality, calculated as the average mortality over the first 5-days, was invariant between all treatments (K-W test: $F = 0.58$, $df = 5$, $P > 0.05$). Mortality parameters were estimated on daily ln(μx) data using both linear and segmental linear regression. All parameters were compared to standard diet by examination of 95% C.I. $^{\#}$ indicates a slope not significantly different from 0, indicating no change in mortality rate with age. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$ 'ns' represents no significant difference between treatments.

5.2.5 Effects of diet sugar content on cellular senescence

Fluorescent AGE pigment content was separately measured in head, thorax and abdomen, at two time points (day 0 and day 35) during the lifespan of flies maintained on different sugar diets. Accumulation of AGE pigments was not uniform across the body segments (Fig. 5.7). AGE pigments accumulated over time only in the head region ($F_{5, 30}$ = 16.77, P <0.0001). Negligible amounts were observed in the thorax and did not increase over time ($F_{5, 30}$ = 2.53, P >0.05). A decrease in AGE pigment content was observed in the abdomen ($F_{5, 30}$ = 13.30, P <0.0001). There was no effect of diet treatment on the relative accumulation in any of the body segments.

Whole body content of AGE pigment was calculated by adding together the AGE pigment content of the separate body segments. Fluorescent AGE pigment accumulated over time in these calculated whole body values ($F_{5, 30}$ = 12.66, P <0.0001, Tukey HSD post-hoc test). However, there were no significant differences seen between the diet treatments by day 35 (Fig. 5.7; $F_{4, 25}$ = 2.06, P >0.05).

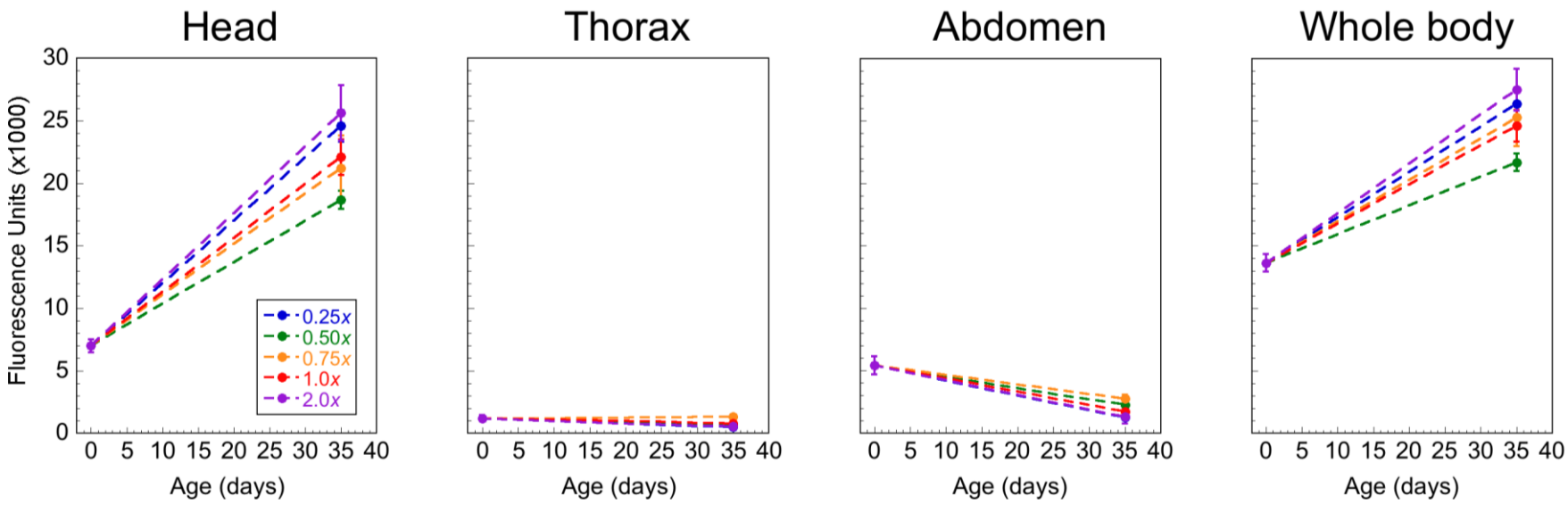

Figure 5.7. Fluorescent AGE pigment accumulation in *Calliphora stygia* given diets of varying sugar content in whole body and separated body parts (head, abdomen and thorax). Data points are means ± SEM (N = 6).

5.3 Discussion

Dietary restriction has been shown to increase longevity and delay the onset of age-related physiological decline in a wide range of organisms (Fontana et al., 2010). The traditional caloric restriction protocol in rodents involves providing animals with less food and, therefore, there is a definite known reduction in food energy consumption. In insects, dietary dilution is used instead of food reduction and it is assumed based on these reduced energy diets, that animals are consuming less energy. In the current study I have measured the food consumption of blowflies given a range of diets. I found that decreasing the food sugar content, and therefore, reducing the energy content of the diet, had a negative effect on longevity. There was no evidence of blowflies increasing consumption of these lower sugar-energy foods, and thus giving blowflies these reduced sugar diets did reduce their energy consumption. This is in contrast to an earlier study on *C. stygia* fed sucrose solutions, which showed that blowflies increased food consumption of a less concentrated sucrose solution, partially compensating for this reduced energy content (Hulbert et al., 2004b). However, those blowflies were not fed yeast and there was no effect on longevity of the different sucrose solutions. Furthermore, Hulbert et al. (2004b) found no correlation between longevity and food consumption of individuals.

Increased food energy consumption in the current study was positively correlated with longevity and blowflies that consumed the greatest amount of energy had significantly extended lifespans. Increased longevity with increased energy consumption is difficult to reconcile in the traditional context of dietary restriction. However, these results are consistent with the more recent investigations of diet

composition on longevity. Three species of insects (two fruit fly species and a species of cricket) fed diets where yeast content was kept constant, have a positive association between longevity and increased sugar consumption (Simpson and Raubenheimer, 2009).

Increases in the average and maximum longevity of flies on the highest sugar diet were not accompanied by any changes to the rate of demographic senescence, but rather were associated with a significantly extended period of slowed senescence. That there was no significant difference in the rate of ageing of either the early or late phase, but a longer delay before the onset of late senescence is consistent with studies of *D. melanogaster*, which have reported no change in the rate of ageing in flies on restricted diets, but a delayed onset of ageing under dietary restriction conditions (Jacobson et al., 2010, Magwere et al., 2004, Mair et al., 2003, Pletcher et al., 2002).

Fluorescent AGE pigment accumulated over time in blowflies at all levels of diet sugar content, however, there was no difference in the rate or level of accumulation between the dietary treatments, nor any evidence of a delayed onset of accumulation that would reflect the demographic senescence observed in the blowflies on the highest sugar diet that had an increased longevity. While correlations between chronological age and AGE pigment concentration have been observed in a range of vertebrates and invertebrates (Terman and Brunk 1998), to date there has been little success in demonstrating a causal link between a reduced rate in the formation of AGEs and longevity extension (Oudes et al., 1998; Gerstbrein et al., 2005). This decoupling of demographic and cellular senescence is consistent with the findings of my previous temperature experiment.

Food energy consumption rates have previously been used to infer metabolic rates in fruit flies in the absence of oxygen consumption measures (Hulbert et al., 2004b). Energy consumption rates can be converted to an approximation of aerobic metabolic rate by assuming 20 kJ is equal to 1 L O_2 consumed (Burton, 2000). Using this conversion, blowflies given the standard diet, who consumed a daily average of 0.18 kJ per fly (average body mass of 80 mg), have an estimated metabolic rate of 4.7 µl $O_2.mg^{-1}.hr^{-1}$, which is consistent with previous oxygen consumption measures in another *Calliphora* species. Active *Calliphora erythrocephala* have metabolic rates ranging between 2.4-8.9 µl $O_2.mg^{-1}.hr^{-1}$, dependant on the age at measurement (Tribe, 1966). This confirms that the food consumption values obtained during the study are a suitable proxy for metabolic rate.

Fruit flies have been shown to consume more food when given carbohydrate rich diets (Edgecomb et al., 1994; Skorupa et al., 2008). Similarly, the amount of food *C. stygia* consumed increased linearly with the sugar content of their food in the current study. Estimating the volumes of food consumed to account for density changes, also shows an increase in the volume of food consumed as the food sugar content increased. *Calliphora stygia* fed the greatest amount of sugar therefore, consumed the greatest amount of food and a greater amount of energy, yet they also had increased average and maximum longevity compared to the blowflies given a standard diet. This presents two main conflicts with the results of traditional dietary restriction studies. Firstly, dietary restriction is often induced in fly models by feeding a diluted diet that contains less calories, and flies have been shown to increase longevity in response to a decreased calorie content of such diets (Edgecomb et al., 1994; Mair et al., 2003; Magwere et al., 2004; Skorupa et al., 2008; Jacobson et al., 2010). Secondly, lifespan extension is

considered to be a result of reduced energy consumption that is demonstrated in rodent models given iso-caloric diets but of reduced volumes, restricting energy intake (McDonald and Ramsey, 2010).

In the first instance, diluted diets are often not measured under corresponding conditions where food consumption is recorded continually, and flies are mostly only assumed to be undergoing dietary restriction. A significant strength of the current study was the measurement of food consumption rate over the entire lifespan of the blowflies. Food consumption was shown to decrease with age, highlighting the insufficiency of a single time-point measure that assumes a constant consumption over the entire lifespan.

Some of the discrepancy between diluted diets causing increasing longevity, and the current findings of increased longevity in *C. stygia* on an energy-dense diet may also be partly explained by the recent findings of Ja et al. (2009) in *D. melanogaster*. They found that fruit flies given a standard diet were suffering dehydration, which negatively affected their longevity, whereas fruit flies given the diluted diets were not under the same water stress. They determined that when fruit flies were provided with water *ad libitum*, flies on the standard diet significantly increased their water consumption, and any longevity differences previously seen between the two treatments were abolished. Therefore, there is the potential that many of the previous studies reporting that diluted diets increase *D. melanogaster* longevity, may be an artefact of dehydration-induced lifespan shortening of fruit flies on the standard diet. As water was provided both *ad libitum* and separately throughout for all dietary treatments, dehydration stress is unlikely to have affected any of my results.

The second conflict, in terms of traditional dietary restriction assumptions, was that blowflies with the highest energy consumption had increased longevity. Mair et al.

(2005) demonstrated in *D. melanogaster*, that diet composition was more important than total calorie content, and that the restriction of yeast had a more significant effect on longevity than did the restriction of sugar. These findings have since been replicated in both *D. melanogaster* (Lee et al., 2008; Skorupa et al., 2008; Ja et al., 2009) and the Queensland fruit fly *B. tryoni* (Fanson et al., 2009; Fanson and Taylor, 2011).

6 EFFECT OF DIET YEAST CONTENT.

6.1 Preface

This chapter aims to further investigate the effects of diet composition on food consumption and demographic and cellular senescence in *C. stygia*, by altering the yeast content of the diet while maintaining the sugar content constant. Yeast is the only source of proteins, vitamins, minerals, and lipids in the SY diet for the blowflies. Therefore, changing the yeast content will affect the provision of each of these nutrients within the diet. I have examined the effects of both an increase and decrease in dietary yeast. Statistical analyses are limited to comparing the different diets to the standard diet, except where otherwise stated. Statistical significance is therefore set at $P < 0.05$.

The 'standard' diet conditions of this chapter are the same as those of the previous chapter, however this experiment was performed at a different time of year and on a different batch of pupae than the previous sugar content experiment. There are small (but significant) differences between the two standard diet populations and as a result these chapters are examined separately (see Appendix D for a comparison of 'control' cohorts between experiments).

6.2 Results

6.2.1 Effects of diet yeast content on food consumption

Compared to the standard fly diet, the energy content of the five diets ranged from an increase of 11% to a decrease of ~30% (Table 6.1). As the energy content of the food was decreased, blowflies changed their feeding behaviour and consumed increasingly greater amounts of food, which peaked when food had an energy content

level of ~6.5 kJ/g food (Fig. 6.1A). This compensatory feeding behaviour resulted in blowflies consuming significantly more food of the 'dietary restriction' treatments that had reduced yeast content (0.5*x* and 0.25*x*). Increasing the yeast content had a suppressive effect on feeding, with blowflies consuming significantly less food on the highest yeast diet. Eliminating the yeast from the diet had no effect on the amount of food consumed when compared to the standard fly food (Table 6.1). Despite these compensatory feeding changes, food consumption showed a similar temporal pattern for all treatments with blowflies decreasing the amount of food consumed with age (Fig. 6.2).

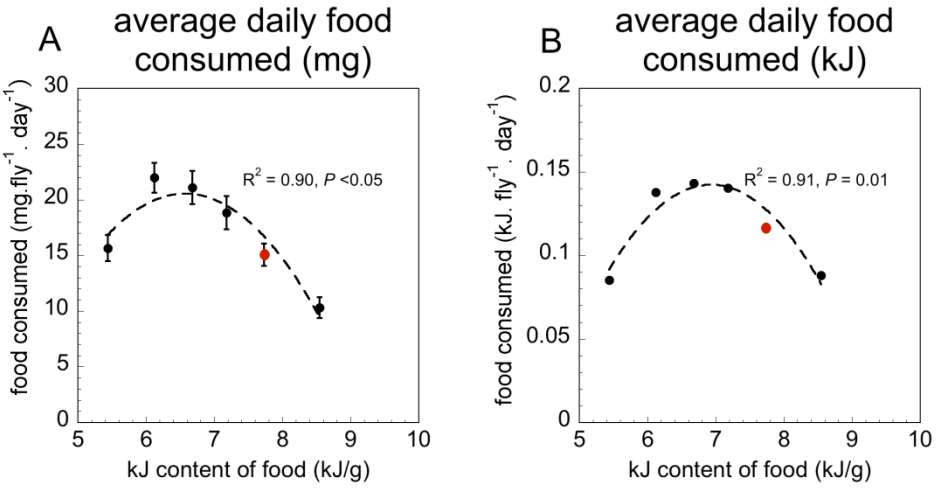

Figure 6.1. The relationship between the energy content of food and the average daily food consumption of *Calliphora stygia* in either: Fig 6.1A. amount of food consumed or Fig. 6.1B. energy consumed from food. Values are means ± SEM (where error bars are absent, error is less than the marker size). Red markers represent the standard treatment.

Table 6.1. Composition and energy content of diets and respective food consumption of *Calliphora stygia*.

Treatment (*x* sugar of standard diet)	Sugar content (g/100g food)	Yeast content (g/100g food)	Energy content (kJ/g)	Density of food (g/ml)	Average daily food consumption (mg.fly^{-1})	(kJ.fly^{-1})	(µl.fly^{-1})$^{\$}$	% Δ in daily kJ consumed from standard diet
0.0	34.0	0.0	5.44	0.41	15.7 ± 1.2^{ns}	0.09 ± 0.01^{ns}	13.1	- 26%
0.25	31.5	7.9	6.12	0.47	22.0 ± 1.3**	0.13 ± 0.01^{ns}	18.4	+15%
0.50	29.3	14.6	6.68	0.52	21.2 ± 1.5*	0.14 ± 0.01^{ns}	17.8	+20%
0.75	27.4	20.5	7.18	0.57	18.8 ± 1.5^{ns}	0.13 ± 0.01^{ns}	15.8	+15%
1.0	**26.1**	**26.1**	**7.73**	**0.60**	**15.1 ± 1.0**	**0.12 ± 0.01**	**13.1**	
1.5	23.5	35.2	8.55	0.67	10.3 ± 0.9*	0.09 ± 0.01^{ns}	9.1	- 25%

Standard diet is in bold. Calculation of energy content is based on final food weight and the relative weight contributions of both sugar and yeast. Kilojoule content of yeast (*Saccharomyces cerevisiae*, Brewers yeast, Enoferm M1, Lallemand, SA) and sugar was based on estimations from Bass et al., (2007) and USDA database (sugar = 16 kJ/100g, yeast = 13.6 kJ/100g). Average daily food consumption (mg and kJ per fly) is calculated as the average over the measurement period from replicate cages. $^{\$}$Average volume of food consumed was estimated from the average daily food consumption in mg and therefore no statistical analysis was performed. * P <0.05, ** P <0.01, *** P <0.0001, 'ns' represents no significant difference between treatments. Sample sizes for each diet population (combined replicate cages) are 0.0*x* N = 734; 0.25*x* N = 742; 0.50*x* N = 758, 0.75*x* N = 746; 1.0*x* N = 758 and 1.5*x* N = 731 flies respectively.

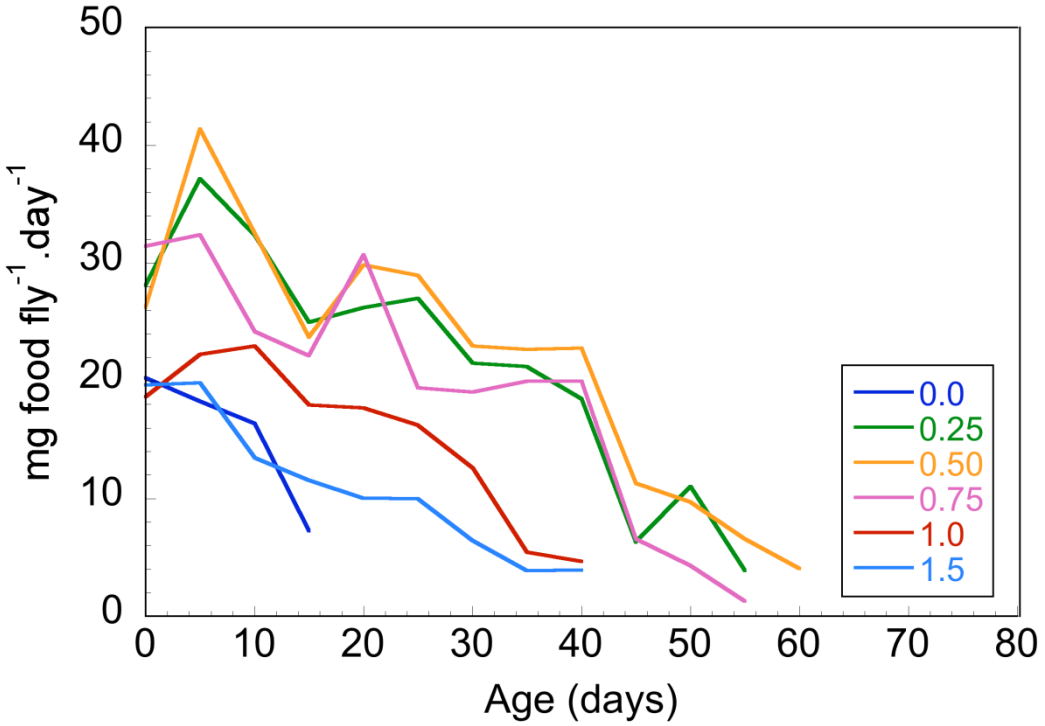

Figure 6.2. Daily food consumption (in mg) of *Calliphora stygia* fed diets varying in yeast content. Food consumption decreased over time for all diets. Data are averages of 5-day periods, with error bars omitted for clarity (overall averages ± SEM are presented in Table 6.1; see Appendix B for daily averages ± SEM).

The increase in food consumption on the restricted-yeast diets resulted in blowflies in all treatments consuming equal amounts of energy compared to the standard diet treatment (Table 6.1). However, when diets were compared over the range of dietary energy content there was a significant peak of energy consumption on the lower yeast diets of which more food was consumed (Fig. 6.1B). Because yeast content was the nutritional component altered in this experiment, for blowflies to consume similar levels of yeast, they had to consume significantly more sugar (Fig. 6.3).

Blowflies given the moderate yeast diets (0.5*x* and 0.75*x*) successfully compensated for the reduced yeast content, and consumed similar amounts of energy from yeast content as those fed the standard diet. For blowflies given the lowest yeast diet (0.25*x*), their increased food consumption did not totally compensate for the reduced availability of yeast. Blowflies consumed, on average, significantly less energy from yeast than those fed the standard diet, and significantly more sugar. Increasing the yeast content of the diet suppressed feeding and blowflies given this diet consumed similar amounts of yeast, but significantly less sugar as a result (Fig. 6.3).

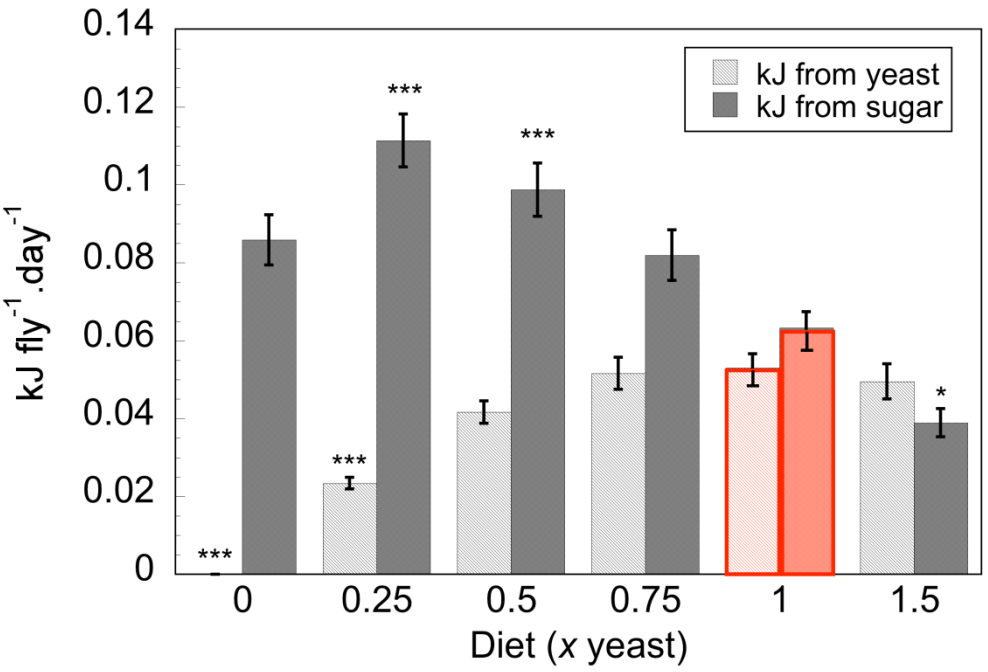

Figure 6.3. Average daily energy consumption of sugar or yeast in *Calliphora stygia* fed diets varying in yeast content. * represents a significant difference between that treatment and the standard diet (1.0*x*); * P <0.05, ** P <0.01, *** P <0.0001. Values are means ± SEM. Red bars represent the standard diet treatment.

6.2.2 Effects of diet yeast content on egg laying

Egg laying was dramatically affected by altering the yeast content of the diet (Fig. 6.4). Yeast was necessary for egg laying, with blowflies given diets containing less than 50% standard yeast content (0.5*x* and below), having a reduced egg laying frequency with no eggs layed by blowflies on either the very low (0.25*x*) or yeast-free (0.0*x*) diets. The reduced egg laying on the 0.5*x* diet is not associated with yeast consumption. Blowflies consumed a similar average amount of energy from yeast as the other diets that supported normal egg laying behaviour. It may be that high sugar consumption has a suppressive effect on egg laying, as blowflies on the 0.5*x* consumed significantly increased amounts of sugar compared to the other diets (Fig. 6.3). Alternatively, the balance between yeast and sugar in the diet may affect signalling mechanisms that control reproductive behaviour.

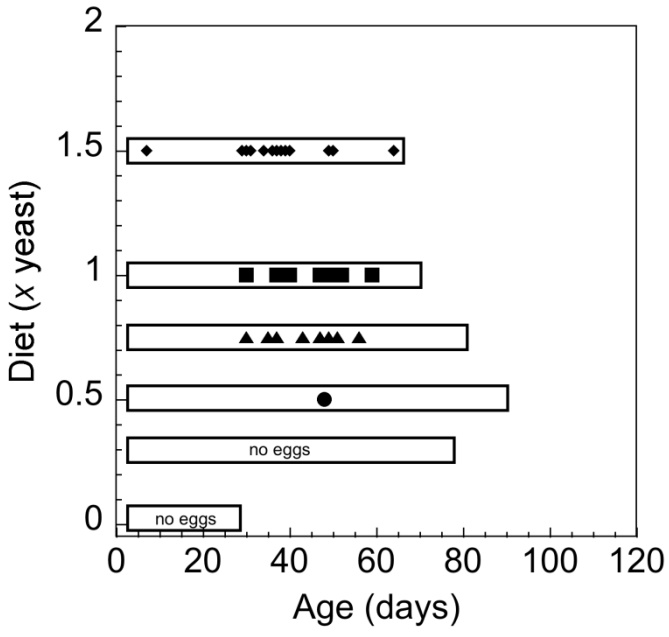

Figure 6.4. Egg laying frequency of *Calliphora stygia* fed diets varying in yeast content. Boxes represent female maximum longevity (calculated as the average of the longest-lived 5% of females). Data points represent each day eggs were found in the cages (*N* = females per treatment, 0.0*x* = 376, 0.25*x* = 378, 0.5*x* = 425, 0.75*x* = 380, 1.0*x* = 412, and 1.5*x* = 346).

6.2.3 Effects of diet yeast content on longevity

Dietary yeast content had a significant effect on the longevity of *C. stygia* (Table 6.2). Blowflies fed the standard diet lived for an average of 42 days, with a maximum longevity of 71 days. Longevity peaked when the yeast content of the diet was halved. This dietary treatment had a much greater effect on maximum longevity (+28%) than on average longevity (+16%). Longevity was also significantly increased on the more moderate reduction of 0.75*x*, with a similar relative increase in both average and maximum longevity.

Longevity was dramatically decreased when yeast was removed from the diet. Both average and maximum longevity were decreased by ~60% on the yeast-free diet (Table 6.2). Increasing the yeast content of the diet above standard diet levels also reduced average longevity, but it had no effect on maximum longevity.

In contrast to predictions that increased energy consumption reduces longevity, there was a significant positive correlation between total energy consumption and both average and maximum longevity (Fig. 6.5A). There was no relationship between sugar energy consumption and longevity (Fig. 6.5B). Although differences in the yeast energy consumption were not statistically significant, the correlation between yeast energy consumption and longevity (Fig. 6.5C) was much greater than that between sugar energy consumption and longevity (R^2 = 0.64 and 0.60 versus R^2 = 0.02 and 0.02 for average and maximum longevity respectively).

Table 6.2. Longevity of adult *Calliphora stygia* maintained on diets of varying yeast content.

Diet (*x* yeast)	**(*N*)**	**Average longevity (days)**	**% Δ from standard diet**	**Maximum longevity (days)**	**% Δ from standard diet**
0.0	734	$17.0 \pm 0.2^{***}$	- 59%	$27.0 \pm 0.6^{***}$	- 62%
0.25	742	44.2 ± 0.7^{ns}	+ 7%	$77.7 \pm 1.0^{**}$	+10%
0.5	758	$48.0 \pm 0.9^{***}$	+16%	$90.6 \pm 1.0^{***}$	+28%
0.75	746	$47.0 \pm 0.9^{***}$	+14%	$84.7 \pm 1.1^{***}$	+19%
1.0	**758**	**41.5 ± 0.7**		**70.9 ± 0.8**	
1.5	731	$37.7 \pm 0.7^{**}$	- 9%	68.0 ± 0.6^{ns}	- 4%

All values are averages (± SEM). Maximum longevity is calculated as the average longevity of the 5% longest-lived animals for each group. Average and maximum longevities are compared to the standard diet (1.0*x*) by Kruskal-Wallis ANOVA with pairs selected using Dunn's multiple comparison post-hoc test. * is * P <0.05, ** P <0.001 and *** P <0.0001, 'ns" represents no significant difference.

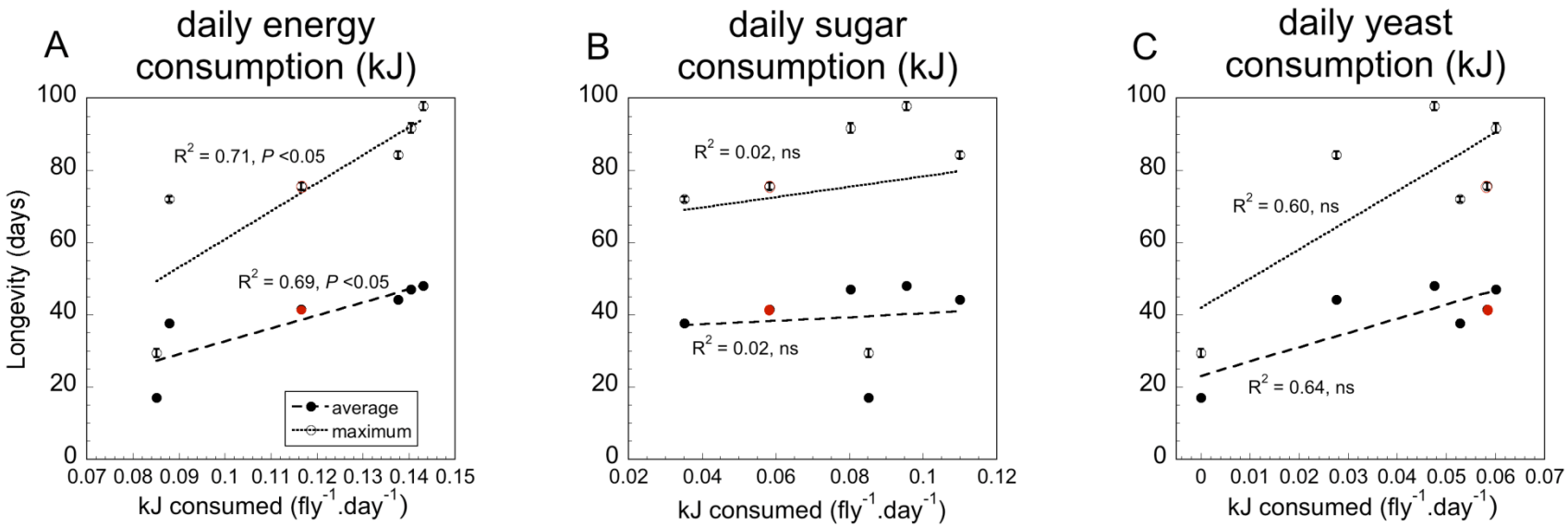

Figure 6.5. Correlation between average and maximum longevity and the daily energy consumption (kJ/g) of *Calliphora stygia* on diets of varying yeast content (Fig. 6.5A). This positive correlation between energy consumption and longevity disappears when energy consumption is described as obtained from either: sugar consumption (Fig. 6.5B), or yeast consumption (Fig. 6.5C). Red markers represent the data points for the standard diet conditions. Values are means ± SEM (where error bars are absent, error is smaller than the marker size).

6.2.4 Effects of diet yeast content on demographic senescence

There were similar levels of initial mortality between flies on all diet treatments (Table 6.3). Blowflies given yeast-free diets had a very rapid rate of ageing during the early phase, which was for a similar length of time as their average longevity (Fig. 6.6). The rate of ageing of blowflies on this treatment was then slowed during the late phase of ageing. Increasing the yeast content of the diet had little effect on the longevity of blowflies and likewise there were no differences in the trajectory of mortality rates between the blowflies given the high-yeast (1.5*x*) and standard diets.

The biphasic mortality pattern (described by the two-phase Gompertz) was absent in the three treatments where yeast content was reduced (Fig. 6.6). Yeast, therefore, appears to be necessary for this early phase of slow ageing. A single Gompertz was fit to these treatments, and the two treatments with significantly increased average and maximum longevities had a corresponding decreased rate of ageing (Table 6.3). The lowest yeast treatment (0.25*x*) had a rate of ageing not significantly different from the standard dietary treatment, despite a 10% increase in maximum longevity compared to the standard diet treatment.

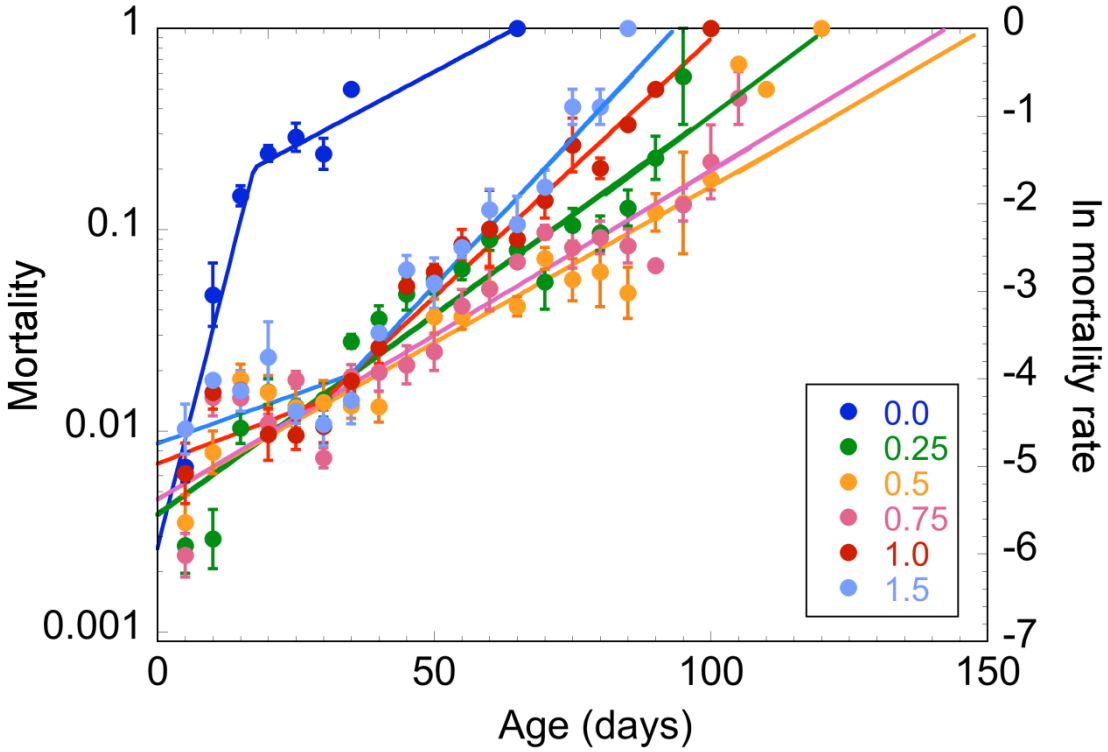

Figure 6.6. Mortality curves of *Calliphora stygia* fed diets varying in yeast content. Both single and two-phase Gompertz equations were fit to the ln(μx) of each diet treatment, with standard, 1.5*x* and 0.0*x* diets being best fit by a two-phase Gompertz and the restricted-yeast diets of 0.25*x*, 0.5*x* and 0.75*x* being best-fit by a single Gompertz (see Table 6.3 for parameter estimates and correlation coefficients). Values are averaged over a 5-day period, error bars are ± one SEM.

