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THE EFFECT OF DIET ON FOOD PREFERENCES AND LIFE HISTORY TRAITS IN *DROSOPHILA MELANOGASTER*

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Abstract

Gustation underlies much insect behaviour, including finding and assessing food and egg laying sites. *Drosophila* are capable of learning sensory information about their environment, however, whether they are capable of learning gustatory information about their larval environment and using this information to modify their adult behaviour is as yet unknown. A behavioural assay was therefore designed to try to resolve this issue.

Varying the concentration of carbohydrate in the larval diet had varying effects on adult food preference depending on carbohydrate type. When sucrose and glucose concentrations in the larval diet were reduced, adults were more likely to feed on these reduced carbohydrate content media but for sucrose were less likely to lay their eggs on it. When trehalose and fructose concentration in the larval diet was reduced, adults showed no preference for either food medium to feed and lay their eggs on. Reducing the protein concentration in the larval diet led the adults having a preference for the high yeast medium to both feed and lay their eggs on. Finally, increasing concentrations of NaCl in the larval diet had an affect on adult feeding preferences at some concentrations although no clear pattern was apparent.

When sucrose concentration in the larval diet of the learning and memory mutants *dunce* and *rutabaga* were varied there was no subsequent affect on adult food preferences. This was also the case when the mushroom bodies (the putative centres for learning and memory in insects) were ablated. This provides strong evidence that cAMP and MB's are involved in the effect of larval diet on adult food choice, although exactly how this effect is achieved remains unclear. One possibility is that learning and memory may be involved, although further experiments would be required to determine whether this is indeed the case.

The food choices an individual makes will have a huge impact on its health and survival. Recent studies in mammals have also shown that this impact can continue into subsequent generations. The aim of the final chapter of this thesis was therefore to determine the affects of the food types used in the choice experiments on the health of the flies over 5 generations. Results showed that diet effected several traits in *Drosophila*, including fecundity, development time, survival and larval locomotion. Diet had no effect on starvation resistance. In addition, high concentrations of NaCl in the diet reduced larval fitness increasingly over 5 generations and low levels of carbohydrate in the diet increased survival in subsequent generations. These results therefore provide a strong case for the use of *Drosophila* as a model organism for gaining a further understanding of the developmental origins of health and disease in humans.

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Abbreviations

11βHSD	11 β hydroxysteroid dehydrogenase
ACOX	Acyl CoA oxidase
ANOVA	Analysis of variance
ATP	Adenosine tri-phosphate
CPT	Carnitine: palmitoyl transferase
cAMP	Cyclic adenosine mono-phosphate
CREB	cAMP response element binding protein
DNA	Deoxyribonucleic acid
Dnmt	DNA methyltransferase
DOHaD	Developmental Origins of Health and Disease
ER	Endoplasmic reticulum
FOAD	Foetal Origins of Adult Disease
GH	Growth hormone
GLUT	Glucose transporter
GR	Glucocorticoid receptor
HU	Hudroxyurea
IGF	Insulin-like growth factor
IR	Insulin receptor
LPL	Lipoprotein lipase
LR	Leptin receptor
MBs	Mushroom bodies
NHT	Nicotine hydrogen tartrate
NOS	Nitric oxide synthase
PEPCK	Phosphoenopyruvate carboxykinase
PPARα	Peroxisomal proliforator - activated receptor alpha
PPARγ	Peroxisomal proliforator - activated receptor gamma
PKA	Protein kinase A
RNA	Ribonucleic acid
TNFα	Tumour necrosis factor alpha

Chapter 1. General Introduction

1.1 Chemosensory Modulation – Gustation and Olfaction

1.1.1 What is Gustation?

There are two main mechanisms whereby animals can detect the properties of their environment. The first is via the physical senses, using either touch or vision. The second is to detect molecules within the environment via the chemical senses. This includes both the sense of smell (olfaction) and the sense of taste (gustation). Olfaction involves the detection of molecules in the atmosphere, whereas gustation involves the detection of molecules dissolved in solution (Boudreau and Tsuchitani, 1973; Dunipace *et al*, 2001; Singh, 1997). Both of these senses are important to the animal in order to find and assess the quality of a potential food source (Baker *et al*, 2002; Simmonds *et al*, 1992; White and Chapman, 1990), to avoid noxious and harmful chemicals in the environment (Hunt *et al*, 1999; Meunier *et al*, 2003), in the finding of potential mates (Fukaya *et al*, 2004; Savarit *et al*, 1999) and to assess the suitability of potential oviposition, or egg laying, sites (Baur *et al*, 1998; Chess and Ringo, 1985; Oyeyele and Zalucki, 1990).

1.1.2 Detection of Chemosensory Stimuli

The sensory organ involved in chemoreception in insects is the basiconic sensillum. Basiconic sensilla are diverse structures, but are commonly sensory hairs or pegs innervated by the dendrites of chemosensory neurons (Dethier, 1955). The number of chemosensory neurons per sensillum varies greatly and can be anything between two and fifty neurons (Chapman, 1982; Ochieng *et al,* 1998). The number and distribution of sensilla on an insect also varies depending on species, size, sex and age. Caterpillars, for example, have very few sensilla (Faucheux, 1995) whereas other insects such as beetles can have thousands (Zacharuk, 1985). Sensilla can be found all over an insect's body but are usually concentrated in specific regions. These regions are most commonly found on the tarsi, antennae, maxillae, labium, labrum, and cibarial cavity (Rogers and Newland, 2003).

Chemosensory sensilla can be sub-divided into two groups based on their structure and function (Chapman, 1982). The first of these groups contains sensilla involved in the detection of gustatory stimuli. These sensilla are uniporous i.e. they have a single pore at the tip (Fig. 1.1). The dendrites of the chemosensory neurons innervating these uniporous sensilla are usually unbranched fibres (Ochieng *et al*, 1998). Gustatory sensilla have a general distribution over the surface of the body (Rogers and Newland, 2003), but are found at higher densities on the mouthparts where they are involved in feeding and on the ovipositor for oviposition and oviposition site selection (Mitchell *et al*, 1999). The second of the two sub-divisions are the olfactory sensilla. These are generally multiporous and contain chemosensory neurons with highly branched dendrites (Ochieng *et al*, 1998). Many of the olfactory sensilla can be found on the antennae, but they can also be found on the mouthparts (Keil, 1999). In *Drosophila* olfactory sensilla are exclusively found on the third segment of the antennae and the maxillary palp (Dunipace *et al*, 2001; Vosshall *et al*, 2000). In *Drosophila* larvae the dorsal organ is involved in olfaction and the terminal organ in gustation (Heimbeck *et al*, 1999; Oppliger *et al*, 2000).

The dendrites of the chemosensory neurons are surrounded by a dendritic sheath that divides the sensillum into two parts, the dendritic chamber and the sensillar chamber (Fig. 1.1) (Keil, 1999; Mitchell *et al*, 1999). A chemical stimulus from the environment enters the sensillum through the apical pore. Here it moves across the sensillar lymph and comes into contact with the dendrites of the chemosensory neurons. Some chemicals are able to diffuse across the sensillar lymph, but other hydrophobic chemicals are unable to diffuse, therefore carrier proteins present in the lymph transport these chemicals to the dendrites of the chemosensory neurons (Ronnett and Moon, 2002; Shanbhag *et al*, 2001). The dendrites of the chemosensory neurons express receptors for the chemical stimuli. There are at least 54 gustatory receptors (Clyne *et al*, 2000; Dunipace *et al*, 2001; Scott *et al*, 2001) and at least 57 olfactory receptors (Vosshall *et al*, 2000) in *Drosophila*. Individual olfactory neurons are thought to express only one receptor type (Heimbeck *et al*, 1999; Vosshall *et al*, 2000). This information is then transduced into an electrical signal that is conducted via the axons of the chemosensory neurons to the central nervous system for further processing (Lessing and Carlson, 1999).

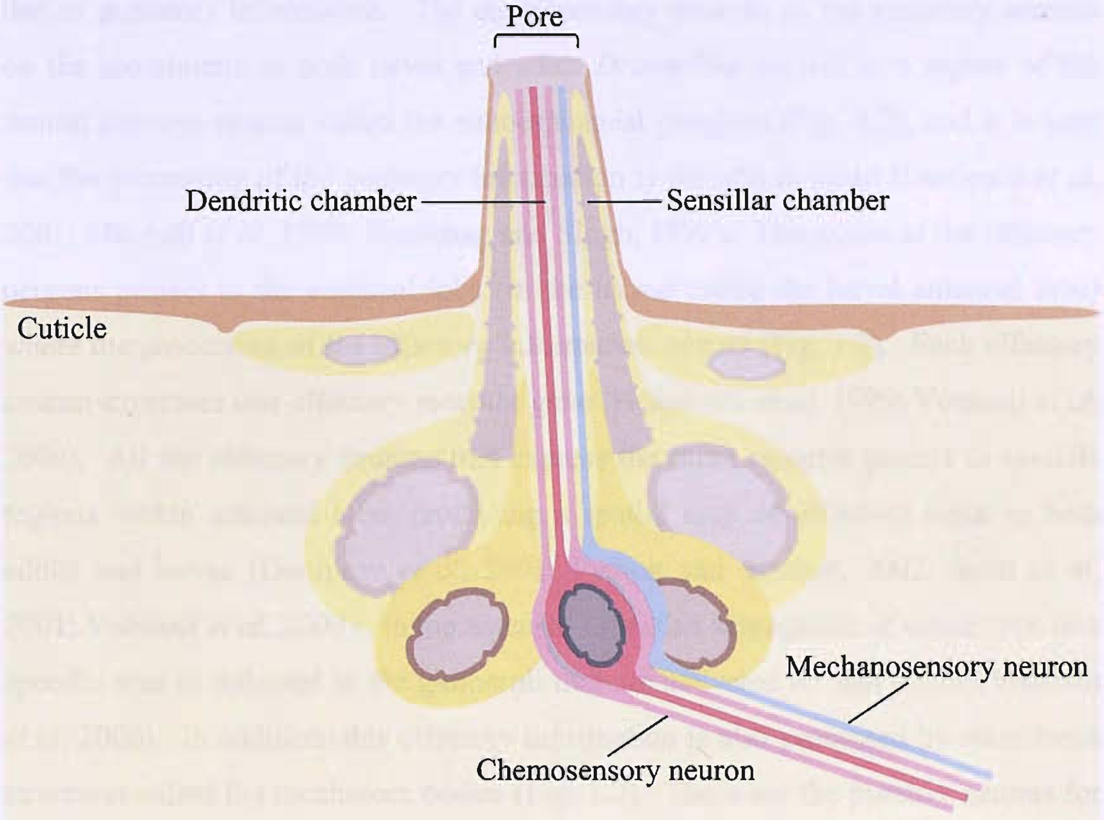


Figure 1.1: Cross-section through a gustatory basiconic sensillum. The sensillum is a specialised structure of the insect cuticle innervated by several chemosensory neurons (pink) and a single mechanosensory neuron (blue). Basiconic sensilla are used by insects to detect both gustatory and tactile information. Chemosensory information enters the sensillum through the apical pore, where it travels across the senisllar chamber and comes into contact with the dendrites of the chemosensory neurons in the dendritic chamber. The dendrites of the chemosensory neurons have receptors on their surface which are able to detect this chemosensory information and transduce it to the central nervous system.

1.1.3 Processing of Chemosensory Information within the Central Nervous System

Much more is known about the central processing of olfactory information than that of gustatory information. The chemosensory neurons of the gustatory sensilla on the mouthparts in both larval and adult *Drosophila* project to a region of the central nervous system called the suboesphageal ganglion (Fig. 1.2), and it is here that the processing of the gustatory information is thought to occur (Dunipace *et al*, 2001; Mitchell *et al*, 1999; Shanbhag and Singh, 1992). The axons of the olfactory neurons project to the antennal lobe (in the larvae called the larval antennal lobe) where the processing of the olfactory information occurs (Fig. 1.2). Each olfactory neuron expresses one olfactory receptor gene (Heimbeck *et al*, 1999; Vosshall *et al*, 2000). All the olfactory neurons that express the same receptor project to specific regions within antennal lobe, producing a spatial map of olfactory input in both adults and larvae (Dunipace *et al*, 2001; Python and Stocker, 2002; Scott *et al*, 2001; Vosshall *et al*, 2000). In the antennal lobe, this segregation of odour type to a specific area is reflected in the glomeruli that are activated by that odour (Vosshall *et al*, 2000). In addition, this olfactory information is also processed by other brain structures called the mushroom bodies (Fig. 1.2). These are the putative centres for olfactory learning and memory (de Belle and Heisenberg, 1994; Lessing and Carlson, 1999). Gustatory information is probably also sent to higher centres of the insect brain, although definitive evidence for this is lacking (Mitchell *et al*, 1999).

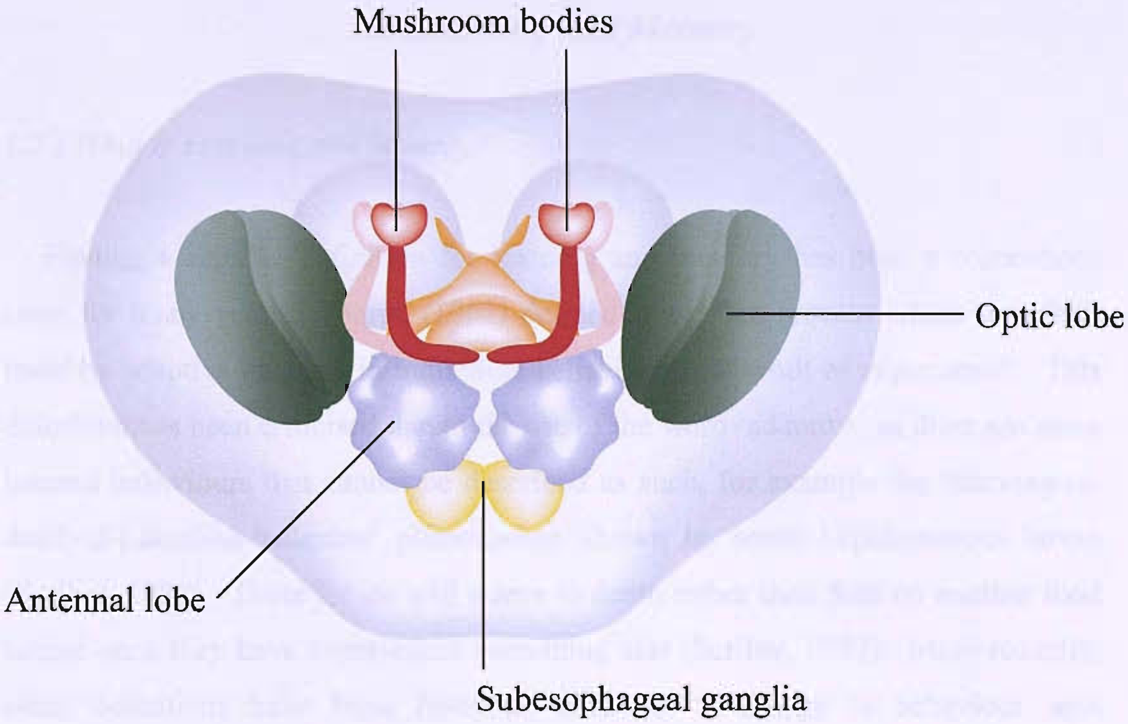


Figure 1.2: The adult Drosophila *brain (adapted from Gerber* et al, *2004a). Olfactory neurons project to the antennal lobe (blue) and the gustatory neurons to the subesophageal ganglion (yellow), where the chemosensory information is further processed. The mushroom bodies (red) are the putative centres for learning and memory in insects.*

1.2 Learning and Memory

1.2.1 What is Learning and Memory?

Finding a suitable definition for learning and memory has been a contentious issue for many years. Thorpe (1963) defined it as “that process which manifests itself by adaptive changes in individual behaviour as a result of experience”. This definition has been criticised due to the use of the word ‘adaptive’ as there are some learned behaviours that cannot be described as such, for example the ‘starving-to-death-at-Lucullian-banquets’ phenomenon shown by some Lepidopterous larvae (Scriber, 1982). These larvae will starve to death rather than feed on another food source once they have experienced something else (Scriber, 1982). More recently, other definitions have been favoured, such as “a change in behaviour with experience” suggested by Szentesi and Jermy in 1990. It is this latter definition that will be used to define ‘learning’ in this thesis.

There has been much research into learning and memory in insects and there are many examples of learning in the literature. For example, when honeybees were offered a sucrose solution paired with an odour, they quickly learnt to extend their proboscis in response to the odour alone (Bitterman *et al*, 1983). In *Drosophila,* when an odour is paired with an electric shock, the flies quickly learn to avoid that odour (Quinn *et al*, 1974). Insects have also been shown to learn information about their environment and use this to modify their behaviour, for example: crab spiders (*Misumena vatia*) have been shown to choose their hunting site based on previous experience (Morse, 1999). Previous experience can also affect choice of egg laying site (Jaenike, 1982; Jaenike, 1983) or a food source (de Boer and Hanson, 1984; Jermy *et al*, 1968; Rowell – Rahier, 1984) (see Chapters 3 and 4).

1.2.2 Classifications of Learning

Learning is generally divided into two major types: ‘non-associative’ learning involves the presentation of one stimulus alone, and is further divided into habituation and sensitisation; 'associative learning' involves the presentation of one stimulus in relation to another. These types of learning are described below.

Habituation is the waning of a response to a specific stimulus after repeated exposure (Papaj and Prokopy, 1989; Szentesi and Jermy, 1990). For example, fifth instar larvae of *Schistocerca gregaria* fed only on the feeding deterrent, nicotine hydrogen tartrate (NHT), over several days ate significantly more NHT contaminated leaves than naive individuals (Szentesi and Bernays, 1984).

Sensitisation is a type of learning that involves an increase in the response to a stimulus but does not involve associating the stimulus with another stimulus (Papaj and Prokopy, 1989; Szentesi and Jermy, 1990). For example, upon tactile stimulation *Aplysia* will withdraw its gill. If for several days prior to this stimulus an individual is given electric shocks, the withdrawal time will be longer than for those that have not previously been shocked (Pinsker *et al*, 1973). This type of learning may therefore account for the effects of experience on behaviour.

Associative learning involves the association of one stimulus with another stimulus or response (Papaj and Prokopy, 1989; Szentesi and Jermy, 1990). For example, experiments using *Drosophila melanogaster* paired an electric shock with a specific odour (Quinn *et al*, 1974). When then given the choice of two odours the flies avoided the odour that had been previously associated with the electric shock.

1.2.3 Memory Formation

Memory develops over time and different phases take different lengths of time to form (Hammer and Menzel, 1995). There are five distinct phases involved in learning and memory. The first phase is 'acquisition', or 'learning', this is followed by the formation of 'short-term memory', followed by formation of 'medium-term memory', then finally 'Anaesthesia-resistant memory' and 'long term memory' form in parallel (Dubnau and Tully, 1998). Short-term memory decays rapidly, at which point medium-term memory takes over. Medium-term memory is considered an early form of memory (DeZazzo and Tully, 1995). Repeated learning trials then lead to long term memory formation (Hammer and Menzel, 1995). Long term memory is protein synthesis dependent (Agranoff *et al*, 1966; Hyden and Lange, 1970; Montarolo *et al*, 1986; Yin *et al*, 1994) and can last for more than a week in *Drosophila* (Yin *et al*, 1995). When a memory is initially formed it can be disrupted by cold-shock, however, after approximately 2 hrs the memory becomes insensitive to this cold shock. This phenomenon is known as 'anaesthesia-resistant' memory (Tully *et al*, 1994b). This type of memory appears to be protein synthesis independent (Yin *et al*, 1994).

1.2.4 The Evolutionary Benefits of Learning

A common argument for the evolutionary benefit of learning is that the ability to learn which utilisable resource is most abundant will be advantageous as it will reduce search time and hence energy wastage (Papaj, 1986). In addition, it is argued that learning may provide an adaptive benefit to an individual because in an environment where resources are spatially and temporally distributed, utilisation of the substrate that allows the most offspring to survive into the next generation would be advantageous (Mery and Kawecki, 2002; Prokopy *et al*, 1982). Learning may also allow the insect to feed on a suitable substrate that it would normally find deterrent (Szentesi and Jermy, 1990) whilst avoiding food sources that are noxious (Stadler and Hanson, 1978). In addition, some studies have shown that switching between food sources can affect the efficiency of food utilisation (Grabstein and Scriber, 1982; Scriber, 1982).