Table 6.3. Mortality parameter estimates of adult *Calliphora stygia* maintained on diets of varying yeast content.

Diet (*x* Yeast)	**Initial mortality (% day^{-1})**	**Early rate of ageing (% Δ in rate.day^{-1})**	**Length of early phase (days)**	**Late rate of ageing (% Δ in rate.day^{-1})**	**Rate of ageing$^{\$}$ (single Gompertz)**	**Two-phase vs. single Gompertz (R^2)**
0.0	0.5 ± 0.3^{ns}	$25.0 \pm 2.4^{*}$	$17 \pm 1^{*}$	3.4 ± 1.3^{ns}	$11.13 \pm 1.4^{***}$	0.91 vs. 0.69
0.25	0.3 ± 0.4^{ns}				4.6 ± 0.2^{ns}	na vs. 0.83
0.50	0.5 ± 0.2^{ns}				$3.6 \pm 0.2^{***}$	na vs. 0.77
0.75	0.4 ± 0.2^{ns}				$3.8 \pm 0.2^{**}$	na vs. 0.81
1.0	**0.4 ± 0.3^{ns}**	**2.4 ± 1.1**	**30 ± 7**	**5.9 ± 0.5**	**4.9 ± 0.3**	**0.84 vs. 0.82**
1.5	0.4 ± 0.2^{ns}	2.3 ± 1.0^{ns}	35 ± 6^{ns}	6.7 ± 0.6^{ns}	4.9 ± 0.3^{ns}	0.81 vs. 0.77

Initial mortality, calculated as the average mortality over the first 5-days was invariant between treatments (ANOVA; $F = 0.58$, $df = 5$, $P > 0.05$). Mortality parameters were estimated on daily ln(μx) data by both linear (single Gompertz) and segmental-linear (two-phase Gompertz) regression. Where columns are blank a two-phase Gompertz could not be fit to the data and parameters for a single Gompertz only are given. All parameters from the two-phase Gompertz were compared to standard diet by examination of 95% C.I. Slopes from single Gompertz were compared to standard diet by an F-test, to determine differences in the rate of ageing. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$, 'ns' represents no significant difference between treatments.

6.2.5 Effects of diet yeast content on cellular senescence

Fluorescent AGE pigment was measured separately in the head, thorax and abdomen segments at day 55, except for the blowflies on the 0.0*x* diet, where it was only measured at day 16 (Fig. 6.7). As with previous chapters, AGE pigment was only found to accumulate in the head region for all dietary treatments ($F_{6, 35}$ = 11.62, P <0.0001; Dunn's post-hoc test). A significant decrease in AGE pigment was found over time in both the abdominal ($F_{6, 35}$ = 4.22, P <0.01) and thoracic segments ($F_{6, 35}$ = 6.65, P <0.001). At day 55, there was no effect of diet yeast content on the level of AGE pigment ($F_{4, 25}$ = 1.05, P >0.05). The limited sampling times of this study prevents the measurement of the actual rate of pigment accumulation in the head segment. The apparent faster accumulation of AGE pigment in blowflies fed the yeast-free diet may not represent a truly faster rate as it was apparent in the temperature experiment blowflies (Fig. 3.7) that AGE pigment accumulation is not linear and shows a faster accumulation early in adult life.

The values for the three segments were added together to estimate whole body AGE pigment content. There was a significant accumulation of pigment over time for all diets, but diet yeast content did not affect the level of pigment accumulation by day 55 ($F_{4, 25}$ = 1.30, P >0.05).

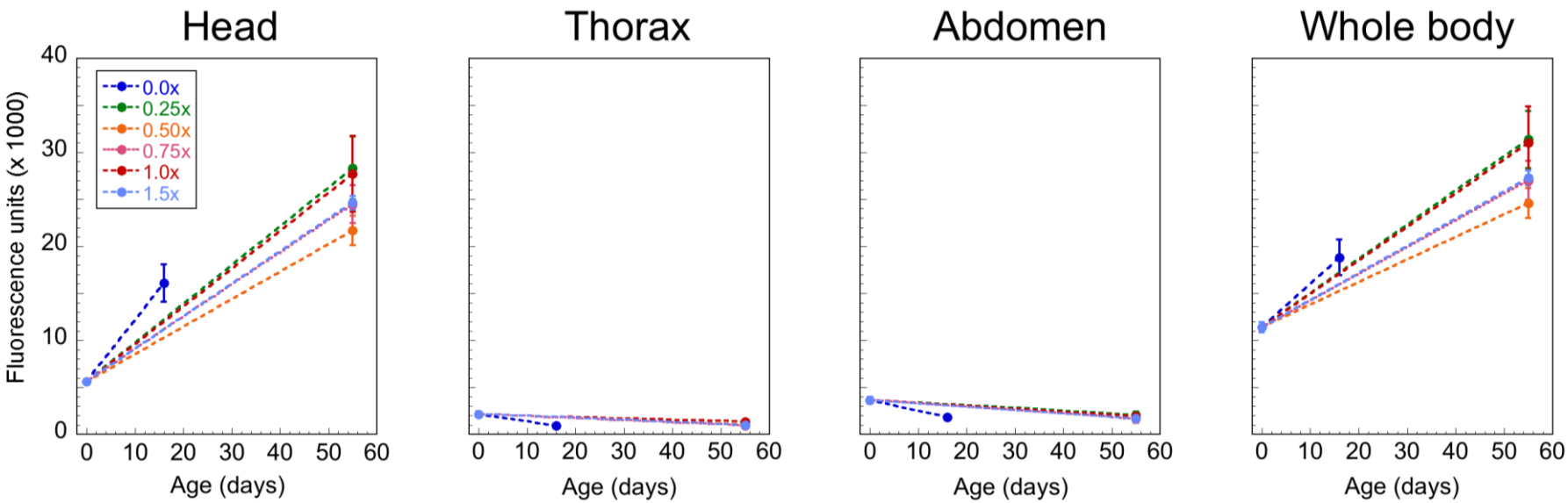

Figure 6.7. Fluorescent AGE pigments found in separated segments and estimated for whole body, of *Calliphora stygia* fed diets of varying yeast content. Values are means ± SEM (*N* = 6). Where error bars are absent, error is less than the marker size.

6.3 Discussion

Consistent with my findings, other invertebrate studies have found that dietary restriction, by reducing yeast alone, is sufficient to extend longevity (Mair et al., 2005; Lee et al., 2008; Fanson et al., 2009; Ja et al., 2009; Fanson and Taylor, 2011). Halving the yeast content of the diet in *C. stygia* increased maximum longevity by 28%, compared to blowflies given the standard diet. Food consumption measures showed that blowflies on the half-yeast diet consumed greater amounts of food than those on the standard diet. This compensatory increase in food consumption resulted in blowflies on the half-yeast treatment consuming similar amounts of both yeast and total energy to those fed the standard fly diet. These findings are in contradiction to energy consumption being the mechanism behind the dietary restriction extension of lifespan. An estimation of metabolic rate can be made from food consumption measures (see previous chapter). Using these conversions, estimated metabolic rate of *C. stygia* at 25°C on standard diet in the current study is 3.13 µl $O_2.mg^{-1}.hr^{-1}$. This is similar to oxygen consumption rates measured in a similar blowfly species during activity (2.4 -8.9 µl $O_2.mg^{-1}.hr^{-1}$; Tribe, 1966), as well as food consumption estimates from the previous chapter (4.7 µl $O_2.mg^{-1}.hr^{-1}$, Chapter 5). This indicates the current measurement of food consumption is an accurate reflection of energy expenditure.

Calliphora stygia responded to changes in dietary content of yeast by altering their food consumption. Blowflies fed on the diet of highest yeast content had the lowest average food consumption, which agrees with studies of *D. melanogaster* showing increased dietary yeast content to suppress feeding rate (Skorupa et al., 2008). Blowflies responded to dietary restriction of yeast by consuming greater amounts of food. The greatest volume of food was consumed on the reduced yeast

diets of 0.25*x* and 0.5*x*, and although it was not statistically significantly different from the blowflies given the standard diet, this increased food consumption resulted in blowflies also having a higher average daily energy intake on these diets.

The most obvious difference between the dietary intake of energy between the treatments was the source of the calories. *Calliphora stygia* given diets with half the relative content of yeast, consumed equal amounts of yeast-based energy as those on the standard diet but significantly more sugar-based energy. Blowflies given the diet with a quarter of the yeast, also consumed significantly more sugar, but significantly less yeast compared to blowflies given standard diets. These treatments with higher energy intake and higher sugar intake also had increased maximum longevity over the blowflies fed a standard diet. This suggests a positive influence on longevity as a result of either an increased sugar intake, or a particular balance between yeast and sugar. Indeed, extended longevity as a result of altering the balance between yeast and sugar has also been determined in fruit flies (Mair et al., 2005; Lee et al., 2008; Skorupa et al., 2008; Fanson et al., 2009; Ja et al., 2009; Fanson and Taylor, 2011).

Extended longevity is often thought to be at the expense of fecundity. A trade-off between reproduction and longevity is considered an evolutionary adaptation that maximises resources during periods of famine (Grandison et al., 2009). During food limited periods, resources are diverted from reproduction towards somatic maintenance, which favours longevity (Mair and Dillin, 2008). Reduced fecundity during dietary restriction has been demonstrated in *D. melanogaster* (Chapman and Partridge, 1996). The provision of a single essential amino acid, methionine, during dietary restriction in fruit flies was sufficient to restore fecundity to levels observed during full feeding, without affecting the extended longevity observed during dietary restriction (Grandison et al., 2009). Rather than any fixed intake requirements,

Grandison et al. (2009) suggest that it is the balance of essential nutrients in the diet that has the greatest effect on fecundity and longevity.

In the current study reproductive output was not measured, however, there was a reduced frequency of egg laying in the blowflies given diet containing half the amount of yeast of standard diet and no eggs laid when blowflies were given diets containing only 25% of the yeast of standard diets. These two treatments had greater longevity than the standard diet, yet this reduced egg laying was not associated with the actual quantities of yeast consumed. Blowflies given the half-yeast diet consumed more energy overall, and in terms of source of energy intake, similar amounts of yeast to the standard diet. Furthermore, blowflies given the modestly reduced yeast diet (75% of standard) had extended longevity compared to those given the standard diet, but a similar frequency of egg laying.

Flies given diet choice experiments voluntarily eat both sugar and protein (provided as yeast) but at levels that maximise reproductive output rather than maximise longevity (Lee et al., 2008). The three diets that resulted in reduced or no egg laying in the current study were all associated with increased sugar intake. Increased dietary sugar content has been shown to suppress egg laying rates in *D. melanogaster* (Skorupa et al., 2008). It is therefore likely that rather than protein deficiency causing reduced reproduction, increased sugar intake has suppressed reproduction. This could be a result of either the sugar alone, or the perceived imbalance of nutrients in the diet (Grandison et al., 2009).

Two previous studies of this species (Part A and Chapter 5) suggest that their life history under standard conditions (standard diet, 25°C) is best described in two phases, with a relatively low rate of change in mortality in the early phase, followed by a faster rate of ageing in the late phase which continues until death. This contrasts

with the pattern resulting from the two-phase Gompertz mortality rates in *Drosophila* that typically describe a deceleration of mortality in old age (Shahrestani et al., 2012). Interestingly, yeast appears essential for the biphasic mortality pattern of *C. stygia*. Blowflies given diets of restricted yeast content did not show the typical two-phases of ageing. Instead, the yeast-restricted diets that extended longevity were best described by a single relationship with a slowed rate of ageing compared to the standard diet. The biological significance of such variation in mortality patterns is unknown (Wilson, 1994), although they are not uncommon within species in response to treatment or between genders, and have been described in *D. melanogaster* (Promislow and Haselkorn, 2002; Bross et al., 2005).

The lack of a biphasic mortality curve in *C. stygia* given yeast-restricted diets could effectively be the result of two changes: i) an increase in their rate of early ageing and ii) a reduction in their late rate of ageing, such that there no longer exists a significant difference between the two phases. These yeast-restricted blowflies had significantly increased average and maximum longevity, despite a lack of reduced early ageing, and the dietary influence on maximum longevity was comparatively greater than on average longevity. This may indicate that increased dietary yeast may have a positive influence on the health of *C. stygia* during early ageing.

These findings that reduced yeast content changes the rate of ageing contrasts with previous findings of dietary restriction-related longevity extension in *D. melanogaster* which has been associated with a delay in the onset of senescence rather than a decreased rate of ageing (Mair et al., 2003). However, the study of *D. melanogaster* was based on diets that had both the sugar and yeast content diluted, rather than any change in the dietary balance between these nutrients. In the current study it was the restriction of dietary yeast content, not actual yeast consumption that

affected the biphasic mortality pattern of *C. stygia*, suggesting that the dietary balance of nutrients may be responsible for this relationship.

Demographic senescence describes the ageing process at a population level, while markers of cellular damage, such as fluorescent AGE pigments may indicate the process of cellular senescence. Fluorescent AGE pigments are implicated in a number of age-related disease pathologies such as diabetes and Alzheimer's (Oudes et al., 1998). As has been observed previously in this thesis, AGE pigment was accumulated only in the head region of the blowfly, with negligible amounts found in the abdomen and thorax that disappear over time. At the cellular level, AGE pigments are thought to accumulate in the lysosomes and are unable to be degraded, accumulating over time in post-mitotic cells (Terman and Brunk, 2004). While mitotic cells can dilute the content of AGE pigments during proliferation, this is unlikely to be the case in *C. stygia*, as most adult cells are postmitotic (Johnson et al., 1999). However, this pattern of segment-specific accumulation has been demonstrated in both the temperature experiments, and the other diet composition experiment in this thesis and may indicate a difference in the susceptibility of different tissues to ageing in the blowfly.

Within the head, AGE pigments accumulated with time for all treatments. As only two time points were measured for each treatment, this limited sampling does not allow for any detailed descriptions of the trajectory of accumulation. Blowflies given yeast-free diets appeared to have a more rapid increase in AGE pigments, however, as was previously demonstrated in Chapter 3, blowflies have more accumulation of AGE pigment initially and this may be what is being observed during this dramatic increase in AGE pigments in the blowflies given the yeast-free diet. It has been theorised that alteration or reduction of AGE pigment formation may

extend longevity and reduce age-related disease (Terman and Brunk, 2004). However, despite slowing the rate of ageing and extending longevity, reduction of dietary yeast had no effect on the accumulation of AGE pigments. This decoupling between demographic senescence and cellular senescence suggests that AGE pigments may be more a consequence of time rather than a cause of ageing.

PART C

DIET FATTY ACID PROFILE, MEMBRANE FATTY ACID COMPOSITION AND LONGEVITY

7 EFFECT OF DIETARY FATTY ACIDS

7.1 Preface

An obvious test of the membrane pacemaker theory of ageing is to experimentally alter membrane fatty acid composition and observe whether this affects longevity. Such alteration of membrane fatty acid composition has been attempted a number of times for *C. stygia* by manipulation of the dietary fatty acid profile. Unfortunately, these attempts have been relatively unsuccessful at altering membrane composition and are currently unpublished (Usher and Hulbert, personal communication). These previous attempts have generally used a variety of oils (both vegetable and fish) to modify larval and adult food. In all of these studies membrane fatty acid composition was only examined at one time point during adult life and then compared to the membrane composition of flies measured on the day of eclosion. The commercial breeder of *C. stygia* from which I have sourced my pupae, rears the blowfly larvae on a diet of fish carcasses obtained from a local abattoir. Thus phospholipids extracted from adult blowflies at the time of eclosion will show the effects of the fish-based larval diet and not that given to the adults.

The results of one of these previous studies, examining the effects of olive oil added to standard adult diet in various amounts, has been published (Ujvari et al., 2009). This study showed that too much added dietary fat (>5 g/ 100 ml diet) dramatically decreased longevity. However, this was not due to differences in membrane fatty acid composition of blowflies on the different diets. It became obvious from this and the other unpublished work that it was necessary to examine in detail the relationship between diet fatty acids and membrane changes during the adult life of *C. stygia*. This chapter reports the results of two experiments examining

the relationship between dietary fatty acid profile and membrane (phospholipid) fatty acid composition of *C. stygia* during their adult life.

Firstly, the effect of individual 18-carbon fatty acids (as methyl esters) added to the standard SY diet at moderate levels (3 g/ 100 ml food) on the fatty acid composition of whole blowfly phospholipids and isolated thoracic mitochondrial phospholipids, was measured over the first four to six weeks of adult life. Secondly, the effects of two highly polyunsaturated oil mixtures (fish oil and krill oil) added to the standard SY diet on whole blowfly phospholipids was measured over the first six weeks of adult life. The emphasis of these two experiments is to look at the effect of diet fatty acid profiles on membrane composition. The expense of the methyl esters added to the diet in the first experiment, limited the numbers of blowflies used in the populations to ~120 blowflies (compared to the ~700 flies of earlier chapters). Longevity and demographic senescence were only measured in the first experiment.

7.2 Results

7.2.1 Effect of dietary fatty acid profile on phospholipid fatty acid composition and longevity of the blowfly

All of the earlier unpublished studies on *C. stygia* have isolated phospholipids from whole blowflies on which to determine membrane fatty acid composition. I have also done this in the current study. As well, I have isolated mitochondria from blowfly thoraxes to determine if mitochondrial membranes show similar changes to whole blowfly phospholipids. Results for both sources of phospholipid are presented in this chapter. The sequence with which I will present the results of the next section is as follows: i) a comparison of the fatty acid composition of phospholipids from

these two sources as extracted from blowflies at the beginning of their adult life (i.e. on the day of eclosion), ii) changes of phospholipid composition during adult life of blowflies fed standard SY diet, iii) effect of 18-carbon fatty acids added to the diet on the composition of phospholipids over the first four to six weeks of adult life and their effects on longevity.

7.2.1.1 Comparison of fatty acid composition of phospholipids from whole blowflies and thoracic mitochondria

The fatty acid composition of phospholipids from whole blowfly and isolated thoracic mitochondria are presented in Table 7.1. These data are from blowflies at the beginning of their adult life (day of eclosion) and there is therefore no influence of the standard adult diet. There are no substantial differences between these two phospholipids from these two sources at this life stage.

In phospholipids from both sources, the dominant fatty acids were the monounsaturated fatty acids (MUFA) 18:1*n*-9 and 16:1*n*-7 (~30% and ~20% total fatty acids respectively), with these two accounting for most of the MUFA content in the phospholipids. Of the saturated fatty acids (SFA), 16:0 was the most common and was responsible for most of the SFA content. Although these blowflies were fed on fish carcasses during the larval stage, there are relatively low amounts of 22:6*n*-3 and 20:5*n*-3 polyunsaturated fatty acids (PUFA), compared to the high levels of these fatty acids in fish (and thus high in the larval diet). Both the *n*-6 and *n*-3 PUFA were relatively equally represented, as shown by the PUFA balance (which is the % of total PUFA that is comprised by *n*-3 PUFA). The *n*-6 PUFA were dominated by

18:2*n*-6, with small amounts of the 20-carbon fatty acid 20:4*n*-6. While the *n*-3 PUFA were dominated by 20:5*n*-3 with small amounts of 18:3*n*-3 and 22:6*n*-3.

The only significant differences between the two sources of phospholipids were seen in two minor SFA, 15:0 and 17:0 (which together account for <1% of total fatty acids). These results suggest that measurements of whole blowfly phospholipids are sufficient to represent membrane fatty acid composition.

7.2.1.2 Changes in phospholipid fatty acid composition of blowflies given standard SY diet during adult life

Yeast contains approximately 1.5 g of lipids for each 100 g of yeast and is the only source of dietary fatty acids for adult flies fed the standard diet. The fatty acids in standard SY food is approximately 75% MUFA, 25% SFA and only 0.2% PUFA (Fig. 7.1). The main MUFA are 16:1*n*-7 and 18:1*n*-9, and the main SFA are 16:0 and 18:0 (Table 7.2). The low PUFA content of this standard adult diet contrasts with the high PUFA content of the larval diet of fish for these blowflies.

Table 7.1 Comparison of whole blowfly phospholipids and thoracic mitochondrial phospholipid fatty acid composition (expressed as % total fatty acids) at day 0 for *Calliphora stygia*.

	Whole fly (*N* = 4)	**Mitochondria (*N* = 3)**	***P* (<0.01)**
14:0	0.7 ± 0.1	0.5 ± 0.1	ns
15:0	0.5 ± 0.0	0.1 ± 0.0	0.0001
16:0	17.4 ± 1.0	17.5 ± 0.3	ns
17:0	0.6 ± 0.0	0.2 ± 0.1	0.004
18:0	1.4 ± 0.3	1.3 ± 0.8	ns
20:0	0.5 ± 0.0	0.0 ± 0.0	#
21:0	0.0 ± 0.0	0.0 ± 0.0	#
22:0	0.3 ± 0.0	0.0 ± 0.0	#
16:1*n*-7	18.5 ± 1.0	20.4 ± 1.0	ns
18:1*n*-9	31.3 ± 0.9	32.1 ± 0.8	ns
18:1*n*-7	0.8 ± 0.3	0.0 ± 0.0	#
18:2*n*-6	7.7 ± 0.4	7.8 ± 0.6	ns
18:3*n*-6	0.7 ± 0.0	0.7 ± 0.0	ns
20:4*n*-6	3.9 ± 0.2	3.6 ± 0.2	ns
18:3*n*-3	1.2 ± 0.1	1.6 ± 0.2	ns
20:5*n*-3	12.6 ± 0.6	13.0 ± 0.5	ns
22:6*n*-3	0.9 ± 0.1	1.1 ± 0.1	ns
SFA	21.5 ± 1.2	19.6 ± 0.9	ns
MUFA	51.6 ± 0.6	52.6 ± 1.5	ns
PUFA	26.9 ± 1.3	27.8 ± 0.6	ns
***n*-6 PUFA**	12.2 ± 0.6	12.1 ± 0.4	ns
***n*-3 PUFA**	14.7 ± 0.7	15.8 ± 0.7	ns
C 20-22	19.2 ± 0.8	17.8 ± 0.8	ns
UI	156.4 ± 4.7	161.5 ± 2.0	ns
PI	111.0 ± 5.1	115.7 ± 4.0	ns
PUFA Balance	54.5 ± 0.6	56.6 ± 1.8	ns

All values but PI and PUFA balance are means ±SEM of % total fatty acids. See methods for calculation of PI. PUFA balance is *n*-3 PUFA as % total PUFA.
represents no t-test performed due to no measurable content in the mitochondrial sample, 'ns' means no significant difference was found.

Table 7.2 Fatty acid content (expressed as % total fatty acids) of standard SY (sugar/yeast) diet.

Fatty acids	Standard diet
14:0	0.2
15:0	0.2
16:0	15.3
17:0	0.6
18:0	8.4
20:0	0.1
21:0	0.3
16:1*n*-7	32.5
17:1*n*-7	0.9
18:1*n*-9	39.2
18:1*n*-7	2.2
20:1*n*-9	0.1
18:2*n*-6	0.1
20:5*n*-3	0.1

Data in table represents all fatty acids present in concentrations ≥0.1%.

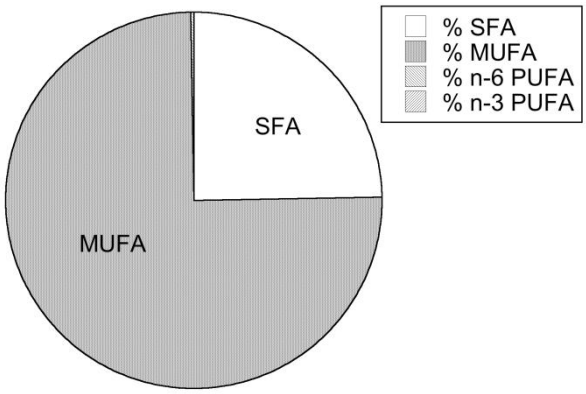

Figure 7.1 Fatty acid content of the standard SY diet.

The changes in fatty acid composition of phospholipids of whole blowflies fed standard SY diet during the first six weeks of adult life is presented in Figures 7.2 and 7.3 (see red symbols only), while the changes in the fatty acids of mitochondrial phospholipids of these blowflies during the first four weeks of adult life is presented in Figures 7.4 and 7.5 (see red symbols only). Over time there was a very small decrease in SFA, a much larger relative decrease in PUFA and these were counterbalanced by an increase in MUFA (Fig. 7.3). These changes are essentially the same as measured in mitochondrial phospholipids (Fig. 7.5).

The decrease in PUFA over time is caused by decreases in both the *n*-6 and *n*-3 PUFA, as the balance between *n*-3 and *n*-6 (PUFA balance) remains relatively constant up to day 42. There is a large regular decrease in the long chain 20-carbon fatty acids (~60% decrease from day zero to day 42). As a result of this continual decline in PUFA content there is also a decline in PI with age (Fig. 7.3 and Fig. 7.5). These decreases in PUFA content with time may be a reflection of the change from a fish-carcass diet fed to the larvae, to the standard SY diet fed to the adults which is very low in PUFA (~0.2%). The increase in MUFA content over time is predominantly a result of a doubling of 16:1*n*-7 over the first six weeks of adult life, with relatively small changes in 18:1*n*-9. The decrease in SFA is a result of a small initial decrease in 16:0.

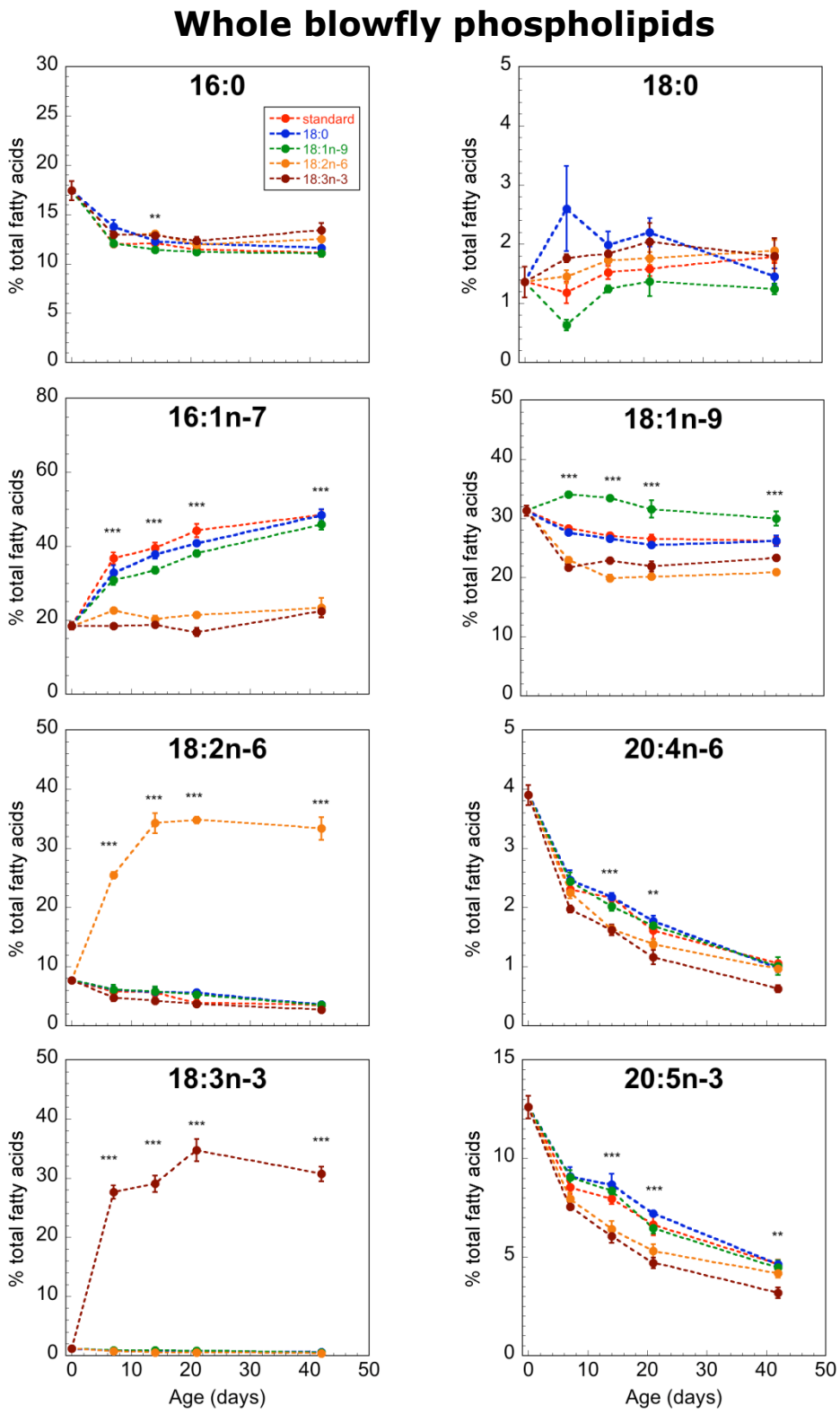

Figure 7.2 The main fatty acids present in whole blowfly phospholipids measured over a period of 42 days for *Calliphora stygia*. * represents a significant difference between diets at that day (* P <0.01, ** P <0.001, ***P <0.0001; see Appendix E for tables). Values are means ± SEM (N = 4).

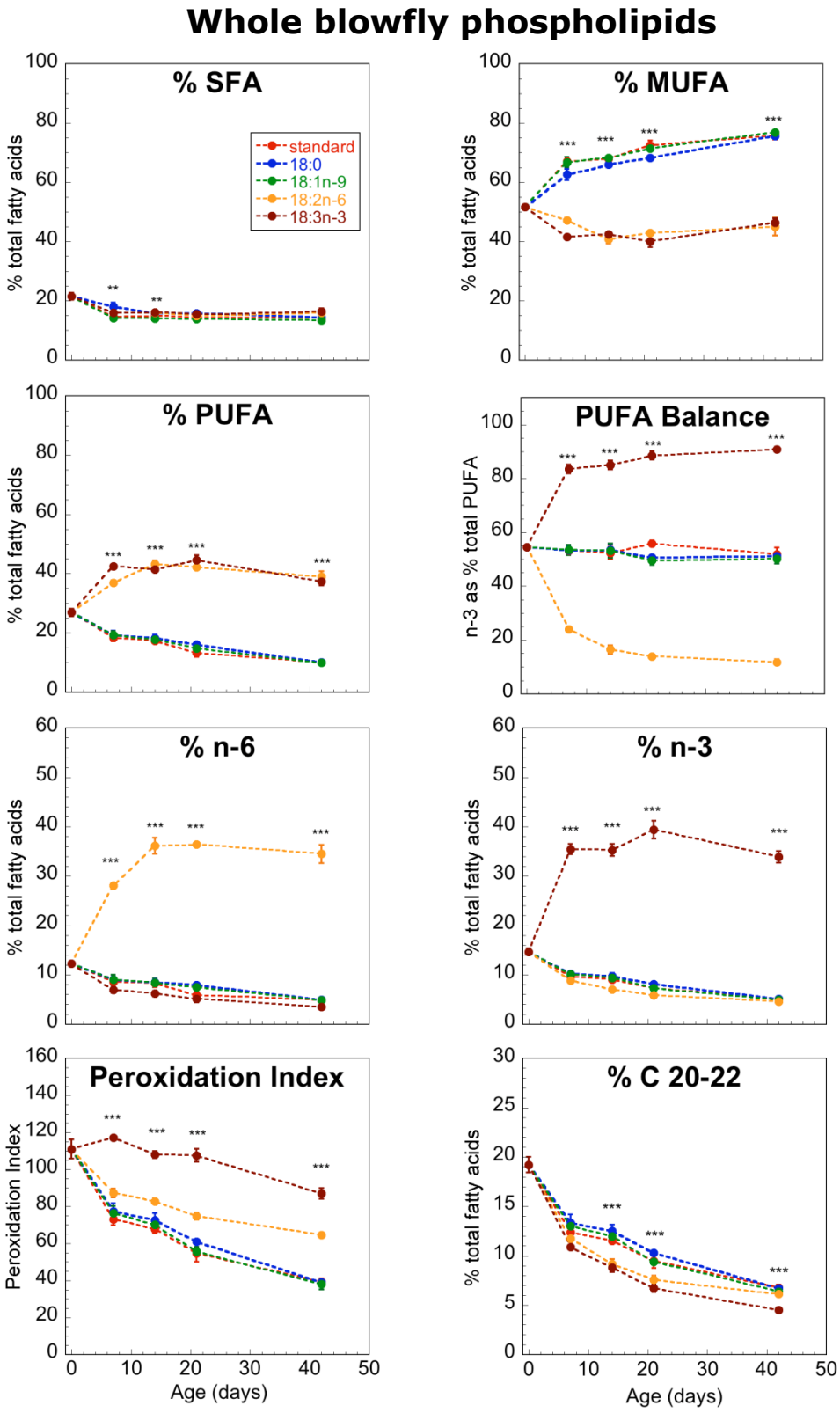

Figure 7.3 Fatty acid composition of whole blowfly phospholipids measured over a 42-day period for *Calliphora stygia*. * represents a significant difference between diets at that day (* P <0.01, ** P <0.001, ***P <0.0001; see Appendix E for tables). Values are means ± SEM (N = 4).