1.3 Learning and Memory through Metamorphosis

There is evidence from many different species that animals are capable of learning and memory, including honeybees (Carew, 2000), rats (Lundy *et al*, 2004), spiders (Punzo, 1988) and humans (Brunstrom, 2004). Learning has also been clearly demonstrated in both larval (Gerber *et al*, 2004a; Neuser *et al*, 2005) and adult *Drosophila* (Le Bourg and Buecher, 2002; Quinn *et al*, 1974). However, an insect's life cycle is punctuated by metamorphosis and whether learning can be retained through metamorphosis is a much more contentious issue. To fully investigate this issue, an understanding of what occurs during metamorphosis is first required.

1.3.1 Metamorphosis

There are two forms of metamorphosis in insects. The first is incomplete metamorphosis, which involves three stages and occurs in insects such as grasshoppers. Initially an egg is laid, which then hatches into a nymph. This nymph appears as an immature adult and undergoes several moults in which wings and sexual organs gradually grow until the imaginal moult turns the nymph into a winged and sexually mature adult (Sehnal *et al*, 1996). The second form of metamorphosis is complete metamorphosis and this occurs in holometabolous insects. In this case an insect transforms from a simple larva to a complex adult, punctuated by a pupal stage (Highman, 1981). *Drosophila* undergo holometabolous development, and a diagram of the *Drosophila* life cycle can be seen in Figure 1.3. The cycle starts with an egg being laid. A larva will hatch from this egg and undergo three larval stages before pupariation occurs and a pupa is formed. It is at this stage of the life cycle that metamorphosis occurs to form an adult fly that emerges during the final imaginal moult (Gilbert, 2000).

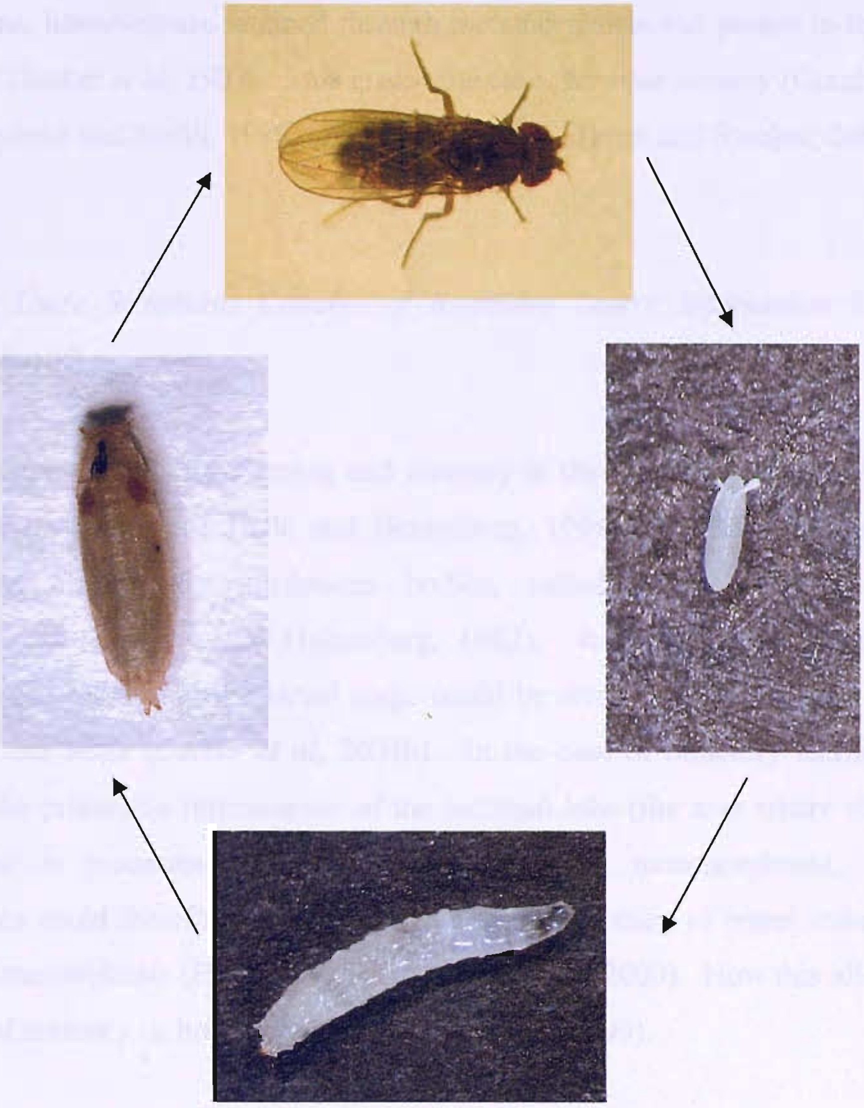


Figure 1.3. Holometabolous development in Drosophila melanogaster. *At 25°C, the 1st instar larvae will hatch from the egg after approximately 21 hrs. 118 hrs after the egg was laid, pupariation will occur to form the pupae. 221 hrs after the initial egg laying event, an adult fly will emerge (Roberts and Standen, 1998).*

Along with the obvious changes in the appearance of the organism, there is also a pronounced rearrangement of the chemosensory system during metamorphosis. Many of the larval sensilla and chemosensory neurons are lost during metamorphosis and are replaced by adult sensilla and chemosensory neurons originating from the imaginal discs (Gendre *et al*, 2004). Many adult interneurons also only differentiate during metamorphosis (Allen *et al*, 1998). Some larval interneurons, however, are retained through metamorphosis and persist in the adult organism (Stocker *et al*, 1997). This is also the case for some sensory (Gendre *et al*, 2004; Shepherd and Smith, 1996) and motor neurons (Tissot and Stocker, 2000).

1.3.2 Are There Structures Capable of Retaining Learnt Information Through Metamorphosis?

The putative centres for learning and memory in the central nervous system are the mushroom bodies (de Belle and Heisenberg, 1994) (Fig. 1.2). A subset of interneurons within the mushroom bodies, called Kenyon cells, survive metamorphosis (Technau and Heisenberg, 1982). It is therefore possible that information learnt during the larval stage could be retained by these cells and so persists in the adult (Gerber *et al*, 2004b). In the case of olfactory learning and memory, the projection interneurons of the antennal lobe (the area where olfactory information is processed) are also retained through metamorphosis. These interneurons could therefore also be involved in the retention of learnt information through metamorphosis (Ray, 1999; Tissot and Stocker, 2000). How this allows for retention of memory is, however, still unknown (Ray, 1999).

1.3.3 Is There Any Evidence for the Retention of Learnt Information Through Metamorphosis?

At a meeting of the American Association of Economic Entomologist's, Hopkins (1917) suggested that adult individuals may have a preference to oviposit upon the host which they fed on as larvae. This is now known as the 'Hopkin's Host Selection Theory' and has since been assumed to depend on learning and memory (Corbet, 1985; Wiklund, 1974). Much research has been conducted to elucidate whether this theory is correct. Several studies using the holometabolous mealworm *Tenebrio molitor* have provided evidence for the retention of learnt information through metamorphosis (Alloway, 1972; Borsellino *et al*, 1970; Somberg *et al*, 1970). Similar results have been found in other insects, such as the parasitic wasp

Hyssopus pallidus (Gandolfi *et al*, 2003), the house fly *Musca domestica* (Ray, 1999) and the locust (Goldsmith *et al*, 1978). For example, fifth instar hoppers (*Locusta migratoria migratorioides*) were trained to maintain leg flexion by being subject to electric shock when the leg was extended. After metamorphosis the adult locusts were then tested for the retention of memory through metamorphosis and results showed when trained as hoppers to maintain leg flexion adults were also much less likely to extend their legs than those that had not be trained as hoppers (Goldsmith *et al*, 1978).

Studies of this phenomenon in *Drosophila* have proved variable. One of the earliest studies was conducted by Thorpe (1939) who raised *Drosophila* larvae on a medium containing 0.5% peppermint essence. At the pupal stage, these individuals were washed and isolated. Upon eclosion, the adult flies were placed into an olfactometer, with one arm containing 0.5% peppermint essence and the other being unscented. Results showed that flies had a much greater acceptance for the 0.5% peppermint essence if they had been raised in the presence of this odour as larvae compared to those who had been raised on an unscented medium. Thorpe concluded that these results provided evidence for the retention of learnt information through metamorphosis. Later repeats of these experiments have shown varied results. In some cases studies have supported Thorpe's results (Hershberger and Smith, 1967; Manning, 1967; Rietdorf and Steidle, 2002). In other cases the results have provided no evidence for the retention of memory through metamorphosis (Jaenike, 1982 and 1983; Monteith, 1962; van Emden *et al*, 1996; Wiklund, 1974). In the case of Jaenike's study (1982), not only was there no observed change in the behaviour of the adults when the larvae were raised on 0.5% peppermint essence, but there was also no change in behaviour when the larvae were raised in the presence of other chemicals, such as sodium chloride. A more recent experiment claiming to provide evidence for the retention of memory through metamorphosis was conducted by Tully *et al* in 1994b. In this case, *Drosophila* larvae were raised in the presence of an odour paired with an electric shock. Results showed that the larvae were able to learn to avoid the odour that was paired with the electric shock, and that this avoidance behaviour could still be detected in the adult flies (Tully *et al*, 1994b).

The experiment by Tully *et al* (1994b) has been highly criticised due to the questionable relevance of an electric shock as a reinforcing stimulus (Barron and Corbett, 1999). Many of the other studies claiming to support the Hopkins Host Selection Theory have also been criticised as there is a possibility that the change of behaviour with larval diet may be due to the newly emerging adults coming into contact with traces of their larval environment on the pupal cases. If this is the case, it would be early adult rather than larval experience affecting adult behaviour. The hypothesis that early adult rather than larval experience affects subsequent behaviour is called the Chemical Legacy Hypothesis (Corbet, 1985). This theory suggests that minute quantities of chemical from the larval environment persist either on or in the insect's body. Upon emergence, the adult comes into contact with these chemical cues which then modify the chemosensory responsiveness of the adult (Corbet, 1985). This theory could therefore explain the effects of larval diet on adult behaviour described above.

1.4 The Effect of Diet on Health

It has long been known that diet can have an impact on health in mammals. For example, fatty acids in the diet have been shown to increase serum cholesterol levels which can significantly increase the risk of coronary heart disease (Amano *et al*, 2004). In addition, high dietary glycaemic load combined with a low intake of cereal fibre significantly elevates the risk of type 2 diabetes (Salmeron *et al*, 1997). Diet has also been implicated in the risk of several different cancers including breast cancer (McCann *et al*, 2007; Silvera *et al*, 2005), ovarian cancer (Silvera *et al*, 2007) and colorectal cancer (McCarl *et al*, 2006). Diet can also affect the risk of haemorrhagic stroke (Levitan *et al*, 2007). The food choices an individual makes can therefore have a major impact on their future health.

More recently evidence has suggested that maternal diet can also influence the health of the offspring (see Chapter 6). For example, poor foetal growth has been linked with an increased risk of glucose intolerance and metabolic syndrome in later life (de Rooij *et al*, 2006). It has also been linked with other adulthood diseases such as cardiovascular disease (Painter *et al*, 2006; Roseboom *et al*, 2000), hypertension (Stein *et al*, 2006), obesity (Ravelli *et al*, 1976; Ravelli *et al*, 1999; Stanner *et al*, 1997; Stein *et al*, 2007), affective disorder (Brown *et al*, 2000), antisocial personality disorder (Neugebauer *et al*, 2004) and schizophrenia (Hoek *et al*, 1998; St Clair *et al*, 2005). This effect has also been shown to continue through to the following generations. For example, a lack of protein *in utero* can affect glucose metabolism for 3 generations in rats (Benyshek *et al*, 2006). The food choice an individual makes will therefore also have a major impact on the health of subsequent generations.

Diet has also been shown to affect several measures of health in insects. These measure include starvation resistance (Burger *et al*, 2007; Chippindale *et al*, 1997), fecundity (Burger *et al*, 2007; Butler and O'Neil, 2007; Kyneb and Toft, 2004; Naya *et al*, 2007; Roff and Gelinas, 2003), development time (Azidah and Sofian-Azirun, 2006; Butler and O'Neil, 2007; Kyneb and Toft, 2004), mortality/survival (Butler and O'Neil, 2007; Kyneb and Toft, 2004) and life span (Burger *et al*, 2007;

Butler and O'Neil, 2007; Chippindale *et al*, 1997). In addition, diet has also been shown to affect some of these characteristics over several generations (Amarillo – Suarez and Fox, 2006; Fox *et al*, 1995; Spitzer, 2004). For example, in the seed-feeding beetle *Stator limbatus* maternal diet was shown to affect development time of offspring irrespective of offspring diet (Amarillo – Suarez and Fox, 2006). The fact that diet can affect life history traits in insects across several generations leads to the possibility that they could be suitable model organisms for gaining an understanding of the underlying mechanisms involved in the effect of diet on future generations.

1.5 Aims and Objectives

The aim of this project was to determine whether diet had an affect on adult behaviour and life history traits in *Drosophila melanogaster*. In Chapter 3 the effect of larval diet on adult oviposition preferences was analysed. The aim of this chapter was to determine whether Hopkins Host Selection Theory (see Section 1.3.3) that larval diet can influence adult oviposition preferences is correct. To do this, *Drosophila* larvae were raised on several diets varying in the concentration of one component and then as adults offered the choice of either the diet upon which they were raised or a standard diet. In parallel with these experiments other larvae were raised on a standard diet and as adults offered the same choices. The results of these experiments could then be compared to determine whether larval diet can indeed affect adult oviposition preferences. In addition, flies were also raised for several generations on specific diets to determine if the oviposition preferences of these adults are affected by diet upon exposure to that diet over several generations. Chapter 4 goes onto look at the effects of varying several components of the diet upon adult feeding preferences using similar methods to those described in Chapter 3.

The aim of Chapter 5 was to gain a further understanding of the possible mechanisms underlying these changes in adult behaviour with varying larval diet. As mentioned in Section 1.3.3, the Hopkins Host Selection Theory suggests that the effects of larval diet on adult behaviour are due to learning and memory. To determine whether this was the case two learning and memory mutants, *dunce* and *rutabaga*, were tested for the effects of larval diet on adult behaviour to see if these mutations had any affect on adult behaviour. In addition the mushroom bodies (MBs) of the flies were ablated to determine whether it is these central structures which are involved in the storage of the information from the larval stages and into the adult.

The final aim of this project was to determine what effects these choices had on several life history characteristics in the flies (Chapter 6). These characteristics included development time, fecundity, starvation resistance, larval fitness and survival. In addition, flies were kept on these diets for 5 generations to determine if there was any change in the life history characteristics when exposed to these diets for several generations. This could lead to the possibility of using insects as a model organism for gaining a better understanding of the effects of diet on health and disease as has been described in mammals.

Chapter 2: General Methods

In this chapter the techniques for routine fly culture and anaesthesia are described, along with methods key to several of the following chapters. Methods specific to different assays are included in the relevant chapters.

2.1 Stock Flies

Throughout this report 'flies' or '*Drosophila*' refers to the Oregon R strain of *Drosophila melanogaster*. This stock was acquired from the Department of Developmental and Cell Biology, University of Southampton and was originally obtained from the Bloomington Stock Centre.

2.2 The Incubator

All flies involved in an experiment were kept in an incubator (LMS 305) maintained at 25.5°C ± 0.2°C. The flies were kept in a L12:D12 light cycle, with the lights coming on at 7:30 am and going off at 7:30 pm. The door was not opened during the dark phase of this cycle. Open containers of water were kept in the incubator, maintaining the humidity between 52% and 62%.

2.3 Handling of the Flies

The flies were handled as gently and as little as possible. Stock flies were maintained in 30 ml vials containing a standard medium (see Section 2.5) adapted from that described by Ashburner and Roote (2000) and transferred to fresh vials regularly. Transferral involved tapping the flies from one vial to another. About twenty flies were kept in each vial, and the flies were periodically anaesthetised to ensure that both sexes were present in the vial and that there was no evidence of unexpected phenotypes.

2.4 Anaesthetising the Flies

Laboratories routinely anaesthetise flies to make the handling of them easier. The three most common anaesthetics used are carbon dioxide (CO_2), ether and chilling (Barron, 2000). All of these methods, however, come with their own problems.

The use of ether is the traditional method of anaesthetising *Drosophila*. Experiments have shown that ether can significantly reduce locomotor activity in flies up to 18 days after the initial anaesthetic (van Dijken *et al*, 1977). The use of ether has also been criticised due to the health and safety risks involved.

Chilling usually involves putting the flies over ice for a short period of time. The use of this method risks over-anaesthetising the flies (Greenspan, 1997), and studies have also shown that chilling can disrupt recently acquired memory (Quinn and Dudai, 1976).

CO_2 is currently the most widely used form of anaesthesia for *Drosophila* (Greenspan, 1997). Anesthetising the flies using this method usually involves inserting a tube with CO_2 flowing through it into the vial containing the flies. Once the flies are anaesthetised they are transferred onto a porous plate that also has CO_2 flowing through it. This keeps the flies anaesthetised until they are removed from the porous plate. There are also several reported problems with this method of anaesthetisation. Experiments by Perron *et al* (1972) have shown that CO_2 can increase mortality in 0 to 3 hr old flies and decrease fecundity if exposed for longer than 15 mins. In addition, carbon dioxide has been shown to increase motor activity, but the effects on locomotion do not last as long as the effects of ether (van Dijken *et al*, 1977).

In this study, the use of anaesthesia was avoided as far as possible. On the occasions where it was required, however, CO_2 was the anaesthetic of choice since the side effects are minimal and there are no reported effects on learning and memory. Individuals involved in an experimental procedure were not anaesthetised at any point during their development, and as adults were not anaesthetised until three days after eclosion. In addition, the length of exposure to CO_2 was kept to a minimum, with the maximum exposure time being 10 mins. In this way, anaesthesia was not used at any point where is has been shown to have a detrimental effect on the flies.

2.5 The Food Media

2.5.1 Ingredients

The food medium used in this study was a recipe adapted from Ashburner and Roote (2000). The ingredients required to prepare 50 ml of this medium are presented in Table 2.1.

Ingredient	**Amount**	**Supplier**
Micro agar	0.3 g	Duchefa Biochemie
Plain flour	2.06 g	McDougalls Foods Ltd
Dried Active Yeast	0.9 g	Allinson, Westmill Foods Ltd.
Distilled Water	50 ml	
Methyl-4-hydroxybenoate (Nipagin)	0.15 g	Sigma
100% Ethanol	1.5 ml	

Table 2.1: The ingredients and quantities required to prepare 50 ml of standard food media.

Carbohydrate was also added to the food, although the carbohydrate type and concentration was varied in these experiments. The carbohydrates used were sucrose, fructose, trehalose or glucose. In order to compare the results from each of these carbohydrates, the concentration of these carbohydrates was kept constant in the standard medium, at 257.2 mM. Table 2.2 shows the amount of the different carbohydrates required to make up 50 ml of standard food at a concentration of 257.2 mM.

Carbohydrate	**Amount**	**Supplier**
Sucrose	4.4 g	Fischer Scientific
D-(-)-Fructose	2.32 g	Sigma
D-(+)-Trehalose Dihydrate	4.87 g	Sigma
D-(+)-Glucose	2.32 g	Sigma

Table 2.2: Amount of each of the carbohydrates required to make up 50 ml of standard medium with 257.2 mM of carbohydrate.

In addition to varying the carbohydrate type, carbohydrate concentration was also varied. The concentrations used were 0%, 12.5%, 25% or 50% of the amount used in the standard media.

Further, in one set of experiments sodium chloride was added to the standard sucrose food medium to determine whether the concentration of NaCl in the diet had an effect on behaviour. The concentrations of NaCl used in these experiments were 13 mM, 25 mM, 50 mM, 100 mM or 200 mM. The amounts of NaCl required to make up 50 ml of food medium are described in Table 2.3.

NaCl Concentration Used	**Amount Required (g)**	**Supplier**
13 mM	0.03799	Fischer Scientific
25 mM	0.0731	
50 mM	0.1461	
100 mM	0.2922	
200 mM	0.5844	

Table 2.3: Amounts of NaCl added to the food media to make 50 ml of food containing the specified concentrations of NaCl.

The final variation made to the food was varying the amount of yeast. The standard amount of yeast added to make 50 vials of food was 7.5 g. As with the carbohydrates, this was reduced to 0%, 12.5%, 25% or 50% of the standard amount of yeast.

It must be noted that only one component of the food was varied at any one time, for example when the concentration of NaCl was varied the concentration of all the other food components were at the concentrations described in the standard medium (see Table 2.1).

2.5.2 Preparing the Food Medium

The agar, flour, carbohydrate, yeast and distilled water were all added to a saucepan and heated. NaCl was also added where required. The mixture was continuously stirred until it came to the boil. The mixture was then removed from the heat and allowed to cool. The methyl-4-hydroxybenoate was dissolved in ethanol and then added to the mixture and thoroughly stirred. This acts as a fungal inhibitor within the food medium. The food was then poured into 30 ml vials (unless otherwise stated), 7 ml per vial. The vials were then plugged with a polyurethane foam plug and placed in a refrigerator at 4°C. The vials were used within two days, and allowed to warm to room temperature for 20 mins before any flies were placed in them.

Chapter 3: Does Larval Diet Influence Adult Egg Laying Preferences?

3.1 Introduction

3.1.1 Egg Laying Behaviour in Insects

Oviposition, or egg laying, involves a complex sequence of behaviours whereby the female has to detect, accept and lay her eggs on a specific host or substrate. Studies have shown that female insects are initially attracted to a possible oviposition substrate by both olfactory (Thorpe and Jones, 1937) and visual (Papaj, 1986; Rausher, 1978) cues. When the substrate or host is reached, the female will then further examine it using gustation (taste) (Bénédet *et al*, 2002). This involves sampling with the legs, mouthparts and ovipositor (Baur *et al*, 1998; Oyeyele and Zalucki, 1990). At any point during these sampling behaviours the female could reject the substrate, but if it is accepted then oviposition occurs. This complex set of behaviours suggests that the female is making a specific decision as to whether the substrate is appropriate for egg laying or not. This therefore suggests that females must have a preference to lay their eggs on some substrates over others.

3.1.2 What is Oviposition Preference and Why is it Important?

Oviposition preference is the likelihood that an organism will lay its eggs on a particular substrate or host when given the choice (Sheeba *et al*, 1998). In their natural environment insects will come across many different potential oviposition sites, and studies have shown that insects often have a hierarchy in their preference for the different oviposition sites available to them (Omkar, 2005; Prokopy *et al*, 1982; Sadeghi and Gilbert, 1999; Wiklund, 1974). Many factors have been implicated that could be used by the female to assess the suitability of an oviposition substrate, including colour (Carpenter *et al*, 2001), texture (Chess and Ringo, 1985) and the presence of other larvae (Chess and Ringo, 1985).

Oviposition preferences are important because possible oviposition sites will vary in their quality. The choice the female makes will therefore affect her offspring's fitness (Chess and Ringo, 1985; Tabashnik, 1981). It has been hypothesised that the female will show a preference for the substrate that the larvae will develop best on, hence increasing its fitness. This is called the Optimal Oviposition Theory (Jaenike, 1978) and the results from several studies support it (Omkar, 2005; Sadheghi and Gilbert, 1999; Smiley, 1978). For example, in a study on the relationship between oviposition preference and offspring performance in a generalist predatory ladybird, a strong positive correlation was found between the preference of the female for various aphid species and the performance of her offspring (Omkar, 2005). However, in many cases there is little or no evidence that female preference has any effect on larval fitness (Pawlitz and Bultman, 2000; Scheirs *et al*, 2004; Smiley, 1978) and in some cases female oviposition preference can actually reduce larval survival (Badenes – Perez *et al*, 2006; Chess and Ringo, 1985; Digweed, 2006; Lu *et al*, 2004). For example, when the diamondback moth (*Plutella xylostella*) was offered a choice of yellow rocket, Chinese cabbage or common cabbage, almost all of the eggs were laid on the yellow rocket. When the survival of these individuals was studied, however, very few of the larvae on the yellow rocket (less than 10%) survived to pupation whereas 87-100% of the larvae on the cabbage plants survived to pupation (Lu *et al*, 2004).

Several possible reasons for this apparent lack of association between female oviposition preferences and larval fitness have been suggested, including selection of enemy free space (Jaenike, 1985; Price *et al*, 1986; Thompson, 1987), competition (Digweed, 2006; Whitham, 1980) and host abundance (Chew, 1977). In addition, in some cases it appears that females simply lay their eggs on the substrate which they themselves feed, independent of whether this is the best substrate for larval development (Jaenike, 1986; Scheirs *et al*, 2000; Scheirs and de Bruyn, 2002; Scheirs *et al*, 2004).

Oviposition preferences vary between species, but studies have shown that oviposition preferences can also vary within species (Sadeghi and Gilbert, 1999; Sheeba *et al*, 1998; Singer, 1983). If there is such a variation in oviposition preferences both within and between species, what is controlling this variation?

Several factors have been described which could be involved in this, and these are introduced below.

3.1.3 Genetic Control of Oviposition Preferences

There is much evidence to support the suggestion that oviposition preferences are genetically controlled. For example, in an experiment using the swallowtail butterfly *Papilio machaon*, larvae were raised on various host plants (Wiklund, 1974). As adults they were provided with a choice of egg laying sites. All of the adults showed a preference for the same host plant irrespective of larval diet. This lack of affect of environmental factors suggests that the oviposition preferences observed must be under genetic control (Wiklund, 1974). Genetic control of oviposition preferences has also been shown in other species of butterfly (Smiley, 1978) as well as other insects including weevils (Reitdorf and Steidle, 2002; Wasserman and Futuyma, 1981), sawflies (Knerer and Atwood, 1973), houseflies (Pimetel *et al*, 1967) and fruit flies (Klaczko *et al*, 1986). Studies in Lepidoptera suggest that oviposition preferences are determined by loci on the X chromosome (Thompson, 1988; Janz, 1998). In addition, olfactory binding proteins have been shown to be important in the selection of oviposition sites in *Drosophila* (Matsuo *et al*, 2007). *Drosophila sechellia* is a specialist of *Morinda citrifolia* but its closest relatives (including *Drosophila melanogaster*) avoid this fruit due to its toxic nature. Genetic manipulation studies have shown that this difference in preference is due to *odourant binding proteins 57d* and *57e* (Matsuo *et al*, 2007).

3.1.4 Learning of Oviposition Preferences

Learning has also been implicated in controlling oviposition preferences. It is argued that learning may provide an adaptive benefit to an individual because in an environment where resources are spatially and temporally distributed learning to lay eggs on the substrate that will allow the most offspring to survive into the next generation would be advantageous (Mery and Kawecki, 2002; Prokopy *et al*, 1982). In addition, it has been argued that resources will often vary in their abundance over time and so the ability to learn which utilisable resource is most abundant will be advantageous as it will reduce search time and hence energy wastage (Papaj, 1986; Davis and Stamps, 2004). There are two main stages during the life cycle of holometabolous insects where learning of oviposition preferences could occur, and these are introduced below.

a) Learning of Oviposition Preferences during the Adult Stage

There is much evidence to suggest that adult experience of a particular host or substrate can affect subsequent adult oviposition behaviour (Cadieu *et al*, 2000; Hoffman, 1985; Jaenike, 1982 and 1983; Mery and Kawecki, 2002; Prokopy *et al*, 1982). The information that is learnt by the adult can be chemical (Mery and Kawecki, 2002) or visual (Papaj, 1986). In some cases experience of a substrate increases the adult preference for that substrate (Akhtar and Isman, 2003; Anderson *et al*, 1995; Cadieu *et al*, 2000; Hoffman, 1985; Jaenike, 1982 and 1983; Prokopy *et al*, 1982; Vet and van Opzeeland, 1984). For example, in *Drosophila* the addition of peppermint oil or ethanol to the food media, or the use of apple and tomato as a food source, increased the preference of the females for these substances as egg laying sites when given a choice (Jaenike, 1982; 1983). However, in others cases preference is decreased by previous exposure (Cadieu *et al,* 2000; Hoffman, 1985; Mery and Kawecki, 2002). For example, in *Drosophila* when offered the choice of apple or orange, previous exposure to apple increased the number of eggs laid on orange (Hoffman, 1985). In other cases, however, there is no evidence for the effect of adult experience on female egg laying preferences (Tabashnik, 1981).

b) Learning of Oviposition Preferences during the Larval Stages

The Hopkins Host Selection Theory suggests that adult individuals will have a preference to oviposit on the host that they fed on as larvae. This theory comes from comments made by Hopkins at a meeting of the American Association of Economic Entomologists (Hopkins, 1917) and has since been assumed to depend on learning and memory (Corbet, 1985; Wiklund, 1974). Several studies claim to support this theory (see Section 1.3.3), however much of this work involved olfactory associative learning and has not looked at the effect of larval experience on adult egg laying choices. Some studies do, however, claim to have shown that larval diet can affect adult oviposition preferences (Craighead, 1921; Cushing, 1941; Smith and Cornell, 1979; Thorpe and Jones, 1937; Vinson *et al*, 1977). For example, in a study using the parasitoid wasp *Nasonia vitripennis,* results showed that when this wasp was reared on *Musca domestica* pupae, these individuals showed a preference to oviposit on this host as adults (Smith and Cornell, 1979). Those studies that have looked at the effect of larval diet on adult oviposition preferences have, however, been criticised as they did not rule out the possibility that any changes in behaviour could have been due to the newly emerged adults coming into contact with traces of their larval environment left on the cases of the pupae and hence be due to early adult learning (Corbet, 1985; Jaenike, 1982 and 1983; van Emden *et al*, 1996). Therefore, although there clearly is evidence that information about the larval environment can be learnt and this information retained through metamorphosis and into the adult, whether this can have an effect on oviposition site selection remains unclear. In addition, other studies have not found any influence of larval diet on adult egg laying preferences (Jaenike, 1982 and 1983; Knerer and Atwood, 1973; Monteith, 1962; Rojas and Wyatt, 1999; Tabashnik, 1981; van Emden *et al,* 1996; Wiklund, 1974).

Even though in some cases larval diet does not seem to have any influence on adult oviposition preferences, it has been argued that in the wild there will be an affect (Corbet, 1985; Jaenike, 1982). As already discussed, there is evidence to suggest that adult experience can affect oviposition preferences. As individuals will often pupate near the site of egg laying, the adult is likely to come into contact with its larval diet upon emergence and hence larval diet could still be an influencing factor (Jaenike, 1982). Residues of larval diet are also often found on or within the pupal case, which could then influence adult behaviour without any larval memory being involved (Corbet, 1985).

3.1.5 What Effects Could These Preferences Have on Future Generations?

As we have seen above, there is mounting evidence for individual variation in oviposition preference within a species and this can be caused by various different factors (see Sections 3.1.3 and 3.1.4). Individual variation in habitat selection can have important consequences for evolutionary and ecological processes (Davis and Stamps, 2004). One such factor is the formation of host races. Host races are defined as populations of the same species that are partially reproductively isolated from each other due to adaptations to different hosts (Diehl and Bush, 1984). In order for this to occur, the female must firstly be attracted to, and then be stimulated to oviposit upon, a new host. The immature stages of the insect must then be able to survive and develop on this new host (Jaenike, 1982). For example, if experience of a normally repellent substrate leads these individuals to no longer find it repellent there could be an increased utilisation of that substrate. This could then lead to changes in oviposition site preferences which can influence host shift and expansion (Craig *et al*, 2001). For example, the freshwater weevil *Euhrychiopsis lecontei* is endemic to Northern America and its native host is the northern watermilfoil *Myriophyllum sibiricum.* The weevil has recently expanded its host range to include the exotic watermilfoil *Myriophyllum spicatum*, and results from oviposition preference studies show that weevils raised on the exotic *M. spicatum* species have a preference for this species as an egg laying site. The weevil has therefore expanded its host range to include the exotic watermilfoil species. The results also suggest that there is the potential for a host shift onto the exotic watermilfoil species as those raised on this species have an increased preference to oviposit on it (Solarz and Newman, 1996; Solarz and Newman, 2001). Other studies have also found evidence for the existence of host races in a variety of species (Abrahamson *et al*, 2003; Emelianoz *et al*, 2001; Emelianoz *et al*, 2004; Martel *et al*, 2003; Via, 1999).

It has been argued that host shifts can lead to sympatric speciation. Sympatric speciation occurs when organisms mate in specific habitats and therefore mate choice is directly associated with habitat choice. If the habitat of choice imposes strong enough fitness trade-offs, genetic changes are likely to occur to increase the fitness of the offspring on that host. These genetic changes can then lead to sympatric speciation (Feder and Forbes, 2007). There is now some evidence to suggest that sympatric speciation has occurred in indigobirds, which are host-specific brood parasites (Sorenson *et al*, 2003). Male indigobirds mimic the song of their host and females choose both their mate and their host from the song of the bird they were reared by. Birds reared by a novel host therefore acquire the song of that host which leads to immediate reproductive isolation once a new host is colonised (Sorenson *et al*, 2003). In addition, sympatric speciation is thought to have occurred in apple maggot flies (*Rhagoletis polmonella*). The ancestral host of these flies is hawthorn, but with the introduction of the domesticated apple a host shift occurred about 150 years ago. These flies mate on or nearby their host plant, to which they are attracted by olfaction (Linn *et al*, 2003; Linn *et al*, 2004). Odour preference has therefore led to reproductive isolation. In addition, F_1 hybrids of the apple and hawthorn host races do not respond to either host fruit volatiles suggesting that hybrids have a fitness disadvantage (Linn *et al*, 2004). The odour preferences of these host races has been shown to have a genetic basis suggesting sympatric speciation is occurring in this species (Dambroski *et al*, 2005). Speciation due to host shifts is also thought to have occurred in other species (Sachet *et al*, 2006). In other studies, however, the initial change in preference was not reinforced over several generations suggesting that changes in preferences may not always lead to speciation (Smith and Cornell, 1979).

3.1.6 Aims and Objectives

As discussed above, oviposition preferences vary both within and between species and this variation could be due to several different factors. It is generally accepted that there is genetic control of oviposition site selection. It is also generally accepted that adult experience can influence oviposition preferences. The issue of whether larval experience can affect adult oviposition preferences is still, however, a contentious issue. This is due to limitations in the previous work that was conducted in this area. The aim of this chapter is to resolve this issue by raising fruit flies on diets varying in several different components and then offering the adults the choice of either the diet they were raised on or a standard diet. As a control, flies were raised on the standard diet and offered the same choices. The results of these experiments were then compared to see if larval diet had any affect on adult food preferences. In addition, to gain a further understanding of how these preferences may affect the population, flies were raised on specific diets over several generations.

3.2 Methods

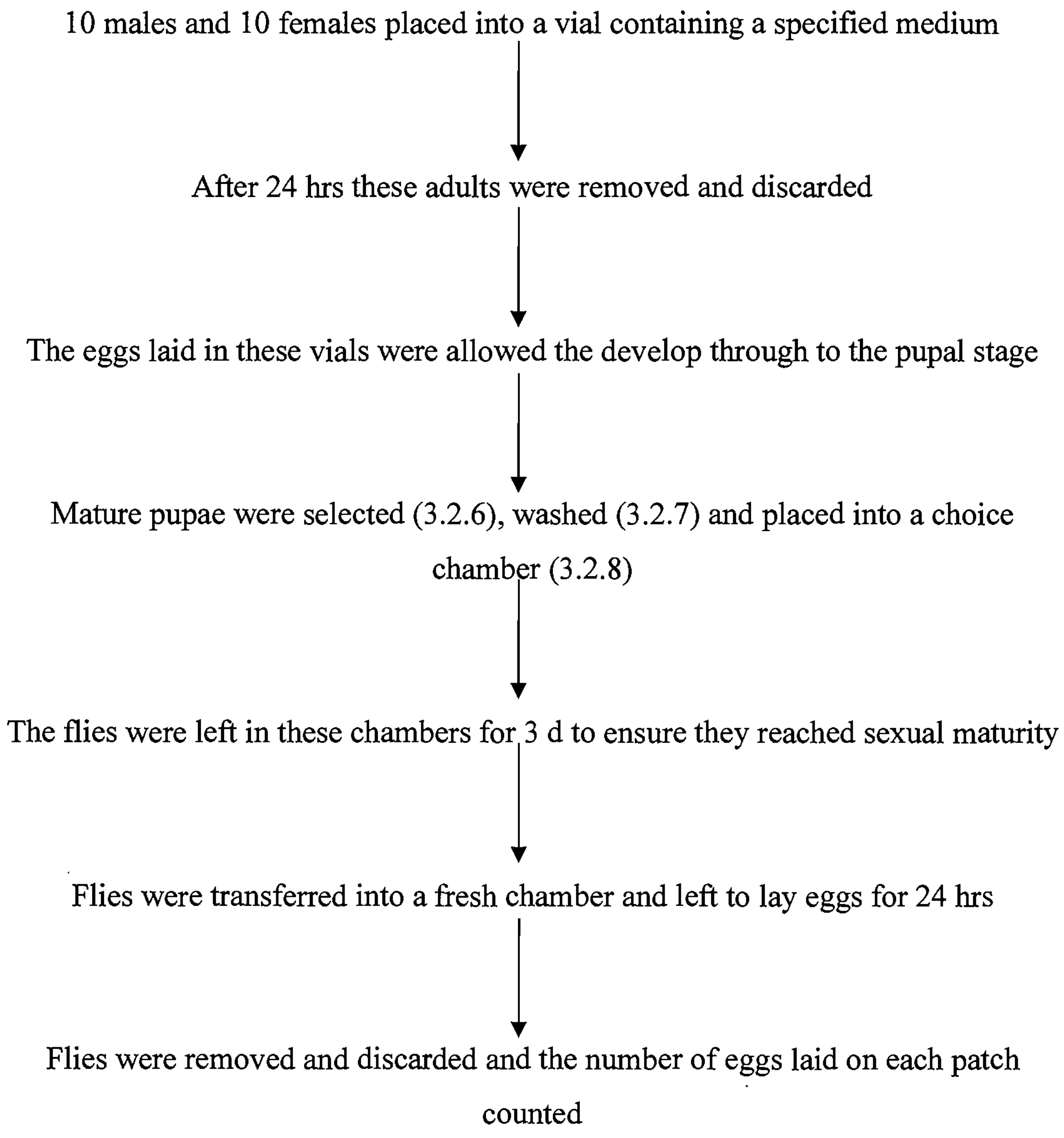
3.2.1. Determining Whether Larval Diet Affects Adult Egg Laying Site Preferences

The aim of these experiments was to determine whether larval diet can affect subsequent adult egg laying preferences. To do this, larvae were raised on varying diets where the concentration of one of the components of the standard diet was varied (see Section 3.2.5). As adults, these individuals were then offered the choice of either the diet upon which they were raised or a standard diet to lay their eggs on. To determine whether any change in preference occurred due to larval diet, control experiments were run in parallel with these experiments where individuals were raised on a standard diet as larvae and then as adults offered the same choices. The results of the two experiments were then compared.

These experiments were complicated by the fact that females do not start laying eggs immediately after eclosion but instead begin at about 1 d of age and peak between 3-10 d of age (Jaenike, 1982). It is therefore not possible to test egg laying preference without the adults firstly being exposed to a food source so that they can feed. The problem with this is that adult experience is known to affect egg laying preference (see Section 3.1.4). Two methods were therefore employed to try to disentangle the effects of larval and adult diets on subsequent adult egg laying preferences. The first method (see Section 3.2.2) involved offering the flies' access to the two food sources that they would be offered when their egg laying preferences were tested (Fig. 3.1). The flies therefore have access to both food sources at all times before their egg laying preferences were tested. In the second method (see Section 3.2.3) larvae were raised on a specified diet as before, but the adults were then fed on only one of the two food sources offered when preferences were determined (Fig. 3.2). In both methods adult diet may have affected egg laying preference but statistical analyses in method 2 allowed the effects of the two feeding stages to be disentangled.

3.2.2. Method 1 – Giving the Adults a Choice of Food Sources Prior to Egg Laying Preference Analysis

The basic outline of this method can be seen in Fig. 3.1. A more detailed description follows.