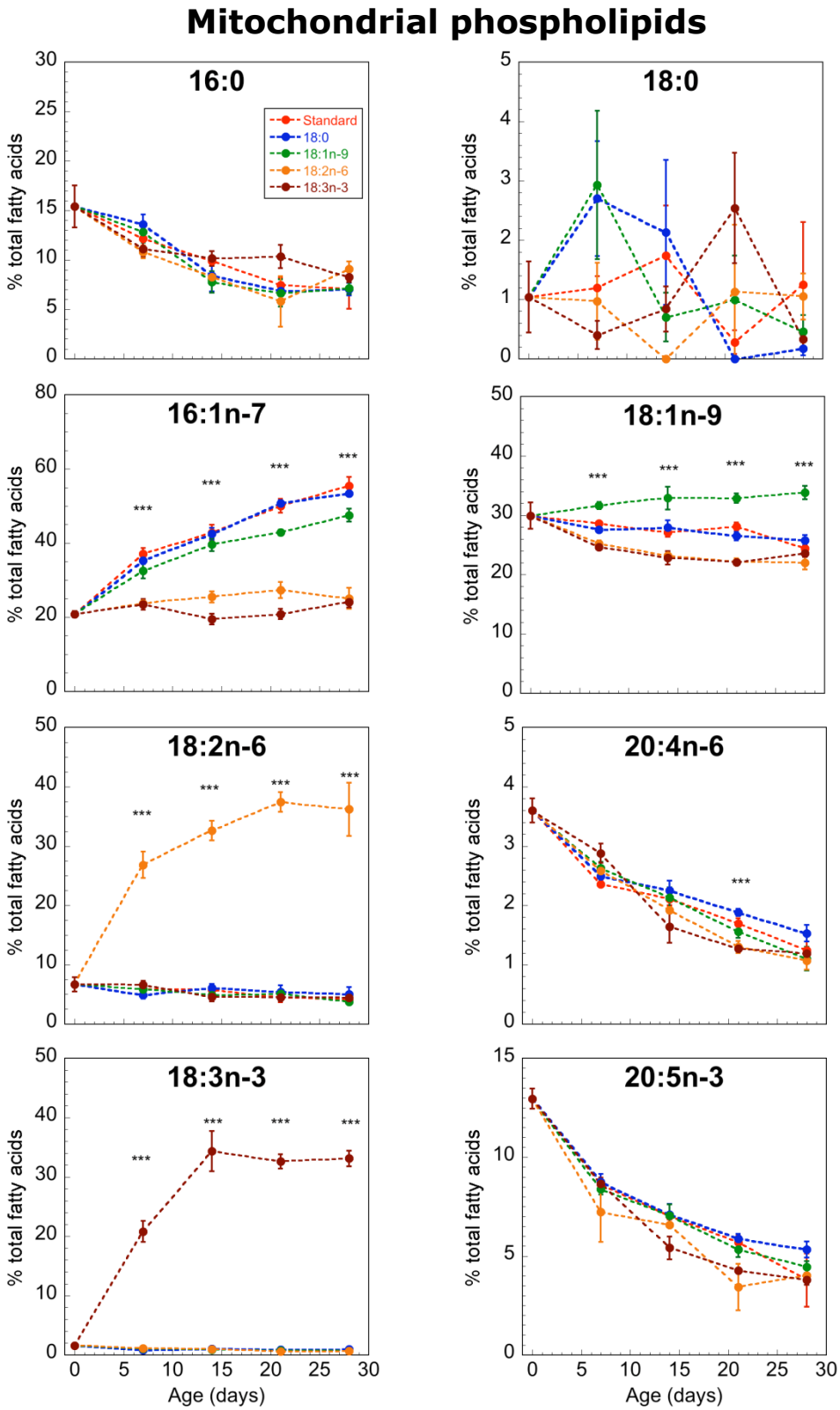

Figure 7.4 The main fatty acids measured in isolated mitochondria (thorax) membranes over a 28-day period for *Calliphora stygia*. * represents a significant difference between diets at that day (* P <0.01, ** P <0.001, ***P <0.0001; see Appendix E for tables). Values are means ± SEM (N = 4, except day 0 where N = 3).

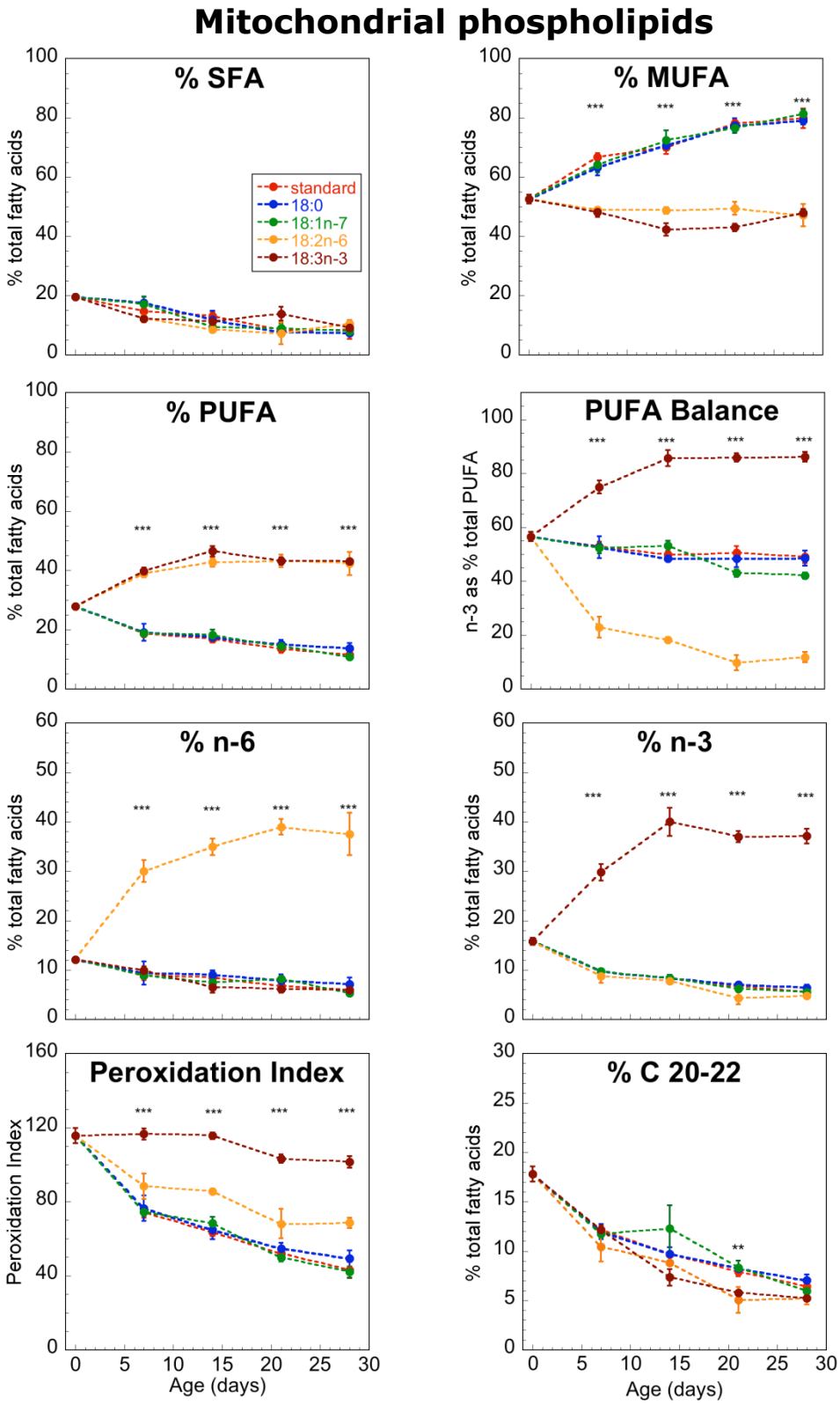

Figure 7.5 Fatty acid composition of mitochondrial membranes measured over a 28-day period for *Calliphora stygia*. * represents a significant difference between diets at that day (* P <0.01, ** P <0.001, ***P <0.0001; see Appendix E for tables). Values are means ± SEM (N = 4, except day 0 where N = 3).

7.2.1.3 Effects of 18-carbon fatty acids added to standard SY diet on whole blowfly and mitochondrial phospholipids

Each of the methyl esters was added at a concentration of 3 g per 100 ml of food, while yeast contains 1.5 g of lipid per 100 g yeast. Of the 18-carbon fatty acids that were examined, only 18:1*n*-9 was already present at high levels ~40% (of the total fatty acids) in the standard SY food. As the addition of 3 g of fatty acids more than triples the amount of lipid present in the food, the various methyl esters are likely to be available in a similar amount between the diets.

Examination of the daily food consumption shows all treatment populations decreased their food consumption over time (Fig. 7.6A). There were no apparent palatability differences in the diets, with blowflies in all treatments having similar average daily food consumption (Fig. 7.6B).

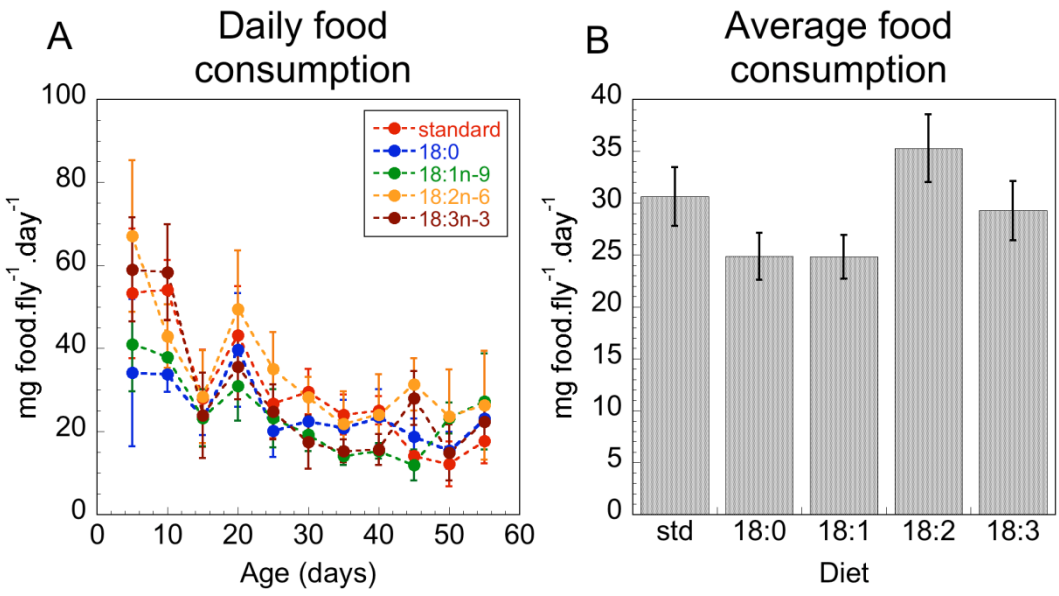

Figure 7.6 Food consumption of *Calliphora stygia* on diets enriched with 18-carbon methyl esters. Fig. 7.6A. Each data point is the means ± SEM over a 5-day period. Fig. 7.6B. Average daily food consumption per fly over the period of measurement as shown in Fig. 7.6A. There are no significant differences between diet treatments. Values are means ± SEM.

7.2.1.4 Enrichment of food with 18:0

Addition of 18:0 (see blue symbols; Fig. 7.2 to Fig. 7.5) in the diet had no significant effect on membrane composition, with both whole blowfly phospholipids and mitochondria membranes having the same profiles as those fed the standard diet.

7.2.1.5 Enrichment of food with 18:1*n*-9

There was a small but significant change in membrane fatty acid profiles when blowflies were provided with 18:1*n*-9 (see green symbols; Fig. 7.2 to Fig. 7.5). Membranes had increased levels of 18:1*n*-9 by day seven, and this remained significantly elevated throughout the 42-day measurement period. This increase in 18:1*n*-9 was at the expense of a decrease in 16:1*n*-7 which was significantly lower than blowflies given standard diet until day 21 (Fig. 7.2 to 7.5).

7.2.1.6 Enrichment of food with 18:2*n*-6

Provision of 18:2*n*-6 (see yellow symbols; Fig. 7.2 and 7.4) in the diet had a dramatic effect on membrane composition. The presence of 18:2*n*-6 in the membranes increased approximately five-fold (from 6% to 25%) by day seven and was the dominant membrane fatty acid throughout the measurement period (Fig. 7.2 and 7.4). These changes in the phospholipid pool were relatively quick, with most changes occurring by day seven. Where in the standard diet phospholipids are dominated by MUFA, due to the large increase in PUFA present these blowflies had equal amounts of MUFA and PUFA (Fig. 7.3 and 7.5). The increase in 18:2*n*-6 resulted in a rapid decrease in the PUFA balance, which was reduced from ~50% to

~20%. There was also a significant increase in the PI of these blowflies, which remained elevated over those given standard diet at day 42.

This huge increase in 18:2*n*-6 was not associated with any elongation and further desaturation to longer chain 20:4*n*-6. In fact, there was actually a decrease in the amount of 20:4*n*-6 present in the membranes, which was also seen in the other long chain PUFA, 20:5*n*-3. The increase in 18:2*n*-6 in the membranes was associated with changes in the amount of 16:1*n*-7 and the presence of long chain PUFA. While standard diet showed 16:1*n*-7 to increase over time, with an associated decrease in 18:1*n*-9, there was no change in the relative percent composition of 16:1*n*-7 over time in blowflies given dietary 18:2*n*-6.

7.2.1.7 Enrichment of food with 18:3*n*-3

Blowflies given 18:3*n*-3 (see burgundy symbols, Fig. 7.2 to Fig. 7.5) in their diet had significantly more of this specific fatty acid incorporated into the phospholipid pool, with an increase from less than 1% to almost 30% by day seven, which remained steady at around ~30% to day 42 (Fig. 7.2 and 7.4). As seen for blowflies given 18:2*n*-6, most of the changes in the phospholipid pool occurred by day seven and the increase in 18:3*n*-3 PUFA resulted in equal amounts of MUFA and PUFA. PUFA balance was rapidly affected, with blowflies increasing PUFA balance from ~50% to ~80% (Fig. 7.3 and 7.5).

Similarly to the flies given 18:2*n*-6, there was no subsequent increase in long chain PUFA associated with the elongation or desaturation of this fatty acid. Both 20:5*n*-3 and 22:6*n*-3 (see Appendix E tables) decreased over time as with the standard and other diets, and in fact long chain PUFA were less than in the standard diet blowflies, as was also seen in the 18:2*n*-6 blowflies. Peroxidation index was

significantly higher in the blowflies given 18:3*n*-3 compared to standard diet over the 42 days, and it was also significantly higher than blowflies given 18:2*n*-6, a result of the higher peroxidisability of 18:3*n*-3 over 18:2*n*-6.

7.2.1.8 Longevity and demographic senescence

Provision of methyl esters in the diet had no effect on the maximum longevity of *C. stygia* ($F_{4, 27}$ = 0.72, P >0.05; Fig. 7.7A). There was also very little effect of diet on measures of demographic senescence of adult blowflies (comparison of 99% confidence intervals; Table 7.4). All populations were best fit by a two-phase Gompertz, dividing senescence into an early phase of slow ageing and a late phase of faster ageing (Fig. 7.7B). Blowflies given the 18:0 enriched diet had a significantly longer period of early slow ageing but they also had a faster rate of late ageing compared to that of those on the standard SY diet. There was no significant difference between 18:0 and any of the other fatty acid enriched diets for any of the mortality parameters.

Blowflies given the diets with added 18:2*n*-6 had a decreased average longevity compared to the standard SY diet, with average longevity decreasing from 37 to 27 days ($F_{4, 301}$ = 12.26, P <0.0001; Fig. 7.7A). A greater proportion of blowflies were dying in early life on the 18:2*n*-6 diet, as indicated by the higher mortality level, however the rate of ageing of these blowflies was similar to those on the other diets (Fig. 7.7B). Blowflies given 18:0, 18:1*n*-9 and 18:3*n*-3 all had a significant ~30% increase in their average longevity compared to blowflies given the standard SY diet (Figure 7.7A).

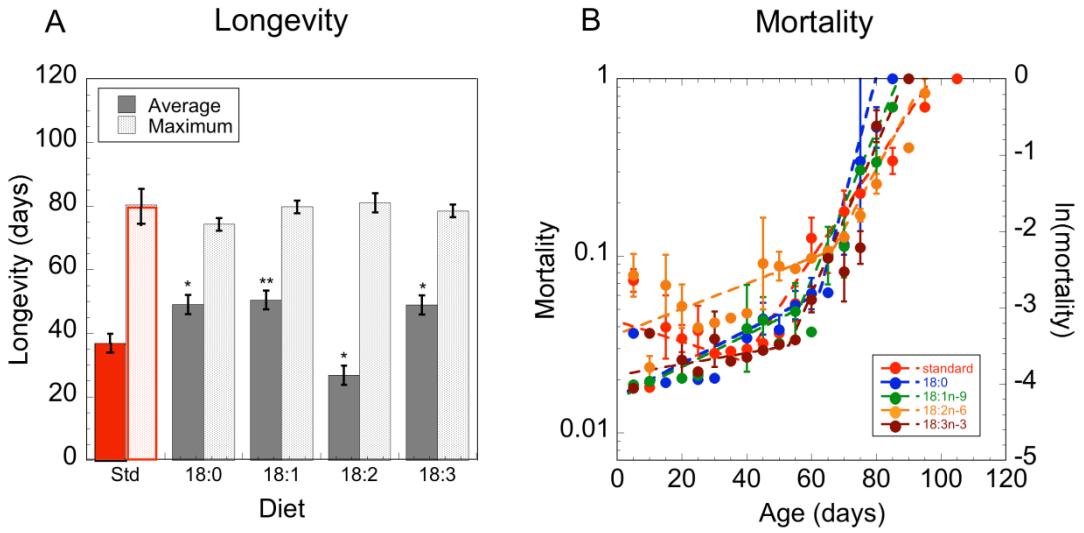

Figure 7.7A. Average and maximum longevity of *Calliphora stygia* given diets enriched with specific fatty acids. Standard diet values are in red. Values are means ± SEM, with maximum longevity determined as the average of the longest-lived 5 % of the population. * represents a significant difference in average or maximum longevity between that diet treatment and the standard diet, * P <0.05, ** P <0.01. (N = 67, 57, 56, 68 and 58 for standard diet to 18:3*n*-3 respectively). Figure 7.8B. Age-specific mortality of flies fit to a two-phase Gompertz (R^2 = 0.83, 0.80, 0.86, 0.73 and 0.85 from standard to 18:3*n*-3 respectively).

7.2.1.9 Peroxidation index of flies in life versus death

Peroxidation index decreased over time for all treatments (Fig. 7.3 and Fig. 7.5). The high initial PI is likely a result of larval blowflies being fed on fish carcasses, with the early decrease in PI a reflection of the high MUFA content of the standard fly diet. These decreases in PUFA and PI in membranes with time may however, be an artefact of selection within the populations, as blowflies with high PI may die early and are therefore, not being measured at the later time points. To answer this question, a small study was performed that measured the fatty acid content of blowflies that were alive, 'dying' or dead.

Table 7.3. Mortality parameter estimates of adult *Calliphora stygia* maintained on diets modified with different specific fatty acids.

Diet (*fatty acid*)	**Initial mortality (% per day)**	**Duration of early ageing (days)**	**Early rate of ageing (% Δ in mortality rate.day^{-1})**	**Late rate of ageing (% Δ in mortality rate.day^{-1})**	**Two-phase vs. single Gompertz (R^2)**
Std	2.4 ± 0.2^{a}	39 ± 4^{a}	-1.3 ± 1.1$^{a\#}$	6.3 ± 0.6^{a}	**0.83** vs. 0.60
18:0	1.8 ± 0.3^{a}	63 ± 2^{b}	2.2 ± 0.6^{a}	16.1 ± 3.0^{b}	**0.80** vs. 0.61
18:1*n*-9	1.3 ± 0.4^{a}	56 ± 4^{ab}	2.1 ± 0.6^{a}	9.7 ± 1.4^{ab}	**0.86** vs. 0.73
18:2*n*-6	1.3 ± 0.4^{a}	67 ± 6^{ab}	1.7 ± 0.5$^{a\#}$	7.8 ± 2.1^{ab}	**0.73** vs. 0.63
18:3*n*-3	1.2 ± 0.6^{a}	53 ± 3^{ab}	0.7 ± 0.6$^{a\#}$	9.7 ± 1.1^{ab}	**0.85** vs. 0.66

Mortality parameters were estimated on daily ln(μx) data using both linear and segmental linear regression. All parameters were compared to standard diet by examination of 99% C.I. Values within columns that do not share the same letter are significantly different. $^{\#}$ indicates a slope not significantly different from zero, indicating no change in mortality rate with age.

Blowflies were collected at age 32 days (similar to average longevity) in one of three conditions; i) alive, ii) ‘dying’, which was characterised as blowflies found lying on their backs on the cage floor and unable to right themselves, and iii) ‘dead’. As ‘dead’ blowflies could have died at any time point over the previous 24 hours, to ensure there were no effects of membrane fatty acid changes post-mortem, blowflies were collected alive and then immediately killed by immersion in liquid nitrogen. These blowflies were left in an empty cage at 25°C for a period of 1, 4, 8, 16 or 24 hours to determine how PI may change over time after death.

There was very little difference in PI regardless of the cause of death or the period of time that had passed after dying before measurement (Fig. 7.8). There is therefore no reason to believe that the decrease in PI seen over time in both the whole blowfly phospholipids and the mitochondria are a result of blowflies with high PI dying earlier. These changes in membrane fatty acid composition over time are therefore more likely to be a result of changes in diet, from the larval fish diet high in PUFA to the SY diet fed to adults that was low in PUFA and high in MUFA.

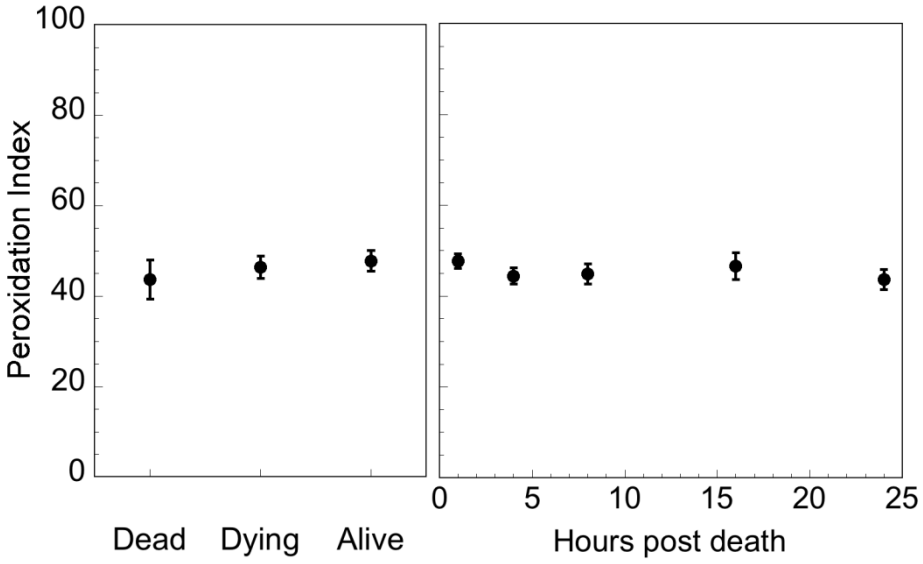

Figure 7.8 Peroxidation index of whole blowfly phospholipids of *Calliphora stygia* at age 32 days that were alive, dead, dying or killed by immersion in liquid nitrogen and left for 1, 4, 8, 16 or 24 hours at 25°C before measurement. There were no significant differences in PI between the various time points or conditions measured. Values are means ± SEM (N = 4).

7.2.2 Effect of fish oil and krill oil-enriched diets during the adult life of blowflies

The relatively large decrease in long chain (20-22 carbon) PUFA observed in the previous section may have been a result of the cessation of fish being the primary food source for larval stages, and totally absent in adult diets. This section reports the result of a preliminary study on a separate batch of pupae, which was an attempt to maintain a high level of PUFA in adult *C. stygia* through the provision of fish oil or krill oil-enriched standard SY food.

Standard fly food is predominantly MUFA (75%) and SFA (25%), and the enrichment of this standard food with three grams of either fish or krill oil significantly altered the fatty acid composition of the food (Table 7.4; Fig. 7.9). The addition of fish oil increased PUFA content to ~15% mainly by the presence of 20:5*n*-3 and 22:6*n*-3, while also increasing SFA to equal proportions with MUFA

(~40% and ~45% respectively). Krill oil is especially high in long chain PUFA and as such, food PUFA content was increased to 50% in this diet, predominantly by high levels of 20:5*n*-3 and 22:6*n*-3. This increase in PUFA content came at the expense of the MUFA 16:1*n*-7 and 18:1*n*-9 which were both reduced to below 10% (compared to > 30% each in standard diet).

Whole blowfly phospholipids were mostly unchanged between the fish oil and krill oil enriched diets (Fig. 7.10 and 7.11). Both populations were still dominated by the MUFA 16:1*n*-7 and 18:1*n*-9, and showed the same patterns of an initial decrease in 16:0 and relatively stable levels of 18:0, 18:2*n*-6 and 18:3*n*-3 over time as shown for whole blowfly phospholipids from the standard diet treatment (Fig. 7.2 and 7.3). The only significant differences between the fish and krill-oil enriched diets for the more common fatty acids were seen in the long chain 20-carbon fatty acids, 20:4*n*-6 and 20:5*n*-3 (Fig. 7.10). The krill oil diet had a significantly higher level of 20:5*n*-3 and a corresponding decrease in the level of 20:4*n*-6. However, while 20:5*n*-3 was the dominant fatty acid available in the krill oil enriched food, the incorporation of this fatty acid into the phospholipids was still relatively minor. 20:5*n*-3 comprised only about 10% of the total fatty acids, only 2% above that of the fish oil enriched diet, in contrast to the 30% increase in 18:2*n*-6 and 18:3*n*-3 seen when these specific fatty acids were added to the diet. There was also little incorporation of 22:6*n*-3 into the phospholipid pool despite the large amount of 22:6*n*-3 present in the krill oil enriched food (15.9%) and the fish oil food (7.7%), remaining at levels below 1% (see Appendix E).

Table 7.4 Fatty acid composition (expressed as % total fatty acids) of fly food that was enriched with either fish or krill oil (3 g /100 ml food).

Fatty Acids	Fish oil diet	Krill oil diet
14:0	5.2	3.0
16:0	34.7	25.3
18:0	0.0	1.4
16:1*n*-7	19.9	3.4
17:1*n*-7	0.4	0.3
18:1*n*-9	22.0	7.0
18:1*n*-7	2.1	5.5
20:1*n*-9	0.8	2.2
22:1*n*-9	0.0	0.8
18:2*n*-6	0.6	1.4
18:3*n*-6	0.0	0.1
20:2*n*-6	0.2	0.1
20:4*n*-6	0.4	0.6
22:4*n*-6	0.1	0.7
18:3*n*-3	0.1	1.0
20:5*n*-3	7.7	29.7
22:5*n*-3	0.8	0.7
22:6*n*-3	4.8	15.9

Only fatty acids present at >0.1% were included above.

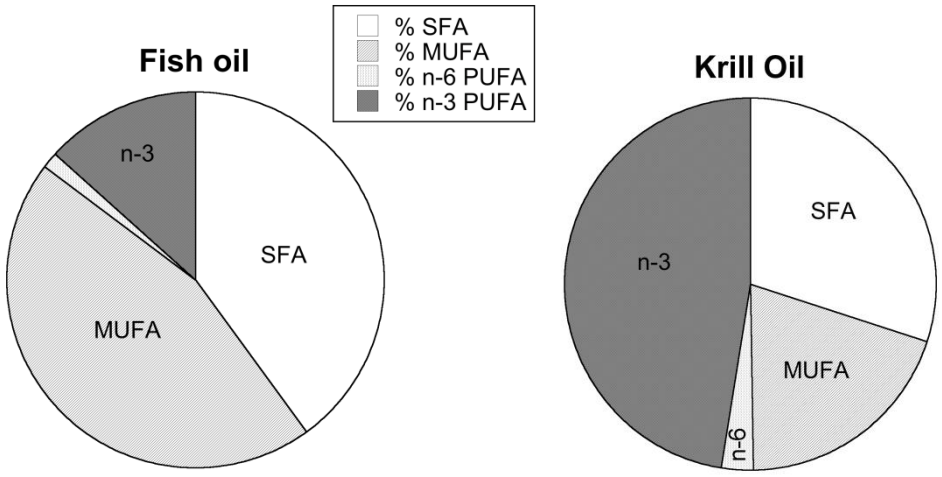

Figure 7.9 The relative proportions of saturated, monounsaturated and polyunsaturated fatty acids in fish-oil enriched and krill-oil enriched diets. Polyunsaturated fatty acids are divided into *n*-6 and *n*-3 PUFA.

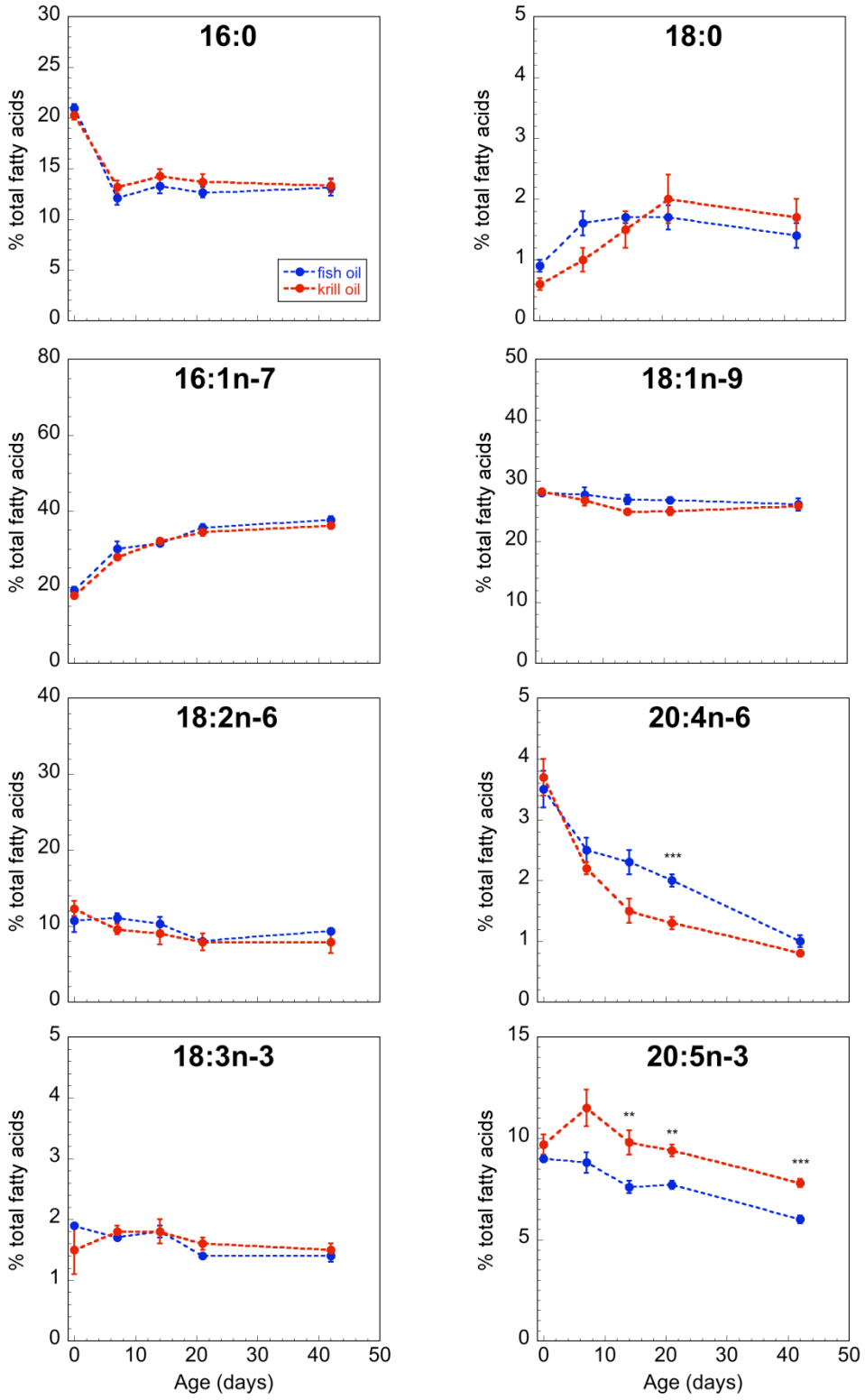

Figure 7.10 The main fatty acids of *Calliphora stygia* measured in whole blowfly phospholipids over a 42-day period on diets enriched with either fish oil or krill oil. * represents a significant difference between diets at that day (* P <0.01, ** P <0.001, ***P <0.0001; see Appendix E for tables). Values are means ± SEM (N = 4).

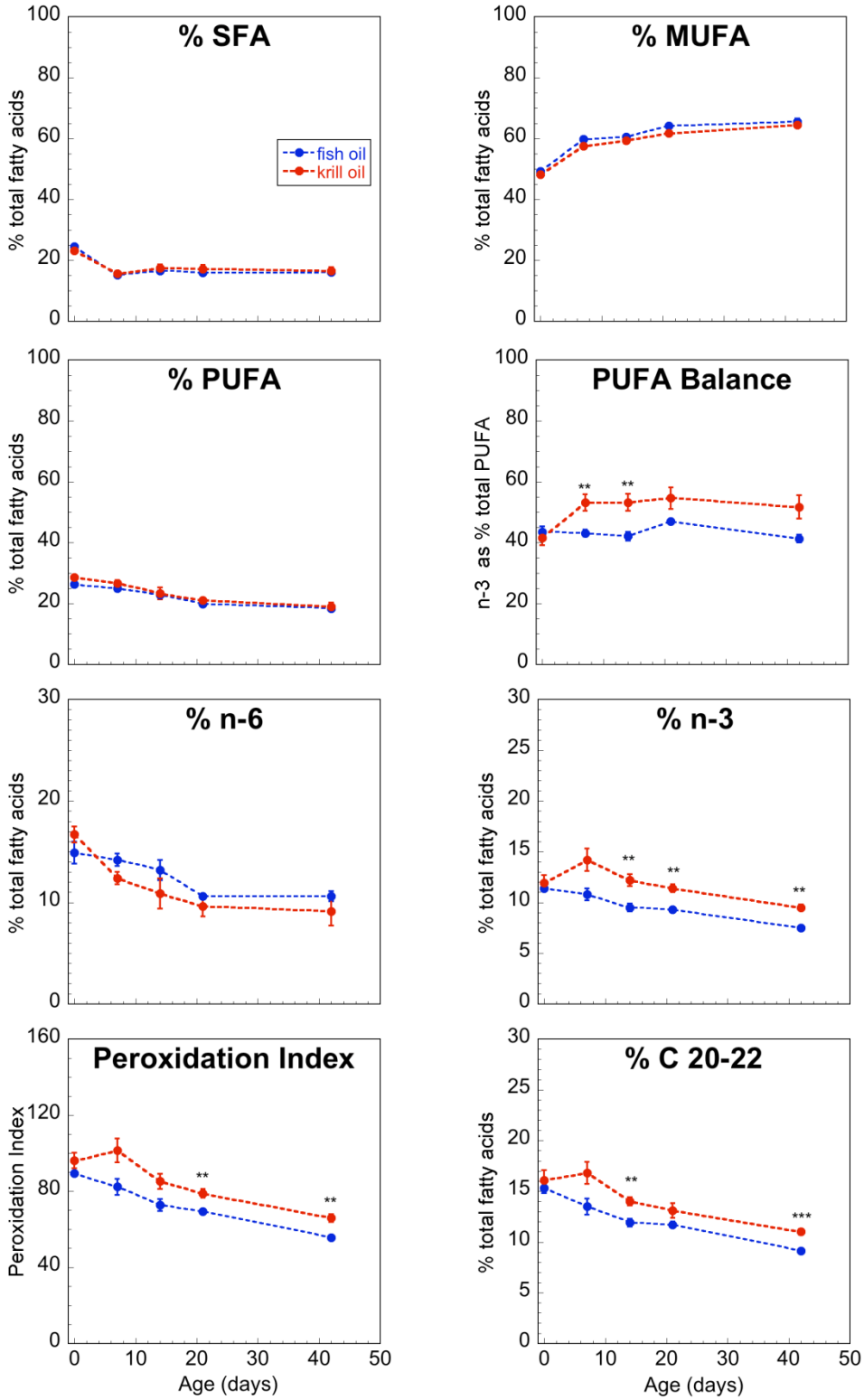

Figure 7.11 Fatty acid composition of whole blowfly phospholipids of *Calliphora stygia* given fish or krill oil enriched diets. * represents a significant difference between diets at that day (* P <0.01, ** P <0.001, ***P <0.0001; see Appendix E for tables). Values are means ± SEM (N = 4).