10 males and 10 females placed into a vial containing a specified medium

After 24 hrs these adults were removed and discarded

The eggs laid in these vials were allowed the develop through to the pupal stage

Mature pupae were selected (3.2.6), washed (3.2.7) and placed into a choice chamber (3.2.8)

The flies were left in these chambers for 3 d to ensure they reached sexual maturity

Flies were transferred into a fresh chamber and left to lay eggs for 24 hrs

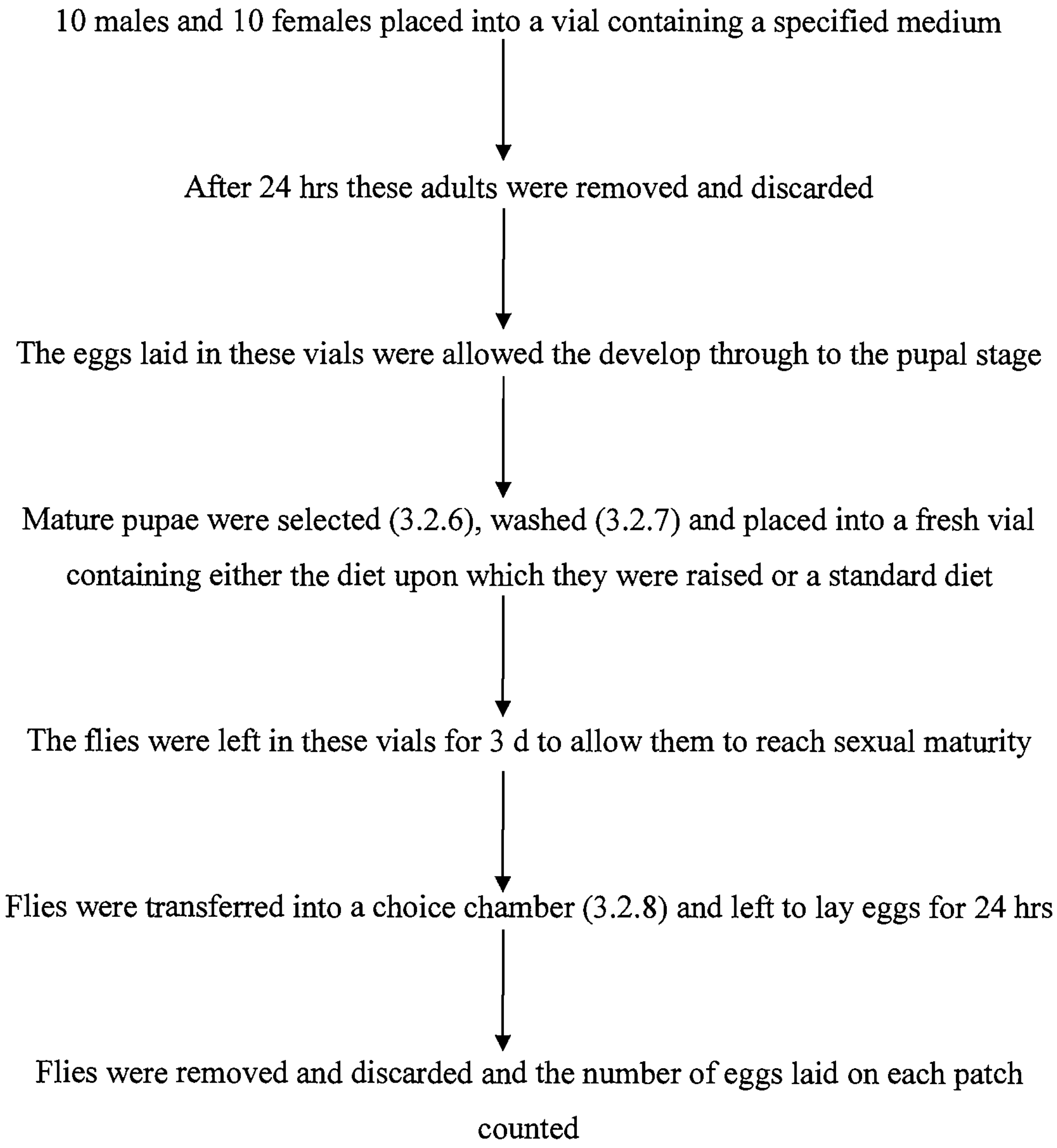
Flies were removed and discarded and the number of eggs laid on each patch counted

Figure 3.1: A flow diagram giving an outline of Method 1, used to determine whether larval diet influences adult food choice. This method involves the adults being exposed to both food sources for the duration of their adult lives until their egg laying preferences are tested.

10 males and 10 females were placed into vials containing a specified medium (see Section 3.2.5), for example a medium containing no added sucrose (0% sucrose). 10 vials were set up per food type. These adults were left in these vials for 24 hrs before being removed and discarded. The eggs laid by these adults were allowed to develop through to the pupal stage. At this point, 200 mature pupae were selected from these vials, washed (see Sections 3.2.6 and 3.2.7 respectively) and transferred into 10 choice chambers (see Section 3.2.8), 20 pupae per chamber. The choice chamber contained two food sources, one the diet upon which they were raised and the other a standard medium. For example, if raised on 0% sucrose as larvae, one of the wells would contain this 0% sucrose medium and the other a standard 100% sucrose medium. These individuals were left in these chambers for three days in order to ensure sexual maturity had been reached. They therefore had a choice of two food types to feed upon during this period. After 3 days the adults were transferred to fresh choice chambers containing the same food choices and left for 24 hrs. After 24 hrs the adults were removed and discarded, and the number of eggs on each food type was counted. To aid accurate counting of egg numbers, minuten pins were placed onto the food patches dividing them into four smaller sections. Running in parallel with these experiments, larvae were also raised on a standard diet and then as adults offered the same choices. For example, in parallel with the 0% sucrose experiments, larvae were also raised on 100% sucrose and then as adults offered the choice of 0% or 100% sucrose to lay their eggs on.

3.2.3 Method 2 - Controlling Adult Diet Prior to Egg Laying Preference Analysis

Method 1 involved varying the diets that the larvae were raised on and then allowing the adults the choice of 2 diets to feed upon for 3 days before egg laying preferences were tested (see Section 3.2.2). This, however, meant that the effect of adult feeding on egg laying preferences could not be determined. Therefore a second method was utilised to determine the effects of both larval and adult diet on subsequent egg laying preferences. An outline of this method can be seen in Fig. 3.2, followed by a more detailed description below.



10 males and 10 females placed into a vial containing a specified medium

After 24 hrs these adults were removed and discarded

The eggs laid in these vials were allowed the develop through to the pupal stage

Mature pupae were selected (3.2.6), washed (3.2.7) and placed into a fresh vial containing either the diet upon which they were raised or a standard diet

The flies were left in these vials for 3 d to allow them to reach sexual maturity

Flies were transferred into a choice chamber (3.2.8) and left to lay eggs for 24 hrs

Flies were removed and discarded and the number of eggs laid on each patch counted

Figure 3.2: A flow diagram giving an outline of method 2 used to determine whether larval diet influences adult food choice. This method involved adults being exposed to only one food source for the duration of their adult lives until their egg laying preferences are tested.

10 males and 10 females were placed into vials containing a specified medium (see Section 3.2.5), for example 0% sucrose. 20 vials were set up per food type. These adults were left in these vials for 24 hrs before being removed and discarded. The eggs that had been laid were allowed to develop through to the pupal stage. 400 mature pupae per food type were then collected and washed as described in Sections 3.2.6 and 3.2.7. This time, however, instead of being placed into a choice chamber as they were Method 1, the pupae were instead placed into fresh vials containing either the diet upon which they were raised or a standard diet. For example, when raised as larvae on 0% sucrose, half of the pupae collected were placed into 10 fresh vials containing 0% sucrose and the other half into 10 vials containing 100% sucrose, 20 pupae per vial. These pupae were allowed to eclose and the adults allowed to feed in these vials for 3 days. The adults therefore did not have a choice of diets to feed upon as they did in Method 1. After 3 days, the adults were collected out of these vials using a pooter and placed into a choice chamber containing both the diet upon which they were raised as larvae and a standard diet. A fresh choice chamber was used for each vial of flies. For example, when raised as larvae on 0% sucrose, the adults were fed on either 0% or 100% sucrose. In the choice chamber the adults were then offered the choice of both 0% and 100% sucrose as an egg laying site. After 24 hrs in the choice chambers, the adults were removed and discarded and the number of eggs on each food type counted. To aid accurate counting of egg numbers, minuten pins were used to divide the food patches into 4 smaller sections. In parallel with these experiments, controls were conducted where larvae were raised on a standard diet and were then subject to the same protocol. For example, in parallel with the experiment where individuals were raised as larvae on 0% sucrose, others were raised on 100% sucrose. When pupae were collected from these 100% sucrose vials they were either placed into vials containing 0% or 100% sucrose. The adults were left to feed on these diets for 3 days. After 3 days, the adults were removed and placed into a choice chamber containing both 0% and 100% sucrose and left to lay eggs for 24 hrs. A comparison could therefore be made of the egg laying preferences of the adults when both larva and adult diet was varied. This method was used with 0% and 25% sucrose along with their 100% sucrose controls.

3.2.4 Does Diet Over Several Generations Affect Oviposition Preference?

In addition to analysing at the effect of larval diet on oviposition preferences in one generation, the effect of diet on egg laying preferences over several generations was determined. This was done by placing 10 males and 10 females into a vial containing a specified food medium. The adults were left for 24 hrs so that eggs would be laid. The adults were then discarded and the eggs allowed to develop until the adult stage. The adults that eclosed were then collected, and 10 males and 10 females were again placed into fresh vials of the same medium and allowed to lay eggs. After 24 hrs these adults were again removed and the eggs again allowed to develop through to adult flies. This was repeated until the flies had gone through three generations on each food type. At generation 3 the egg laying preferences of the adults were determined using Method 1 (see Section 3.2.2). This was done with 0% and 25% sucrose, along with their 100% controls.

3.2.5 Varying the Food Medium

To determine whether gustatory information about larval environment can be learnt and this information retained through metamorphosis, the diets on which larvae were raised was varied. In all cases, the medium used was based upon the standard medium described in Section 2.5. The concentrations of several of the ingredients in this standard medium were, however, varied. These variations included changes in the concentration of carbohydrate or yeast within the medium. The concentrations of carbohydrate and yeast were varied to 0%, 12.5%, 25% and 50% of the amounts used in the standard medium. Carbohydrate type was also varied, including sucrose, fructose, glucose or trehalose. Finally, varying concentrations of sodium chloride (NaCl) were also added to the standard sucrose medium. The concentrations of NaCl used were 13 mM, 25 mM, 50 mM, 100 mM and 200 mM.

3.2.6 Selection of Mature Pupae

To use individuals at a similar developmental stage, only mature pupae were selected from the vials. Mature pupae were recognised by selecting only those that had well-developed eyes (Fig. 3.3). These pupae were removed from the vials using a damp paintbrush.

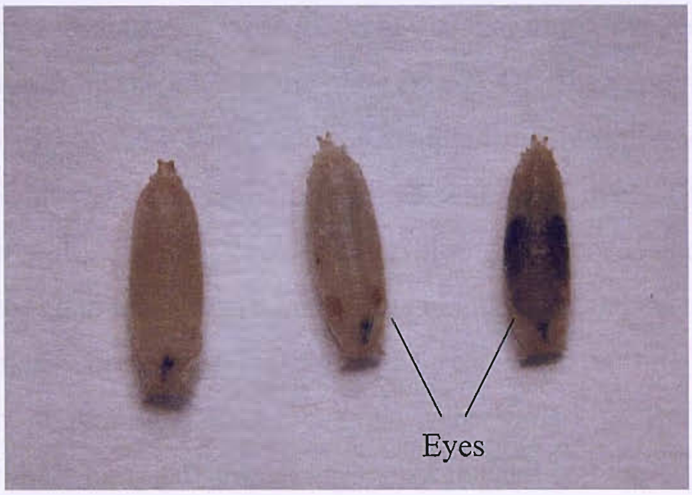


Figure 3.3: The method used to select mature pupae. Early during pupal development, no eye is visible (left). As the pupae mature, so an orange colour can be seen as the eye develops (middle). In mature pupae this colour becomes darker until it becomes a deep red (right). Only pupae with deep red eyes were selected for the experiments.

3.2.7 Washing of the Puparia

Once removed from the vials, the pupae were washed to ensure that no trace of the larval diet was left on the pupal cases. This was carried out to discount the possibility that any changes in adult behaviour could be due to exposure to residues of food left on the pupal case. Once removed from the vials, the pupae were transferred to fine gauze and washed with the aid of a soft brush. Firstly, they were washed in distilled water for 30 s at room temperature, followed by 70% ethanol for 5 s, followed by a further two washes in distilled water for 10 s each. The puparia were then placed on filter paper to dry before being transferred to the choice chamber.

3.2.8 The Choice Chamber

The choice chamber comprised a 90 mm petri dish containing 17 ml of 3% agar. Two wells, 15 mm in diameter, were made in the agar along the central line of the plate, each 15 mm from the edge of the plate (Fig. 3.4). The experimental medium was placed into one of these wells, and the standard medium placed into the other well. These media were differentially marked with food dye. The colours used were red (Supercook), green (Supercook) or blue (Supercook) food dye. Initial experiments were conducted where both wells contained standard media to ensure there was no innate preference for either the red, green or blue food dye. In addition, the concentrations of these dyes were varied to find the optimum concentrations where no innate preferences were present and the eggs could clearly be seen on top of the food medium. To further control for the possible effect of food colour on egg laying preferences during the experimental procedure the media type containing the red dye was varied between the experimental media and the standard media. When the pupae had developed they were washed and 20 mature pupae were arranged along the midline of each choice chamber.

3.2.9 Statistical Analysis

For all experiments conducted using method 1, firstly the affect of diet type on the total number of eggs laid was determined using a three-way ANCOVA, with dye colour and numbers of males and females in each experiment as covariates. A 3-way ANCOVA was also conducted looking at the effect of concentration on the number of eggs laid on the experimental medium. In this case, the number of eggs laid on the experimental medium was first transformed into a binomial distribution. For method 2, where the effect of adult diet was also incorporated into the experimental design, a 2-way ANOVA was used. All statistics were conducted using SPSS version 14.0 for Windows.

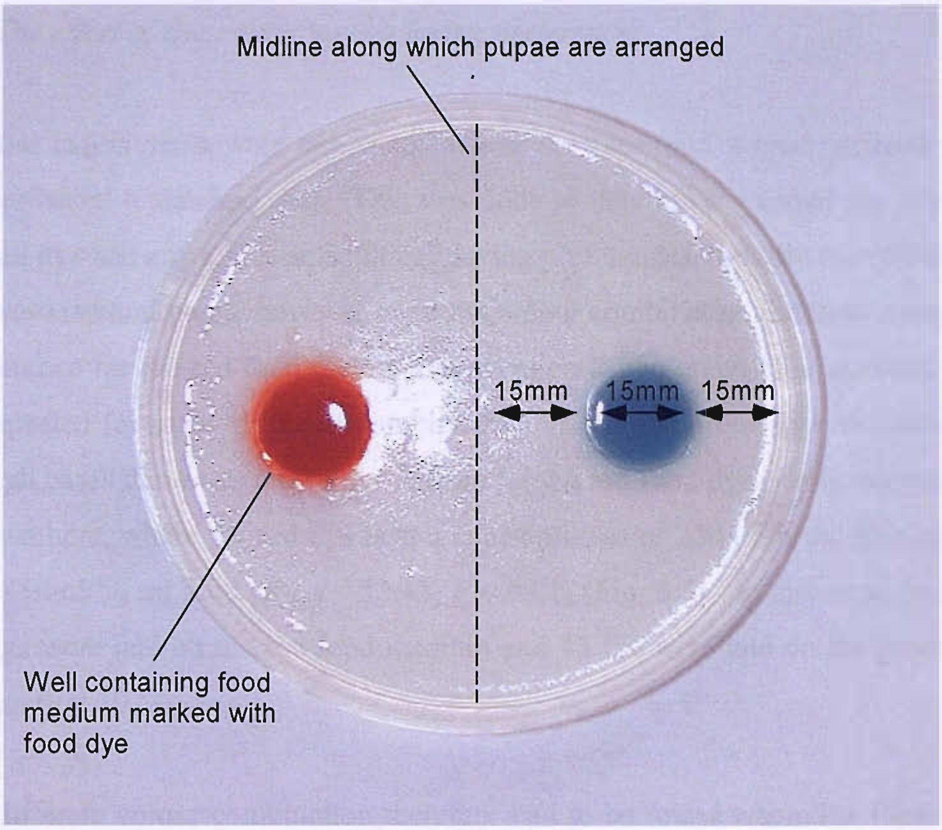


Figure 3.4: The choice chamber. The two wells contain food media, one of which is the medium upon which the larvae were raised and one is a standard medium. Pupae were lined up in a row along the centre of the choice chamber in between the two food sources.

3.3 Results

3.3.1 The effect of dye colour on egg laying preferences

Initial experiments were conducted where the two food sources offered to the flies contained a standard diet. This was done to determine whether the colour of the food dye had any effect on adult egg laying preferences. Initially the two colours used were red and green, however with this colour combination the flies always had a preference for the red food medium/an aversion to the green food medium. This was repeated for many different combinations of red and green dye concentrations but in all cases there was a clear preference for the red food dye. Only one example is shown here, where the red dye is at a concentration of 250 µl/50 ml food and the green 450 µl/50 ml food ($F_{1, 6} = 52.43$, $P < 0.01$) (Fig. 3.5). In this case, 56.9% of the eggs were laid on the red food medium and 43.1% were laid on the green food medium ($n = 1072$).

A different colour combination therefore had to be found where the flies would not show any preference for one particular colour. Red and blue food dye was therefore tested at several different concentrations. The concentrations found to be optimal for observing the eggs clearly on the food patches were 0.75 ml dye/50 ml food for the red dye and 1 ml dye/50 ml food for the blue dye. Results showed no significant preference for either the red or the blue food media as an egg laying site at these concentrations ($F_{1, 18} = 0.004$, $P = 0.952$) (Fig. 3.6). 48.5% of the total eggs were laid on the red food medium and 51.5% were laid on the blue medium ($n = 825$). Red and blue at these concentrations were therefore used throughout the rest of this thesis.

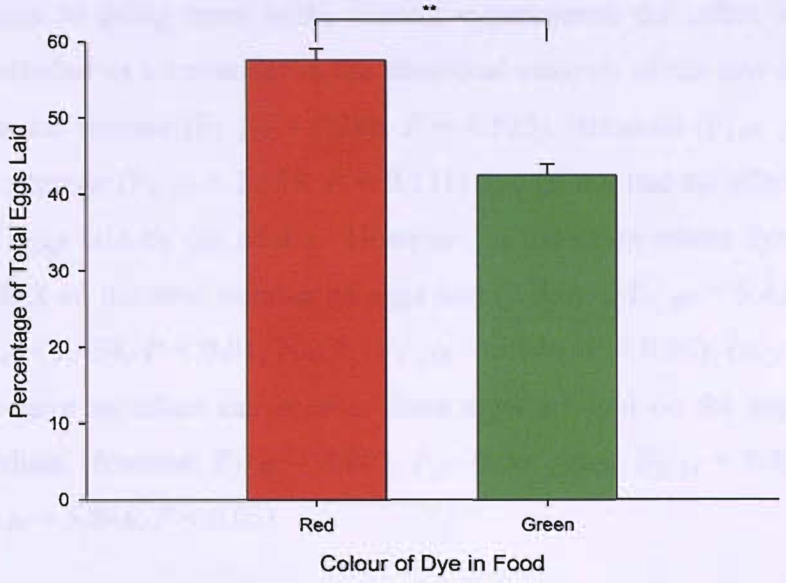


Figure 3.5: The effect of red and green dye on egg laying preferences. A graph showing the results of the control experiment where adult Drosophila *were offered the choice of two standard diets to lay their eggs on. One of the choices contained red food dye and the other contained green food dye. ** indicates a significant difference at the* $P < 0.01$ *level. Flies showed a significant preference for the red food medium as an egg laying site* ($F_{1,6} = 52.43$, $P < 0.01$) *(n = 1072).*

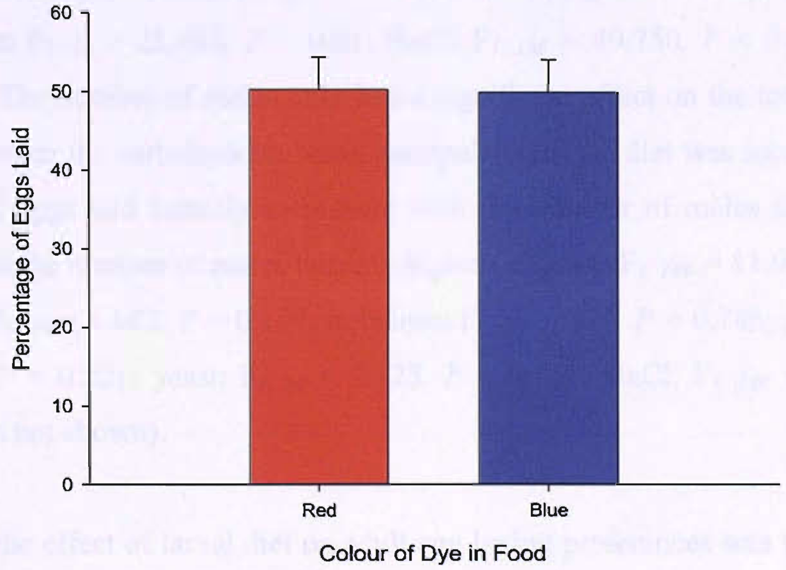


Figure 3.6: The effect of red and blue dye on egg laying preferences. Results of a control experiment where adult Drosophila *were offered the choice of two standard diets to lay their eggs on. One of the choices containing red food dye and the other contained blue food dye. Results showed that the colour of the food had no subsequent affect on egg laying preferences* ($F_{1,18} = 0.004$, $P = 0.952$) *(n = 825).*

In addition to doing these initial control experiments, the effect of dye colour was also included as a covariant in the statistical analysis of the raw data. Results showed that for sucrose ($F_{1,\ 149}$ = 0.386, P = 0.535), trehalose ($F_{1,69}$ = 0.692, P = 0.409) and glucose ($F_{1,\ 69}$ = 2.335, P = 0.131) dye colour had no affect on the total number of eggs laid by the adults. However, in the cases where dye colour does have an affect on the total number of eggs laid (fructose: $F_{1,\ 69}$ = 5.423, $P < 0.05$; yeast: $F_{1,\ 54}$ = 9.438, $P < 0.01$; NaCl: $F_{1,\ 186}$ = 5.846, $P < 0.05$), only for salt does dye colour have an effect on whether these eggs are laid on the experimental or control medium (fructose: $F_{1,\ 69}$ = 3.807, P = 0.06; yeast: $F_{1,\ 54}$ = 9.438, $P < 0.01$; NaCl: $F_{1,\ 186}$ = 5.846, $P < 0.05$).