As seen for all the diets with the addition of specific fatty acids, both 20:4*n*-6 and 20:5*n*-3 decreased over time in the whole fly phospholipid pool (Fig. 7.10). This suggests that the large availability of these fatty acids in the diet does not ensure their incorporation into the phospholipids once they have been consumed. Indeed, PI still declined over time for both diets and while it was significantly higher in flies on the krill oil enriched diet, these differences reflect the small differences in the 20:4*n*-6 and 20:5*n*-3 fatty acids (Fig. 7.11). These results indicate that the changes in membrane fatty acid composition with age are unlikely to be a result of changes to diet fatty acid availability. It is more likely that these changes in composition are a result of some age-related regulation of membrane fatty acid composition.

7.3 Discussion

The membrane pacemaker theory highlights the variation in peroxidation susceptibility between specific membrane fatty acids and predicts that membranes with a high PI, and the resultant increased lipid peroxidation, will be correlated with reduced longevity (Hulbert et al., 2007). As a test of this theory, diet was manipulated by the addition of specific fatty acids to determine whether: i) membrane composition could be altered and ii) if any subsequent changes in PI would affect longevity. These experiments were only partially successful, with membrane fatty acid composition relatively resistant to change through alteration of dietary fatty acids. Of the six fatty acid-enriched diets examined, including four enriched with specific fatty acids and two with oil mixtures, only two diets had large effects on membrane fatty acid composition.

Most animals, including all insects investigated, have been shown to be able to synthesise 16:0, 18:0 and 18:1 *de novo*, however, both 18:2*n*-6 and 18:3*n*-3 are considered essential fatty acids for animals as they are generally unable to synthesise these two lipid species (Hulbert et al., 2005). A number of insect species, such as the cockroach, *P. americana* and the house cricket, *A. domestica*, have been shown to be able to synthesise 18:2*n*-6 *de novo* (Blomquist et al., 1991). This fatty acid is particularly important for both structural properties in membranes and the desaturation into longer chain PUFA, which play a role in cellular metabolism (Hulbert et al., 2005). In insects, the long chain PUFA are also important for the synthesis of various waxes and pheromones, with houseflies shown to transfer 20:4 during copulation (Stanley-Samuelson et al., 1988). Over time, *C. stygia* given the standard diet showed a decrease in 18:2*n*-6. It is unlikely from these observations that *C. stygia* can synthesise 18:2*n*-6, making it an essential dietary fatty acid for *C. stygia*, similar to most animals.

When either 18:2*n*-6 or 18:3*n*-3 were provided in the diet the level of these fatty acids increased dramatically. Both 18:2*n*-6 and 18:3*n*-3 fatty acids were preferentially added to the phospholipid pool over the MUFA 16:1*n*-7 and 18:1*n*-9, which were present in the standard diet, and can also be made from 16:0 and 18:0. All diets examined in this study were low in 18:2*n*-6 and 18:3*n*-3, including the fish oil and krill oil enriched diets, with the obvious exception of the 18:2*n*-6 and 18:3*n*-3 enriched diets. The level of these fatty acids in *C. stygia* phospholipids following feeding with mixed oils containing high levels of PUFA (e.g. safflower oil [*n*-6] and flaxseed oil [*n*-3]) showed that they are incorporated into membranes in similarly large quantities as demonstrated in the current study (Usher and Hulbert, personal communication). These large increases in the *n*-6 and *n*-3 PUFA may reflect the non-

discriminate preferential incorporation of these essential dietary PUFA by acyltransferases into the *sn*-2 position (Lands et al., 1982).

The examination of whole blowfly phospholipids may have resulted in some of this dramatic increase being measured as fatty acids moving into triglycerides or storage fats, and therefore temporarily associated with phosphatidic acid rather than being incorporated into the membranes. However, it would be unlikely for this to be also occurring at the same rate in the isolated mitochondria and the same pattern was evident in both tissues. Phosphatidic acid content is also unlikely to represent a significant proportion of the phospholipids measured, with recent examination of *D. melanogaster* suggesting it makes up roughly 5% of the phospholipid composition of membranes (Carvalho et al., 2012). Furthermore, the large increases were only seen for two of the four diets, which suggests some preference for PUFA over SFA and MUFA. Preliminary unpublished results on feeding mixed oil diets to larval *C. stygia* also suggest a preference for 18:2 and 18:3, which are readily incorporated into the membrane, but blowflies having little ability to metabolise these from longer chain substrates (Hulbert, personal communication).

Until relatively recently insects were not considered to have long chain PUFA in their membranes or require them for metabolic function (Stanley-Samuelson and Dadd, 1983). Long chain PUFA of greater than 18 carbons are incredibly important biosignalling molecules but are not considered essential dietary fatty acids as they are able to be converted from 18:2*n*-6 and 18:3*n*-3 by the elongation and desaturation of these fatty acids to 20:4*n*-6 and 20:5*n*-3 respectively (Hulbert et al., 2005). However, within the insect group there are a number of examples of variations in this general fatty acid metabolism of PUFA. In particular, within the order Diptera, mosquitoes have been shown to be unable to elongate and desaturate these essential

fatty acids and as such also require dietary long chain PUFA, similar to cats which are obligate carnivores (Rivers et al., 1975; MacDonald et al., 1983; Stanley-Samuelson et al., 1988). While the fruit fly *D. melanogaster* is unique in that it appears to neither biosynthesise or require long chain PUFA, as evidenced by flies being reared on a synthetic fatty acid-free diet for over 10 generations (Rapport et al., 1983). Dietary supplementation with long chain PUFA resulted in little incorporation of long chain (>20-carbon) PUFA into fly total lipids of *D. melanogaster* (Shen et al., 2010). An in depth analysis of fatty acid composition of *D. melanogaster* recently confirmed this lack of long chain PUFA, finding 18 carbons to be the longest chains present for all species including cardiolipin, PE, PC and ceraminides (Hammad et al., 2011).

In contrast to *D. melanogaster* I found a number of long chain PUFA present in the both whole blowfly and mitochondrial phospholipids. These long chain PUFA were predominately 20:5*n*-3 and 20:4*n*-6 with small amounts of 22:6*n*-3 and the SFA 20:0, 21:0 and 22:0. All long chain PUFA decreased with age, regardless of whether they had high levels of the fatty acid precursors from dietary methyl esters, or high levels of the long chain PUFA present in the diet (as on the fish or krill diets). There is therefore the question of whether *C. stygia* can elongate and desaturate 18:2*n*-6 or 18:3*n*-3 to form these long chain PUFA.

There was no obvious evidence of *C. stygia* converting 18:2*n*-6 or 18:3*n*-3 to long chain PUFA, with the dramatic increases in 18:2*n*-6 and 18:3*n*-3 phospholipids not associated with any concurrent increases in 20:4*n*-6 or 20:5*n*-3. This suggests that similar to other insects, they have an inability to deacylate dietary long chain PUFA (Stanley-Samuelson and Dadd, 1983). Indeed, a recent investigation of *D.*

melanogaster found no evidence of genes encoding for the desaturases (Δ5 and Δ6) that are involved in the desaturation of PUFA in *D. melanogaster* (Shen et al., 2010).

Following provision of dietary 18:2*n*-6 and 18:3*n*-3 there was actually a decrease in these long chain PUFA, suggesting that, not only are they not being replaced after conversion into biologically active molecules, they may be actively displaced by 18:2*n*-6 and 18:3*n*-3. Larvae *C. stygia* were fed on fish carcasses prior to eclosion and there was the possibility that the change in fatty acid content from the fish diet to the adult diet was responsible for much of this decline in PUFA over time. However, even when long chain PUFA were provided in high levels in the diet, as when fed krill oil, they were only partially replaced and did not appear to be preferentially incorporated as there was still an overall decrease in PUFA and increase in MUFA with age in these blowflies. By contrast, those provided with linoleic and linolenic acids showed a more stable PUFA content over time, with no increase in MUFA content. Further, there seems to be little evidence of long chain PUFA being converted into the 18 carbon fatty acids. The large increases in 18 carbon PUFA when provided as specific fatty acids, were not replicated when given as long chain PUFA in krill or fish oil,

Changing dietary fatty acid composition did have an affect on average longevity, with blowflies provided with dietary 18:0, 18:1*n*-9 and 18:3*n*-3 all showing significant increases in average longevity, but no changes in rate of ageing or maximum longevity. Changes to average longevity are often thought to be due to decreases in initial mortality, which may be a result of increased health and delayed ageing (Pletcher et al., 2000). Indeed membrane fatty acid composition has been shown to affect the health of vertebrates, with increases in *n*-3 fatty acids and maintaining a high PUFA balance important for metabolic and mental health

(Hulbert et al., 2005). Increasing dietary 18:3*n*-3 increased average longevity, however as 18:0 and 18:1*n*-9 had similar effects on longevity, but neither had significant effects on membrane composition, the longevity benefits from dietary provision of 18:0, 18:1*n*-9 and 18:3*n*-3 are probably more related to the availability of excess dietary fats rather than the specific fatty acid moiety. Sugar/yeast foods fed to the flies are generally low in dietary fat (1.5 g/100 ml). Flies are able to sense and preferentially feed on specific fatty acids, and their fatty acid requirements have been shown to change during development (Fougeron et al., 2011), and so it is possible that the traditional diets have insufficient levels of dietary fatty acids.

Peroxidation index represents the relative peroxidative potential of a membrane based on calculations of the oxygen consumption of individual fatty acids (Harman, 1956; Hulbert et al., 2007). While increasing the number of double bonds in a fatty acid increases its susceptibility to peroxidation, the formation of lipid peroxidation end products is influenced by many factors. For example, while carbonyl formation was found to be highly dependent on the degree of membrane unsaturation (Refsgaard et al., 2000), formation of HNE only occurs from the peroxidation of *n*-6 PUFA, not *n*-3 PUFA (Pryor and Porter, 1990). Further the reactivity of these lipid peroxidation products such as MDA or HNE with proteins and nucleic acids also varies (Refsgaard et al., 2000; Blair, 2001). Likewise the abilities of antioxidant and repair mechanisms acting upon peroxides and damaged biomolecules. Therefore, there is any number of other determinants of the degree of damage incurred by lipid peroxidation in addition to the peroxidation susceptibility of the membrane.

Peroxidation index was increased in only two of the diets, those provided with linoleic and linolenic acids, and the changes in PI were solely through the

subsequent increase in these fatty acids in the membranes, with no additional increases in long chain PUFA as might have been expected from the desaturation and elongation of these 18-carbon precursors. Therefore, while the PI was increased, particular PUFA fatty acids may be more important in lipid peroxidation pathways and subsequent effects on longevity. The increase in PI as a result of increases in 18:2*n*-6 and 18:3*n*-3 may not result in as much damage as were the increases to result from a longer chain PUFA such as 20:4*n*-6. Arachidonic acid is often considered responsible for most peroxidative damage to lipids as its high density of double bonds makes it very susceptible to peroxidation (Hulbert et al., 2007). However, while much of the investigation into lipid damage is based around the products of arachidonic acid, linoleic acid is generally the most common membrane lipid in vertebrates and it is likely that these linoleic acid-derived hydroperoxides would be the greatest contributor to damage (Spiteller, 2001).

8 EFFECT OF ENVIRONMENTAL TEMPERATURE, AND DIET SUGAR AND YEAST CONTENT ON MEMBRANE FATTY ACID COMPOSITION

8.1 Preface

This chapter investigates whether treatments that altered the longevity of blowflies (reported in Chapters 3, 5, and 6) were associated with changes in the membrane fatty acid composition of blowflies. During those experiments, live blowflies were collected at a number of different time points in adult life and stored at -80°C for later fatty acid analyses of whole blowfly phospholipids.

8.2 Results

8.2.1 Effects of temperature on membrane fatty acid composition

Temperature had a small but significant effect on membrane fatty acid composition in adult *C. stygia*. Blowflies kept at 25°C showed an age-related decrease in 16:0 and the long chain PUFA 20:4*n*-6 and 20:5*n*-3, while increasing 16:1*n*-7 which resulted in an overall decrease in PUFA content and an increase in MUFA content (Fig. 8.1 and 8.2). This pattern of change in membrane composition was seen at all temperatures, with the greatest effect of temperature being on the rate of change of membrane composition, which was reduced at low temperatures. The response to temperature was relatively small, with a Q_{10} of ~1.5 calculated for the rate of decrease in PUFA content (18:2*n*-6, 20:4*n*-6 and 20:5*n*-3 having Q_{10}'s of 1.6, 1.4 and 1.4 respectively).

Homeoviscous adaptation of membrane fatty acid composition in response to reduced temperature involves increases in the number of double bonds to maintain membrane fluidity (Crockett, 2008). There was no evidence of any rapid changes to membrane fatty acid composition in response to temperature. Membrane fatty acid composition was similar between all temperature treatments after 24 hours, and there were only minor differences apparent after one week (see Appendix E tables). In contrast to homeoviscous adaptation expectations, instead of PUFA content increasing in response to cold temperature, blowflies maintained at cold temperatures showed slower age-related membrane composition changes when compared to those at high temperatures (Fig. 8.1 and 8.2).

8.2.2 Effect of diet sugar content on membrane fatty acid composition

Diet sugar content had no effect on membrane fatty acid composition. Blowflies on the standard diet showed an age-related change in membrane composition that is the same as shown previously for blowflies maintained at 25°C on standard diet. Whole blowfly phospholipids had reduced PUFA and SFA, and increased 16:1*n*-7 (Fig. 8.3 and 8.4). Initial membrane fatty acid changes, measured immediately post-eclosion, were rapid with the largest changes in 16:0, 18:1*n*-9 and 16:1*n*-7 occurring by day eight. The remaining age-related membrane changes, most notably the decreased PUFA content, occurred more slowly over the 35 days measured (Fig. 8.4).

8.2.3 Effect of diet yeast content on membrane fatty acid composition

Blowflies given diets of varying yeast content showed relatively few diet-related changes in membrane fatty acid composition. Blowflies that were fed yeast-free diets (0.0*x*) showed a different profile to those with yeast included in the diet (Fig. 8.5 and 8.6). In contrast to the other treatments containing yeast, whole blowfly phospholipids on the yeast-free diet showed less change over time, with levels remaining relatively stable over the first 16 days.

Similar to the previous treatments, the most significant changes in membrane composition occurred over time, with a decrease in SFA and PUFA and an increase in MUFA, mostly as a result of increases in 16:1*n*-7 (Fig. 8.5 and 8.6). In contrast to blowflies fed diets of varying sugar content, there were some small effects of dietary yeast content on the membrane fatty acid composition. Blowflies on the restricted yeast diets, 0.25*x* and 0.5*x*, had increased levels of 20:5*n*-3 at 35 days, and subsequently also increased long chain 20-carbon fatty acids (Fig. 8.5). Peroxidation index was also increased at day 35 due to this increase in long chain PUFA (Fig. 8.6).

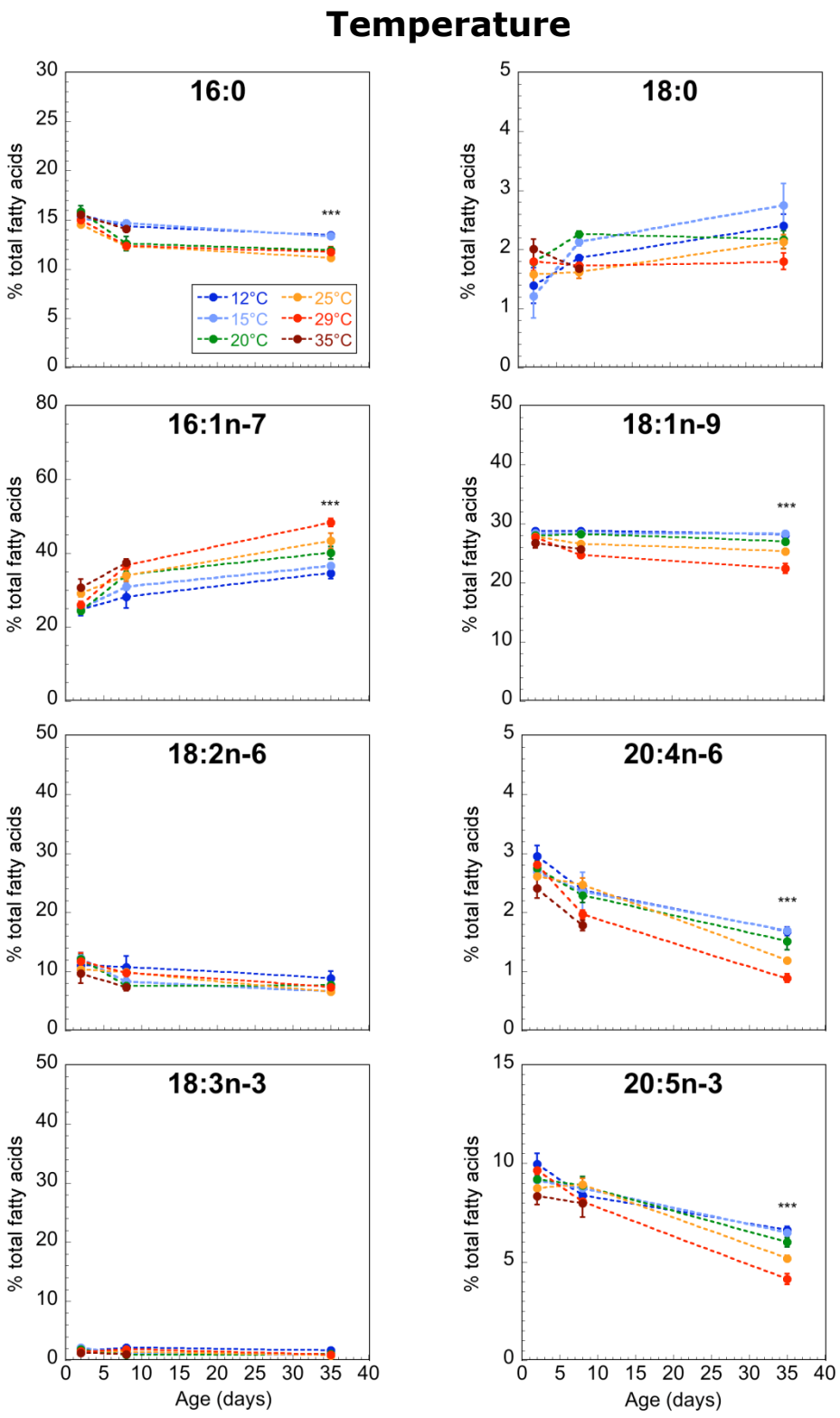

Figure 8.1 The main fatty acids present in whole blowfly phospholipids of adult *Calliphora stygia* kept at different temperatures (from 12°C to 34°C). Values are means ± SEM (N = 4, except day 8 where N = 2 for all temperatures and data presented are means ± SD). * represents a significant difference between temperature treatments at that day (* P <0.01, ** P <0.001, ***P <0.0001 (see Appendix E for tables).

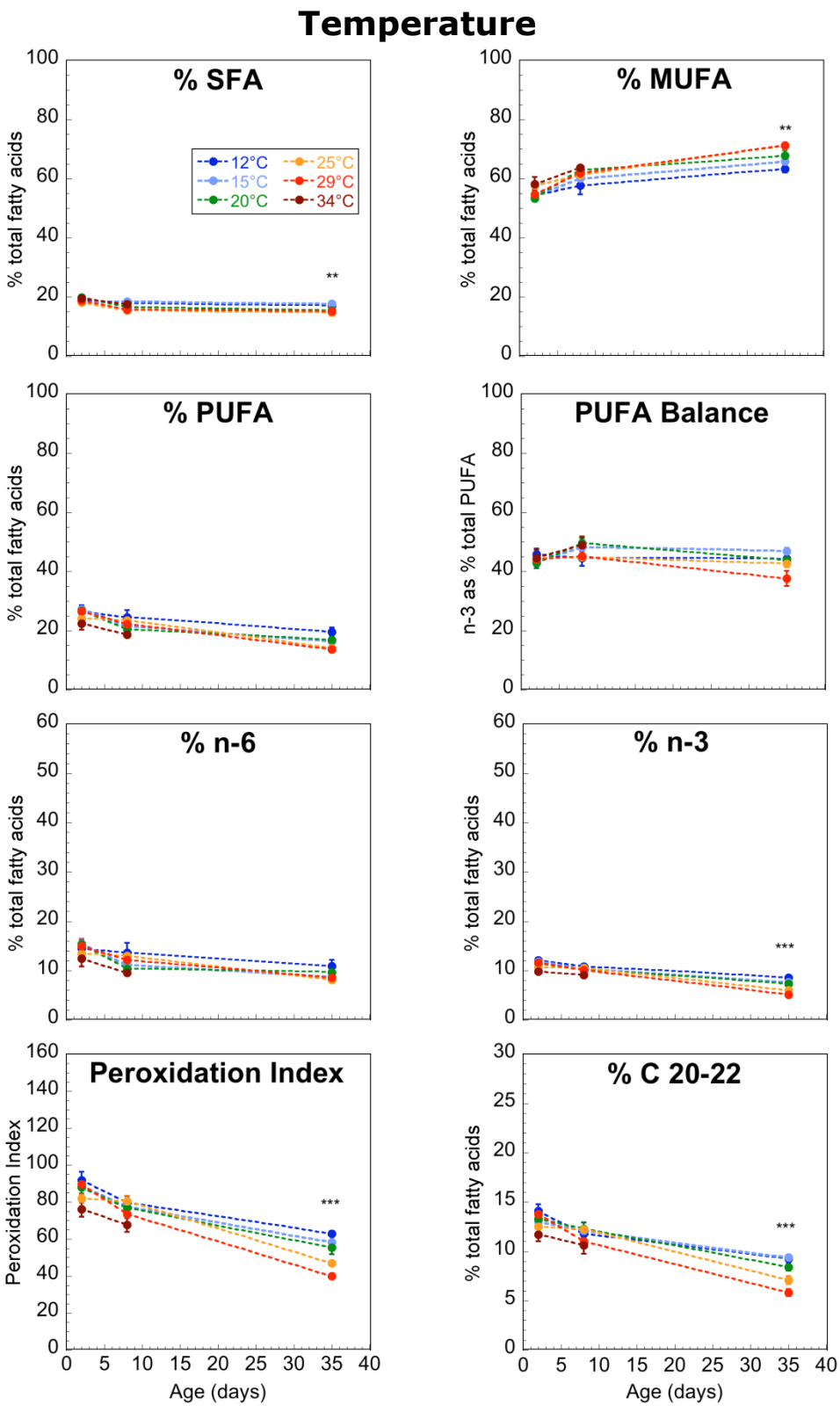

Figure 8.2 Membrane fatty acid composition of adult *Calliphora stygia* kept at different temperatures (from 12°C to 34°C). Values are means ± SEM (*N* = 4, except day 8 where values are means ± SD, *N* = 2 for all temperatures). * represents a significant difference between temperature treatments at that day (* *P* <0.01, ** *P* <0.001, ****P* <0.0001 (see Appendix E for tables).

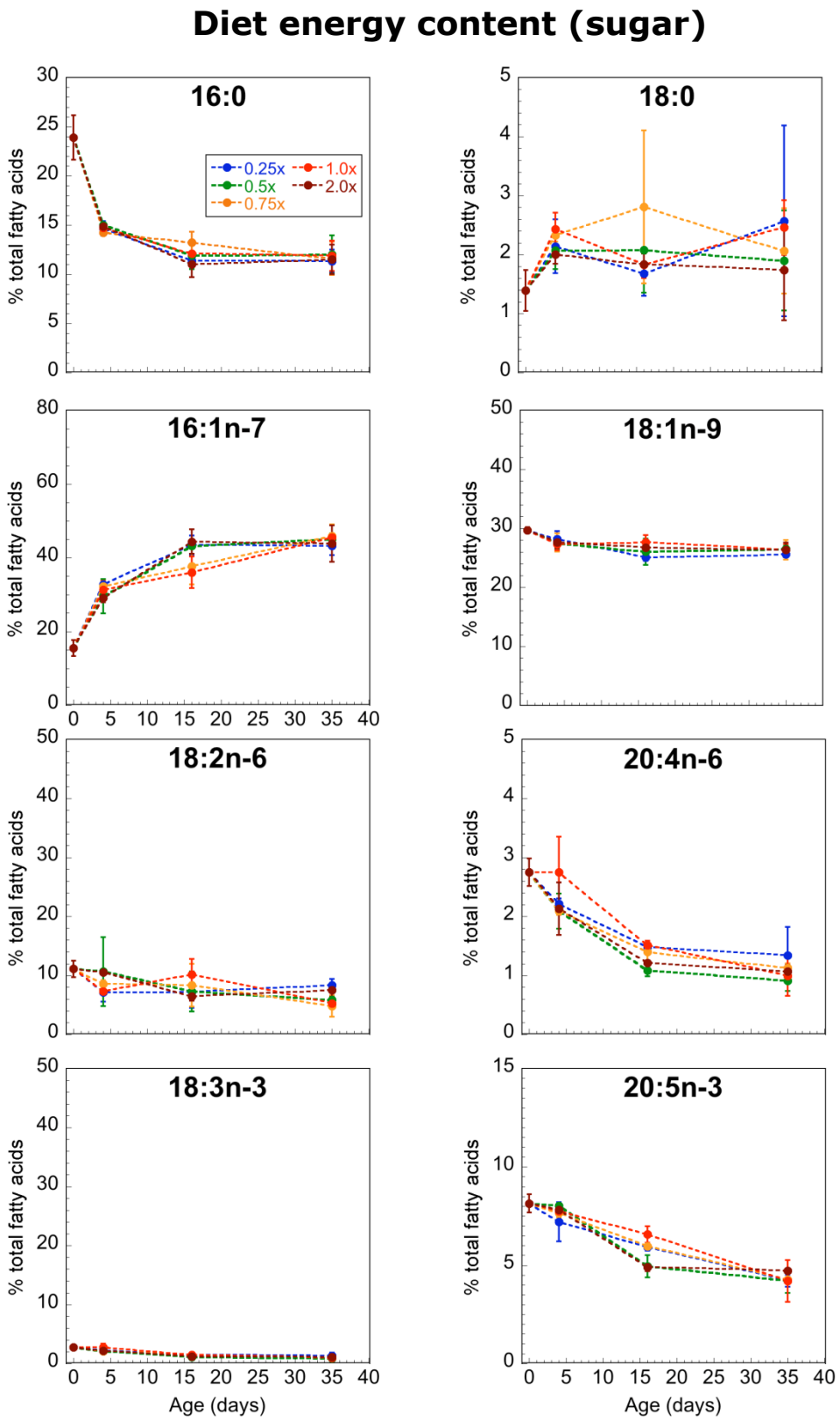

Figure 8.3 The main fatty acids present in whole blowfly phospholipids of adult *Calliphora stygia* kept on diets of varying energy (sugar) content (from 0.25*x* to 2.0*x* standard diet levels). Values are means ± SD (N = 2, except day 0 where N = 6 for all diets). * represents a significant difference between dietary treatments at that day (* P <0.01, ** P <0.001, ***P <0.0001 (see Appendix E for tables).

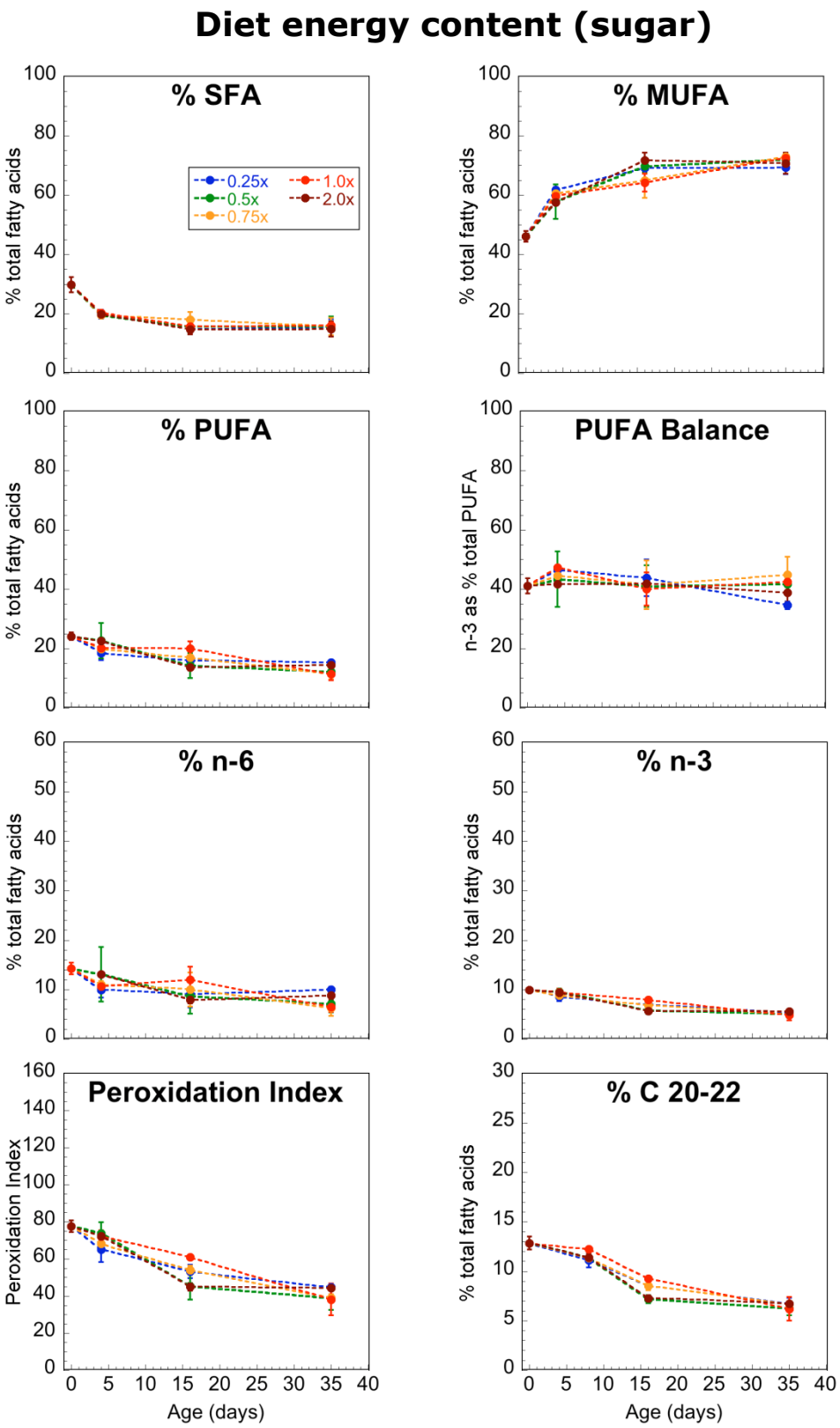

Figure 8.4 Membrane fatty acid composition of adult *Calliphora stygia* kept on diets of varying energy (sugar) content (from 0.25*x* to 2.0*x* standard diet levels). Values are means ± SD (*N* = 2, except day 0 where *N* = 6 for all diets. * represents a significant difference between dietary treatments at that day (* *P* <0.01, ** *P* <0.001, ****P* <0.0001 (see Appendix E for tables).