3.3.2 Does the proportion of males and females affect egg laying numbers and preferences?

Results showed that all cases the number of females had a significant affect on the total number of eggs laid (sucrose: $F_{1,\ 149}$ = 43.920, $P < 0.01$; fructose: $F_{1,\ 69}$ = 8.639, $P < 0.01$; trehalose: $F_{1,\ 69}$ = 8.844, $P < 0.01$; glucose: $F_{1,\ 69}$ = 5.290, $P < 0.05$; yeast: $F_{1,\ 54}$ = 21.488, $P < 0.01$; NaCl: $F_{1,\ 186}$ = 49.750, $P < 0.01$) (data not shown). The number of males only had a significant affect on the total number of eggs laid when the carbohydrate being manipulated in the diet was sucrose, with the number of eggs laid initially increasing with the number of males followed by a decrease as the number of males became higher (sucrose: $F_{1,\ 149}$ = 11.992, $P < 0.05$; fructose: $F_{1,\ 69}$ = 1.682, P = 0.199; trehalose: $F_{1,\ 69}$ = 0.74, P = 0.786; glucose: $F_{1,\ 69}$ = 1.525, P = 0.221; yeast: $F_{1,\ 54}$ = 2.125, P = 0.151; NaCl: $F_{1,\ 186}$ = 2.764, P = 0.09) (data not shown).

When the effect of larval diet on adult egg laying preferences was analysed with the number of males and females as a covariate results showed that for glucose and yeast the number of females present had a significant affect on the egg laying preferences (glucose: $F_{1,\ 69}$ = 4.402, $P < 0.05$; yeast: $F_{1,\ 54}$ = 4.656, $P < 0.05$). In both cases there was a decrease in the number of eggs laid on the experimental medium as the number females increased. In all other cases there was no affect of the number of females present on egg laying preferences (sucrose: $F_{1,\ 149}$ = 1.352, P

= 0.247; fructose: $F_{1, 69} = 0.341$, $P = 0.561$; trehalose: $F_{1, 69} = 2.518$, $P = 0.117$; NaCl: $F_{1, 186} = 0.034$, $P = 0.855$). The number of males present only had a significant affect on the egg laying preferences of the females for the yeast and NaCl experiments (sucrose: $F_{1, 149} = 0.069$, $P = 0.793$; fructose: $F_{1, 69} = 1.975$ $P = 0.164$; trehalose: $F_{1, 69} = 1.729$, $P = 0.193$; glucose: $F_{1, 69} = 0.565$, $P = 0.455$; yeast: $F_{1, 54} = 6.622$, $P < 0.05$; NaCl: $F_{1, 186} = 4.103$, $P < 0.05$). For both the yeast and NaCl data, as the number of males increased so the proportion of eggs laid on the experimental medium decreased (data not shown).

3.3.3 Does Larval Diet Influence Adult Food Choice – Results From Method 1

When sucrose concentration was varied in the diet, larval diet had no significant affect on adult egg laying preference using a 3-way ANCOVA ($F_{1, 149} = 1.331$, $P = 0.250$) (Fig. 3.7). There was, however, a significant affect of the concentration of sucrose in the diet irrespective of larval diet ($F_{3, 149} = 6.653$, $P < 0.01$). As the concentration of sucrose in the experimental medium increased so the number of eggs laid on that medium also increased (Fig. 3.7). There was no significant interaction between larval diet and sucrose concentration in the experimental medium on egg laying preference ($F_{3, 149} = 1.635$, $P = 0.184$). No effect of larval diet was observed for the total number of eggs laid ($F_{1, 149} = 0.070$, $P = 0.781$), however, as sucrose concentration in the experimental medium increased, so the total number of eggs laid decreased, irrespective of larval diet ($F_{3, 149} = 9.309$, $P < 0.01$) (data not shown).

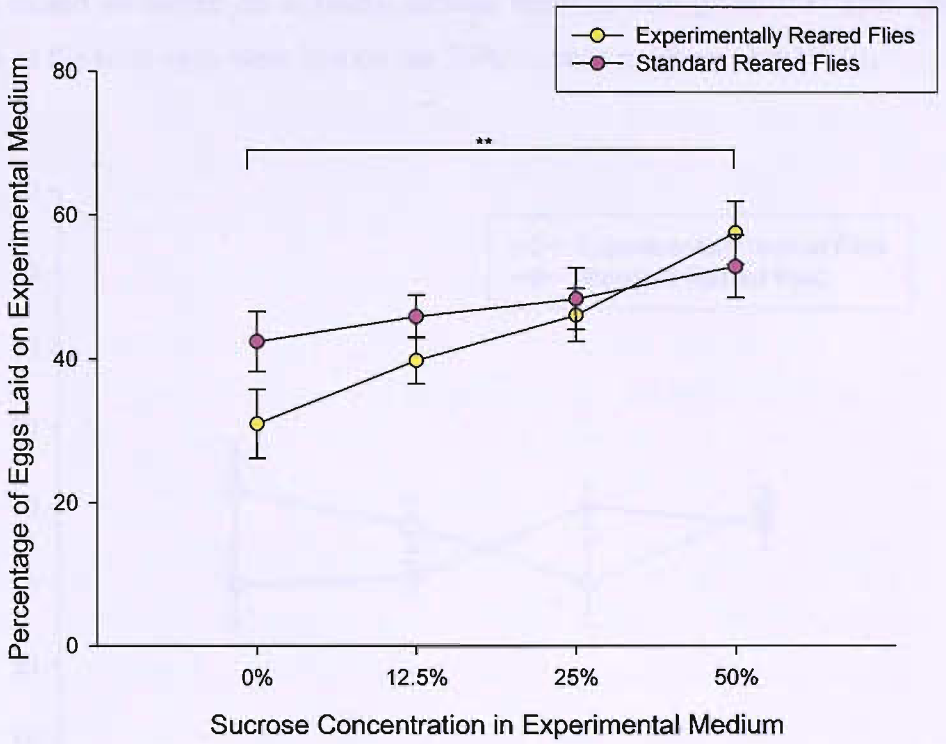


*Figure 3.7: The effect of sucrose concentration in the larval diet on adult egg laying preference. Means ± standard errors for the number of eggs laid on the reduced sucrose content medium when raised as larvae on that reduced sucrose content medium (yellow circles) or on the standard, 100% sucrose, medium (pink circles). ** indicates a significant difference at the* P < *0.01 level. Larval diet had no significant affect on adult egg laying preference, however sucrose concentration in the diet did with an increasing number of eggs being laid on the experimental medium as the sucrose concentration in that medium increased (n (0% sucrose = 2151; n (12.5% sucrose) = 4006; n (25% sucrose) = 3824; n (50% sucrose) = 2846).*

When raised as larvae on a 0% sucrose medium, adults laid slightly less eggs on this medium (30.4% of the total eggs laid; n = 954) than those raised as larvae on a 100% sucrose and subsequently given the same choices (42.7% of the total eggs laid; n = 1197) (Fig. 3.7). When larvae were raised on 12.5%, 25% or 50% sucrose, adults laid just under half of their eggs on the reduced sucrose content medium irrespective of larval diet (Fig. 3.7). For example, when raised as larvae on a 25% sucrose medium, 42.8% of the total eggs were laid on this medium (n = 1855).

When raised as larvae on a 100% sucrose medium and given the same choices, 46.0% of the total eggs were laid on the 25% sucrose medium (n = 1969).

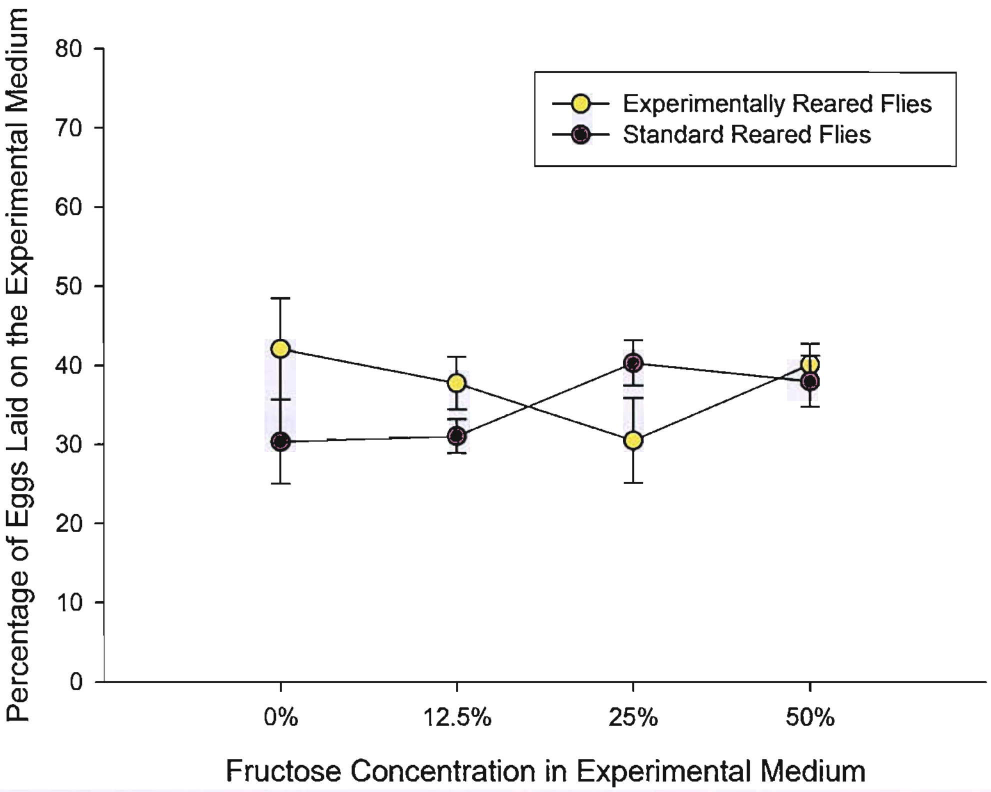


Figure 3.8: The effect of fructose concentration in the larval diet on adult egg laying preference. Means ± standard errors for the number of eggs laid on the reduced fructose content medium when raised as larvae on that reduced fructose content medium (yellow circles) or the standard, 100% fructose, medium (pink circles). Varying the fructose concentration in the larval diet had no subsequent affect on adult egg laying preference at any of the concentrations tested (n (0% fructose) = 2287; n (12.5% fructose) = 3118; n (25% fructose) = 2499; n (50% fructose) = 3462).

Varying the concentration of fructose in the larval diet had no affect on adult egg laying preference ($F_{1, 69}$ = 5.032, P = 0.280). In all cases adults showed a preference to lay their eggs on the 100% fructose medium irrespective of larval diet (Fig. 3.8). For example, when raised as larvae on a diet containing 12.5% fructose, 37.1% (n = 2019) of the total eggs were laid on this medium. When raised on 100%

fructose as larvae and subsequently given the same choices, 31.2% (n = 1099) of the total eggs were laid on the 12.5% fructose medium. The concentration of fructose in the diet also had no significant affect on the number of eggs laid on the experimental medium ($F_{3,\ 69} = 0.718$, $P = 0.545$). There was also no significant interaction between these two factors ($F_{3,\ 69} = 2.027$, $P = 0.118$). Larval diet also had no affect on the total number of eggs laid by the adults ($F_{1,\ 69} = 5.032$, $P = 0.280$) but the diet irrespective of larval diet does ($F_{3,\ 69} = 5.072$, $P < 0.05$) with an increase in the number of eggs laid as the fructose concentration in the larval diet increased (data not shown).

Varying the concentration of trehalose in the larval diet had no significant affect on adult egg laying preference ($F_{1,\ 69} = 0.230$, $P = 0.633$). In all cases, adults laid under half of their eggs on the reduced trehalose content medium irrespective of larval diet (Fig. 3.9). For example, when raised as larvae on a 25% trehalose medium, 29.4% of the total eggs were laid on this medium (n = 1525). When raised as larvae on a 100% trehalose medium and given the same choices, 31.1% of the total eggs were laid on the 25% trehalose medium (n = 1158). The concentration of trehalose in the diet did have a significant affect on the number of eggs laid on the experimental medium ($F_{3,\ 69} = 4.759$, $P < 0.01$), irrespective of larval diet. There was also a significant interaction of larval diet and trehalose concentration in the experimental medium on the egg laying preferences of the females ($F_{3,\ 69} = 2.912$, $P < 0.05$). As the concentration of trehalose in the experimental medium increased so the percentage of eggs laid on that medium increased if raised on the 100% trehalose medium but decreased if raised on the reduced trehalose medium (Fig. 3.9). Larval diet did have an effect on the total number of eggs laid ($F_{1,\ 69} = 8.085$, $P < 0.05$), with a slow increase in the number of eggs laid up to 25% trehalose and then a sharp decrease for 50% trehalose (data not shown).

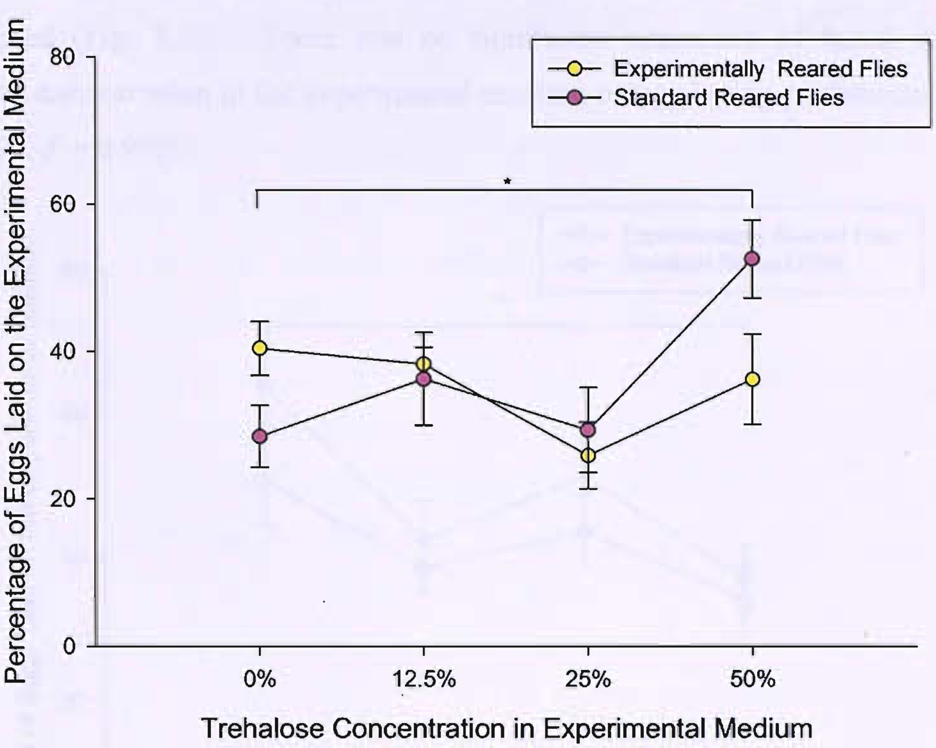


*Figure 3.9: The effect of trehalose concentration in the larval diet on adult egg laying preference. Means ± standard errors for the number of eggs laid on the reduced trehalose content medium when raised as larvae on that reduced trehalose content medium (yellow circles) or on the standard, 100% trehalose, medium (pink circles). * indicates a significant difference at the* $P < 0.05$ *level. When raised as larvae on the reduced trehalose content medium there was an initial decrease in the total number of eggs laid but a subsequent increase for 50% trehalose (n (0% trehalose) = 2335; n (12.5% trehalose) = 2391; n (25% trehalose) = 2683; n (50% trehalose) = 1798).*

Varying the concentration of glucose in the larval diet had no affect on adult egg laying preferences ($F_{1, 69} = 3.801$, $P = 0.06$). For example, when raised as larvae on a diet containing 25% glucose, 45.1% (n = 497) of the total eggs were laid on this medium. When raised on 100% glucose as larvae and subsequently given the same choices, 49.7% (n = 382) of the total eggs were laid on the 25% glucose medium. The concentration of glucose in the diet had a significant affect on egg laying preferences irrespective of larval diet ($F_{3, 69} = 8.424$, $P < 0.01$). As glucose concentration in the diet increased so the number of eggs laid on this medium decreased (Fig. 3.10). There was no significant interaction of larval diet and glucose concentration in the experimental medium on egg laying preferences ($F_{3,\ 69} = 0.191$, $P = 0.902$).

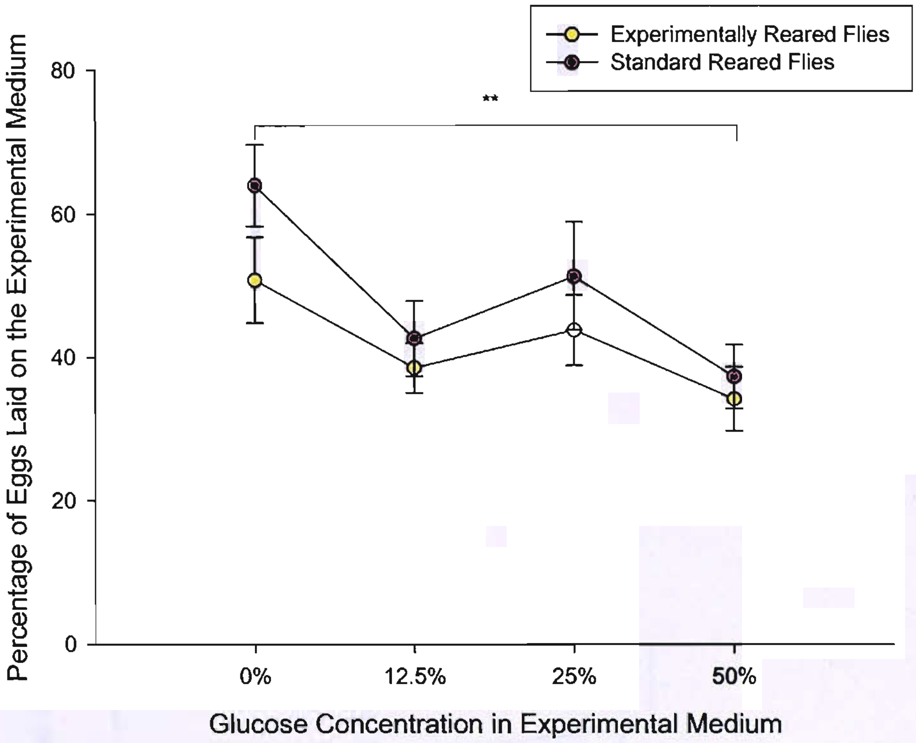


Figure 3.10: The effect of glucose concentration in the larval diet on adult egg laying preference. Means ± standard errors for the number of eggs laid on the reduced glucose content medium when raised as larvae on that reduced glucose content medium (yellow circles) or the standard, 100% glucose, medium (pink circles). Varying glucose concentration in the larval diet had no subsequent affect on adult egg laying preference at any of the concentrations tested (n (0% glucose) = 893; n (12.5% glucose) = 1573; n (25% glucose) = 879; n (50% glucose) = 1402). The glucose concentration in the diet did have an effect on egg laying preference, irrespective of larval diet, with the number of eggs being laid on the experimental medium decreasing as the glucose concentration in the diet increased.