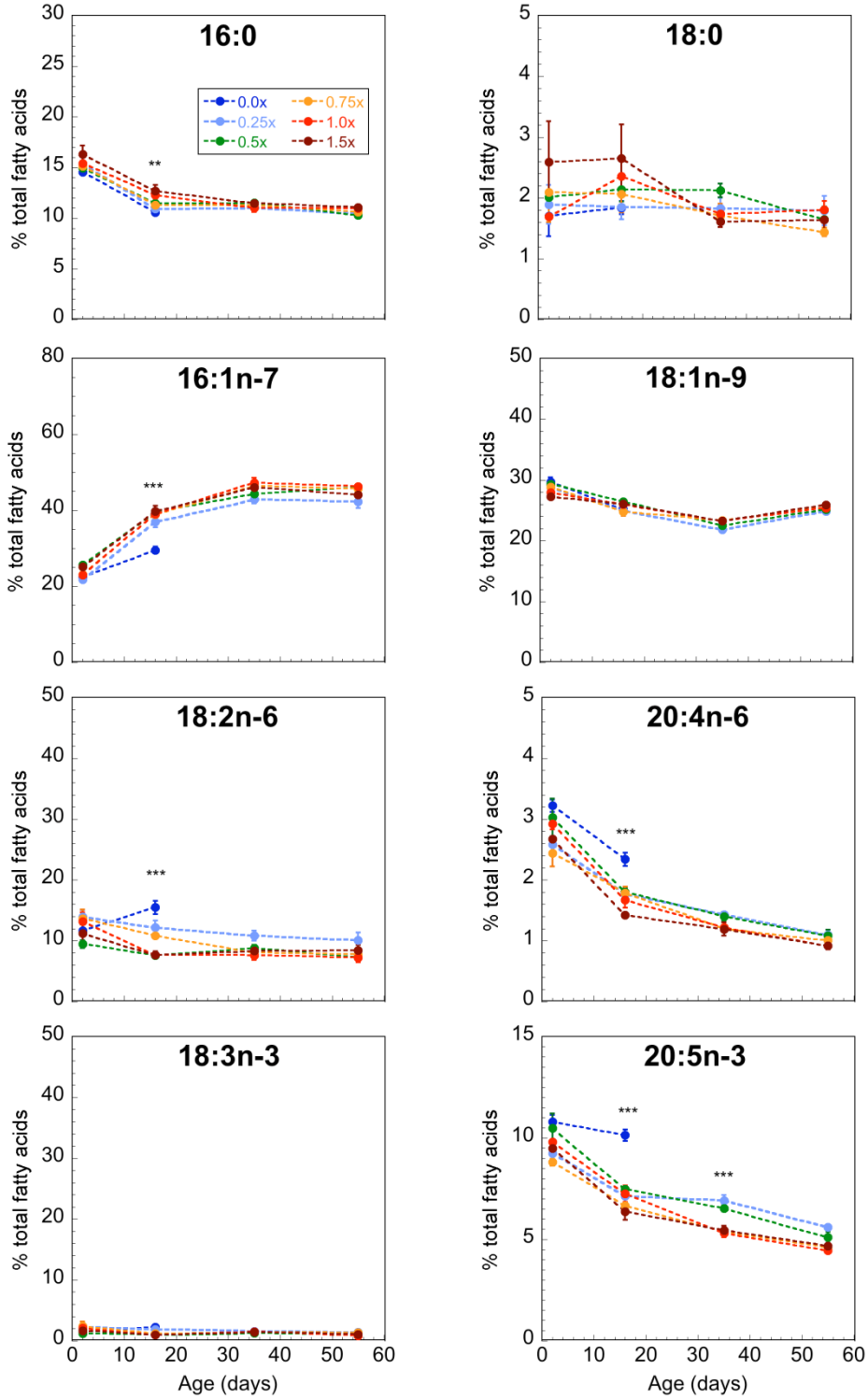

Figure 8.5 The main fatty acids present in whole blowfly phospholipids of adult *Calliphora stygia* kept on diets of varying yeast (nutrient) content (from 0.0*x* to 1.5*x* standard diet levels). Values are means ± SEM (N = 4). * represents a significant difference between dietary treatments at that day (* P <0.01, ** P <0.001, ***P <0.0001 (see Appendix E for tables).

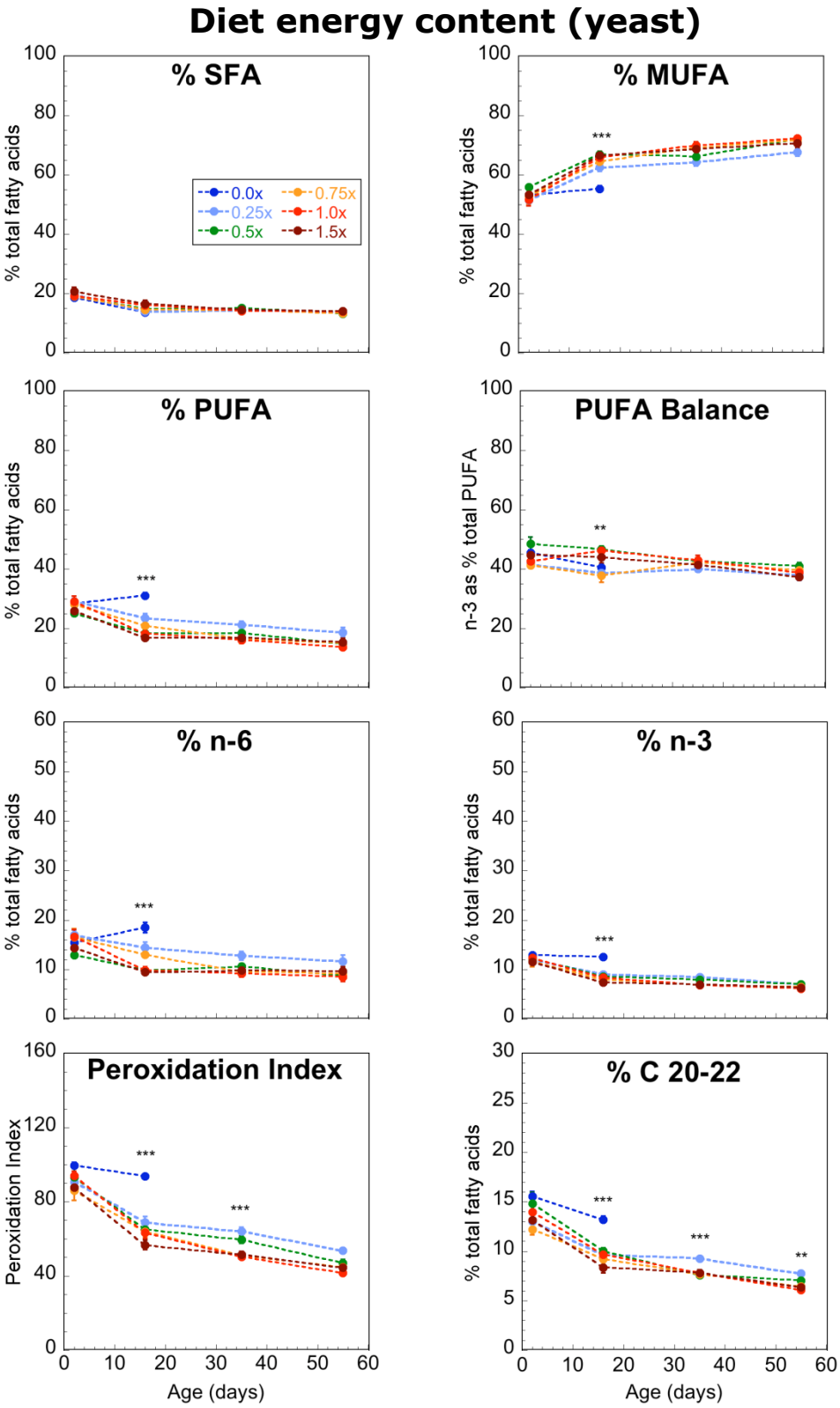

Figure 8.6 Membrane fatty acid composition of adult *Calliphora stygia* kept on diets of varying yeast (nutrient) content (from 0.0*x* to 1.5*x* standard diet levels). Values are means ± SEM (N = 4). * represents a significant difference between dietary treatments at that day (* P <0.01, ** P <0.001, ***P <0.0001 (see Appendix E for tables).

8.3 Discussion

Extension of longevity through environmental manipulation is largely considered to be a result of reduced ROS-related damage in accordance with the 'oxidative stress theory of ageing' (Sohal and Orr, 2012). The 'membrane pacemaker theory' adds an additional parameter to this, in that altering membrane fatty acid composition to reduce membrane peroxidation potential is likely to influence longevity by reducing lipid peroxidation products which are themselves potent ROS, and as a result of a positive feedback loop, significantly increase ROS-related damage (Hulbert et al., 2007). However, there was very little evidence that the differences in longevity due to either effects of environmental temperature or diet energy content were as a consequence of changes to membrane fatty acid composition.

8.3.1 Low temperature slows age-related changes in membrane fatty acid composition.

Lipid restructuring in response to temperature changes has been demonstrated in all ectothermic taxonomic groups examined (Crockett, 2008). One of the main effects of temperature is on the physical properties of membranes, with a tendency to replace SFA in their membranes with more unsaturated fatty acids to maintain fluidity when temperatures decrease (Hazel, 1995). Cold-acclimated fish and oysters have been shown to have large increases in long chain PUFA (Käkelä et al., 2008; Pernet et al., 2008). There was no obvious membrane restructuring in *C. stygia* maintained at temperatures over the range of 12°C to 34°C. Over time, blowflies kept at 25°C, showed a decrease in SFA and PUFA, and an increase in MUFA,

which was mainly contributable to an increase in 16:1*n*-7 and a generalised decrease in all PUFA. All temperature treatments showed the same pattern of membrane composition with age, with changes occurring more slowly in those blowflies kept at low temperatures. The temperature-induced differences appear to be then more a reflection of metabolic rate effects, rather than homeoviscous adaptation. Similar small but directional changes were seen in fruit flies when the effects of larval thermal acclimation on adult *D. melanogaster* membrane phospholipid composition were examined (Overgaard et al., 2008). Cold temperature (15°C) acclimation was characterised by reduced membrane 16:1 and increased 18:2 content compared to those maintained at the higher temperature (25°C). These changes were similar to those shown previously for rapid cold hardening in *D. melanogaster* (Overgaard et al., 2005), and are the same as seen in *C. stygia* with age. It is therefore possible that the changes observed in *D. melanogaster* are also due to slowed age-related compositional changes in response to cold temperatures rather than any temperature-induced membrane restructuring.

However, the analysis of only phospholipids in the current study may have masked some of the temperature changes to head groups or other aspects of membrane remodelling. Overgaard et al. (2008) examined the PE and PC head groups and found increased proportions of PE compared to PC in cold-acclimated flies as well as increased 18:2 at the s*n*-2 position, which has been shown to be more influential on membrane fluidity. These changes may reflect temperature-induced membrane remodelling, however, they could also be a result of age with temperature affecting the rate of ageing and therefore fatty acid composition. Studies of warm-acclimation in trout, *O. mykiss* have shown increased formation of plasmalogens,

which due to their antioxidant capacities may offer increased protection against lipid peroxidation at higher temperatures (Kraffe et al., 2007).

8.3.2 Diet energy content has little effect on membrane fatty acid composition of *C. stygia.*

Altering either the energy content of the diet by changing sugar or yeast content had very little effect on membrane fatty acid composition. Reduction in diet energy content results in significant lifespan extension across a range of organisms (Masoro, 2000). Laganiere and Yu (1989) showed membrane fatty acid of liver mitochondria became more peroxidation resistant under conditions of food restriction. A later comprehensive examination of caloric restriction treatments in mice showed similar results and highlighted the tissue specificity of membrane fatty acid changes under caloric restriction (Faulks et al., 2006). Although brain and skeletal muscle showed less changes, all tissues showed a similar decrease in the very long chain PUFA (>20 carbons), particularly arachidonic acid, which was replaced by the more peroxidation resistant 18:2*n*-6 (Faulks et al., 2006). However, *C. stygia* given diets varying in sugar content showed no changes between treatments in their membrane fatty acid composition, suggesting that dietary energy intake had little effect on membrane phospholipid composition, even though total energy intake was significantly higher on some diets.

The focus of dietary restriction studies has more recently moved from the importance of energy content towards the contribution of dietary balance of nutrients on longevity in both vertebrates and invertebrates (Simpson and Raubenheimer, 2009; Fanson and Taylor, 2012). There is increasing evidence from invertebrate

models that it is the ratio of yeast: sugar (or protein: carbohydrate), rather than calories *per se* that affects longevity (Mair et al., 2005; Lee et al., 2008; Maklakov et al., 2008; Skorupa et al., 2008; Fanson et al., 2009; Ja et al., 2009; Maklakov et al., 2009; Simpson and Raubenheimer, 2009; Fanson and Taylor, 2011). Altering the protein content of the diet has been shown to alter membrane fatty acid composition and PI in rat liver as well as the content of liver mitochondria complex I (Ayala et al., 2007). Restricting the yeast content of the diet of *C. stygia* however, resulted in few changes to membrane fatty acids. Diets with reduced yeast: sugar ratios as a result of yeast restriction had a reduced decline in 20:5*n*-3, however while this difference was significant at 35 days, all diets had similar levels of 20:5*n*-3 by day 55. This small change in 20:5*n*-3 increased the PI of the restricted diets, however, again the effect was transitory. Overall I would conclude that the diet yeast content effects on longevity in *C. stygia* are not a result of membrane fatty acid composition changes.

In contrast to these small changes seen with reduced yeast content, the yeast-free diet, and therefore, no external source of dietary fatty acids, had a dramatic effect on membrane fatty acid composition. Instead of an increase in MUFA and decrease in PUFA, flies given the yeast-free diet had relatively stable membrane fatty acid composition rather than the early changes seen for the other diets. It is therefore likely that the early modifications of membrane fatty acids seen with age in the temperature and diet energy content studies are partially a result of the change in diet, from the larval diet of fish carcass to the adult yeast-based agar food, which is high in the SFA 16:0 and the MUFAs 16:1*n*-7 and 18:1*n*-9.

Interestingly, the long chain PUFA 20:4*n*-6 is still consumed rapidly when fatty acids are absent in the diet, but 20:5*n*-3 was maintained at high levels. These long chain PUFA are important precursor molecules in insects for a variety of

metabolic functions (Stanley, 2006). Arachidonic acid is a precursor to a number of active metabolites in vertebrates (Kuehl Jr. and Egan, 1980), and while in general the levels of long chain PUFA are greatly reduced in insects compared to vertebrates, insects also utilise arachidonic acid as a precursor for biosynthesis of waxes, pheromones, and prostaglandins (Stanley-Samuelson et al., 1988). However, 20:5*n*-3 is also an important eicanosoid precursor, although less so than 20:4*n*-6 (Tapiero et al., 2002). The stable levels of 20:5*n*-3 in *C. stygia* when dietary fatty acids were absent may reflect a preference for 20:4*n*-6 metabolism or a specific signalling for 20:5*n*-3 metabolism which is influenced by the presence of dietary fatty acids. Radiolabelling studies could give insight into the fatty acid metabolism of *C. stygia*, and while these studies have been performed for a number of invertebrate species, little is known about the fatty acid metabolism of many Dipteran species (Stanley-Samuelson et al., 1988; Ghioni et al., 1996).

9 GENERAL DISCUSSION

This thesis experimentally examined the effects of three environmental variables on various parameters of adult longevity and senescence in the blowfly, *Calliphora stygia.* The variables examined were: i) environmental temperature, ii) altering the energy content of the diet (by separately altering the sugar or yeast content), and iii) dietary fatty acid profiles (as an attempt to manipulate membrane fatty acids composition). The first two experimental treatments are often used to manipulate longevity for the investigation of the processes of ageing in other species. The third treatment has not previously been used in invertebrates (to my knowledge) and was an attempt to directly test the 'membrane pacemaker theory of ageing'.

Before a treatment can be considered to affect the ageing process it must be shown to extend the maximum lifespan of a wide range of organisms to ensure its universality. Extension of lifespan is an important criterion, as many treatments (e.g. poisons) can shorten lifespan, but these are not necessarily acting on the ageing process. Similarly, treatments that affect the average lifespan, but not the maximum lifespan of a population, may only be affecting the relative health of those organisms, but not their lifespan potential.

Of the treatments I have investigated, manipulations of environmental temperature and diet energy content meet these criteria, as they were successful in extending both average and maximum longevity. By contrast, my attempt to alter membrane fatty acid composition by altering diet fatty acid profile was relatively unsuccessful. Membrane fatty acid composition changed temporally over the adult lifespan, predominantly displaying a decrease in 20-carbon PUFA and an increase in MUFA that was minimally affected by diet fatty acid profile.

9.1 A comparison of cellular and demographic senescence.

Senescence (or biological ageing) means to 'grow old'. However senescence is more than just a record of time since birth (or eclosion), it is associated with cumulative changes that lead to the inability to physiologically maintain homeostasis and ultimately death. Thus to measure the rate of senescence (the rate of ageing) one can measure either cumulative changes at a cellular level, or the final product of senescence, i.e. mortality, at the population level (demographically).

I measured both demographic and cellular measures of senescence in *C. stygia*. Demographic senescence was measured as the change in age-specific mortality of the populations, with 'ageing' indicated by an increase in the age-specific mortality rates with time (i.e. an increased risk of death with age). Ageing at the cellular level was examined by the fluorescent measurement of AGE pigments. These auto-fluorescent compounds consist of a complex mixture of oxidatively damaged biomolecules and have been shown to accumulate irreversibly with age (see introduction). They are also associated with a number of age-related diseases (Oudes et al., 1998), and although it has been proposed that these AGE pigments contribute to the process of ageing this has not been validated (Gerstbrein et al., 2005). If AGE pigment accumulation is indeed a mechanism of ageing, I expected that both demographic and cellular senescence would show similar patterns with age. A key finding in both Part A and Part B was that there was no concurrence between demographic senescence and cellular senescence (as measured by AGE pigment accumulation). This was especially evident with the low temperature treatments. These treatments showed an extended period of negligible demographic senescence

early in adult life, while at the same time showing a rapid accumulation of AGE pigments (especially in the head) indicating significant cellular senescence. This disparity was also evident in the diet energy content treatments (Part B). Blowflies given diets varying in energy density by the alteration of either sugar or yeast content, showed a similar rate of AGE pigment accumulation for all treatments. Thus there were no differences in the level of AGE accumulation between the treatments when measured at the same chronological age, despite significant extension of longevity being evident in some treatments. These results suggest that the accumulation of AGE pigments occurs with time, but has very little association with the ageing process.

There is the potential that limited sampling times in these experiments may have masked some dynamic aspects of AGE pigment accumulation. It is possible that AGE pigment accumulation is a slow cumulative process which gradually causes ageing, but then suddenly increases just before the onset of death. Because of my limited sampling I did not measure individuals that were particularly close to death, which potentially misses near-death increases in AGE pigments. Attempts to examine this on a small scale in *C. stygia* were somewhat inconclusive. Comparison of AGE pigment levels of blowflies that were ‘dead’ or ‘almost dead’ (found unable to right themselves from lying on their backs) did not differ at either 16 or 35 days. By contrast, AGE pigment levels were significantly higher in these groups than ‘alive’ (captured flying) blowflies at 16 days, but these differences were no longer significant by day 35. Fluorescent AGE pigment accumulation in *C. elegans* shows a similar pattern close to death, in that worms of the same chronological age but determined to have different ‘physiological’ ages showed significant differences in their levels of pigment accumulation. Those that were ‘severely impaired’ had higher

levels of pigment than the mildly impaired or healthy individuals (Gerstbrein et al., 2005). Both of these studies thus suggest that AGE pigment may accumulate more rapidly closer to death, but whether this directly affects mortality or the process of ageing is unclear.

9.2 Use of a two-phase Gompertz equation to describe differences in early and late rates of ageing in *C. stygia* and its dependence on yeast content

Over 180 years ago Benjamin Gompertz showed that the adult mortality rate of the human population increased exponentially with age (Gompertz, 1825). This is the basis of the Gompertz equation that describes adult mortality by a single linear relationship between the natural logarithm of mortality rate and age (Carey, 1998). There are some animals, for example some turtles, deep sea fish and naked mole rats, that do not show an increase in mortality rates with time and are thus said to have 'negligible' senescence (Finch, 2009). It was obvious in the low temperature treatments that the early adult life of *C. stygia* also displayed 'negligible' senescence followed by normal senescence. Blowflies maintained at high temperature showed the opposite relationship, having a faster rate of ageing during early adult life that slowed with old age. The two-phase Gompertz equation has been used to describe this fast early ageing and slowed senescence during old age in species ranging from humans to flies (Vaupel et al., 1998; Shahrestani et al., 2012). To my knowledge, however, this equation has not previously been used to describe the slowed early phase of ageing that I found in the low temperature blowflies.

The mortality rates of the 'control' population groups (standard SY diet, held at 25°C) for all experiments showed this biphasic pattern, with the early ageing rate

being significantly slower than that of late ageing (at 25°C). The two-phase Gompertz was capable of distinguishing this slowed and extended early phase of ageing from the increased rate of the late phase, which previously has only been inferred from observation of a right shifting of mortality or survival curves. There are many ways that treatments can affect demographic ageing. Firstly, treatments may extend longevity by reducing the rate of ageing of either the early or the late phases of ageing. Secondly, treatments may extend longevity by extending the period of slowed early ageing delaying the onset of more rapid ageing with old age.

Extended periods of slowed early senescence was particularly obvious in blowflies kept at low temperatures. *Calliphora stygia* kept at low temperatures had a significantly extended average (three-fold) and maximum (two-fold) longevities compared to blowflies kept at 25°C. The early phase of ageing proved to be the most temperature sensitive part of ageing. Blowflies at high temperatures showed a fast rate of ageing during the early phase, which slowed during old age. By contrast, blowflies kept at low temperatures (≤20°C) had a significantly longer period of slow senescence during the early phase of ageing, with their rate of ageing of this early period not differing significantly from zero. Because ageing is associated with an increase in mortality rates, cold-exposed blowflies appear to experience minimal senescence during this period. This negligible senescence was also apparent in blowflies transferred from high to low temperatures 14 days after eclosion. Thermal history dictated both the length of time of this suspended senescence and the mortality level at which it occurred, although the late rate of ageing was similar between flies during old age regardless of previous thermal history.

Altering the dietary content of sugar or yeast also significantly affected longevity and demographic senescence. This was not a function of the energy content

of the diet, however, as the two treatments had almost opposing effects. When sugar content of the diet was increased, this increased both the energy content of the diet and longevity. The opposite occurred when the dietary yeast content was altered. Longevity peaked as diet energy content was reduced to moderate levels, but then was decreased when yeast levels were reduced further.

These different dietary treatments also changed how demographic senescence was affected (these effects on longevity in the context of dietary restriction studies will be discussed later; see section 9.3). As already mentioned, the mortality rates of blowfly populations maintained on standard diet at 25°C, resembled those of blowflies held at moderate to low temperatures, with a biphasic mortality pattern, consisting of a period of significantly slower ageing during the early phase, followed by a faster rate of ageing during the late phase. Environmental temperature more strongly affected this early phase, by both extending its duration, and reducing the rate of ageing. This was also the case for the extended longevity seen in the sugar dietary treatment, with blowflies getting the highest sugar diet having a longer period of slowed early ageing. By contrast, blowflies consuming diets with reduced yeast content demonstrated no biphasic relationship between age and mortality rates and there was no period of slow senescence early in adult life.

The diet treatments with 25 -75% of normal yeast content were associated with an extension of lifespan within that experiment (Chapter 6). This extended longevity was associated with a slower rate of ageing over the complete adult lifespan and was not due to an extended period of early slowed senescence. This contrasts with findings in *D. melanogaster*, which showed that dietary restriction extended longevity by delaying the onset of senescence rather than altering the rate of senescence (Mair et al., 2003). However, fruit flies in that study were given

diluted diets of equal sugar and yeast content, rather than reduced yeast as in this experiment.

The biphasic mortality pattern of a slower initial ageing rate followed by a higher subsequent rate that was observed in *C. stygia* appeared to require yeast in the diet. In the temperature experiments blowflies were given diets of equal sugar and yeast content. In the diet sugar experiments the yeast content was kept constant while the sugar content was increased or decreased. The two phases of ageing were also manifest in blowflies in the diet fatty acid profile experiment (Part C), in which all treatments received equal amounts of sugar and yeast. It was only the blowflies given reduced yeast content in the yeast dietary experiment that did not show a slower initial rate of ageing.

Interestingly, a period of reduced or negligible senescence could be induced following transfer to low temperature part way through the adult lifespan. This suspension of ageing is therefore likely a period of either increased repair rate, or reduced rate of damage (possibly by increased antioxidant mechanisms), or a combination of both. What this may mean is that periods of negligible or suspended senescence requires adequate supply of some essential nutrient found in yeast (for example, an essential amino acid, mineral, vitamin or fatty acid), which may explain why this period is absent from the low yeast treatments.

9.3 Diet energy content: the balance of nutrients is more important than energy content.

There are three important factors to consider when evaluating dietary manipulation experiments and their effects on longevity. Firstly, manipulating diet to

extend longevity of insects is often performed through dilution of a standard diet that results in the insect consuming food of lesser calorie content. An overarching assumption of such a treatment is that insects will consume similar amounts of diluted food as those given standard diets, and thus consume fewer calories. However, compensatory feeding has been shown in a number of insect species, through both increasing the amounts of food consumed (Carvalho et al., 2005; Lee et al., 2008; Ja et al., 2009), or by increasing the frequency of feeding (Simpson and Abisgold, 1985). A strength of my study, therefore, is that total food consumption was measured over the lifetime of each population. This allows determination of the effect that altering dietary components had on the amount of food consumed by the populations, whether *C. stygia* compensated for the reduced or increased caloric or nutrient content of the food, as well as the amount of each component they actually consumed.

Secondly, while energy restriction is mostly considered to be the cause of extended longevity, there is increasing support for the dietary restriction of certain nutrients being more important than the amount of energy consumed (Mair et al., 2005; Grandison et al., 2009; Simpson and Raubenheimer, 2009). Therefore, in addition to measuring food consumption, the diet components were altered separately to distinguish the effects of differing dietary sugar and yeast contents on longevity.

Thirdly, an important, yet often less considered, aspect of dietary manipulation studies, is that the baseline diet is often an arbitrarily defined diet. That is, a lab-made food type that has been previously successful in rearing and maintaining lab populations. This presumes that lab-based diets are optimal, but this is rarely validated. While such diets may be adequate for particular aspects of an animal's existence, they do not necessarily maximise longevity. Therefore dietary

manipulations, particularly dietary restriction, may not represent a lifespan extension effect, but rather result from the standard diets being nutritionally inferior to the experimentally modified ones in permitting maximal longevity.

This last point was well illustrated by Clancy et al. (2002), who examined the longevities of the *chico*^{1} mutant and wild-type *D. melanogaster* on a range of diets. *Chico*^{1} mutants are dwarf fruit flies that are long-lived when fed standard diets, and this mutation affects the insulin-insulin-like signalling (IIS) pathways. Therefore, this mutant can be used to determine the influence of the IIS pathway on longevity and whether this pathway is directly linked with the longevity effects resulting from dietary restriction. Comparison of longevity of these two strains fed the standard diet, showed that the *chico*^{1} mutants were naturally dietary restricted and had an extended longevity over the wild type fruit flies. Furthermore, they also demonstrated that wild type fruit flies had an advantage over the *chico*^{1} mutants when food concentration was at lower levels and that the strains showed a similar peak in longevity but at a different food concentration. These findings demonstrated that the pathways involved in dietary restriction and the *chico*^{1} mutation were overlapping, but this could only be determined because longevity was maximised for both strains. In my experiments, the standard diet was not assumed to be the optimal diet for this species, and I both increased and decreased the two diet components to ensure a greater range of diets.

While my two diet manipulation studies were performed on two separate batches of pupae and at different times of year, I have plotted the two experiments together (Fig. 9A-C) to examine the overall effects of food energy content on longevity. What is immediately evident from Figure 9.1A, is that longevity was significantly affected by diet energy content. However, the extent of this effect was

highly dependent on which diet component was altered. *Calliphora stygia* given diets varying in yeast content had a more traditional bell-shaped curve, showing a peak of longevity at an intermediate level of energy content, as expected by a traditional dietary restriction protocol. However, blowflies given diets varying in sugar content had increased longevity with increasing dietary sugar.

In addition, *C. stygia* altered their food consumption in response to changed dietary content. Blowflies consumed more food as sugar content was increased, while feeding was suppressed in response to increased yeast content. Similar patterns of food consumption have also been shown in *D. melanogaster* (Skorupa et al., 2008). Due to these changes in food consumption, longevity was positively correlated with total energy consumed for both experiments (Fig. 9.1B). Increased longevity with increased energy consumption is in contradiction to the original premise of limiting caloric intake by dietary restriction. These results illustrate the importance of measuring food consumption when examining the effects of dietary restriction.

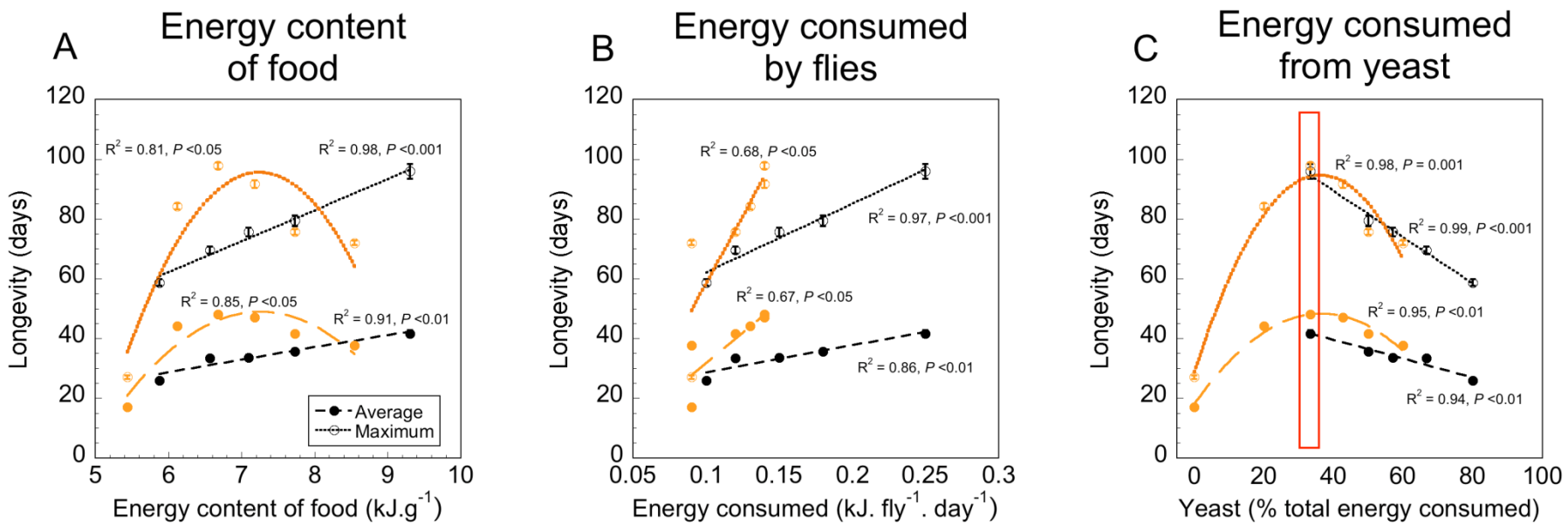

Figure 9.1. Average and maximum longevity of *Calliphora stygia* given diets varying in either sugar or yeast content. Fig. 9.1A. The relationship between longevity and energy content of the food was different depending on the diet component that was altered. Fig. 9.1B. The relationship between longevity and the average daily energy consumption of blowflies showed a significant positive correlation. Fig. 9.1C. The relationship between the energy consumed from yeast as a percentage of the total energy consumed and the longevity of blowflies. Regardless of how the balance between yeast and sugar components were obtained, longevity was optimal when yeast consumption was at ~33% (shown by the red box). Data points are means ± SEM. Data from the sugar experiment are shown in black, data from the yeast experiment are shown in yellow. Open circles represent maximum longevity and closed circles signify average longevity. Where no error bars are shown, the error is less than the size of the symbol.

The two dietary experiments show very different responses of food consumption dependent on the dietary element manipulated. The range of energy content of the diet was very similar over the two experiments, however, this is not true for energy consumption. The blowflies given the diets varying in yeast content appear to more strongly regulate the amount of energy consumed, with a much smaller range between the treatments. These results suggest that both sugar and yeast can affect longevity, but that they most likely do so through different mechanisms.

Both diet manipulation experiments had one thing in common, both were based on adjustments of yeast relative to sugar. Alterations in the ratios of yeast to sugar has more recently been shown to be the main driver of dietary restriction effects on longevity in the cricket, *T. commondus* (Maklakov et al., 2008; Maklakov et al., 2009) and the fruit fly species *D. melanogaster* (Mair et al., 2005; Bass et al., 2007; Lee et al., 2008; Ja et al., 2009; Lushchak et al., 2012), and *B. tryoni* (Fanson et al., 2009; Fanson and Taylor, 2011, 2012). Examining my results as the balance between energy consumed from yeast to total energy consumed, the two dietary experiments show a striking similarity (Fig. 9.1C). The optimal balance of energy obtained from yeast appears to be ~33%, regardless of the energy content of the overall diet (6.68 kJ/ 100 g for the yeast treatment that halved yeast content, and 9.31 kJ/ 100 g for the sugar treatment that doubled sugar content). When the relative levels of the contribution of yeast to energy intake are above or below this optimum proportion, longevity is reduced.

Some studies have described this balance as the protein: carbohydrate (P: C) ratio (Lee et al., 2008; Fanson et al., 2009; Fanson and Taylor, 2011; Lushchak et al., 2012). Yet in many insect models, it is not protein that is altered, but rather the levels of

yeast to sugar. Yeast contains many more nutrients than simply protein, as it is also the only source of vitamins, minerals, essential amino acids and fatty acids, as well as an additional source of carbohydrates in yeast/sugar diets. Consequently, any of these constituents in yeast may contribute to the nutritional importance of yeast in promoting longevity. Chemical-based diets that manipulate nutrients independently have been examined in the fruit fly *B. tryoni* (Fanson and Taylor, 2012). They found that it was not only dietary protein that affected longevity, but also minerals. Furthermore, they also showed that combinations of particular nutrients also affected longevity, with addition of specific amino acids resulting in a reduced longevity, whereas combinations of amino acids and other nutrients increased longevity. Therefore, Fanson and Taylor (2012) suggest exercising caution when presuming that the longevity effects of variation in yeast and sugar content of diets are due solely to differences in their protein and carbohydrate relationship. Therefore, the current study provides substantial evidence that it is not the energy consumption that affects longevity, but rather the balance of yeast in the diet that is the most likely modulator of lifespan. Further studies using chemically-defined diets along with measurement of food consumption rates and fecundity in a range of organisms are needed to better understand the roles of specific nutrients, as well as various combinations, in the extension of lifespan.

9.4 Membrane fatty acid composition is resistant to change and is not responsible for the longevity extension due to temperature and diet energy content

The ‘membrane pacemaker theory of ageing’ proposes that the susceptibility of specific fatty acids to peroxidation, and the autocatalytic nature and wide-ranging damage caused by this process, places membrane fatty acid composition as a very likely candidate for the modulation of lifespan (Hulbert, 2008b). Although my attempts to directly test this theory by varying dietary fatty acids was relatively unsuccessful, it was nonetheless insightful.

Membrane fatty acid composition in *C. stygia* was quite regulated and hard to change by diet modification. Studies in rats have shown that most tissues have a strong regulatory setpoint for the fatty acid composition of membranes, even over a wide range of diets (Abbott et al., 2010; Abbott et al., 2012). This was also true for *C. stygia*. While the two essential fatty acids, 18:2*n*-6 and 18:3*n*-3, were readily incorporated into the phospholipid pool, provision of 18:0 or 18:1*n*-9 had little effect on membrane fatty acid composition. This was further shown when marine oil mixtures were added to foods, with little change in membrane composition even when high amounts of long chain PUFA were readily available in the diet. *Calliphora stygia* also showed very little evidence of fatty acid metabolism of long chain PUFA, in that the increases in 18:2*n*-6 and 18:3*n*-3 were not associated with any concurrent increase in 20:4*n*-6 or 22:6*n*-3. This implies that elongation and desaturation of these essential fatty acids was lacking in adult blowflies. Thus, these blowflies may be similar to cats in that they lack the desaturases required to elongate the precursor fatty acids and are therefore obligate carnivores requiring ready-made dietary long chain PUFA from their prey (Rivers et al.,

1975). This period of obligate carnivory most likely occurs during the larval phase of *C. stygia*, as the age-related change in membrane composition were strongly regulated in adults, regardless of their dietary fatty acid profile.

There were no significant effects on mortality or maximum longevity between the fatty acid enriched diet treatments. *Calliphora stygia* given the 18:2*n*-6 fatty acid enriched diet had a reduced average longevity, possibly suggesting a negative affect of this particular fatty acid on the health of blowflies. By contrast, blowflies given 18:0, 18:1*n*-9 and 18:3*n*-3 enriched diets had an increase in average longevity of ~30% compared to blowflies receiving no added fats. The similarity between the lifespan effects of these latter fatty acids indicates that the provision of excess dietary fat is beneficial to the health of blowflies. The lack of benefit to average longevity by 18:2*n*-6 may suggest either 18:2*n*-6 has a negative effect on blowfly health or that 18:2*n*-6 is not required for these dietary effects on longevity.

The examination of membrane fatty acid composition of *C. stygia* under the different temperature and diet composition treatments suggests that membrane phospholipid composition was not the mechanism by which longevity was modulated. In all cases, there was very little difference in membrane fatty acid composition in response to treatments. This indicates that any affects of these treatments on longevity were not a result of changes to membrane fatty acid profiles.

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APPENDIX A

Gender differences in longevity

Table 10.1. Longevity comparison between genders of *Calliphora stygia* for all experimental treatment conditions.

	Average longevity (males vs. females) [t-test (Welch-corrected)]	Maximum longevity (males vs. females) [t-test (Welch-corrected)]
Temperature		
12°C	↓ **	↓***
15°C	↓***	↓***
20°C	↓***	↓***
25°C	ns	ns
29°C	ns	↑ **
34°C	ns	ns
Diet sugar content		
0.25*x*	ns	↑ **
0.50*x*	↑ *	↑***
0.75*x*	ns	ns
1.00*x*	ns	↑ **
2.00*x*	↑ *	ns
Diet yeast content		
0.00*x*	ns	ns
0.25*x*	ns	ns
0.50*x*	↓ **	ns
0.75*x*	ns	↑***
1.00*x*	ns	↑ **
1.50*x*	ns	↑ **

Arrows indicate male longevity compared to female longevity. Maximum longevity is calculated as the mean longevity of the longest-lived 5% of the population * represents $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$, 'ns' shows no significant difference.

APPENDIX B

Food consumption tables

Table 10.2. Average daily food consumption of *Calliphora stygia* kept at different temperatures.

Days	12°C (mg.fly^{-1})	15°C (mg.fly^{-1})	20°C (mg.fly^{-1})	25°C (mg.fly^{-1})	29°C (mg.fly^{-1})	34°C (mg.fly^{-1})
0-4	2.3 ± 0.2	8.4 ± 3.0	14.1 ± 1.5	23.2 ± 1.0	27.5 ± 0.6	17.2 ± 3.4
5-9	3.1 ± 1.0	9.3 ± 0.9	17.7 ± 1.0	20.4 ± 1.4	25.8 ± 0.7	13.6 ± 0.6
10-14	7.0 ± 0.7	12.6 ± 0.9	19.2 ± 1.4	19.3 ± 0.8	22.6 ± 3.4	14.4 ± 2.5
15-19	4.6 ± 1.5	13.9 ± 1.2	18.8 ± 1.3	23.9 ± 1.2	16.1 ± 2.6	11.6 ± 4.3
20-24	8.0 ± 2.3	9.1 ± 1.5	15.7 ± 0.6	14.6 ± 3.0	21.9 ± 6.2	11.7 ± 5.7
25-29	8.2 ± 1.7	6.6 ± 1.7	16.3 ± 3.0	16.8 ± 2.6	8.1 ± 4.4	11.0 ± 3.8
30-34	6.1 ± 2.5	4.7 ± 1.0	15.7 ± 1.9	14.7 ± 1.0		
35-39	2.4 ± 2.2	5.7 ± 1.2	11.0 ± 1.5	10.9 ± 1.4		
40-44	5.6 ± 2.2	6.2 ± 1.1	15.5 ± 4.1	10.8 ± 1.8		
45-49	2.0 ± 2.4	3.4 ± 1.5	13.3 ± 2.8	5.4 ± 2.4		
50-54	3.1 ± 1.3	4.8 ± 0.7	17.6 ± 2.8	9.1 ± 1.0		
55-59	1.6 ± 0.7	3.8 ± 0.6	10.7 ± 1.2	8.1 ± 2.3		
60-64	2.3 ± 0.7	3.1 ± 0.8	7.1 ± 0.6			
65-69	1.6 ± 1.0	3.7 ± 0.8	5.6 ± 1.2			
70-74	2.1 ± 0.5	4.0 ± 0.6	5.7 ± 1.0			
75-79	1.7 ± 1.0	3.2 ± 0.4				
80-84	2.1 ± 0.7	4.2 ± 0.5				
85-89	1.2 ± 0.5	2.7 ± 0.4				
90-94	0.4 ± 1.4	4.8 ± 1.3				
95-99		4.0 ± 0.3				
100-104		4.6 ± 0.4				
105-109		3.1 ± 0.4				
110-115		0.6 ± 0.9				

Values are means ± SEM, averaged over a 5-day period from pooled replicate cages. As population sizes decreased, food consumption became more variable, and only positive values are included in this table.

Table 10.3. Average daily food consumption of *Calliphora stygia* given diets of varying sugar content.

Days	**0.25*x*** **(mg.fly^{-1})**	**0.5*x*** **(mg.fly^{-1})**	**0.75*x*** **(mg.fly^{-1})**	**1.0*x*** **(mg.fly^{-1})**	**2.0*x*** **(mg.fly^{-1})**
1	20.0 ± 0.3	22.2 ± 3.2	29.0 ± 0.5	45.4 ± 2.3	42.2 ± 2.2
3	21.7 ± 5.2	38.8 ± 13.1	44.7 ± 13.8	56.4 ± 16.8	61.7 ± 2.3
5	25.6 ± 3.3	37.7 ± 6.4	41.8 ± 5.9	42.0 ± 3.3	60.8 ± 3.9
7	32.1 ± 0.0	25.1 ± 3.0	37.9 ± 4.8	43.4 ± 1.3	43.9 ± 0.3
9	18.4 ± 3.4	26.1 ± 12.7	35.7 ± 23.0	41.9 ± 1.9	42.0 ± 0.2
11	10.0 ± 0.0	30.8 ± 4.8	39.0 ± 12.0	33.5 ± 2.4	35.4 ± 4.5
13	16.8 ± 2.9	25.0 ± 3.0	40.8 ± 1.2	32.8 ± 4.5	33.0 ± 1.1
15	15.5 ± 3.1	39.9 ± 12.2	38.8 ± 5.9	49.5 ± 21.9	32.1 ± 1.6
17	22.0 ± 2.4	2.3 ± 3.9	38.4 ± 3.2	37.1 ± 2.9	41.8 ± 10.4
26	-5.5 ± 4.5	11.8 ± 1.1	11.8 ± 0.7	14.5 ± 0.8	25.2 ± 0.5
28	2.4 ± 5.2	15.2 ± 15.0	16.7 ± 2.5	20.0 ± 0.9	29.2 ± 4.5
30		6.8 ± 4.6	12.0 ± 1.3	21.1 ± 3.8	25.0 ± 2.1
32		4.6 ± 1.0	8.4 ± 6.6	18.2 ± 3.2	16.5 ± 1.1
34		9.1 ± 8.3	9.9 ± 0.1	18.4 ± 2.9	17.2 ± 2.6
36		6.7 ± 1.0	7.9 ± 5.9	17.0 ± 2.6	16.3 ± 9.2
38		3.9 ± 0.8	7.5 ± 1.2	13.2 ± 1.4	18.4 ± 1.1
40		3.4 ± 1.0	6.8 ± 5.2	17.9 ± 3.7	19.1 ± 1.1
42		2.1 ± 9.0	12.9 ± 3.1	23.8 ± 10.6	24.3 ± 3.0
44			10.8 ± 16.0	18.2 ± 0.7	21.0 ± 2.0
46			8.7 ± 5.0	-1.2 ± 22.9	16.8 ± 0.4
48			6.2 ± 0.3	17.1 ± 3.8	16.5 ± 1.1
50			3.5 ± 2.8	0.4 ± 6.3	14.2 ± 2.4
52				0.4 ± 0.9	17.4 ± 3.0
54				4.5 ± 16.2	16.8 ± 3.7
56				7.4 ± 8.2	14.3 ± 4.0
58					22.7 ± 5.5
60					14.1 ± 7.4
62					13.5 ± 14.0
64					24.6 ± 6.8

Values are means ± SEM, measured every second day and averaged over replicate cages. As population sizes decreased, food consumption became more variable, and only positive values are included in this table.

Table 10.4. Average daily food consumption of *Calliphora stygia* given diets of varying yeast content.

Days	**0.0*x*** **(mg.fly^{-1})**	**0.25*x*** **(mg.fly^{-1})**	**0.5*x*** **(mg.fly^{-1})**	**0.75*x*** **(mg.fly^{-1})**	**1.0*x*** **(mg.fly^{-1})**	**1.5*x*** **(mg.fly^{-1})**
0-4	20.3 ± 3.5	28.1 ± 1.3	26.3 ± 1.2	31.4 ± 3.0	18.7 ± 1.4	19.6 ± 1.1
5-9	18.3 ± 1.7	37.2 ± 2.9	41.4 ± 2.3	32.4 ± 1.9	22.2 ± 1.5	19.8 ± 1.2
10-14	16.4 ± 1.4	32.4 ± 1.9	32.5 ± 3.9	24.2 ± 2.5	23.0 ± 0.3	13.5 ± 1.1
15-19	7.3 ± 7.6	24.5 ± 1.3	23.7 ± 3.6	22.1 ± 3.2	17.9 ± 1.0	11.5 ± 1.0
20-24		26.2 ± 0.3	29.8 ± 2.6	30.7 ± 3.2	17.7 ± 2.1	10.0 ± 1.2
25-29		27.0 ± 2.1	29.0 ± 2.1	19.4 ± 1.3	16.3 ± 0.9	10.0 ± 0.7
30-34		21.5 ± 3.0	22.9 ± 4.1	19.1 ± 1.6	12.6 ± 1.7	6.4 ± 1.6
35-39		21.2 ± 3.8	22.7 ± 3.2	20.0 ± 4.6	5.5 ± 1.7	3.9 ± 2.4
40-44		18.5 ± 1.1	22.8 ± 1.5	20.0 ± 2.2	4.7 ± 1.2	3.9 ± 1.7
45-49		6.3 ± 4.7	11.3 ± 4.1	6.6 ± 2.9		
50-54		11.0 ± 1.6	9.7 ± 1.7	4.3 ± 1.0		
55-59		3.9 ± 3.2	6.6 ± 1.3	1.3 ± 1.9		
60-64			4.1 ± 0.9			

Values are means ± SEM, averaged over a 5-day period from pooled replicate cages. As population sizes decreased, food consumption became more variable, and only positive values are included in this table.

APPENDIX C

Fluorescent AGE Pigment

(alive, 'dying' and dead comparison)

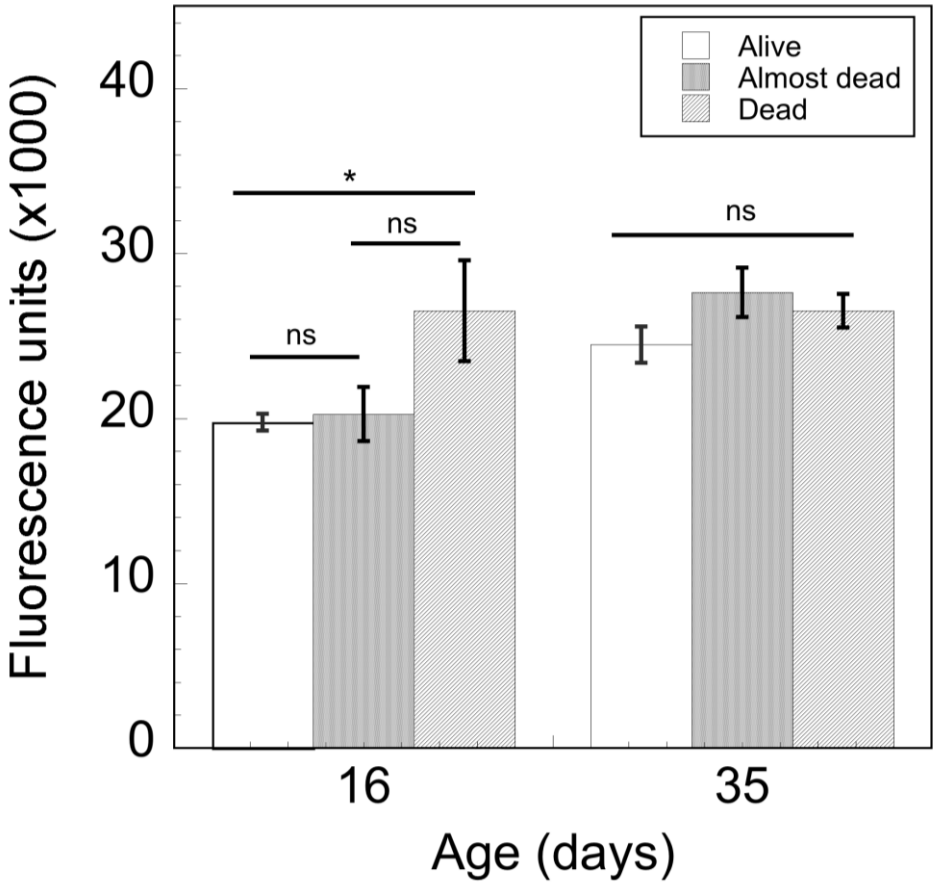

Figure 10.1. Fluorescent AGE pigment of *Calliphora stygia* held at 25°C and collected as alive, dead or 'dying' at days 16, and 35. At day 16, dead blowflies had a significantly higher level of AGE pigment than blowflies collected alive, but was not statistically different to those collected 'dying'. Both the alive and 'dying' blowflies had an increase in AGE pigment by day 35 ($P < 0.01$ for both), yet there was no increase demonstrated in dead blowflies ($P > 0.05$). As a result of this, there was no statistical significance between the groups measured at day 35. 'Dying' blowflies were determined as blowflies that were found on their backs and could not right themselves. Dead blowflies were found dead within the cage and could have died at any point within the previous 24 hours. Values are means ± SEM ($N = 8$ for dead and alive blowflies measured at days 16 and 35, $N = 6$ for 'dying' blowflies at both 16 and 35 days). * represents a significant difference at $P < 0.05$, 'ns' indicates no significant difference.

APPENDIX D

Control comparisons between different experiments

Table 10.5. Comparison of various parameters of 'control' cohorts between experiments.

	Part A (Ch. 3) (25 °C)	Part B (Ch. 5) (Sugar 1.0*x*)	Part B (Ch. 6) (Yeast 1.0*x*)
Food consumption ($mg.fly^{-1}.day^{-1}$)	15.3 ± 0.9^{A}	23.7 ± 3.3^{B}	15.1 ± 1.0^{A}
Average longevity (days)	27.7 ± 0.7^{A}	35.6 ± 1.0^{B}	41.5 ± 0.7^{C}
Maximum longevity (days)	66.9 ± 0.7^{A}	79.4 ± 1.8^{B}	75.6 ± 1.0^{B}

Comparisons between cohorts made by ANOVA (post-hoc test Tukeys HSD). Rows where values do not share the same letter indicate a significant difference between those cohorts ($P < 0.05$). Values are means ± SEM

APPENDIX E

Fatty acid tables

Table 10.6 *Calliphora stygia* whole body phospholipids fatty acid composition (expressed as % of total fatty acids) fed diets enriched with pure methyl esters from day 7-42.

Day 7 – Whole body phospholipids

Diet	**Standard**	**18:0**	**18:1*n*-9**	**18:2*n*-6**	**18:3*n*-3**	***P***
(N =)	*(4)*	*(4)*	*(4)*	*(4)*	*(4)*	*(< 0.01)*
14:0	0.4 ± 0.0^{ab}	0.5 ± 0.0^{b}	0.4 ± 0.0^{ac}	0.4 ± 0.0^{ac}	0.3 ± 0.0^{bc}	<0.0001
15:0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	ns
16:0	12.0 ± 0.3	13.8 ± 0.7	12.1 ± 0.4	13.0 ± 0.1	13.0 ± 0.4	ns
17:0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	ns
18:0	1.2 ± 0.2	2.6 ± 0.7	0.6 ± 0.1	1.5 ± 0.1	1.8 ± 0.1	ns
20:0	0.3 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	ns
21:0	0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	ns
22:0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	ns
16:1*n*-7	36.7 ± 1.6^{a}	32.9 ± 1.9^{ab}	30.8 ± 1.3^{b}	22.6 ± 0.4^{c}	18.4 ± 0.3^{c}	< 0.0001
18:1*n*-9	28.3 ± 0.4^{a}	27.6 ± 0.2^{a}	34.0 ± 0.5^{b}	23.0 ± 0.5^{c}	21.7 ± 0.4^{c}	< 0.0001
18:1*n*-7	0.7 ± 0.0^{a}	0.6 ± 0.0^{a}	0.5 ± 0.0^{ab}	0.4 ± 0.0^{b}	0.5 ± 0.0^{b}	<0.0001
18:2*n*-6	5.8 ± 0.8^{a}	6.0 ± 0.7^{a}	6.1 ± 0.9^{a}	25.4 ± 0.6^{b}	4.7 ± 0.6^{a}	< 0.0001
18:3*n*-6	0.5 ± 0.0	0.5 ± 0.1	0.5 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	ns
20:4*n*-6	2.3 ± 0.1	2.5 ± 0.2	2.4 ± 0.2	2.3 ± 0.1	2.0 ± 0.1	ns
18:3*n*-3	0.8 ± 0.1^{a}	0.8 ± 0.1^{a}	0.9 ± 0.1^{a}	0.7 ± 0.1^{a}	27.6 ± 1.1^{b}	< 0.0001
20:5*n*-3	8.5 ± 0.4	9.1 ± 0.5	9.0 ± 0.4	8.0 ± 0.3	7.5 ± 0.1	ns
22:6*n*-3	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	ns
SFA	14.6 ± 0.4^{a}	18.1 ± 1.4^{b}	14.0 ± 0.3^{a}	15.9 ± 0.2^{ab}	16.0 ± 0.3^{ab}	0.0081
MUFA	67.1 ± 1.4^{a}	62.6 ± 1.9^{a}	66.8 ± 1.7^{a}	47.2 ± 0.8^{b}	41.6 ± 0.5^{b}	< 0.0001
PUFA	18.3 ± 1.2^{a}	19.3 ± 1.5^{a}	19.2 ± 1.5^{a}	36.9 ± 0.8^{b}	42.4 ± 0.8^{c}	< 0.0001
***n*-6 PUFA**	8.6 ± 0.9^{a}	9.0 ± 0.9^{a}	9.0 ± 1.1^{a}	28.1 ± 0.6^{b}	7.0 ± 0.7^{a}	< 0.0001
***n*-3 PUFA**	9.6 ± 0.4^{a}	10.2 ± 0.5^{a}	10.2 ± 0.5^{a}	8.9 ± 0.3^{a}	35.5 ± 1.0^{b}	< 0.0001
C 20-22	12.4 ± 0.5^{ab}	13.3 ± 0.9^{a}	13.0 ± 0.5^{a}	11.7 ± 0.4^{bc}	10.9 ± 0.2^{c}	< 0.0001
PI	72.9 ± 3.1^{a}	77.3 ± 4.5^{a}	76.4 ± 3.7^{a}	87.3 ± 2.5^{b}	117.1 ± 1.2^{c}	< 0.0001
UI	136.4 ± 2.3^{a}	136.1 ± 3.9^{a}	139.5 ± 2.7^{a}	151.4 ± 1.9^{b}	182.1 ± 1.7^{c}	< 0.0001
PUFA Balance	53.2 ± 1.7^{a}	53.4 ± 1.6^{a}	53.4 ± 2.0^{a}	24.0 ± 0.4^{b}	83.5 ± 1.6^{c}	< 0.0001

Values are means ± SEM. Only fatty acids present at > 0.1% are included in the above table.

Day 14 – Whole body phospholipids

Diet	**Standard**	**18:0**	**18:1*n*-9**	**18:2*n*-6**	**18:3*n*-3**	***P***
(N =)	*(4)*	*(4)*	*(4)*	*(4)*	*(4)*	*(< 0.01)*
14:0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	ns
15:0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	
16:0	12.1 ± 0.4^{abc}	12.3 ± 0.3^{abc}	11.4 ± 0.2^{b}	13.0 ± 0.3^{c}	12.9 ± 0.1^{abc}	0.004
17:0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	ns
18:0	1.5 ± 0.1	2.0 ± 0.2	1.2 ± 0.1	1.7 ± 0.2	1.8 ± 0.2	ns
20:0	0.3 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	ns
21:0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	
22:0	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	ns
16:1*n*-7	39.5 ± 1.5^{a}	37.9 ± 1.2^{ab}	33.5 ± 0.9^{b}	20.2 ± 1.0^{c}	18.7 ± 0.5^{c}	<0.0001
18:1*n*-9	27.1 ± 0.5^{a}	26.6 ± 0.1^{a}	33.4 ± 0.3^{b}	19.9 ± 0.6^{c}	22.9 ± 0.4^{d}	<0.0001
18:1*n*-7	0.6 ± 0.0^{a}	0.6 ± 0.0^{a}	0.5 ± 0.0^{a}	0.3 ± 0.0^{b}	0.5 ± 0.0^{ab}	<0.0001
18:2*n*-6	5.5 ± 0.7^{a}	5.7 ± 0.8^{a}	5.8 ± 0.8^{a}	34.2 ± 1.7^{b}	4.2 ± 0.5^{a}	<0.0001
18:3*n*-6	0.6 ± 0.0^{a}	0.6 ± 0.0^{a}	0.6 ± 0.0^{a}	0.3 ± 0.0^{b}	0.3 ± 0.0^{b}	<0.0001
20:4*n*-6	2.2 ± 0.1	2.2 ± 0.1	2.0 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	ns
18:3*n*-3	0.9 ± 0.1^{a}	0.9 ± 0.1^{a}	0.9 ± 0.1^{a}	0.5 ± 0.1^{a}	29.1 ± 1.4^{b}	<0.0001
20:5*n*-3	7.9 ± 0.3^{abc}	8.7 ± 0.5^{b}	8.3 ± 0.2^{bc}	6.4 ± 0.4^{ab}	6.0 ± 0.3^{ac}	0.0003
22:6*n*-3	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	ns
SFA	14.9 ± 0.3^{ab}	15.8 ± 0.5^{ab}	14.0 ± 0.3^{a}	15.9 ± 0.5^{ab}	16.1 ± 0.2^{b}	0.004
MUFA	67.9 ± 1.1^{a}	66.0 ± 1.1^{a}	68.2 ± 0.8^{a}	40.9 ± 1.6^{b}	42.5 ± 0.8^{b}	<0.0001
PUFA	17.2 ± 0.8^{a}	18.2 ± 1.2^{a}	17.8 ± 0.9^{a}	43.2 ± 1.2^{b}	41.4 ± 0.7^{b}	<0.0001
***n*-6 PUFA**	8.3 ± 0.8^{a}	8.5 ± 0.8^{a}	8.4 ± 0.9^{a}	36.1 ± 1.6^{b}	6.2 ± 0.6^{a}	<0.0001
***n*-3 PUFA**	9.0 ± 0.3^{a}	9.7 ± 0.6^{a}	9.4 ± 0.2^{a}	7.0 ± 0.5^{a}	35.3 ± 1.2^{b}	<0.0001
C 20-22	11.5 ± 0.3^{a}	12.5 ± 0.6^{a}	12.0 ± 0.2^{a}	9.1 ± 0.5^{b}	8.8 ± 0.4^{b}	<0.0001
PI	67.7 ± 2.0^{a}	72.5 ± 3.9^{ab}	70.0 ± 1.5^{ab}	82.7 ± 1.5^{b}	108.0 ± 2.0^{c}	<0.0001
UI	132.6 ± 1.3^{a}	135.0 ± 2.8^{a}	135.1 ± 1.3^{a}	151.0 ± 0.8^{b}	176.7 ± 1.6^{c}	<0.0001
PUFA Balance	52.3 ± 2.4^{a}	53.4 ± 2.2^{a}	53.2 ± 2.6^{a}	16.4 ± 1.6^{b}	85.0 ± 1.7^{c}	<0.0001

Values are means ± SEM. Only fatty acids present at > 0.1% are included in the above table.

Day 21 – Whole body phospholipids

Diet	**Standard**	**18:0**	**18:1*n*-9**	**18:2*n*-6**	**18:3*n*-3**	***P***
(N =)	*(4)*	*(4)*	*(4)*	*(4)*	*(4)*	*(< 0.01)*
14:0	0.5 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.1	0.5 ± 0.0	ns
15:0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	ns
16:0	11.4 ± 0.4	12.1 ± 0.1	11.2 ± 0.2	11.9 ± 0.7	12.3 ± 0.4	ns
17:0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	ns
18:0	1.6 ± 0.1	2.2 ± 0.2	1.4 ± 0.3	1.8 ± 0.1	2.0 ± 0.3	ns
20:0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	ns
21:0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	
22:0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	ns
16:1*n*-7	44.2 ± 1.8^{a}	40.9 ± 0.4^{a}	38.1 ± 0.8^{a}	21.4 ± 0.6^{b}	16.7 ± 1.2^{b}	< 0.0001
18:1*n*-9	26.5 ± 0.8^{a}	25.5 ± 0.2^{a}	31.6 ± 1.5^{b}	20.2 ± 0.3^{c}	21.9 ± 0.9^{ac}	< 0.0001
18:1*n*-7	0.6 ± 0.0^{a}	0.5 ± 0.0^{ab}	0.4 ± 0.0^{ab}	0.4 ± 0.0^{b}	0.4 ± 0.0^{ab}	0.0025
18:2*n*-6	3.8 ± 0.6^{a}	5.6 ± 0.4^{a}	5.3 ± 0.6^{a}	34.8 ± 0.5^{b}	3.7 ± 0.5^{a}	< 0.0001
18:3*n*-6	0.4 ± 0.1^{ab}	0.5 ± 0.0^{a}	0.5 ± 0.0^{a}	0.3 ± 0.0^{b}	0.2 ± 0.0^{b}	0.0002
20:4*n*-6	1.6 ± 0.1^{ab}	1.8 ± 0.1^{a}	1.7 ± 0.1^{ab}	1.4 ± 0.1^{ab}	1.2 ± 0.1^{b}	0.005
18:3*n*-3	0.6 ± 0.1^{a}	0.8 ± 0.0^{a}	0.8 ± 0.1^{a}	0.5 ± 0.0^{a}	34.7 ± 1.9^{b}	< 0.0001
20:5*n*-3	6.6 ± 0.5^{ab}	7.2 ± 0.1^{a}	6.5 ± 0.3^{abc}	5.3 ± 0.3^{bc}	4.7 ± 0.3^{c}	0.0004
22:6*n*-3	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	ns
SFA	14.3 ± 0.5	15.7 ± 0.3	13.9 ± 0.3	14.9 ± 0.8	15.5 ± 0.7	ns
MUFA	72.5 ± 1.6^{a}	68.2 ± 0.6^{a}	71.4 ± 0.7^{a}	42.9 ± 0.9^{b}	40.0 ± 2.0^{b}	< 0.0001
PUFA	13.2 ± 1.4^{a}	16.0 ± 0.4^{a}	14.8 ± 0.9^{a}	42.2 ± 0.1^{b}	44.5 ± 1.7^{b}	< 0.0001
***n*-6 PUFA**	5.9 ± 0.7^{a}	7.9 ± 0.3^{a}	7.5 ± 0.6^{a}	36.4 ± 0.5^{b}	5.1 ± 0.7^{a}	< 0.0001
***n*-3 PUFA**	7.3 ± 0.6^{a}	8.1 ± 0.1^{a}	7.3 ± 0.3^{a}	5.8 ± 0.4^{a}	39.4 ± 1.8^{b}	< 0.0001
C 20-22	9.5 ± 0.7^{abc}	10.3 ± 0.2^{a}	9.4 ± 0.3^{bc}	7.6 ± 0.4^{cd}	6.7 ± 0.4^{d}	0.0002
PI	54.6 ± 4.6^{a}	61.0 ± 1.0^{ab}	55.7 ± 2.5^{a}	74.8 ± 1.9^{b}	107.6 ± 3.5^{c}	< 0.0001
UI	123.4 ± 3.3^{a}	127.1 ± 0.8^{a}	125.2 ± 2.1^{a}	146.8 ± 1.8^{b}	180.5 ± 3.6^{c}	< 0.0001
PUFA Balance	55.8 ± 1.1^{a}	50.6 ± 1.1^{a}	49.6 ± 1.7^{a}	13.8 ± 0.9^{b}	88.5 ± 1.6^{c}	< 0.0001

Values are means ± SEM. Only fatty acids present at > 0.1% are included in the above table.

Day 42 – Whole body phospholipids

Diet	**Standard**	**18:0**	**18:1*n*-9**	**18:2*n*-6**	**18:3*n*-3**	***P***
(N =)	*(4)*	*(4)*	*(4)*	*(4)*	*(4)*	*(< 0.01)*
14:0	0.6 ± 0.1	0.6 ± 0.0	0.6 ± 0.0	0.7 ± 0.1	0.6 ± 0.1	ns
15:0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	ns
16:0	11.1 ± 0.4	11.6 ± 0.8	11.1 ± 0.3	12.5 ± 1.1	13.4 ± 0.7	ns
17:0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	ns
18:0	1.8 ± 0.1	1.5 ± 0.1	1.2 ± 0.1	1.9 ± 0.2	1.8 ± 0.3	ns
20:0	0.4 ± 0.0	0.3 ± 0.1	0.3 ± 0.0	0.4 ± 0.1	0.3 ± 0.1	ns
21:0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	
22:0	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.1	0.3 ± 0.0	0.2 ± 0.0	ns
16:1*n*-7	48.4 ± 1.5^{a}	48.4 ± 1.6^{a}	45.9 ± 1.5^{a}	23.4 ± 2.5^{b}	22.3 ± 1.6^{b}	< 0.0001
18:1*n*-9	26.2 ± 0.7^{a}	26.2 ± 0.9^{a}	30.0 ± 1.2^{b}	21.0 ± 0.4^{c}	23.4 ± 0.2^{ac}	< 0.0001
18:1*n*-7	0.5 ± 0.1^{a}	0.4 ± 0.0^{ab}	0.4 ± 0.0^{ab}	0.4 ± 0.0^{b}	0.4 ± 0.0^{b}	0.0057
18:2*n*-6	3.5 ± 0.5^{a}	3.6 ± 0.2^{a}	3.5 ± 0.1^{a}	33.3 ± 1.9^{b}	2.7 ± 0.4^{a}	< 0.0001
18:3*n*-6	0.3 ± 0.0^{a}	0.3 ± 0.0^{a}	0.4 ± 0.1^{a}	0.2 ± 0.0^{b}	0.1 ± 0.0	<0.0001
20:4*n*-6	1.0 ± 0.o	1.0 ± 0.0	1.0 ± 0.2	1.0 ± 0.1	0.6 ± 0.1	ns
18:3*n*-3	0.5 ± 0.1^{a}	0.5 ± 0.1^{a}	0.5 ± 0.0^{a}	0.4 ± 0.0^{a}	30.7 ± 1.2^{b}	< 0.0001
20:5*n*-3	4.6 ± 0.2^{a}	4.6 ± 0.1^{a}	4.5 ± 0.4^{ab}	4.2 ± 0.2^{ab}	3.2 ± 0.3^{b}	0.0063
22:6*n*-3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	
SFA	14.2 ± 0.6	14.3 ± 1.0	13.4 ± 0.5	16.0 ± 1.5	16.3 ± 1.2	ns
MUFA	75.7 ± 1.2^{a}	75.6 ± 0.7^{a}	76.8 ± 0.5^{a}	45.0 ± 3.0^{b}	46.3 ± 1.5^{b}	< 0.0001
PUFA	10.1 ± 0.8^{a}	10.1 ± 0.4^{a}	9.9 ± 0.5^{a}	39.0 ± 1.7^{b}	37.3 ± 1.4^{b}	< 0.0001
***n*-6 PUFA**	4.9 ± 0.5^{a}	4.9 ± 0.2^{a}	4.9 ± 0.2^{a}	34.5 ± 1.9^{b}	3.5 ± 0.5^{a}	< 0.0001
***n*-3 PUFA**	5.2 ± 0.3^{a}	5.2 ± 0.2^{a}	5.0 ± 0.4^{a}	4.6 ± 0.2^{a}	33.9 ± 1.2^{b}	< 0.0001
C 20-22	6.8 ± 0.3^{a}	6.7 ± 0.1^{a}	6.4 ± 0.5^{a}	6.1 ± 0.2^{ab}	4.5 ± 0.3^{b}	0.0005
PI	39.2 ± 2.0^{a}	39.0 ± 1.2^{ab}	38.0 ± 2.8^{a}	64.5 ± 1.3^{b}	87.1 ± 2.9^{c}	< 0.0001
UI	112.7 ± 1.4^{a}	112.5 ± 1.8^{a}	112.7 ± 2.3^{a}	138.1 ± 1.6^{b}	162.6 ± 3.4^{c}	< 0.0001
PUFA Balance	51.9 ± 2.4^{a}	51.2 ± 1.2^{a}	50.3 ± 1.8^{a}	11.8 ± 1.0^{b}	90.8 ± 1.0^{c}	< 0.0001

Values are means ± SEM. Only fatty acids present at > 0.1% are included in the above table.