Flies were raised as larvae on diets varying in yeast concentration (0%, 12.5%, 25% or 50% yeast). At 0% and 12.5% yeast, however, very few pupae developed and therefore the experiments at these concentrations could not be conducted. At

25% and 50% yeast sufficient pupae developed and results showed that varying the concentration of yeast within the larval diet did have a significant affect on adult egg laying preferences ($F_{1, 54} = 4.566$, $P < 0.05$). When raised as larvae on a diet containing reduced yeast concentrations, adults were less likely to lay their eggs on this medium than if raised on 100% yeast and subsequently given the same choices (Fig. 3.11). For example, when raised as larvae on 25% yeast, 31.7% (n = 1355) of the total eggs were laid on this medium whereas when raised as larvae on 100% yeast, 40.1% (n = 843) of the total eggs were laid on the 25% yeast medium. There was also a significant affect of diet on egg laying preference irrespective of larval diet ($F_{1, 54} = 5.384$, $P < 0.05$), with more eggs being laid on the reduced yeast content medium for 50% yeast than for 25% yeast (Fig. 3.11).

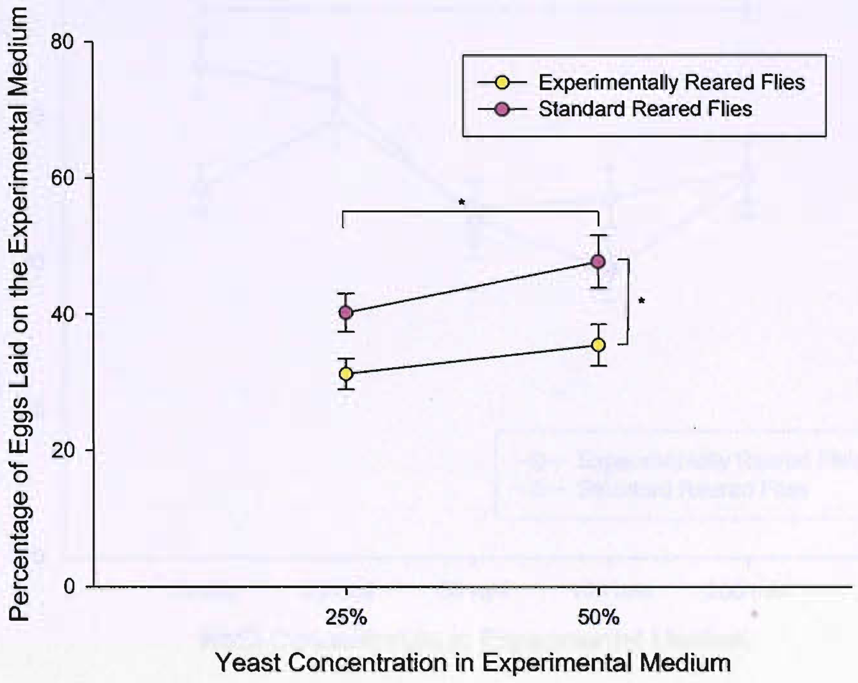


*Figure 3.11: The effect of yeast concentration in the larval diet on adult egg laying preference. Means ± standard errors for the number of eggs laid on the reduced yeast content medium when raised as larvae on that reduced yeast content medium (yellow circles) or the standard, 100% yeast, medium (pink circles). * indicates a significant difference at the* $P < 0.05$ *level. Flies raised as larvae on reduced yeast content media were less likely to lay their eggs on this media than those raised on a 100% yeast diet and given the same choices. In addition, the higher the concentration of yeast in the experimental medium, the more eggs laid on it, irrespective of larval diet (n (25% yeast) = 2198; n (50% yeast) = 2849).*

The concentration of NaCl in the larval diet was varied, there was no significant affect on adult egg laying preferences ($F_{1, 186} = 0.158$, $P = 0.691$). For example, when raised as larvae on a 100 mM NaCl supplemented medium, 46.8% (n = 1960) of the total eggs laid were laid on this medium. When raised as larvae on a standard diet without any additional NaCl, 40.9% (n = 2113) of the total eggs were laid on the 100 mM NaCl medium. Ignoring larval diet, the concentration in the experimental medium did have a significant affect on egg laying preference ($F_{4, 186} = 6.846$, $P < 0.01$) with the number of eggs being laid on the experimental medium decreasing as the NaCl concentration in the medium increased (Fig. 3.12).

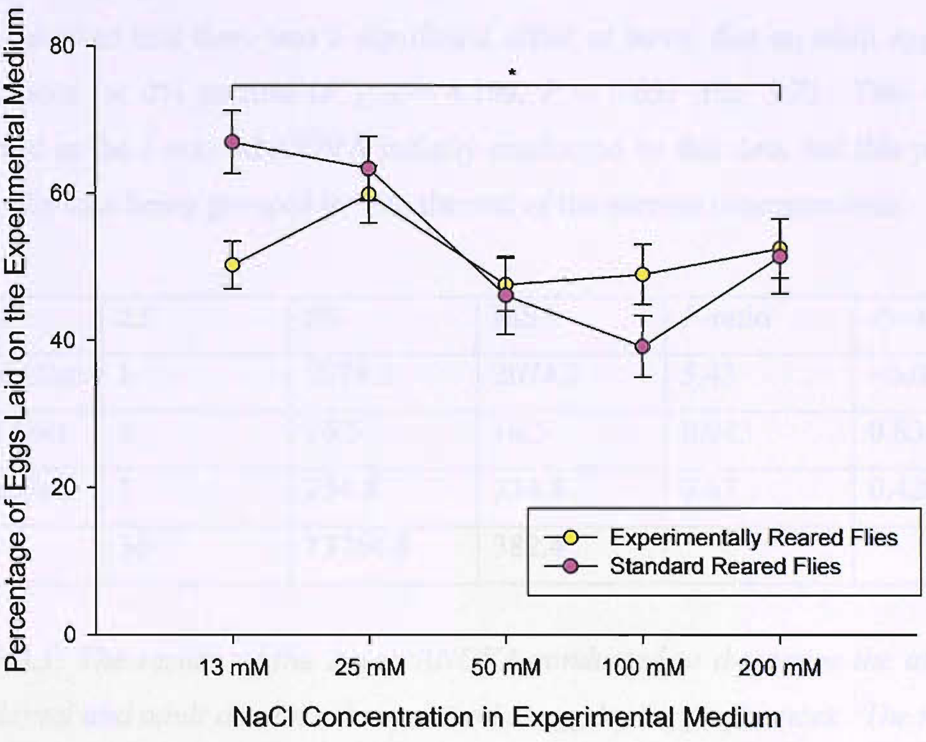


Figure 3.12: The effect of varying NaCl concentration in the larval diet on adult egg laying preference. Means ± *standard errors for the number of eggs laid on the NaCl supplemented medium when raised as larvae on that medium (yellow circles) or on the standard, non supplemented medium (pink circles). * indicates a significant difference at the* $P < 0.05$ *level. Larval diet had no subsequent effect on adult egg laying preference but NaCl concentration in the experimental medium did affect the number of eggs laid on this medium with the number of eggs being laid on the NaCl containing medium decreasing with increasing NaCl concentration (n (13 mM = 4792); n (25 mM NaCl) = 5591; n (50 mM NaCl) = 4585; n (100 mM NaCl) = 4073); n (200 mM NaCl) = 3635).*

3.3.4 Does Larval Diet Influence Adult Food Choice – Results From Method 2

Method 2 involved controlling the diet of both the larvae and the adults. Results showed that for 0% sucrose, larval diet had a significant affect on adult egg laying preferences (Table 3.1). Adult diet did not have any affect on egg laying preferences. In addition, there was no significant interaction of the two factors on egg laying preference (Table 3.1). Flies raised as larvae on 0% sucrose laid significantly less eggs on this medium as adult when compared to those raised as larvae on 100% sucrose (Fig. 3.13). In order to compare this to the 0% sucrose data when employing method 1, a one-way ANOVA was conducted on this data and results showed that there was a significant affect of larval diet on adult egg laying preferences for 0% sucrose ($F_{1, 38} = 4.198$, $P < 0.05$) (Fig. 3.7). This was not observed in the 3-way ANCOVA initially conducted on this data, but this probably due to the data being grouped in with the rest of the sucrose concentrations.

Factor	d.f	SS	MS	*F*-ratio	*P*-value
Larval Diet	1	2074.2	2074.2	5.43	<0.05
Adult Diet	1	16.5	16.5	0.043	0.836
Interaction	1	254.8	254.8	0.67	0.420
Error	36	13764.8	382.4		

Table 3.1: The results of the 2-way ANOVA conducted to determine the affects of both larval and adult diet on subsequent adult egg laying preferences. The result of the statistical analysis show that for 0% sucrose only larval diet had a significant affect on adult egg laying preferences.

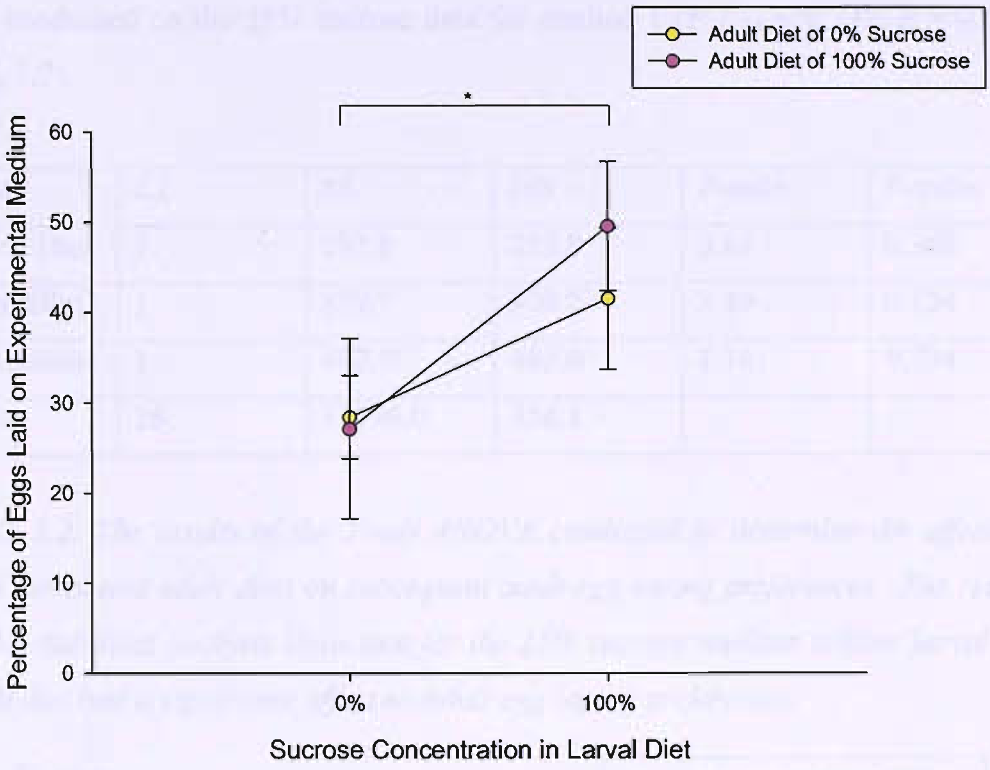


*Figure 3.13: The effect of sucrose concentration in the larval diet on adult egg laying preference using method 2 for 0% sucrose. Means ± standard errors for the percentage of eggs laid on the 0% sucrose medium when raised on either a 0% or a 100% sucrose medium as larvae and then as adults fed either a 0% sucrose (yellow circles) or a 100% sucrose (pink circles) medium. * indicates a significant difference at the* P < 0.05 *level. A larval diet of 0% sucrose significantly decreased the number of eggs laid on this medium when compared to those raised as larvae on the 100% sucrose medium. Adult diet had no affect on subsequent egg laying preferences (n = 268).*

When the concentration of sucrose in the media was reduced to 25% of the standard concentration, neither larval diet or adult diet had a significant affect on adult egg laying preferences (see Table 3.2). In addition, there was no significant interaction of the two factors on egg laying preference (Table 3.2). In all cases flies laid the majority of their eggs on the 100% sucrose medium independent of any previous exposure (Fig. 3.14). Similar results were found when a one-way ANOVA was conducted on the 25% sucrose data for method 1 ($F_{1,\ 38} = 0.222$, $P = 0.640$) (Fig. 3.7).

Factor	d.f	SS	MS	*F*-ratio	*P*-value
Larval Diet	1	293.8	293.8	0.83	0.368
Adult Diet	1	879.7	879.7	2.49	0.124
Interaction	1	402.0	402.0	1.14	0.294
Error	36	12746.0	354.1		

Table 3.2: The results of the 2-way ANOVA conducted to determine the affects of both larval and adult diets on subsequent adult egg laying preferences. The results of the statistical analysis show that for the 25% sucrose medium neither larval nor adult diet had a significant affect on adult egg laying preferences.

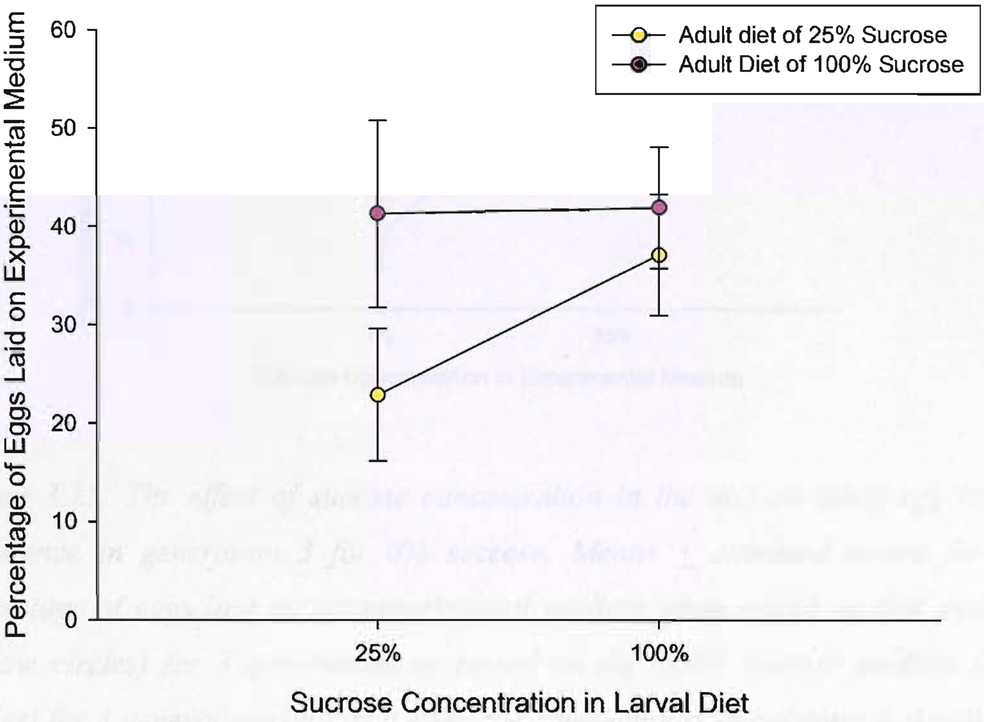


Figure 3.14: The effect of sucrose concentration in the larval diet on adult egg laying preference using method 2 for 25% sucrose. Means ± standard errors for the percentage of eggs laid on 25% sucrose when raised on either the 25% or the 100% sucrose medium as larvae and then as adults fed either 25% sucrose (yellow circles) or 100% sucrose (pink circles). Neither larval nor adult diet had any affect on subsequent egg laying preferences (n = 282).

3.3.5 *Does Diet Influence Egg Laying Preferences Over Several Generations?*

When flies were raised for several generations on either 0% or 25% sucrose and then their egg laying preferences tested at generation 3, results showed that a 0% sucrose diet had a significant affect on adult egg laying preferences ($F_{1, 18} = 12.42$, $P < 0.05$) (Fig. 3.15). For 25% sucrose, 3 generations of feeding on this media had no subsequent affect on adult egg laying behaviour ($F_{1, 18} = 0.513$, $P = 0.483$) (Fig. 3.15).

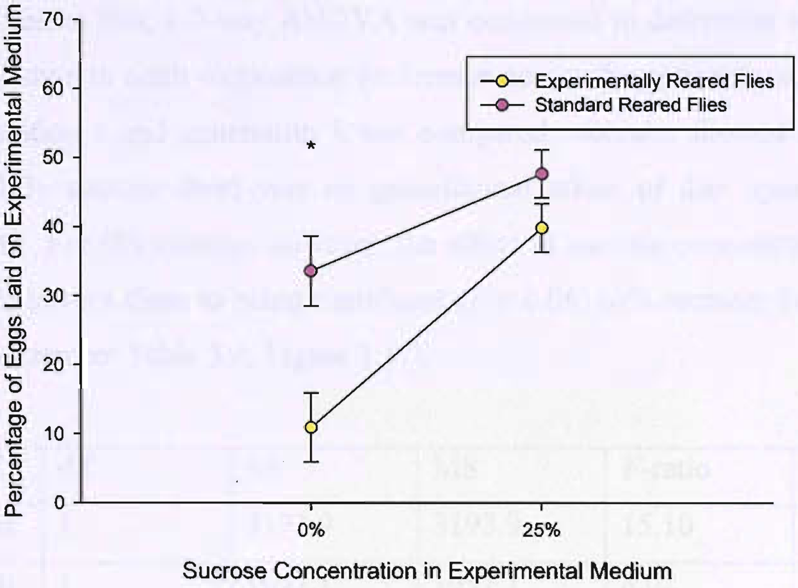


*Figure 3.15: The effect of sucrose concentration in the diet on adult egg laying preference in generation 3 for 0% sucrose. Means ± standard errors for the percentage of eggs laid on the experimental medium when raised on that medium (yellow circles) for 3 generations or raised on the 100% sucrose medium (pink circles) for 3 generations and then given the same choices. * indicates a significant difference at the* P *< 0.05 level. When raised for 3 generations on a 0% sucrose medium, flies laid significantly less eggs on the 0% sucrose medium than those raised for 3 generations on 100% sucrose and subsequently given the same choices (n = 1850). A diet of 25% sucrose for 3 generations had no affect on subsequent egg laying preferences (n = 1261).*

When raised for 3 generations on a 0% sucrose medium, adults laid less eggs on this medium (10.8% of the total eggs laid, n = 420) than those raised as larvae on a 100% sucrose and subsequently given the same choices (34.1% of the total eggs laid, n = 1430) (Fig. 3.15). When raised on a 25% sucrose medium for 3 generations, adults showed no significant preference for food type depending on larval diet (Fig. 3.15), with 39.8% of the eggs being laid on the 25% sucrose medium if raised on it as larvae compared to 47.6% when raised as larvae on 100% sucrose. These results are the same as those found for generation 1 (see Section 3.3.1).

In addition to this, a 2-way ANOVA was conducted to determine whether there was any change in adult oviposition preference across the generations, i.e. the data from generation 1 and generation 3 was compared. Results showed that for both 0% and 25% sucrose there was no generational affect of diet upon egg laying preferences. For 0% sucrose, however, the effect of sucrose concentration over the generations is very close to being significant ($P = 0.06$) (0% sucrose: Table 3.3; Fig. 3.16; 25% sucrose: Table 3.4; Figure 3.17).

Factor	d.f	SS	MS	F-ratio	P-value
Larval Diet	1	3193.9	3193.9	15.10	<0.01
Generation	1	1697.1	1697.1	8.02	0.06
Interaction	1	439.6	439.6	2.08	0.155
Error	56	11842.7	211.5		

Table 3.3: The results of the 2-way ANOVA conducted to determine the affects of both diet and generation on subsequent adult egg laying preferences. The result of the statistical analysis show that for the 0% sucrose medium larval diet did have a significant affect on egg laying preferences but generation did not.