Table 10.7. *Calliphora stygia* thoracic mitochondria phospholipid fatty acid composition (% of total fatty acids) fed diets enriched with pure methyl esters from day 7 to 28.

Day 7 – Mitochondrial phospholipids

Diet	**Standard**	**18:0**	**18:1*n*-9**	**18:2*n*-6**	**18:3*n*-3**	***P***
(N =)	*(4)*	*(4)*	*(4)*	*(4)*	*(4)*	*(< 0.01)*
14:0	0.4 ± 0.0	0.6 ± 0.1	0.7 ± 0.2	0.2 ± 0.1	0.2 ± 0.1	ns
15:0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.1	0.0 ± 0.0	0.1 ± 0.0	ns
16:0	12.1 ± 0.3	13.6 ± 1.0	12.8 ± 1.0	10.8 ± 0.7	11.1 ± 0.8	ns
17:0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	ns
18:0	1.2 ± 0.2	2.7 ± 1.0	2.9 ± 1.2	1.0 ± 0.6	0.4 ± 0.2	ns
20:0	0.3 ± 0.0^{a}	0.2 ± 0.1^{ab}	0.2 ± 0.0^{b}	0.1 ± 0.0^{b}	0.1 ± 0.0^{b}	<0.0001
21:0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	ns
22:0	0.3 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	ns
16:1*n*-7	37.1 ± 1.6^{a}	35.3 ± 2.6^{ab}	32.5 ± 2.0^{b}	23.7 ± 0.4^{c}	23.4 ± 1.4^{c}	< 0.0001
18:1*n*-9	28.6 ± 0.4^{a}	27.6 ± 0.3^{a}	31.6 ± 0.6^{b}	25.2 ± 0.5^{c}	24.6 ± 0.2^{c}	< 0.0001
18:1*n*-7	0.7 ± 0.0	0.3 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	ns
18:2*n*-6	5.8 ± 0.8^{a}	4.9 ± 0.6^{a}	5.9 ± 0.4^{a}	26.8 ± 2.2^{b}	6.6 ± 0.7^{a}	< 0.0001
18:3*n*-6	0.5 ± 0.0	0.2 ± 0.1	0.4 ± 0.1	0.6 ± 0.0	0.4 ± 0.2	ns
20:4*n*-6	2.4 ± 0.1	2.5 ± 0.1	2.6 ± 0.1	2.6 ± 0.1	2.9 ± 0.2	ns
18:3*n*-3	0.8 ± 0.1^{a}	0.8 ± 0.0^{a}	1.0 ± 0.0^{a}	1.1 ± 0.1^{a}	20.8 ± 1.8^{b}	< 0.0001
20:5*n*-3	8.6 ± 0.3	6.6 ± 2.2	8.4 ± 0.3	7.2 ± 1.5	8.7 ± 0.3	ns
22:6*n*-3	0.3 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	ns
SFA	14.7 ± 0.4^{a}	17.6 ± 2.0^{b}	17.1 ± 2.6^{a}	12.3 ± 1.3^{ab}	12.1 ± 1.0^{ab}	ns
MUFA	66.7 ± 1.4^{a}	63.3 ± 2.7^{a}	64.1 ± 2.5^{a}	48.9 ± 0.1^{b}	48.2 ± 1.6^{b}	< 0.0001
PUFA	18.6 ± 1.2^{a}	19.1 ± 2.8^{a}	18.8 ± 0.4^{a}	38.8 ± 1.2^{b}	39.7 ± 1.2^{c}	< 0.0001
***n*-6 PUFA**	8.8 ± 0.9^{a}	9.4 ± 2.3^{a}	8.9 ± 0.5^{a}	30.0 ± 2.2^{b}	9.9 ± 0.9^{a}	< 0.0001
***n*-3 PUFA**	9.8 ± 0.4^{a}	9.7 ± 0.5^{a}	9.8 ± 0.3^{a}	8.8 ± 1.4^{a}	29.8 ± 1.7^{b}	< 0.0001
C 20-22	12.2 ± 0.4	12.0 ± 0.8	11.7 ± 0.4	10.4 ± 1.5	12.1 ± 0.5	ns
PI	74.4 ± 3.1^{a}	76.5 ± 7.0^{a}	74.4 ± 2.0^{a}	88.3 ± 6.9^{b}	116.5 ± 3.0^{c}	< 0.0001
UI	137.4 ± 2.4^{a}	136.5 ± 7.0^{a}	135.1 ± 3.8^{a}	156.7 ± 3.8^{b}	182.0 ± 3.0^{c}	< 0.0001
PUFA Balance	52.9 ± 1.7^{a}	52.6 ± 4.1^{a}	52.3 ± 2.0^{a}	22.8 ± 3.9^{b}	75.0 ± 2.5^{c}	< 0.0001

Values are means ± SEM. Only fatty acids present at > 0.1% are included in the above table.

Day 14 – Mitochondrial phospholipids

Diet	**Standard**	**18:0**	**18:1*n*-9**	**18:2*n*-6**	**18:3*n*-3**	***P***
(N =)	*(4)*	*(4)*	*(4)*	*(4)*	*(4)*	*(< 0.01)*
14:0	0.8 ± 0.1	0.8 ± 0.1	0.3 ± 0.3	0.2 ± 0.1	0.1 ± 0.0	ns
15:0	0.2 ± 0.0	0.3 ± 0.2	0.2 ± 0.2	0.1 ± 0.1	0.1 ± 0.0	ns
16:0	9.9 ± 0.5^{abc}	8.4 ± 1.6^{abc}	7.8 ± 1.1^{b}	8.3 ± 0.4^{c}	10.2 ± 0.7^{abc}	0.004
17:0	0.2 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	ns
18:0	1.7 ± 0.8	2.0 ± 1.2	0.7 ± 0.4	0.0 ± 0.0	0.8 ± 0.4	ns
20:0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	ns
21:0	0.1 ± 0.0	0.0 ± 0.0	0.4 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	ns
22:0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	ns
16:1*n*-7	42.8 ± 2.2^{a}	42.6 ± 0.5^{ab}	39.6 ± 1.8^{b}	25.5 ± 1.5^{c}	19.5 ± 1.5^{c}	<0.0001
18:1*n*-9	27.1 ± 0.8^{a}	27.9 ± 1.2^{a}	32.9 ± 1.9^{b}	23.2 ± 0.7^{c}	22.8 ± 1.1^{d}	<0.0001
18:1*n*-7	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	ns
18:2*n*-6	5.8 ± 0.9^{a}	6.0 ± 0.7^{a}	4.9 ± 0.6^{a}	32.6 ± 1.7^{b}	4.6 ± 0.8^{a}	<0.0001
18:3*n*-6	0.6 ± 0.0	0.7 ± 0.1	0.5 ± 0.2	0.5 ± 0.0	0.3 ± 0.0	ns
20:4*n*-6	2.1 ± 0.1	2.3 ± 0.2	2.1 ± 0.2	1.9 ± 0.0	1.6 ± 0.3	ns
18:3*n*-3	1.0 ± 0.1^{a}	1.0 ± 0.1^{a}	0.9 ± 0.1^{a}	0.9 ± 0.1^{a}	34.3 ± 3.4^{b}	<0.0001
20:5*n*-3	7.0 ± 0.2^{abc}	7.1 ± 0.5^{b}	5.0 ± 1.7^{bc}	6.6 ± 0.1^{ab}	5.4 ± 0.6^{ac}	0.0003
22:6*n*-3	0.3 ± 0.0	0.2 ± 0.0	0.6 ± 0.3	0.2 ± 0.0	0.2 ± 0.0	ns
SFA	13.1 ± 1.5	11.9 ± 2.9	9.4 ± 1.9	8.5 ± 0.5	11.2 ± 1.2	ns
MUFA	70.1 ± 2.3^{a}	70.7 ± 1.6^{a}	72.5 ± 3.3^{a}	48.7 ± 1.1^{b}	42.3 ± 2.0^{b}	<0.0001
PUFA	16.9 ± 1.2^{a}	17.4 ± 1.6^{a}	18.1 ± 1.8^{a}	42.7 ± 1.5^{b}	46.5 ± 1.8^{b}	<0.0001
***n*-6 PUFA**	8.5 ± 1.0^{a}	9.0 ± 0.9^{a}	7.5 ± 0.9^{a}	36.1 ± 1.6^{b}	6.5 ± 1.1^{a}	<0.0001
***n*-3 PUFA**	8.3 ± 0.3^{a}	8.4 ± 0.6^{a}	8.4 ± 0.3^{a}	7.0 ± 0.5^{a}	40.0 ± 2.8^{b}	<0.0001
C 20-22	9.7 ± 0.3	9.7 ± 0.7	12.3 ± 2.3	9.1 ± 0.5	7.3 ± 0.8	ns
PI	63.7 ± 2.2^{a}	64.7 ± 5.0^{ab}	68.5 ± 3.4^{ab}	82.7 ± 1.5^{b}	115.7 ± 1.8^{c}	<0.0001
UI	131.9 ± 2.1^{a}	134.0 ± 6.5^{a}	139.7 ± 3.0^{a}	151.0 ± 0.8^{b}	190.3 ± 3.4^{c}	<0.0001
PUFA Balance	49.9 ± 2.5^{a}	48.4 ± 0.8^{a}	53.1 ± 1.9^{a}	16.4 ± 1.6^{b}	85.7 ± 3.0^{c}	<0.0001

Values are means ± SEM. Only fatty acids present at > 0.1% are included in the above table.

Day 21 - Mitochondrial phospholipids

Diet	**Standard**	**18:0**	**18:1*n*-9**	**18:2*n*-6**	**18:3*n*-3**	***P***
(N =)	*(4)*	*(4)*	*(4)*	*(4)*	*(4)*	*(< 0.01)*
14:0	0.4 ± 0.1	0.5 ± 0.1	0.6 ± 0.0	0.2 ± 0.1	0.6 ± 0.1	ns
15:0	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	ns
16:0	7.5 ± 0.7	6.8 ± 0.8	6.7 ± 1.4	5.8 ± 2.5	10.4 ± 1.2	ns
17:0	0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	ns
18:0	0.3 ± 0.2	0.0 ± 0.0	1.0 ± 0.8	1.1 ± 1.1	2.5 ± 0.9	ns
20:0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	ns
21:0	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	ns
22:0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	
16:1*n*-7	50.0 ± 1.9^{a}	50.8 ± 1.8^{a}	42.9 ± 0.8^{a}	27.3 ± 2.2^{b}	20.9 ± 1.4^{b}	< 0.0001
18:1*n*-9	28.0 ± 0.7^{a}	26.5 ± 0.7^{a}	32.9 ± 0.8^{b}	22.1 ± 0.6^{c}	22.0 ± 0.4^{ac}	< 0.0001
18:1*n*-7	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	
18:2*n*-6	4.5 ± 0.8^{a}	5.4 ± 1.1^{a}	5.1 ± 0.7^{a}	37.4 ± 1.7^{b}	4.5 ± 0.7^{a}	< 0.0001
18:3*n*-6	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	ns
20:4*n*-6	1.7 ± 0.1^{ab}	1.9 ± 0.1^{a}	1.6 ± 0.1^{ab}	1.3 ± 0.1^{ab}	1.3 ± 0.0^{b}	0.005
18:3*n*-3	0.8 ± 0.2^{a}	0.9 ± 0.2^{a}	0.7 ± 0.3^{a}	0.6 ± 0.0^{a}	32.6 ± 1.2^{b}	< 0.0001
20:5*n*-3	5.7 ± 0.4^{ab}	5.9 ± 0.2^{a}	5.3 ± 0.4^{abc}	3.4 ± 1.2^{bc}	4.3 ± 0.1^{c}	0.0004
22:6*n*-3	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	ns
SFA	8.3 ± 1.0	7.7 ± 0.9	9.0 ± 1.7	7.2 ± 3.7	13.9 ± 2.3	ns
MUFA	78.1 ± 1.4^{a}	77.4 ± 2.5^{a}	76.5 ± 1.5^{a}	49.5 ± 2.2^{b}	43.0 ± 1.3^{b}	< 0.0001
PUFA	13.5 ± 1.4^{a}	14.9 ± 1.6^{a}	14.5 ± 0.3^{a}	43.3 ± 2.1^{b}	43.2 ± 1.3^{b}	< 0.0001
***n*-6 PUFA**	6.7 ± 0.9^{a}	7.8 ± 1.3^{a}	8.2 ± 0.4^{a}	39.0 ± 1.6^{b}	6.1 ± 0.7^{a}	< 0.0001
***n*-3 PUFA**	6.7 ± 0.5^{a}	7.0 ± 0.4^{a}	6.2 ± 0.1^{a}	4.3 ± 1.2^{a}	37.0 ± 1.2^{b}	< 0.0001
C 20-22	7.9 ± 0.5^{abc}	8.2 ± 0.3^{a}	8.3 ± 0.8^{bc}	5.0 ± 1.3^{cd}	5.8 ± 0.1^{d}	0.0031
PI	51.9 ± 3.4^{a}	54.7 ± 3.0^{ab}	49.9 ± 2.1^{a}	68.1 ± 8.0^{b}	103.2 ± 2.3^{c}	< 0.0001
UI	128.0 ± 3.2^{a}	131.0 ± 1.7^{a}	126.4 ± 1.9^{a}	150.7 ± 9.0^{b}	178.1 ± 4.4^{c}	< 0.0001
PUFA Balance	50.6 ± 2.4^{a}	48.4 ± 3.1^{a}	43.0 ± 1.5^{a}	9.7 ± 2.8^{b}	85.9 ± 1.5^{c}	< 0.0001

Values are means ± SEM. Only fatty acids present at > 0.1% are included in the above table.

Day 28 - Mitochondrial phospholipids

Diet	**Standard**	**18:0**	**18:1*n*-9**	**18:2*n*-6**	**18:3 -3**	***P***
(N =)	*(4)*	*(4)*	*(4)*	*(4)*	*(4)*	*(< 0.01)*
14:0	0.2 ± 0.1	0.0 ± 0.0	0.3 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	ns
15:0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	ns
16:0	7.0 ± 2.0	7.0 ± 0.6	7.1 ± 0.6	9.1 ± 0.7	8.3 ± 0.5	ns
17:0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	ns
18:0	1.2 ± 1.0	0.2 ± 0.1	0.5 ± 0.3	1.1 ± 0.4	0.3 ± 0.2	ns
20:0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	ns
21:0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	
22:0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	
16:1*n*-7	55.4 ± 2.4^{a}	53.3 ± 2.3^{a}	47.5 ± 1.7^{a}	25.1 ± 2.8^{b}	24.1 ± 1.4^{b}	< 0.0001
18:1*n*-9	24.4 ± 1.0^{a}	25.8 ± 0.9^{a}	33.8 ± 1.1^{b}	22.0 ± 1.2^{c}	23.6 ± 0.6^{ac}	< 0.0001
18:1*n*-7	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	
18:2*n*-6	4.1 ± 0.5^{a}	5.0 ± 1.2^{a}	3.7 ± 0.3^{a}	36.2 ± 4.5^{b}	4.4 ± 0.6^{a}	< 0.0001
18:3*n*-6	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	ns
20:4*n*-6	1.2 ± 0.2^{ab}	1.5 ± 0.1^{a}	1.1 ± 0.2^{ab}	1.1 ± 0.1^{ab}	1.2 ± 0.1^{b}	0.005
18:3*n*-3	0.7 ± 0.0^{a}	1.0 ± 0.2^{a}	0.6 ± 0.1^{a}	0.6 ± 0.1^{a}	33.1 ± 1.3^{b}	< 0.0001
20:5*n*-3	3.8 ± 1.3^{ab}	5.3 ± 0.4^{a}	4.4 ± 0.3^{abc}	4.0 ± 0.5^{bc}	3.8 ± 0.2^{c}	0.0004
22:6*n*-3	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.1	0.1 ± 0.0	0.2 ± 0.0	ns
SFA	8.6 ± 3.1	7.3 ± 0.8	7.9 ± 1.0	10.6 ± 1.1	9.2 ± 0.5	ns
MUFA	79.9 ± 3.4^{a}	79.1 ± 1.5^{a}	81.4 ± 1.6^{a}	47.1 ± 3.8^{b}	47.8 ± 1.4^{b}	< 0.0001
PUFA	11.6 ± 1.2^{a}	13.6 ± 1.9^{a}	10.8 ± 0.9^{a}	42.4 ± 3.9^{b}	43.0 ± 0.9^{b}	< 0.0001
***n*-6 PUFA**	5.9 ± 0.7^{a}	7.1 ± 1.3^{a}	5.3 ± 0.5^{a}	37.5 ± 4.3^{b}	5.9 ± 0.7^{a}	< 0.0001
***n*-3 PUFA**	5.6 ± 0.6^{a}	6.4 ± 0.6^{a}	5.5 ± 0.4^{a}	4.8 ± 0.6^{a}	37.1 ± 1.5^{b}	< 0.0001
C 20-22	6.4 ± 0.6	7.1 ± 0.6	6.0 ± 0.5	5.2 ± 0.6	5.2 ± 0.3	ns
PI	43.1 ± 4.3^{a}	49.3 ± 4.4^{ab}	42.0 ± 3.0^{a}	68.6 ± 2.8^{b}	101.5 ± 3.1^{c}	< 0.0001
UI	121.5 ± 4.8^{a}	127.5 ± 4.1^{a}	121.0 ± 2.1^{a}	147.4 ± 3.4^{b}	181.8 ± 2.2^{c}	< 0.0001
PUFA Balance	49.0 ± 2.3^{a}	48.5 ± 2.8^{a}	51.2 ± 1.2^{a}	11.7 ± 1.9^{b}	86.2 ± 1.8^{c}	< 0.0001

Values are means ± SEM. Only fatty acids present at > 0.1% are included in the above table.

Table 10.8. *Calliphora stygia* whole body phospholipids fatty acid composition (% of total fatty acids) fed diets enriched with fish or krill oil.

	Day 0		Day 7		Day 14		Day 21		Day 42	
	Fish	**Krill**	**Fish**	**Krill**	**Fish**	**Krill**	**Fish**	**Krill**	**Fish**	**Krill**
14:0	0.9 ± 0.0	0.9 ± 0.1	0.6 ± 0.0	0.7 ± 0.1	0.5 ± 0.1	0.7 ± 0.1	0.5 ± 0.0	0.7 ± 0.2	0.5 ± 0.1	0.6 ± 0.1
15:0	0.4 ± 0.1	0.3 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
16:0	21.0 ± 0.4	20.3 ± 0.5	12.1 ± 0.7	13.2 ± 0.6	13.3 ± 0.7	14.3 ± 0.7	12.7 ± 0.5	13.7 ± 0.8	13.2 ± 0.8	13.3 ± 0.7
17:0	0.3 ± 0.1	0.2 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
18:0	0.9 ± 0.1	0.6 ± 0.1	1.6 ± 0.2	1.0 ± 0.2	1.7 ± 0.1	1.5 ± 0.3	1.7 ± 0.2	2.0 ± 0.4	1.4 ± 0.2	1.7 ± 0.3
20:0	0.5 ± 0.0	0.4 ± 0.2	0.4 ± 0.1	0.4 ± 0.0	0.4 ± 0.0	0.3 ± 0.1	0.4 ± 0.0	0.3 ± 0.0	0.4 ± 0.1	0.4 ± 0.0
21:0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
22:0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
16:1*n*-7	19.2 ± 0.9	17.8 ± 0.9	30.2 ± 1.9	27.9 ± 0.8	31.6 ± 0.7	32.2 ± 0.7	35.6 ± 1.0	34.5 ± 1.1	37.7 ± 1.0	36.3 ± 0.9
18:1*n*-9	28.0 ± 0.3	28.2 ± 0.2	27.7 ± 1.2	26.8 ± 0.9	26.9 ± 0.8	25.0 ± 0.4	26.8 ± 0.5	25.0 ± 0.7	26.1 ± 1.0	25.9 ± 0.6
18:1*n*-7	0.5 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.7 ± 0.1	0.3 ± 0.0	0.6 ± 0.0***	0.4 ± 0.1	0.6 ± 0.1	0.3 ± 0.1	0.5 ± 0.1
18:2*n*-6	10.7 ± 1.5	12.4 ± 1.1	11.1 ± 0.6	9.6 ± 0.7	10.3 ± 1.0	9.0 ± 1.4	8.0 ± 0.2	7.9 ± 1.1	9.3 ± 0.4	7.9 ± 1.5
18:3*n*-6	0.6 ± 0.1	0.6 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.6 ± 0.1	0.4 ± 0.0	0.6 ± 0.0	0.4 ± 0.1	0.3 ± 0.0	0.4 ± 0.0
20:4*n*-6	3.5 ± 0.3	3.7 ± 0.3	2.5 ± 0.2	2.2 ± 0.1	2.3 ± 0.2	1.5 ± 0.2	2.0 ± 0.1	1.3 ± 0.1***	1.0 ± 0.2	0.8 ± 0.0
18:3*n*-3	1.9 ± 0.0	1.5 ± 0.4	1.7 ± 0.0	1.8 ± 0.1	1.8 ± 0.1	1.8 ± 0.2	1.4 ± 0.1	1.6 ± 0.1	1.4 ± 0.1	1.5 ± 0.1
20:5*n*-3	9.0 ± 0.1	9.7 ± 0.6	8.8 ± 0.5	11.5 ± 0.9	7.6 ± 0.3	9.9 ± 0.6**	7.7 ± 0.2	9.4 ± 0.3**	6.0 ± 0.2	7.8 ± 0.2***
22:6*n*-3	0.5 ± 0.0	0.6 ± 0.1	0.3 ± 0.0	0.9 ± 0.1**	0.1 ± 0.0	0.6 ± 0.1**	0.1 ± 0.0	0.5 ± 0.2	0.1 ± 0.0	0.3 ± 0.1
SFA	24.5 ± 0.4	23.2 ± 0.4	15.2 ± 0.7	15.7 ± 0.8	16.6 ± 0.9	17.4 ± 1.2	15.9 ± 0.8	17.2 ± 1.3	16.1 ± 1.0	16.6 ± 1.2
MUFA	49.2 ± 0.8	48.2 ± 0.7	59.8 ± 0.9	57.6 ± 0.5	60.6 ± 0.5	59.4 ± 1.1	64.1 ± 1.0	61.7 ± 0.8	65.5 ± 1.0	64.5 ± 0.5
PUFA	26.3 ± 1.1	28.6 ± 0.4	25.0 ± 1.0	26.7 ± 1.0	22.8 ± 1.4	23.3 ± 1.9	20.0 ± 0.5	21.1 ± 0.6	18.4 ± 0.5	18.9 ± 1.4
***n*-6 PUFA**	14.9 ± 1.1	16.7 ± 0.8	14.2 ± 0.6	12.4 ± 0.6	13.2 ± 1.0	11.0 ± 1.5	10.6 ± 0.3	9.6 ± 1.0	10.6 ± 0.5	9.1 ± 1.4
***n*-3 PUFA**	11.4 ± 0.2	11.9 ± 0.8	10.8 ± 0.6	14.2 ± 1.1	9.5 ± 0.4	12.2 ± 0.6**	9.3 ± 0.2	11.4 ± 0.4**	7.5 ± 0.2	9.5 ± 0.3***
C 20-22	15.3 ± 0.5	16.1 ± 1.0	13.5 ± 0.8	16.8 ± 1.1	11.9 ± 0.4	14.0 ± 0.4**	11.7 ± 0.3	13.1 ± 0.7	9.1 ± 0.2	11.0 ± 0.3***
UI	140.5 ± 1.2	146.8 ± 2.4	144.7 ± 3.5	156.2 ± 4.6	136.7 ± 3.4	143.3 ± 4.1	133.9 ± 1.4	138.6 ± 0.7	124.7 ± 1.6	130.5 ± 2.6
PI	89.3 ± 1.1	96.2 ± 4.1	82.3 ± 4.3	101.4 ± 6.3	72.8 ± 3.3	85.2 ± 4.0	69.3 ± 1.6	78.8 ± 2.3**	55.6 ± 1.1	65.9 ± 2.1**
PUFA Balance	43.5 ± 1.9	41.6 ± 2.5	43.1 ±1.1	53.2 ± 2.7**	42.1 ± 1.5	53.2 ± 2.8**	46.9 ± 0.2	54.6 ± 3.6	41.3 ± 1.3	51.7 ± 3.9

Values are means ± SEM. Only fatty acids present at ≥ 0.1 % were included in the above table. ***** Values are significantly different between the two diets as determined by a t-test (** $P < 0.01$, ***$P < 0.001$).

Table 10.9 *Calliphora stygia* whole body phospholipids fatty acid composition (% of total fatty acids) when kept at different ambient temperatures from day 2 to 35.

Day 2

Temperature	**12°C**	**15 °C**	**20 °C**	**25 °C**	**29 °C**	**34 °C**	***P***
(N =)	*(4)*	*(4)*	*(4)*	*(4)*	*(4)*	*(4)*	(< 0.01)
14:0	0.7 ± 0.0	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.5 ± 0.2	0.7 ± 0.1	ns
15:0	0.4 ± 0.0	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	ns
16:0	15.4 ± 0.5	15.2 ± 0.6	15.9 ± 0.6	14.6 ± 0.2	15.0 ± 0.3	15.6 ± 0.4	ns
17:0	0.4 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	ns
18:0	1.4 ± 0.3	1.2 ± 0.4	1.8 ± 0.3	1.6 ± 0.2	1.8 ± 0.2	2.0 ± 0.2	ns
20:0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	ns
21:0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	ns
22:0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	ns
16:1*n*-7	24.8 ± 1.7	25.1 ± 1.6	24.4 ± 0.9	29.1 ± 1.0	26.0 ± 1.0	30.7 ± 2.3	ns
18:1*n*-9	28.7 ± 0.4	28.4 ± 0.3	28.0 ± 0.6	27.8 ± 0.2	27.7 ± 0.2	26.7 ± 0.8	ns
18:1*n*-7	0.7 ± 0.0	0.8 ± 0.1	0.7 ± 0.1	0.7 ± 0.0	0.8 ± 0.0	0.6 ± 0.1	ns
18:2*n*-6	11.0 ± 1.4	12.3 ± 1.0	12.1 ± 0.9	10.3 ± 1.2	11.7 ± 1.4	9.7 ± 1.6	ns
18:3*n*-6	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.1	0.4 ± 0.0	ns
20:4*n*-6	3.0 ± 0.2	2.7 ± 0.1	2.8 ± 0.1	2.6 ± 0.2	2.8 ± 0.1	2.4 ± 0.2	ns
18:3*n*-3	1.6 ± 0.3	2.2 ± 0.2	1.8 ± 0.5	1.5 ± 0.3	1.6 ± 0.2	1.3 ± 0.3	ns
20:5*n*-3	10.0 ± 0.5	9.2 ± 0.4	9.2 ± 0.5	8.8 ± 0.4	9.6 ± 0.2	8.3 ± 0.4	ns
22:6*n*-3	0.4 ± 0.0	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.0	0.3 ± 0.1	ns
SFA	19.0 ± 0.6	18.4 ± 1.0	19.9 ± 0.7	18.2 ± 0.4	18.7 ± 0.3	19.5 ± 0.5	ns
MUFA	54.4 ± 1.9	54.4 ± 1.4	53.3 ± 1.0	57.7 ± 0.9	54.6 ± 1.2	58.1 ± 2.4	ns
PUFA	26.5 ± 2.0	27.2 ± 1.0	26.8 ± 1.2	24.1 ± 1.1	26.7 ± 1.3	22.4 ± 2.0	ns
***n*-6 PUFA**	14.5 ± 1.6	15.4 ± 1.1	15.4 ± 0.9	13.5 ± 1.2	15.0 ± 1.3	12.5 ± 1.7	ns
***n*-3 PUFA**	12.0 ± 0.6	11.8 ± 0.3	11.5 ± 0.6	10.7 ± 0.3	11.7 ± 0.1	9.9 ± 0.5	ns
C20-22	14.1 ± 0.7	13.0 ± 0.4	13.3 ± 0.6	12.5 ± 0.5	13.8 ± 0.4	11.7 ± 0.7	ns
PUFA Balance	45.8 ± 2.0	43.5 ± 2.2	42.9 ± 1.7	44.5 ± 2.3	44.1 ± 2.1	44.7 ± 2.7	ns
PI	91.8 ± 4.6	88.4 ± 1.5	88.0 ± 3.4	81.8 ± 2.3	89.7 ± 1.6	76.2 ± 4.3	ns
UI	147.2 ± 3.7	146.3 ± 1.4	144.2 ± 2.8	140.9 ± 1.7	146.2 ± 1.4	135.4 ± 2.9	ns

Values are means ± SEM. Only fatty acids present at > 0.1% are included in the above table.

Day 8

Temperature	**12°C**	**15 °C**	**20 °C**	**25 °C**	**29 °C**	**34 °C**	***P***
(N =)	*(2)*	*(2)*	*(2)*	*(2)*	*(2)*	*(2)*	(< 0.01)
14:0	0.5 ± 0.1^{a}	0.5 ± 0.0^{ab}	0.4 ± 0.0^{a}	0.5 ± 0.0^{a}	0.5 ± 0.0^{a}	0.7 ± 0.0^{b}	0.005
15:0	0.2 ± 0.0^{a}	0.2 ± 0.0^{ab}	0.2 ± 0.0^{ab}	0.1 ± 0.0^{ab}	0.1 ± 0.0^{b}	0.2 ± 0.0^{b}	0.003
16:0	14.4 ± 0.6	14.7 ± 0.0	12.6 ± 0.7	12.4 ± 0.2	12.4 ± 0.3	14.1 ± 0.1	ns
17:0	0.2 ± 0.1	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	ns
18:0	1.9 ± 0.0^{abc}	2.1 ± 0.1^{ac}	2.3 ± 0.1^{a}	1.6 ± 0.1^{b}	1.7 ± 0.0^{bc}	1.7 ± 0.0^{bc}	0.001
20:0	0.4 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	ns
21:0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.0	0.0 ± 0.0	ns
22:0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	ns
16:1*n*-7	28.2 ± 3.0	31.0 ± 0.9	34.1 ± 1.0	34.0 ± 1.6	36.7 ± 0.5	37.5 ± 1.0	ns
18:1*n*-9	28.8 ± 0.1^{a}	28.4 ± 0.1^{a}	28.2 ± 0.2^{a}	26.6 ± 0.0^{a}	24.7 ± 0.6^{b}	25.7 ± 0.8^{ab}	0.002
18:1*n*-7	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.0	0.6 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	ns
18:2*n*-6	10.8 ± 1.9	8.4 ± 1.4	7.6 ± 0.4	9.9 ± 1.2	9.8 ± 0.2	7.4 ± 0.6	ns
18:3*n*-6	0.5 ± 0.0	0.4 ± 0.1	0.5 ± 0.1	0.6 ± 0.0	0.5 ± 0.0	0.4 ± 0.0	ns
20:4*n*-6	2.4 ± 0.1	2.4 ± 0.3	2.3 ± 0.1	2.5 ± 0.1	2.0 ± 0.1	1.8 ± 0.1	ns
18:3*n*-3	2.2 ± 0.3	1.3 ± 0.3	1.0 ± 0.0	1.4 ± 0.3	1.9 ± 0.5	1.0 ± 0.1	ns
20:5*n*-3	8.4 ± 0.1	8.7 ± 0.5	8.9 ± 0.5	8.9 ± 0.3	8.1 ± 0.2	8.0 ± 0.7	ns
22:6*n*-3	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	ns
SFA	17.9 ± 0.6	18.4 ± 0.0	16.5 ± 0.9	15.4 ± 0.0	15.7 ± 0.3	17.6 ± 0.1	ns
MUFA	57.7 ± 3.0	60.1 ± 0.7	63.0 ± 1.2	61.1 ± 1.6	62.0 ± 1.0	63.7 ± 0.2	ns
PUFA	24.5 ± 2.4	21.5 ± 0.8	20.5 ± 0.3	23.5 ± 1.6	22.3 ± 0.8	18.6 ± 0.1	ns
***n*-6 PUFA**	13.6 ± 2.0	11.2 ± 1.0	10.3 ± 0.2	13.0 ± 1.0	12.2 ± 0.0	9.5 ± 0.5	ns
***n*-3 PUFA**	10.9 ± 0.4	10.3 ± 0.3	10.2 ± 0.5	10.5 ± 0.6	10.1 ± 0.7	9.1 ± 0.6	ns
C20-22	11.8 ± 0.2	12.1 ± 0.8	12.3 ± 0.7	12.3 ± 0.1	11.0 ± 0.1	10.6 ± 0.9	ns
PUFA Balance	44.6 ± 2.7	48.2 ± 2.9	49.6 ± 1.8	44.9 ± 0.6	45.1 ± 1.7	48.9 ± 2.9	ns
PI	79.7 ± 3.5	77.7 ± 2.8	76.9 ± 3.4	80.6 ± 2.9	73.4 ± 2.1	67.7 ± 3.9	ns
UI	140.4 ± 2.7	137.0 ± 1.3	137.9 ± 1.4	142.6 ± 2.4	137.6 ± 1.5	130.4 ± 2.2	ns

Values are means ± SEM. Only fatty acids present at > 0.1% are included in the above table.

Day 35

Temperature	**12°C**	**15 °C**	**20 °C**	**25 °C**	**29 °C**	***P***
(N =)	*(3)*	*(4)*	*(4)*	*(4)*	*(4)*	(< 0.01)
14:0	0.3 ± 0.0^{a}	0.4 ± 0.0^{a}	0.4 ± 0.0^{a}	0.4 ± 0.0^{a}	0.7 ± 0.0^{b}	< 0.0001
15:0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	ns
16:0	13.5 ± 0.1^{a}	13.4 ± 0.3^{a}	11.9 ± 0.3^{ab}	10.7 ± 0.5^{b}	11.8 ± 0.5^{ab}	0.0009
17:0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	ns
18:0	2.4 ± 0.2	2.8 ± 0.4	2.2 ± 0.2	2.1 ± 0.2	1.8 ± 0.1	ns
20:0	0.4 ± 0.0^{ab}	0.5 ± 0.0^{a}	0.3 ± 0.0^{b}	0.4 ± 0.0^{ab}	0.4 ± 0.0^{ab}	0.001
21:0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	ns
22:0	0.2 ± 0.0^{a}	0.3 ± 0.0^{ab}	0.2 ± 0.0^{a}	0.3 ± 0.0^{ab}	0.3 ± 0.0^{b}	0.001
16:1*n*-7	34.6 ± 1.5^{a}	36.7 ± 0.7^{a}	40.1 ± 1.7^{ab}	43.4 ± 2.1^{bc}	48.3 ± 1.1^{c}	< 0.0001
18:1*n*-9	28.1 ± 0.2^{ab}	28.3 ± 0.5^{a}	27.0 ± 0.3^{ab}	24.3 ± 0.9^{b}	22.5 ± 0.8^{c}	< 0.0001
18:1*n*-7	0.6 ± 0.0^{ab}	0.6 ± 0.0^{a}	0.6 ± 0.0^{ab}	0.5 ± 0.0^{ab}	0.4 ± 0.0^{b}	0.003
18:2*n*-6	8.9 ± 1.2	6.7 ± 0.6	7.7 ± 1.3	10.2 ± 4.0	7.4 ± 0.7	ns
18:3*n*-6	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	ns
20:4*n*-6	1.7 ± 0.1^{a}	1.7 ± 0.0^{a}	1.5 ± 0.1^{ab}	1.2 ± 0.1^{bc}	0.9 ± 0.1^{c}	< 0.0001
18:3*n*-3	1.7 ± 0.5	1.0 ± 0.2	1.1 ± 0.2	0.8 ± 0.0	1.0 ± 0.2	ns
20:5*n*-3	6.6 ± 0.2^{a}	6.5 ± 0.1^{a}	6.0 ± 0.2^{ab}	5.0 ± 0.3^{bc}	4.1 ± 0.3^{c}	< 0.0001
22:6*n*-3	0.2 ± 0.0^{a}	0.2 ± 0.0^{a}	0.1 ± 0.0^{ab}	0.1 ± 0.0^{bc}	0.0 ± 0.0^{c}	< 0.0001
SFA	17.2 ± 0.3^{ab}	17.8 ± 0.7^{a}	15.4 ± 0.5^{ab}	14.2 ± 0.7^{b}	15.1 ± 0.7^{ab}	0.004
MUFA	63.3 ± 1.3^{a}	65.8 ± 0.7^{ab}	67.8 ± 1.8^{ab}	68.3 ± 3.0^{b}	71.2 ± 0.7^{b}	0.0007
PUFA	19.5 ± 1.5	16.4 ± 0.9	16.9 ± 1.9	17.5 ± 3.7	13.7 ± 0.6	ns
***n*-6 PUFA**	10.9 ± 1.3	8.8 ± 0.6	9.6 ± 1.5	11.7 ± 3.9	8.6 ± 0.7	ns
***n*-3 PUFA**	8.6 ± 0.3^{a}	7.7 ± 0.3^{a}	7.3 ± 0.5^{ab}	5.8 ± 0.3^{bc}	5.1 ± 0.2^{c}	< 0.0001
C20-22	9.3 ± 0.2^{a}	9.4 ± 0.1^{a}	8.4 ± 0.2^{ab}	7.4 ± 0.2^{b}	5.8 ± 0.4^{c}	< 0.0001
PUFA Balance	44.3 ± 2.1	46.8 ± 1.2	43.8 ± 2.3	37.2 ± 6.5	37.6 ± 2.6	ns
PI	63.0 ± 1.2^{a}	58.2 ± 1.4^{ab}	55.4 ± 3.7^{ab}	49.0 ± 2.5^{bc}	40.0 ± 1.2^{c}	<0.0001
UI	128.6 ± 1.8^{a}	123.6 ± 1.9^{ab}	124.4 ± 3.3^{ab}	121.9 ± 3.6^{ab}	114.0 ± 1.3^{b}	0.002

Values are means ± SEM. Only fatty acids present at > 0.1% are included in the above table.

Table 10.10. Whole body phospholipid fatty acid composition (% of total fatty acids) of *Calliphora stygia* fed diets varying in energy (sugar) content from day 4 to 35.

Day 4

Diet	**Initial**	**0.25*x***	**0.5*x***	**0.75*x***	**1.0*x***	**2.0*x***
(N =)	*(6)*	*(2)*	*(2)*	*(2)*	*(2)*	*(2)*
14:0	1.2 ± 0.1	0.6 ± 0.1	0.6 ± 0.0	0.7 ± 0.0	0.7 ± 0.1	0.7 ± 0.0
15:0	0.9 ± 0.2	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.0	0.5 ± 0.0	0.6 ± 0.1
16:0	23.9 ± 2.3	14.7 ± 0.7	15.0 ± 0.3	14.2 ± 0.1	14.7 ± 0.4	14.9 ± 0.3
17:0	0.7 ± 0.2	0.3 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.4 ± 0.1
18:0	1.4 ± 0.3	2.1 ± 0.5	2.1 ± 0.3	2.3 ± 0.1	2.4 ± 0.3	2.0 ± 0.2
20:0	0.8 ± 0.2	0.5 ± 0.0	0.3 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.4 ± 0.1
21:0	0.3 ± 0.2	0.7 ± 0.0	0.4 ± 0.2	0.6 ± 0.1	0.5 ± 0.3	0.6 ± 0.0
22:0	0.5 ± 0.1	0.3± 0.1	0.2 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.3 ± 0.1
16:1*n*-7	15.5 ± 2.2	32.8 ± 0.5	29.6 ± 4.7	32.2 ± 0.9	31.3 ± 2.4	29.2 ± 1.2
18:1*n*-9	29.7 ± 0.5	28.1 ± 1.4	27.5 ± 1.1	27.6 ± 1.5	27.4 ± 1.1	27.6 ± 0.9
18:1*n*-7	0.9 ± 0.1	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.1	0.9 ± 0.1	0.8 ± 0.1
18:2*n*-6	11.1 ± 1.4	7.1 ± 1.6	10.6 ± 5.9	8.6 ± 0.6	7.3 ± 0.1	10.5 ± 0.4
18:3*n*-6	0.4 ± 0.0	0.5 ± 0.0	0.4 ± 0.1	0.4 ± 0.1	0.6 ± 0.2	0.5 ± 0.0
20:4*n*-6	2.8 ± 0.2	2.2 ± 0.1	2.1 ± 0.3	2.1 ± 0.1	2.7 ± 0.6	2.1 ± 0.4
18:3*n*-3	1.6 ± 0.1	1.3 ± 0.0	1.3 ± 0.3	1.1 ± 0.2	1.5 ± 0.8	1.4 ± 0.0
20:5*n*-3	8.1 ± 0.5	7.2 ± 1.0	8.0 ± 0.1	7.7 ± 0.2	7.8 ± 0.2	7.8 ± 0.2
22:6*n*-3	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.2	0.1 ± 0.1	0.2 ± 0.0	0.1 ± 0.0
SFA	29.8 ± 2.5	19.8 ± 1.5	19.6 ± 0.1	19.5 ± 0.1	20.2 ± 0.2	19.9 ± 0.1
MUFA	46.1 ± 1.8	61.8 ± 0.9	57.8 ± 5.8	60.6 ± 0.8	59.7 ± 1.1	57.6 ± 0.4
PUFA	24.1 ± 1.2	18.5 ± 2.4	22.6 ± 5.9	19.9 ± 0.7	20.1 ± 1.3	22.5 ± 0.3
***n*-6 PUFA**	14.2 ± 1.2	9.9 ± 1.5	13.1 ± 5.5	11.0 ± 0.7	10.6 ± 0.7	13.1 ± 0.1
***n*-3 PUFA**	9.9 ± 0.6	8.6 ± 0.9	9.5 ± 0.5	8.9 ± 0.1	9.5 ± 0.6	9.4 ± 0.2
C20-22	12.8 ± 0.7	11.1 ± 0.7	11.4 ± 0.2	11.3 ± 0.2	12.2 ± 0.1	11.4 ± 0.4
UI	127.0 ± 3.5	126.8 ± 6.4	133.9 ± 6.2	129.4 ± 0.0	131.6 ± 3.1	132.7 ± 1.7
PI	77.5 ± 3.2	65.1 ± 6.7	73.6 ± 6.0	68.1 ± 0.4	72.1 ± 3.1	72.2 ± 2.7
PUFA balance	41.1 ± 2.6	46.5 ± 1.0	43.4 ± 9.4	44.6 ± 1.9	47.3 ± 0.1	41.7 ± 0.4

Values are means ± SD. Only fatty acids ≥ 0.1 % total fatty acids are included. No statistical analyses were performed due to low sample size.

Day 16

Diet	**0.25*x***	**0.5*x***	**0.75*x***	**1.0*x***	**2.0*x***
(N =)	*(2)*	*(2)*	*(2)*	*(2)*	*(2)*
14:0	0.5 ± 0.0	0.5 ± 0.0	0.6 ± 0.2	0.4 ± 0.0	0.5 ± 0.0
15:0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.0
16:0	11.4 ± 0.5	11.9 ± 1.4	13.2 ± 1.1	12.1 ± 0.2	11.0 ± 1.3
17:0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	0.1 ± 0.0
18:0	1.7 ± 0.4	2.1 ± 0.7	2.8 ± 1.3	1.8 ± 0.2	1.8 ± 0.2
20:0	0.3 ± 0.1	0.4 ± 0.1	0.4 ± 0.0	0.4 ± 0.2	0.4 ± 0.1
21:0	0.4 ± 0.1	0.3 ± 0.0	0.3 ± 0.1	0.3 ± 0.2	0.4 ± 0.0
22:0	0.3 ± 0.1	0.3± 0.1	0.3 ± 0.0	0.3 ± 0.2	0.3 ± 0.0
16:1*n*-7	43.5 ± 2.6	43.3 ± 0.4	37.7 ± 5.0	36.0 ± 4.3	44.4 ± 3.3
18:1*n*-9	25.1 ± 0.6	26.0 ± 2.2	26.7 ± 1.1	27.6 ± 1.2	26.7 ± 0.8
18:1*n*-7	0.5 ± 0.0	0.5 ± 0.1	0.6 ± 0.0	0.5 ± 0.1	0.5 ± 0.1
18:2*n*-6	7.2 ± 2.7	7.3 ± 3.4	8.3 ± 3.6	10.2 ± 2.7	6.5 ± 0.8
18:3*n*-6	0.4 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
20:4*n*-6	1.5 ± 0.0	1.0 ± 0.1	1.4 ± 0.2	1.5 ± 0.1	1.2 ± 0.0
18:3*n*-3	0.9 ± 0.2	0.8 ± 0.2	0.9 ± 0.2	1.3 ± 0.3	0.8 ± 0.1
20:5*n*-3	5.9 ± 0.2	4.9 ± 0.6	5.9 ± 0.1	6.6 ± 0.4	4.9 ± 0.2
22:6*n*-3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0
SFA	14.9 ± 1.0	15.9 ± 2.2	18.1 ± 2.5	15.8 ± 0.6	14.7 ± 1.6
MUFA	69.2 ± 2.0	69.8 ± 2.0	65.0 ± 6.0	64.2 ± 3.0	71.6 ± 2.7
PUFA	16.0 ± 3.0	14.3 ± 4.2	16.9 ± 3.5	20.0 ± 2.5	13.7 ± 1.1
***n*-6 PUFA**	9.1 ± 2.7	8.6 ± 3.5	10.0 ± 3.4	12.0 ± 2.6	8.0 ± 0.8
***n*-3 PUFA**	6.9 ± 0.3	5.7 ± 0.7	6.9 ± 0.1	7.9 ± 0.1	5.7 ± 0.3
C20-22	8.5 ± 0.0	7.2 ± 0.4	8.5 ± 0.5	9.2 ± 0.2	7.3 ± 0.3
UI	123.2 ± 4.6	116.6 ± 8.4	120.8 ± 0.5	128.7 ± 0.9	117.3 ± 0.0
PI	53.2 ± 3.7	45.2 ± 7.3	53.8 ± 2.5	61.0 ± 0.6	44.9 ± 2.0
PUFA balance	43.8 ± 6.1	41.0 ± 7.0	41.5 ± 8.2	40.1 ± 5.5	41.8 ± 1.2

Values are means ± SD. Only fatty acids ≥ 0.1 % total fatty acids are included. No statistical analyses were performed due to low sample size.

Day 35

Diet	**0.25*x***	**0.5*x***	**0.75*x***	**1.0*x***	**2.0*x***
(N =)	*(2)*	*(2)*	*(2)*	*(2)*	*(2)*
14:0	0.4 ± 0.0	0.7 ± 0.3	0.6 ± 0.4	0.6 ± 0.1	0.5 ± 0.2
15:0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
16:0	11.3 ± 1.1	12.0 ± 2.0	11.6 ± 1.7	11.9 ± 1.5	11.5 ± 1.5
17:0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
18:0	2.6 ± 1.6	1.9 ± 0.8	2.1 ± 0.7	2.5 ± 0.5	1.7 ± 0.9
20:0	0.3 ± 0.0	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.3 ± 0.1
21:0	0.3 ± 0.0	0.3 ± 0.1	0.4 ± 0.2	0.3 ± 0.0	0.3 ± 0.1
22:0	0.3 ± 0.1	0.3± 0.1	0.3 ± 0.0	0.3 ± 0.2	0.3 ± 0.0
16:1*n*-7	43.2 ± 2.5	45.1 ± 1.6	45.9 ± 3.1	45.6 ± 0.2	43.8 ± 4.9
18:1*n*-9	25.6 ± 0.3	26.5 ± 0.7	26.4 ± 1.7	26.4 ± 0.9	26.3 ± 1.3
18:1*n*-7	0.5 ± 0.0	0.5 ± 0.1	0.5 ± 0.2	0.5 ± 0.1	0.5 ± 0.0
18:2*n*-6	8.4 ± 1.1	5.9 ± 1.5	4.8 ± 1.8	5.2 ± 0.6	7.5 ± 1.1
18:3*n*-6	0.3 ± 0.0	0.3 ± 0.1	0.4 ± 0.1	0.3 ± 0.0	0.3 ± 0.0
20:4*n*-6	1.3 ± 0.5	0.9 ± 0.2	1.1 ± 0.1	1.0 ± 0.3	1.1 ± 0.1
18:3*n*-3	0.9 ± 0.2	0.8 ± 0.2	0.9 ± 0.2	1.3 ± 0.3	0.8 ± 0.1
20:5*n*-3	4.2 ± 0.3	4.2 ± 0.6	4.3 ± 0.1	4.2 ± 1.1	4.7 ± 0.1
22:6*n*-3	0.2 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
SFA	15.4 ± 2.8	15.8 ± 3.2	15.7 ± 3.0	16.1 ± 1.3	14.9 ± 2.6
MUFA	69.3 ± 2.2	72.1 ± 0.7	72.9 ± 1.2	72.5 ± 0.9	70.7 ± 3.5
PUFA	15.3 ± 0.6	12.1 ± 2.5	11.4 ± 1.7	11.4 ± 2.2	14.4 ± 0.9
***n*-6 PUFA**	10.0 ± 0.5	7.1 ± 1.7	6.3 ± 1.6	6.5 ± 1.0	8.9 ± 1.0
***n*-3 PUFA**	5.3 ± 0.0	5.0 ± 0.8	5.1 ± 0.1	4.9 ± 1.1	5.6 ± 0.1
C20-22	6.7 ± 0.6	6.2 ± 0.7	6.7 ± 0.2	6.2 ± 1.2	6.7 ± 0.1
UI	117.2 ± 2.0	111.8 ± 8.1	111.9 ± 4.7	110.7 ± 7.4	117.0 ± 2.1
PI	44.8 ± 1.5	38.8 ± 6.4	39.1 ± 1.9	38.1 ± 8.5	44.2 ± 0.2
PUFA balance	34.5 ± 1.2	41.6 ± 2.2	44.9 ± 5.9	42.5 ± 1.9	38.8 ± 3.0

Values are means ± SD. Only fatty acids ≥ 0.1 % total fatty acids are included. No statistical analyses were performed due to low sample size.

Table 10.11. Whole body phospholipids fatty acid composition (% of total fatty acids) of *Calliphora stygia* fed diets of varying yeast (nutrient) content from day 2 to 55.

Day 2

Diet	**0.0*x***	**0.25*x***	**0.5*x***	**0.75*x***	**1.0*x***	**1.5*x***	***P***
(N =)	*(2)*	*(2)*	*(2)*	*(2)*	*(2)*	*(2)*	(< 0.01)
14:0	0.74 ± 0.05	0.77 ± 0.10	0.63 ± 0.03	0.65 ± 0.04	0.62 ± 0.01	0.69 ± 0.03	ns
15:0	0.34 ± 0.06	0.37 ± 0.02	0.29 ± 0.01	0.31 ± 0.04	0.37 ± 0.03	0.32 ± 0.02	ns
16:0	14.57 ± 0.24	15.41 ± 0.72	14.97 ± 0.21	15.12 ± 0.37	15.41 ± 0.28	16.30 ± 0.87	ns
17:0	0.43 ± 0.04	0.40 ± 0.03	0.40 ± 0.05	0.34 ± 0.01	0.42 ± 0.02	0.36 ± 0.02	ns
18:0	1.70 ± 0.33	1.89 ± 0.32	2.01 ± 0.07	2.09 ± 0.49	1.69 ± 0.03	2.59 ± 0.67	ns
20:0	0.34 ± 0.04	0.30 ± 0.03	0.33 ± 0.03	0.30 ± 0.04	0.35 ± 0.06	0.27 ± 0.03	ns
21:0	0.12 ± 0.03	0.12 ± 0.01	0.10 ± 0.01	0.09 ± 0.00	0.12 ± 0.00	0.05 ± 0.05	ns
22:0	0.25 ± 0.03	0.19 ± 0.00	0.25 ± 0.00	0.20 ± 0.02	0.22 ± 0.081	0.08 ± 0.08	ns
16:1*n*-7	22.51 ± 0.04	21.81 ± 0.10	25.62 ± 0.09	23.15 ± 1.73	23.04 ± 1.40	25.15 ± 0.39	ns
18:1*n*-9	29.69 ± 0.76	28.78 ± 0.32	29.27 ± 0.61	28.83 ± 1.00	27.95 ± 0.84	27.73 ± 0.11	ns
18:1*n*-7	0.78 ± 0.04	0.83 ± 0.08	0.85 ± 0.05	0.79 ± 0.02	0.76 ± 0.03	0.81 ± 0.03	ns
18:2*n*-6	11.64 ± 0.20	13.90 ± 1.12	9.44 ± 0.65	13.59 ± 1.55	13.19 ± 1.48	11.24 ± 1.39	ns
18:3*n*-6	0.56 ± 0.07	0.50 ± 0.01	0.47 ± 0.01	0.45 ± 0.01	0.54 ± 0.02	0.47 ± 0.04	ns
20:4*n*-6	3.22 ± 0.10	2.58 ± 0.10	3.03 ± 0.31	2.44 ± 0.22	2.92 ± 0.09	2.67 ± 0.01	ns
18:3*n*-3	1.47 ± 0.03	2.26 ± 0.22	1.21 ± 0.06	2.42 ± 0.76	2.05 ± 0.42	1.67 ± 0.54	ns
20:5*n*-3	10.8 ± 0.3	9.3 ± 0.0	10.5 ± 0.7	8.8 ± 0.2	9.8 ± 0.0	9.5 ± 0.1	ns
22:6*n*-3	0.55 ± 0.04	0.48 ± 0.08	0.51 ± 0.17	0.33 ± 0.05	0.45 ± 0.05	0.40 ± 0.03	ns
SFA	18.67 ± 0.57	19.49 ± 1.20	19.04 ± 0.18	19.12 ± 0.09	19.28 ± 0.27	20.75 ± 1.37	ns
MUFA	53.02 ± 0.72	51.49 ± 0.06	55.82 ± 0.67	52.81 ± 2.68	51.73 ± 2.17	53.31 ± 0.47	ns
PUFA	28.31 ± 0.14	29.01 ± 1.13	25.14 ± 0.50	28.08 ± 2.77	28.99 ± 1.90	25.95 ± 1.84	ns
***n*-6 PUFA**	15.42 ± 0.17	16.98 ± 1.01	12.94 ± 0.34	16.49 ± 1.76	16.65 ± 1.41	14.39 ± 1.41	ns
***n*-3 PUFA**	12.89 ± 0.31	12.03 ± 0.12	12.20 ± 0.83	11.59 ± 1.01	12.33 ± 0.49	11.56 ± 0.43	ns
C 20-22	15.5 ± 0.5	13.0 ± 0.2	14.8 ± 1.1	12.2 ± 0.6	14.0 ± 0.0	13.1 ± 0.0	ns
PUFA Balance	45.53 ± 0.86	41.51 ± 1.19	48.46 ± 2.35	41.33 ± 0.47	42.62 ± 1.10	44.67 ± 1.52	ns
PI	99.55 ± 1.69	90.62 ± 0.40	93.24 ± 6.13	86.10 ± 5.46	93.97 ± 2.48	87.68 ± 1.88	ns
UI	152.93 ± 0.52	147.23 ± 1.97	147.30 ± 3.72	144.56 ± 4.84	149.46 ± 2.16	142.76 ± 3.47	ns

Values are means ± SEM. Only fatty acids present at > 0.1% are included in the above table.

Day 16

Diet	**0.0*x***	**0.25*x***	**0.5*x***	**0.75*x***	**1.0*x***	**1.5*x***	***P***
(N =)	*(4)*	*(4)*	*(4)*	*(4)*	*(4)*	*(4)*	(< 0.01)
14:0	0.5 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	0.5 ± 0.1	ns
15:0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	ns
16:0	10.6 ± 0.1^{a}	10.9 ± 0.1^{ab}	11.5 ± 0.3^{ab}	11.3 ± 0.3^{ab}	12.3 ± 0.5^{ab}	12.7 ± 0.6^{b}	0.0035
17:0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	ns
18:0	1.8 ± 0.1	1.9 ± 0.2	2.1 ± 0.2	2.1 ± 0.3	2.4 ± 0.3	2.7 ± 0.6	ns
20:0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.1	0.3 ± 0.0	ns
21:0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	ns
22:0	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	ns
16:1*n*-7	29.5 ± 0.9^{a}	37.0 ± 1.5^{b}	39.6 ± 0.6^{b}	39.0 ± 1.1^{b}	39.1 ± 0.7^{b}	39.7 ± 1.5^{b}	< 0.0001
18:1*n*-9	25.1 ± 0.5	24.9 ± 0.2	26.4 ± 0.2	24.8 ± 0.6	26.0 ± 0.3	26.1 ± 0.4	ns
18:1*n*-7	0.5 ± 0.1	0.5 ± 0.0	0.7 ± 0.1	0.5 ± 0.0	0.6 ± 0.1	0.5 ± 0.0	ns
18:2*n*-6	15.5 ± 1.1^{a}	12.2 ± 1.1^{ac}	7.6 ± 0.5^{b}	10.8 ± 0.4^{bc}	7.7 ± 0.6^{b}	7.7 ± 0.6^{b}	< 0.0001
18:3*n*-6	0.7 ± 0.1	0.5 ± 0.0	0.5 ± 0.0	0.4 ± 0.0	0.4 ± 0.1	0.4 ± 0.0	ns
20:4*n*-6	2.3 ± 0.1^{a}	1.8 ± 0.1^{b}	1.8 ± 0.1^{b}	1.8 ± 0.1^{b}	1.7 ± 0.1^{b}	1.4 ± 0.1^{b}	< 0.0001
18:3*n*-3	2.3 ± 0.3	1.8 ± 0.3	1.0 ± 0.1	1.2 ± 0.4	1.0 ± 0.2	0.9 ± 0.1	ns
20:5*n*-3	10.1 ± 0.3^{a}	7.1 ± 0.4^{b}	7.5 ± 0.2^{b}	6.7 ± 0.3^{b}	7.3 ± 0.4^{b}	6.4 ± 0.4^{b}	< 0.0001
22:6*n*-3	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	ns
SFA	13.7 ± 0.2	14.0 ± 0.2	14.8 ± 0.4	14.6 ± 0.7	16.0 ± 0.7	16.5 ± 1.2	ns
MUFA	55.3 ± 1.0^{a}	62.6 ± 1.4^{b}	66.8 ± 0.7^{b}	64.5 ± 1.4^{b}	65.8 ± 0.7^{b}	66.6 ± 1.4^{b}	< 0.0001
PUFA	31.0 ± 1.1^{a}	23.5 ± 1.5^{b}	18.4 ± 0.8^{bc}	21.0 ± 0.9^{bc}	18.2 ± 1.1^{bc}	16.9 ± 0.7^{c}	< 0.0001
***n*-9**	25.2 ± 0.6	25.0 ± 0.2	26.6 ± 0.2	24.9 ± 0.6	26.2 ± 0.3	26.3 ± 0.4	ns
***n*-7**	30.1 ± 0.9^{a}	37.5 ± 1.5^{b}	40.3 ± 0.7^{b}	39.5 ± 1.1^{b}	39.7 ± 0.7^{b}	40.3 ± 1.5^{b}	< 0.0001
***n*-6 PUFA**	18.5 ± 1.0^{a}	14.4 ± 1.1^{ab}	9.8 ± 0.6^{cd}	13.0 ± 0.4^{bc}	9.8 ± 0.7^{cd}	9.5 ± 0.6^{d}	< 0.0001
***n*-3 PUFA**	12.5 ± 0.1^{a}	9.1 ± 0.4^{b}	8.6 ± 0.2^{b}	8.0 ± 0.7^{b}	8.4 ± 0.4^{b}	7.4 ± 0.4^{b}	< 0.0001
C 20-22	13.2 ± 0.3^{a}	9.6 ± 0.4^{b}	10.0 ± 0.3^{b}	9.2 ± 0.6^{b}	9.7 ± 0.3^{b}	8.4 ± 0.5^{b}	< 0.0001
PUFA Balance	40.5 ± 1.2	38.7 ± 1.2	46.7 ± 1.1	37.8 ± 2.3	46.2 ± 1.1	43.9 ± 1.0	ns
PI	93.9 ± 1.3^{a}	69.0 ± 3.1^{b}	65.1 ± 1.8^{b}	63.8 ± 3.1^{b}	63.4 ± 3.2^{b}	56.6 ± 2.5^{b}	< 0.0001
UI	155.8 ± 1.2^{a}	137.1 ± 2.6^{b}	131.5 ± 1.7^{bc}	132.1 ± 1.9^{bc}	129.2 ± 2.9^{bc}	124.0 ± 1.3^{c}	< 0.0001

Values are means ± SEM. Only fatty acids present at > 0.1% are included in the above table.

Day 35

Diet	**0.25*x***	**0.5*x***	**0.75*x***	**1.0*x***	**1.5*x***	***P***
(N =)	*(4)*	*(4)*	*(4)*	*(4)*	*(4)*	(< 0.01)
14:0	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	ns
15:0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	ns
16:0	11.0 ± 0.2	11.5 ± 0.1	11.3 ± 0.2	11.0 ± 0.4	11.5 ± 0.2	ns
17:0	0.2 ± 0.0[a]	0.1 ± 0.0[ab]	0.1 ± 0.0[b]	0.1 ± 0.0[b]	0.1 ± 0.0[ab]	0.0016
18:0	1.8 ± 0.2	2.1 ± 0.1	1.7 ± 0.2	1.7 ± 0.1	1.6 ± 0.1	ns
20:0	0.5 ± 0.0	0.6 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	ns
21:0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	ns
22:0	0.4 ± 0.0[a]	0.4 ± 0.0[a]	0.3 ± 0.0[b]	0.3 ± 0.0[ab]	0.4 ± 0.0[ab]	0.0013
16:1*n*-7	42.9 ± 1.1	44.4 ± 0.4	46.4 ± 1.3	47.2 ± 1.3	46.0 ± 0.9	ns
18:1*n*-9	21.8 ± 0.3	22.5 ± 0.5	23.3 ± 0.2	23.3 ± 0.4	23.3 ± 0.4	ns
18:1*n*-7	0.4 ± 0.0[a]	0.4 ± 0.0[a]	0.3 ± 0.0[b]	0.3 ± 0.0[ab]	0.3 ± 0.0[b]	0.0007
18:2*n*-6	10.8 ± 0.8	8.8 ± 0.5	8.1 ± 1.2	7.6 ± 0.8	8.4 ± 0.7	ns
18:3*n*-6	0.5 ± 0.0[a]	0.5 ± 0.0[a]	0.3 ± 0.0[b]	0.3 ± 0.0[b]	0.3 ± 0.0[b]	<0.0001
20:4*n*-6	1.4 ± 0.0	1.4 ± 0.1	1.2 ± 0.1	1.2 ± 0.0	1.2 ± 0.1	ns
18:3*n*-3	1.5 ± 0.3	1.3 ± 0.2	1.4 ± 0.5	1.5 ± 0.4	1.4 ± 0.2	ns
20:5*n*-3	6.9 ± 0.3[a]	6.5 ± 0.2[a]	5.4 ± 0.1[b]	5.3 ± 0.2[b]	5.5 ± 0.2[b]	<0.0001
22:6*n*-3	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	ns
SFA	14.4 ± 0.1	15.2 ± 0.2	14.3 ± 0.4	14.1 ± 0.3	14.6 ± 0.3	ns
MUFA	64.3 ± 1.3	66.2 ± 0.7	69.2 ± 1.3	69.9 ± 1.2	68.6 ± 0.7	ns
PUFA	21.3 ± 1.2	18.6 ± 0.8	16.5 ± 1.6	16.0 ± 0.9	16.8 ± 0.6	ns
***n*-9**	21.0 ± 0.3	21.4 ± 0.5	22.5 ± 0.2	22.3 ± 0.4	22.3 ± 0.4	ns
***n*-7**	43.3 ± 1.0	44.8 ± 0.4	46.7 ± 1.3	47.6 ± 1.3	46.3 ± 0.9	ns
***n*-6 PUFA**	12.8 ± 0.8	10.7 ± 0.5	9.6 ± 1.2	9.2 ± 0.8	9.9 ± 0.6	ns
***n*-3 PUFA**	8.5 ± 0.4	7.9 ± 0.3	6.9 ± 0.4	6.8 ± 0.3	7.0 ± 0.3	ns
C 20-22	9.6 ± 0.3[a]	9.3 ± 0.2[a]	7.6 ± 0.2[b]	7.7 ± 0.2[b]	7.8 ± 0.3[b]	<0.0001
PUFA Balance	40.0 ± 0.9	45.3 ± 0.5	42.1 ± 1.8	42.8 ± 1.7	41.5 ± 2.2	ns
PI	64.1 ± 2.3[a]	59.5 ± 1.9[a]	51.0 ± 1.6[b]	50.3 ± 1.2[b]	51.6 ± 1.6[b]	0.0001
UI	132.7 ± 1.8[a]	127.8 ± 1.7[ab]	122.7 ± 2.1[b]	122.4 ± 0.8[b]	123.0 ± 1.00[b]	0.001

Values are means ± SEM. Only fatty acids present at > 0.1% are included in the above table.

Day 55

Diet	**0.25*x***	**0.5*x***	**0.75*x***	**1.0*x***	**1.5*x***	***P***
(N =)	*(3)*	*(4)*	*(4)*	*(4)*	*(4)*	(< 0.01)
14:0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	ns
15:0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	ns
16:0	10.4 ± 0.4	10.3 ± 0.2	10.6 ± 0.4	11.0 ± 0.2	11.1 ± 0.3	ns
17:0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	ns
18:0	1.8 ± 0.2	1.7 ± 0.2	1.4 ± 0.1	1.8 ± 0.1	1.6 ± 0.2	ns
20:0	0.4 ± 0.0	0.4 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	ns
21:0	0.2 ± 0.0[a]	0.1 ± 0.0[ab]	0.1 ± 0.0[ab]	0.1 ± 0.0[b]	0.1 ± 0.0[ab]	0.0092
22:0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	ns
16:1*n*-7	42.3 ± 1.7	46.1 ± 0.5	45.6 ± 0.6	46.2 ± 0.8	44.1 ± 1.3	ns
18:1*n*-9	24.8 ± 0.1	25.1 ± 0.5	25.5 ± 0.5	25.4 ± 0.4	25.9 ± 0.2	ns
18:1*n*-7	0.5 ± 0.2	0.6 ± 0.0	0.5 ± 0.0	0.6 ± 0.1	0.5 ± 0.0	ns
18:2*n*-6	10.1 ± 1.2	7.3 ± 0.4	7.7 ± 0.4	7.2 ± 0.7	8.4 ± 0.9	ns
18:3*n*-6	0.4 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	ns
20:4*n*-6	1.1 ± 0.0	1.1 ± 0.1	1.0 ± 0.0	0.9 ± 0.1	0.9 ± 0.1	ns
18:3*n*-3	1.4 ± 0.3	1.0 ± 0.0	1.3 ± 0.3	0.8 ± 0.1	1.0 ± 0.2	ns
20:5*n*-3	5.6 ± 0.1	5.1 ± 0.3	4.6 ± 0.2	4.4 ± 0.2	4.7 ± 0.3	ns
22:6*n*-3	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	ns
SFA	13.7 ± 0.3	13.2 ± 0.2	13.3 ± 0.3	14.0 ± 0.3	14.1 ± 0.4	ns
MUFA	67.6 ± 1.4	71.9 ± 0.6	71.6 ± 1.1	72.3 ± 0.7	70.6 ± 1.5	ns
PUFA	18.7 ± 1.5	14.8 ± 0.6	15.1 ± 0.9	13.7 ± 0.9	15.4 ± 1.3	ns
***n*-9**	25.0 ± 0.1	25.3 ± 0.5	25.6 ± 0.5	25.5 ± 0.4	26.0 ± 0.2	ns
***n*-7**	42.6 ± 1.5	46.7 ± 0.4	46.1 ± 0.6	46.8 ± 0.8	44.6 ± 1.3	ns
***n*-6 PUFA**	11.6 ± 1.3	8.8 ± 0.5	9.1 ± 0.4	8.4 ± 0.8	9.7 ± 0.9	ns
***n*-3 PUFA**	7.0 ± 0.3	6.1 ± 0.3	6.0 ± 0.5	5.3 ± 0.2	5.7 ± 0.5	ns
C 20-22	7.8 ± 0.1[a]	7.0 ± 0.4[ab]	6.5 ± 0.3[ab]	6.1 ± 0.2[b]	6.3 ± 0.3[ab]	0.0093
PUFA Balance	37.9 ± 1.8	41.0 ± 1.2	39.7 ± 1.0	38.8 ± 2.3	37.3 ± 1.0	ns
PI	53.6 ± 1.7	46.8 ± 2.1	44.9 ± 2.3	41.7 ± 1.5	44.6 ± 3.0	ns
UI	125.9 ± 2.0	120.5 ± 1.6	119.4 ± 1.6	116.0 ± 1.5	118.5 ± 2.3	ns

Values are means ± SEM. Only fatty acids present at > 0.1% are included in the above table.