Factor	d.f	SS	MS	F-ratio	P-value
Larval Diet	1	132.38	132.38	1.402	0.241
Generation	1	61.62	61.62	0.652	0.423
Interaction	1	31.06	31.06	0.329	0.569
Error	56	5289.12	94.45		

Table 3.4: The results of the 2-way ANOVA conducted to determine the affects of both diet and generation on subsequent adult egg laying preferences. The result of the statistical analysis show that for the 25% sucrose medium neither larval diet nor generation had a significant affect on egg laying preferences.

The results of the 2-way ANOVA show that when raised as larvae on a 0% sucrose medium, adults laid significantly fewer eggs on this medium as adult compared to those raised as larvae on a 100% sucrose medium and subsequently given the same choices (Fig. 3.16). This is the case for both generation 1 and generation 3. There is not, however, a significant difference in the number of eggs laid on the 0% sucrose medium when the results from generation 1 and generation 3 were compared (Fig. 3.16).

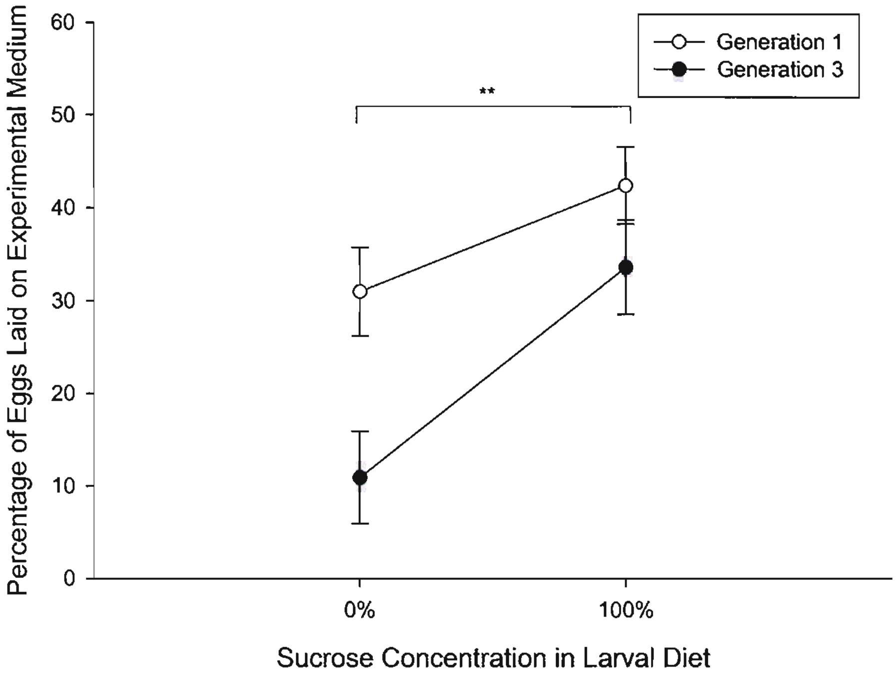


*Figure 3.16: The effect of sucrose concentration in the diet on adult egg laying preference over 3 generations for 0% sucrose. Means ± standard errors for the percentage of eggs laid on the 0% sucrose medium when fed on either a 0% or 100% sucrose medium for 1 generation (white circles) or 3 generations (green circles). ** indicates a significant difference at the* P *< 0.01 level. When raised on a 0% sucrose medium, flies laid less eggs on the 0% sucrose medium than those raised on a 100% sucrose medium and subsequently given the same choices for both generation 1 and 3. No significant difference was found in the number of eggs laid on the 0% sucrose medium when comparing generation 1 with generation 3 (n = 4001).*

When flies were raised for 3 generations on a 25% sucrose medium, results showed that this had no subsequent affect on adult egg laying preferences (Fig. 3.17). There was also no significant difference in the number of eggs laid on the 25% sucrose medium when generation 1 and generation 3 were compared (Fig. 3.17).

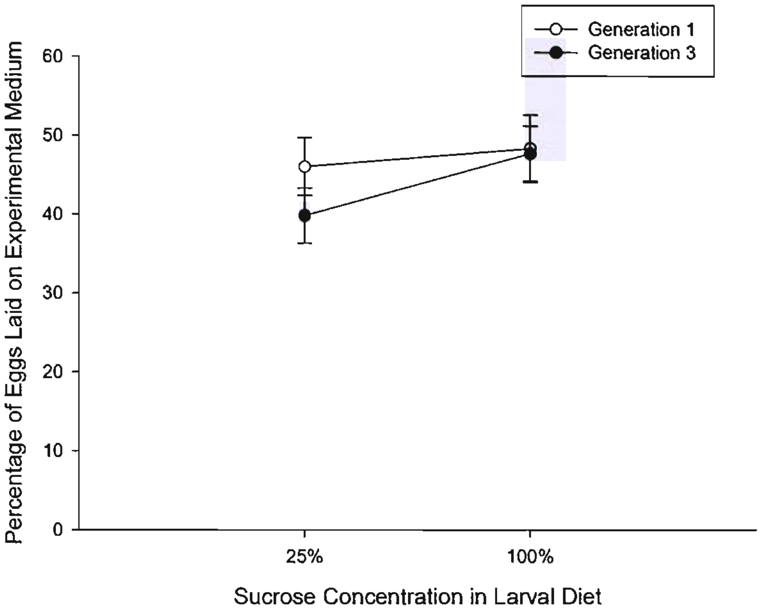


Figure 3.17: The effect of sucrose concentration in the diet on adult egg laying preference in over 3 generations for 25% sucrose. Means ± standard errors for the percentage of eggs laid on the 25% sucrose medium when fed on either a 25% or a 100% sucrose medium for 1 generation (white circles) or 3 generations (green circles). No significant difference was found in the number of eggs laid on the 25% sucrose medium for either generation 1 or generation 3. In addition, there was no significant difference in the number of eggs laid on the 25% sucrose medium when the results from generation 1 and generation 3 were compared (n =5085).

3.4 Discussion

3.4.1 The effect of dye colour on egg laying

Before these experiments could begin initial control experiments were required to determine whether the flies had an innate preference to lay their eggs on a medium dependent on its colour. Flies were therefore offered the choice to lay their eggs on either a red or a green medium, both media being 100% sucrose. The results of this experiment showed that the flies had a preference to lay their eggs on the red food medium when given this choice. The combination of red and green food dye was initially chosen as previous data from the lab (A. Massey, unpublished data) had shown that flies had no innate preference for either red or green food media as an egg laying site. The reason for this discrepancy in results is unclear but may be due to additives in the green dye that were aversive to the flies. It was decided, however, that a different colour combination should be found. Red and blue was therefore tested. This combination was chosen as several other studies had found that flies have no innate preference for either colour when testing feeding preferences (Meunier *et al*, 2003; Tanimura *et al*, 1982). The results of this experiment showed that colour had no effect on adult egg laying behaviour and therefore it was this colour combination that was used in the rest of this study.

In addition to these control experiments, the effect of dye colour was also included as a covariant in the analysis of the effects of diet on the total number of eggs laid and number of eggs laid on the experimental medium when larval diet was varied. For the sucrose, trehalose and glucose experiments there was no effect of dye colour on the number of eggs laid. For the fructose and yeast experiments, there was a significant effect of dye colour on the total number of eggs laid but not on the choice of diet these eggs were laid on. Therefore, in these cases, although there may have been an effect of dye colour on the total number of eggs laid this would not have had an effect on the results of the experiments looking at the effect of larval diet on adult egg laying preferences as dye colour did not affect the choices made by the adults. Finally, for the NaCl experiments, there was a significant effect of dye colour on both the total number of eggs laid and the choice of egg laying site made by the female. Why this was the case is not clear, but this does highlight the importance of analysing the effect of dye colour in similar experiments.

3.4.2 Does the proportion of males and females affect egg laying numbers and preferences?

For all food types, there was an increase in the number of eggs laid as the number of females within the choice chamber increased. It is of no surprise that as the number of females increased so the number of eggs laid also increased. The number of males present in the choice chamber only had a significant affect on the number of eggs laid for the sucrose experiment. In this case there was an initial increase in the number of eggs laid as the number of males increased followed by a decrease as the number of males passed 13.

The number of males and females present also had a predictable effect on the number of eggs laid on the experimental medium. In most cases there was no effect but for the glucose and NaCl experiments there was a significant affect of the number of females and for yeast and NaCl a significant effect of the number of males present. In all cases as the proportion of the respective sex increased so the number of eggs laid on the experimental medium decreased. This is highly likely to be due to competition leading to the females having to choose a less attractive medium as the food source will only be able to provide for a finite number of larvae (Chess and Ringo, 1985).

3.4.3 Does the Method Used Affect the Results of the Preference Tests?

As mentioned in Section 3.2.1, there is a major problem to overcome when trying to determine the affect of larval diet on adult egg laying preferences. This problem is that adult *Drosophila* do not start laying eggs until about 3 days of age (Jaenike, 1982). In order to survive, however, the flies have to feed between eclosion and egg laying and this feeding could affect their egg laying preferences (see Section 3.1.4). To overcome this problem, two different methods were utilised in this study. The first method involved raising larvae on a specified medium and then providing the adults with both of the food sources they would be offered when their egg laying preferences were tested (see Section 3.2.2). In the second method adult diet was controlled, with the adults being fed only one of the two food sources they would be offered when their egg laying preferences were tested (see Section 3.2.3). The first method is therefore more reflective of what would occur in the fly's natural environment, where after eclosion the adults disperse and therefore be likely to come into contact with other food sources as well as the diet they fed on as larvae. The second method, however, allowed for the relative affects of the two feeding stages on egg laying preferences to be determined. When comparing the results of the two methods (Method 2 was only conducted on 0% and 25% sucrose and therefore only this data was compared), it is clear that both methods give the same results i.e. that a larval diet of 0% sucrose can affect adult egg laying preferences whereas a larval diet of 25% sucrose has no subsequent affect on egg laying preferences (see Section 3.4.3 for a more detailed discussion of these results). The two methods therefore provide the same results.

The results of Method 2 showed that adult diet had no affect on later egg laying preferences. This is contradictory to several other studies in insects (Prokopy *et al*, 1982) including *Drosophila* (Cadieu *et al*, 2000; Hoffman, 1985; Jaenike, 1982 and 1983; Mery and Kawecki, 2002). It does, however, support other studies where there has been no evidence for the affect of adult diet upon subsequent egg laying preferences (Jaenike, 1983; Tabashnik, 1981). The variable results of the studies looking at the effects of adult diet on egg laying preferences may be due to the type of food used in that study. For example, in a study looking at the effects of adult exposure to several different diets on subsequent egg laying preferences in *Drosophila*, results showed that previous exposure to grape increased adult preference for grape as an egg laying site whereas previous exposure to apple had no subsequent affect on egg laying preferences (Jaenike, 1983). It is therefore possible that the results of this study are due to the type of diet being used rather than suggesting that adult diet never has an affect on subsequent egg laying preferences.

3.4.4 Does Larval Diet Influence Adult Food Choice? – Varying Carbohydrate Type and Concentration in the Larval Diet

The effect of varying carbohydrate type and concentration in the diet on adult egg laying preferences was dependent on the carbohydrate type and concentration used. When the concentrations of sucrose and trehalose were varied in the diet, a higher percentage of eggs were laid on the experimental medium when the carbohydrate concentration in this medium was higher. For the fructose experiments, there was no affect of fructose concentration on the egg laying preference with the adults always showing a preference for the 100% fructose medium. Finally, when glucose concentration was varied in the diet, fewer eggs were laid on the experimental medium as the concentration of glucose in this medium increased. Only for sucrose was there a significant affect of larval diet on adult egg laying preference (Section 3.3.3) with adults showing a preference for the 0% sucrose if raised on it as larvae. This suggests that oviposition preferences for fructose, glucose and trehalose within the medium are innate but that oviposition preferences for sucrose can be influenced by larval environment.

The difference in response depending on the carbohydrate type being varied when larval diet was manipulated may be reflective of the concentrations found in the flies' natural diet. *Drosophila* feed on rotting fruit in their natural environment. Most fruits contain fructose and glucose at relatively high concentrations and most also contain sucrose, but generally at lower concentrations (Adao and Gloria, 2005; Li *et al*, 2002; Ordonez *et al*, 2005). In addition, concentrations of fructose and glucose increase as the fruit ripens (Adao and Gloria, 2005; Ordonez *et al*, 2005). Trehalose, which is present in yeast, will also increase in concentration as the yeast concentration increases (Chyb *et al*, 2003). The fact, therefore, that larval diet had no influence on adult egg laying preferences for fructose, glucose and trehalose may be because in their natural environment these two carbohydrates are good indicators of a suitable food source and are therefore innate.

Varying the concentration of sucrose in the larval diet only had a significant affect on adult egg laying preferences when sucrose concentration was reduced to 0%. When flies were raised on 0% sucrose as larvae they were less likely to lay their eggs on this medium than if they were raised on 100% sucrose and subsequently given the same choices. There are several possible reasons for this. Firstly, when raised as larvae on a 0% sucrose medium the adults may be more sensitive to sucrose due to a lack of carbohydrate in the larval diet and are therefore more attracted to the high sucrose content medium than those raised on diets containing high concentrations of sucrose. This has been described in several species when looking at feeding preference (Abisgold and Simpson, 1987; Amawaka, 2001; Edgecomb *et al*, 1994; Friedman *et al*, 1991; Schiff *et al*, 1988; Simmonds *et al*, 1992; Telang *et al*, 2001). For example, when *Spodoptera littoralis* larvae were raised on a carbohydrate deficient diet and then given the choice of a carbohydrate or a protein rich diet, they showed a preference for the carbohydrate rich diet as a food source (Simmonds *et al*, 1992). In this study, when larvae were raised on a carbohydrate deficient diet (0% sucrose) and then as adults offered either a carbohydrate deficient (0% sucrose) or sucrose rich (100% sucrose) diet, they showed a preference to lay their eggs on the carbohydrate rich diet. The results of this experiment are therefore similar to that described for feeding preference when carbohydrate in the diet is firstly restricted. This difference in food choice depending on previous diet is thought to be controlled by the monitoring of nutrients in the haemolymph (Abisgold and Simpson, 1987; Amawaka, 2001; Friedman *et al*, 1991; Schiff *et al*, 1988; Thompson, 2003). Another possibility is that sucrose concentration in the diet is learnt during the larval stages and this information retained through metamorphosis into the adult fly, where it then has an impact on egg laying preferences. Carbohydrate concentration can be quite variable between fruit types (Li *et al*, 2002) and may therefore be a good component of the fruits chemistry to learn about in order to recognise the same fruit when living in a highly complex environment. This may help to decide what food source out of all the possible options is appropriate as an egg laying site. In addition, high sucrose levels could be indicative of a fruit being a suitable site for larval development in the future, as in some fruits the concentration of sucrose decreases during ripening (Adao and Gloria, 2005). Therefore if the egg is laid on a fruit high in sucrose content, by the time the egg hatches and the larvae go through the three larval stages the fruit will begin to rot and so becomes a suitable site for larval development.

3.4.5 Does Larval Diet Influence Adult Food Choice? - Varying the Concentration of Yeast in the Larval Diet

When the concentration of yeast in the larval diet was reduced to 0% or 12.5% of the standard concentrations no pupae developed. A lack of yeast is known to arrest reproduction in *Drosophila* (Good and Tatar, 2001). The lack of pupae in the 0% and 12.5% yeast experiments is therefore probably due to very few eggs being laid by the adults at the beginning of the experiment.

Reducing the concentration of yeast in the larval diet to 25% or 50% of the standard concentration had a significant effect on subsequent adult egg laying preferences for both concentrations. In both cases, when the flies were raised on the reduced yeast concentration medium they were less likely to lay their eggs on this medium as adults compared to those raised on 100% yeast and given the same choices. As with the sucrose data, this may be due either a change in sensitivity to yeast (Abisgold and Simpson, 1987; Amawaka, 2001; Edgecomb *et al*, 1994; Friedman *et al*, 1991; Schiff *et al*, 1988; Simmonds *et al*, 1992; Telang *et al*, 2001) due to larval diet or due to the flies learning about the nutritional quality of the diet as larvae and using this information to decide on a suitable egg laying site as adults (see Section 3.4.3). As these results do not parallel those found when varying the concentration of trehalose in the larval diet, other taste qualities as well as trehalose within the yeast must be processed by the flies.

3.4.6 Does Larval Diet Influence Adult Food Choice? - Varying the Concentration of Salt in the Larval Diet

Salt is important at low concentrations in the diets of animals, including insects, but is aversive at high concentrations (Hiroi *et al*, 2004; Liu *et al*, 2003). It was important therefore, to assess the role of salt in adult egg laying preferences. In this study, varying the concentration of NaCl followed this pattern, with the number of eggs being laid on the NaCl containing medium decreasing as the NaCl concentration increased. There was no affect of larval diet on adult egg laying preferences.

Low concentrations of NaCl (below 200 mM) are attractive to *Drosophila* (Hiroi *et al*, 2004; Liu *et al*, 2003) and studies have shown responses to NaCl at concentrations as low as 10 mM NaCl (Liu *et al*, 2003). Therefore all of the concentrations used in this study should have been detectable and attractive to the flies. In this study, at the two lowest concentrations of NaCl used, a higher percentage chose to lay their eggs on this diet than on the diet containing no supplementary NaCl. For the three higher concentrations (50 mM, 100 mM and 200 mM) the eggs were laid approximately equally on the two food sources, suggesting the adults have no preference for either food type. In this study, therefore, it was only for 13 mM and 25 mM NaCl that the adults showed a preference for the NaCl supplemented diet. This difference in results compared to previous studies (Hiroi *et al*, 2004; Liu *et al*, 2003) may be due to many generations of these flies being raised on a diet containing no supplementary NaCl. They may therefore have evolved to not require as much salt as the wild flies that have not been bred for many generations in the laboratory.

3.4.7 Does Diet Over Several Generations Affect Egg Laying Preference?

In addition to analysing whether larval diet influences egg laying preferences in one generation, flies were kept on either 0%, 25% or 100% sucrose media for 3 generations and then their egg laying preferences tested to determine whether diet over several generations can affect egg laying preferences. Results (see Section 3.3.4) showed that as for generation 1, if raised on 0% sucrose media for 3 generations flies were less likely to lay their eggs on this media as adults than those raised on 100% sucrose and subsequently given the same choices. In addition, as with generation 1 a diet of 25% sucrose for 3 generations had no subsequent affect on adult egg laying preferences. When the data from generations 1 and 3 for both 0% and 25% sucrose were compared no significant difference in the number of eggs laid on the experimental medium between the different generations was observed.

It has been argued that changes in egg laying preferences with either adult or larval diet can lead to the formation of host races and eventually lead to speciation

(Craig *et al*, 2001) (see Section 3.1.5). In this case only a larval diet of 0% sucrose had a subsequent affect on adult egg laying preference and this is unlikely to lead to host race formation as the effect of a larval diet of 0% sucrose actually decreases the likelihood of adults laying their eggs on this medium. Several generations of being raised on this diet did not change the preference of the adults, and they still laid fewer eggs on this medium when raised on it as larvae. So, in this case, it would appear that although results show larval diet can affect adult egg laying preferences this is not likely to have an affect on host race formation or speciation. However, this study only looked at three generations and it is possible that if raised on these diets for a longer period of time that changes in preference could occur.

3.4.8 Hopkins' Host Selection Theory

The results of this study clearly show that the effect of larval diet on adult oviposition preferences depends on the chemical and concentration in question. This could therefore explain why in some cases in the literature there has been no evidence for the effect of larval diet on adult egg laying preferences (see Section 3.1.4). For example, Jaenike (1982) added 171 mM NaCl to the larval diet of *Drosophila* and then tested the effect of this on subsequent adult egg laying preferences. Results showed no significant affect of larval diet on subsequent adult egg laying preferences. Jaenike (1982) argued that this disproved the Hopkin's Host Selection Theory. The results of this thesis, however, show that at lower concentrations NaCl in the larval diet can affect subsequent adult egg laying preferences.

The results of this study support the Hopkin's Host Selection Theory that larval diet can influence adult egg laying preferences. The fact that in this study the pupae were thoroughly washed before the adults emerged make it unlikely that the changes in egg laying behaviour are due to the exposure of the emerging adults to residues of their larval environment on the pupal case (Corbet, 1985). This, however, cannot be completely discounted as undetectable levels of contamination may still have remained, though this is very unlikely.

3.4.9 Summary

The results from this chapter show that the effect of larval diet on adult oviposition preferences depends on the chemical and concentration in question. For some chemicals (i.e. fructose and glucose) no effect of larval diet on adult oviposition preferences was observed. For other chemicals, an effect was only observed at certain concentrations (i.e. sucrose and trehalose at a concentration of 0%; NaCl at 13 mM). Finally, when the concentration of yeast in the larval diet was varied, this affected adult oviposition preferences at both concentrations tested. The change in adult egg laying preference when sucrose concentration was varied in the larval diet was also observed even when the methodology was changed. In addition, adult diet did not have any affect on subsequent egg laying preferences for both 0% and 25% sucrose.

The fact that the effect of larval diet on adult oviposition preferences depended on the chemical and concentration in question suggests that the behavioural responses to some chemicals may be under genetic control whereas the behavioural responses to others may be learnt. This may therefore explain why in the literature there is conflicting evidence about the effects of larval diet on subsequent adult oviposition preferences as different studies have used different chemicals at different concentrations. This may also explain why in this study no effect of adult diet on subsequent egg laying preferences was found.

Varying the concentration of some chemicals in the larval diet increased the female's preference for it as an oviposition site whereas other chemicals decreased the female's preference for it. This suggests that the females may be able to learn whether their larval environment is suitable for their offspring and hence adapt their behaviour accordingly. Another possibility is that there is a feedback mechanism affecting the adults sensitivity to certain components of the diet and therefore if the females are deficient in carbohydrate, for example, they will be attracted to a carbohydrate rich diet as adults whereas if deficient in protein they will be attracted to a high protein diet. The feedback mechanism is therefore specific to the chemical. The issue of whether the change in egg laying preference with varying sucrose concentration in the larval diet is due to learning is investigated in Chapter 5.

Varying the concentration of sucrose in the diet for 3 generations had the same effect on egg laying preference as was seen in generation 1. No change in egg laying preferences was observed when the results of generation 1 and generation 3 were compared. This suggests that in this case the change in egg laying preference with larval diet is unlikely to lead to the formation of host races and possible speciation.

Chapter 4: Does Larval Diet Influence Adult Feeding Preferences?

4.1 Introduction

4.1.1 Feeding Behaviour in Insects

In order to feed insects must first detect and then accept a food source. Studies have shown that individuals are initially attracted to a possible food source by both olfactory (Devaud, 2003; Hiroi *et al*, 2004) and visual cues (Dethier, 1954). When the substrate is reached it is further examined using gustation (taste) (Dethier, 1954). Generally, the first step in the gustatory examination of the food source involves contact with the taste sensilla of the legs. At this stage if the food is considered acceptable the insect will extend its proboscis exposing the taste sensilla on the mouthparts to the food, and again if it is considered acceptable, feeding occurs (Dethier, 1955; Edgecomb *et al*, 1994; Pollack and Balakrishnan, 1997). At any point during these sampling behaviours the food could be rejected (Szentesi and Bernays, 1984). This sequence of behaviours is quite complex, suggesting that the insect is making a specific decision about whether the food source is suitable or not and this in turn suggests that an insect may have specific preferences to feed on some substrates more than others.

4.1.2 What is Feeding Preference and Why is it Important?

Different insect species have different strategies for feeding. Some are monophagus, feeding on only one food type. Some are oligophagus, feeding on a small group of food sources and some are polyphagus, feeding on many different food sources (Dethier, 1954). Those species that are either oligophagus or polyphagus often have hierarchies in their feeding preferences (de Boer and Hanson, 1984). This would be advantageous to an individual as different food sources vary in their quality and hence affect the fitness of the individual. Although host plant chemistry and nutritional quality is known to affect feeding preference (Dethier, 1954; Ehrlich and Raven, 1964) other factors are also thought to drive the evolution of feeding preferences. These include predation (Bernays and Graham, 1988), parasitism (Jaenike, 1985; Price *et al*, 1986), host abundance (Dethier, 1954), and competition (Dethier, 1954). Feeding preferences are extremely important to an individual because not only will type of diet affect survival (Jones and Widemo, 2005) but it can also affect lifetime fecundity (Vandekerkhove *et al*, 2006) and the survival of the offspring (Jones and Widemo, 2005).

Evidence suggests that feeding preferences are under genetic control (Forister *et al*, 2007; Sotka, 2003; Stadler and Hanson, 1978). Some factors, however, can influence the food choice that an insect will make and these include factors such as nutritional status and previous experience and are discussed below.

4.1.3 Nutritional Control of Feeding Preferences

Insects require many different nutrients in order to survive (Chapman, 2003), but not all food sources will contain the right balance of nutrients required by the insect. After feeding, therefore, the insect may still be deficient in certain nutrients. The 'geometric framework model' describes the optimal food choices that an individual should make on a nutritional basis in order to reach an ideal nutritional state (Raubenheimer and Simpson, 1999; Simpson and Raubenheimer, 1993; Simpson and Raubenheimer, 1999). For example, in a study by Simmonds *et al* (1992) *Spodoptera littoralis* larvae were raised on diets deficient in either protein or carbohydrate. They were then given a choice of either a protein or carbohydrate rich diet. When raised on a diet deficient in protein, the larvae chose to feed on the high protein diet, whereas when raised on a diet deficient in carbohydrate the larvae fed preferentially on the carbohydrate rich diet. This phenomenon is known as 'dietary self selection' (Thompson, 2003), and has been described in many different species of insect (Abisgold and Simpson, 1987; Amawaka, 2001; Friedman *et al*, 1991; Schiff *et al*, 1988; Telang *et al*, 2001) including *Drosophila* (Edgecomb *et al*, 1994). Similar results have also been gained from studies looking at dietary self selection in mammals (Dibattista and Holder, 1998). This change in responsiveness to certain nutrients with varying nutritional state is thought to be regulated by the monitoring of nutrients within the haemolymph, although the underlying mechanism is at yet unknown (Abisgold and Simpson, 1987; Amawaka, 2001; Friedman *et al*, 1991; Schiff *et al*, 1988; Thompson, 2003). It may, however, be partly mediated by nitric oxide (Schuppe *et al*, 2007).

4.1.4 Control of Feeding Preferences by Experience within a Life History Stage

Previous experience has been implicated in contributing to the control of feeding preferences in insects. For example, in a study by Stadler and Hanson (1978), larvae of the tobacco hornworm *Manduca sexta* were raised until the forth larval instar stage on a specified medium. The preferences of the fifth instar larvae were then tested, and results showed that the larvae had a preference to feed upon the diet upon which they were raised (Stadler and Hanson, 1978). This 'induction of preference' has been described in many insects, including both oligophagus (de Boer and Hanson, 1984; Jermy *et al*, 1968; Rowell – Rahier, 1984) and polyphagus species (Grabstein and Scriber, 1982; Jermy *et al*, 1968; Pszczolkowski and Brown, 2005; Scriber, 1982). These feeding preferences have been shown to be retained through several larval moults (Jermy *et al*, 1968) and in some insects once induction has occurred the insect will starve to death rather than consume another host plant (Scriber, 1982). In other cases, however, no induction is observed (Boer and Hanson, 1984; Chew, 1980; Rowell – Rahier, 1984). This may be due to induction of feeding preference being variable depending on the food source used (de Boer and Hanson, 1984; Jermy *et al*, 1968). Previous experience can also have the opposite affect on subsequent feeding preferences. For example, when larvae of the codling moth *(Cydia pomonella)* were exposed to *Gingko* leaves treated with saccharine this caused the larvae to regurgitate. When subsequently offered a choice of saccharin treated or untreated fruit these larvae avoided the saccharin treated fruit and had therefore learnt to associate saccharin with a deleterious effect and therefore avoided it (Pszczolkowski and Brown, 2005). Changes in feeding preferences dependent on previous experience has also been described in other species including snakes (Krause and Burghardt, 2001), spiders (Punzo, 2002), snails (Desbuquois and Degunzan, 1995), nudibranchs (Avila, 1998), turtles (Berghardt and Hess, 1966) and mammals (Distel and Provenza, 1991; Whitney and Olson, 2006).

The mechanisms involved in the induction of feeding preferences are as yet not clear, although it has been suggested that it could be due to habituation or sensitisation (Glendinning *et al,* 2001; Stadler and Hanson, 1978; Szentesi and Bernays, 1984). Other studies suggest it may be due to changes in the chemosensory system (Abisgold and Simpson, 1988; Simmonds *et al*, 1992) or associative learning (Behmer *et al*, 1999; Blaney and Simmonds, 1985; Lee and Bernays, 1990).

4.1.5 Control of Feeding Preferences by Experience between Life History Stages

The possibility that learning could be involved in feeding preferences leads to the interesting question of whether these preferences can be retained through metamorphosis and into the adult organism. This idea is similar to the Hopkins' Host Selection Theory which suggests that larval diet can affect subsequent adult oviposition preferences (see Section 3.1.4). As previously discussed, there is much evidence to suggest that information about olfactory and visual cues during the larval stages can be learnt and this information retained through metamorphosis (Alloway, 1972; Borsellino *et al*, 1970; Gandolfi *et al*, 2003; Hershberger and Smith, 1967; Manning, 1967; Ray, 1999; Rietdorf and Steidle, 2002; Somberg *et al,* 1970; Thorpe, 1939; Tully *et al*, 1994b). In addition, there is some evidence to suggest that larval diet can affect subsequent adult oviposition preferences (see Section 3.1.4). Others, however, have found no evidence for the retention of memory through metamorphosis (Jaenike, 1982 and 1983; Monteith, 1962; van Emden *et al*, 1996; Wiklund, 1974). No work, however, has ever looked at whether feeding preferences learnt during the larval stages have any effect on subsequent adult feeding preferences.

4.1.6 What Effects Could These Preferences Have on Future Generations?

If feeding experience affects subsequent feeding preferences and this is continued through the following generations this can have a huge impact on the species. Firstly, when the adults disperse if they have a preference for their larval habitat then dispersal between patches will be dependent on these patches containing the same food source as the larval diet (Hanski and Singer, 2001). Thus diet may affect dispersal of the adults and subsequent colonisation of new patches.

In mammals, much research has looked at the effects of diet during pregnancy on the health of the offspring in later life (see Section 6.1.3). In these experiments, results have shown that maternal diet can have an affect on birth weight (Stein and Lumley, 2000) and the likelihood of cardiovascular disease (Kaati *et al*, 2002) and diabetes mellitus (Boloker *et al*, 2002; Kaati *et al*, 2002) in later life. In insects diet has also been shown to affect several traits including fecundity (Burger *et al*, 2007; Butler and Robert, 2007; Kyneb and Toft, 2004; Naya *et al*, 2007), life span (Butler and Robert, 2007; Kyneb and Toft, 2004) and survival (Burger *et al*, 2007; Butler and Robert, 2007). Therefore if feeding preferences are affected by larval diet this could have a huge impact on the subsequent success of future generations.

4.1.7 What are the Benefits of Learning Food Preferences?

The learning of feeding preferences may be advantageous for several reasons. Firstly, it may help the insect to find suitable food sources at a later date (Grabstein and Scriber, 1982; Szentesi and Jermy, 1990). Learning may also allow the insect to feed on a suitable substrate that it would normally find deterrent (Szentesi and Jermy, 1990) and avoid food sources that are noxious (Stadler and Hanson, 1978). In addition, some studies have shown that switching between food sources can affect the efficiency of food utilisation (Grabstein and Scriber, 1982; Scriber, 1982).

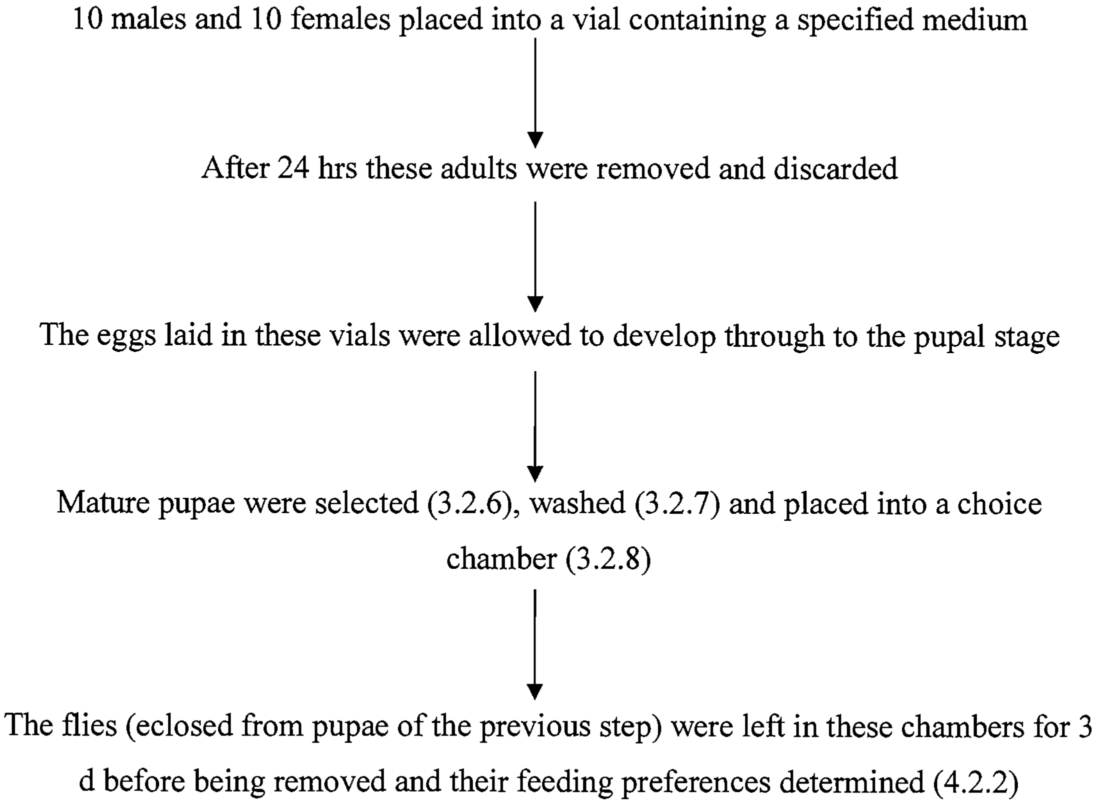
4.1.8 Aims and Objectives

As we can see, feeding preferences within a species can vary for several different reasons. It is generally accepted that nutritional status and previous experience within a life history stage can affect feeding preferences. The issue of whether larval experience can affect adult feeding preferences is still, however, a contentious issue. In addition, many of the studies looking at the induction of feeding preferences have used plants to induce the feeding preferences, which have made it difficult to determine which constituents of the diet have induced the preferences observed (Szentesi and Jermy, 1990). This chapter will aim to resolve both of these issues by raising *Drosophila* larvae on a defined medium of known content and subsequently as adults test their feeding preferences. In addition, work in both mammals and insects suggests that maternal food choice can affect the health and success of both her offspring and that of subsequent generations and flies will therefore also be raised for several generations on several different diets to determine whether diet can also affect feeding preferences over several generations.

4.2 Methods

4.2.1. Determining Whether Larval Diet Affects Adult Feeding Preferences

The aim of these experiments was to determine whether larval diet can affect subsequent adult feeding preferences. To do this, larvae were raised on varying diets where the concentration of one of the components of the standard diet was varied (see Section 3.2.5). As adults, these individuals were then offered the choice of either the diet upon which they were raised or a standard diet to feed upon. To determine whether any change in preference occurred due to larval diet, control experiments were run in parallel with these experiments where individuals were raised on a standard diet as larvae and then as adults offered the same choices. The results of the two experiments were then compared. An outline of the method used can be seen in Fig. 4.1 followed by a more detailed description of each of the steps involved.



10 males and 10 females placed into a vial containing a specified medium

After 24 hrs these adults were removed and discarded

The eggs laid in these vials were allowed to develop through to the pupal stage

Mature pupae were selected (3.2.6), washed (3.2.7) and placed into a choice chamber (3.2.8)

The flies (eclosed from pupae of the previous step) were left in these chambers for 3 d before being removed and their feeding preferences determined (4.2.2)

Figure 4.1: A flow diagram giving an outline of the method used to determine whether larval diet can influence adult feeding preferences.

10 males and 10 females aged between 3 – 10 d were placed into a vial containing a specified medium (see Section 3.2.5), for example a medium containing no added sucrose (0% sucrose). 10 vials were set up for each food type. These adults were left in these vials for 24 hrs before being removed and discarded. The eggs laid by these adults were allowed to develop through to the pupal stage. At this point, 200 mature pupae per food type were selected from the vials, washed (see Sections 3.2.6 and 3.2.7 respectively) and transferred into 10 choice chambers (see Section 3.2.8), 20 pupae per chamber. The choice chamber contained two food sources, one the diet upon which they were raised and the other a standard medium. For example, if raised on 0% sucrose as larvae one of the wells would contain this 0% sucrose medium and the other a standard 100% sucrose medium. These individuals were left in these chambers for three days to feed on the diet of their choice. After 3 days the adults were removed from these chambers and examined under a dissection microscope to determine their feeding preferences (see Section 4.2.2). Running in parallel with these experiments, larvae were also raised on a standard diet and then as adults offered the same choices. For example, in parallel with the 0% sucrose experiments, larvae were also raised on 100% sucrose and then as adults offered the choice of 0% or 100% sucrose to feed upon. These experiments were conducted with all the food types described in Section 3.2.5.

4.2.2 Determining Feeding Preferences

To determine the feeding preferences of the flies, the two wells of food offered in the choice chamber (see Section 3.2.8) were differentially marked with food dye. The colours used were red (Supercook), green (Supercook) or blue (Supercook) food dye. Initial experiments were conducted where both wells contained standard (100% sucrose) media to ensure there was no innate preference for either the red, green or blue food dye. In addition, the concentrations of these dyes were varied to find the optimum concentrations where no innate preference for either colour was present and the feeding preference of the flies could be easily determined. To further control for the possible effect of food colour on feeding preferences during the experimental procedure the media type containing the red dye was varied between the experimental media and the standard media.

After 3 days within the choice chamber, the feeding preferences of the flies were determined by drilling a small hole in the top of the choice chamber. This allowed for a hose with carbon dioxide flowing through it to be inserted into the choice chamber to anesthetise the flies. While anaesthetised, the flies could then be examined under a microscope to determine the feeding preferences as the food dye present in the food medium was clearly visible within the abdomens of the flies (Fig. 4.2). The number of flies feeding on each food type was counted. The number that had fed on a mixture of the two media (i.e. both colours visible in the gut or a dark purple colour if the food was mixed) was also counted. This method for determining feeding preferences was initially described by Tanimura *et al* (1982).

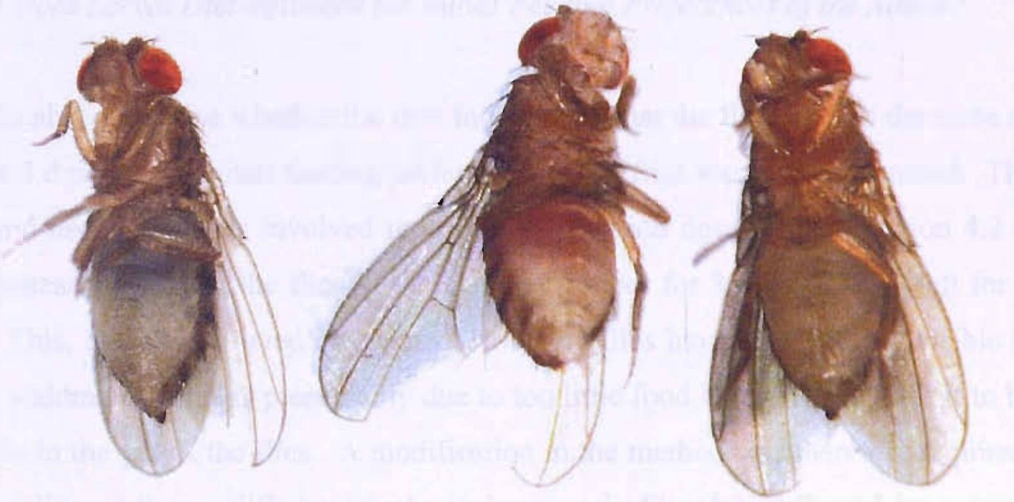


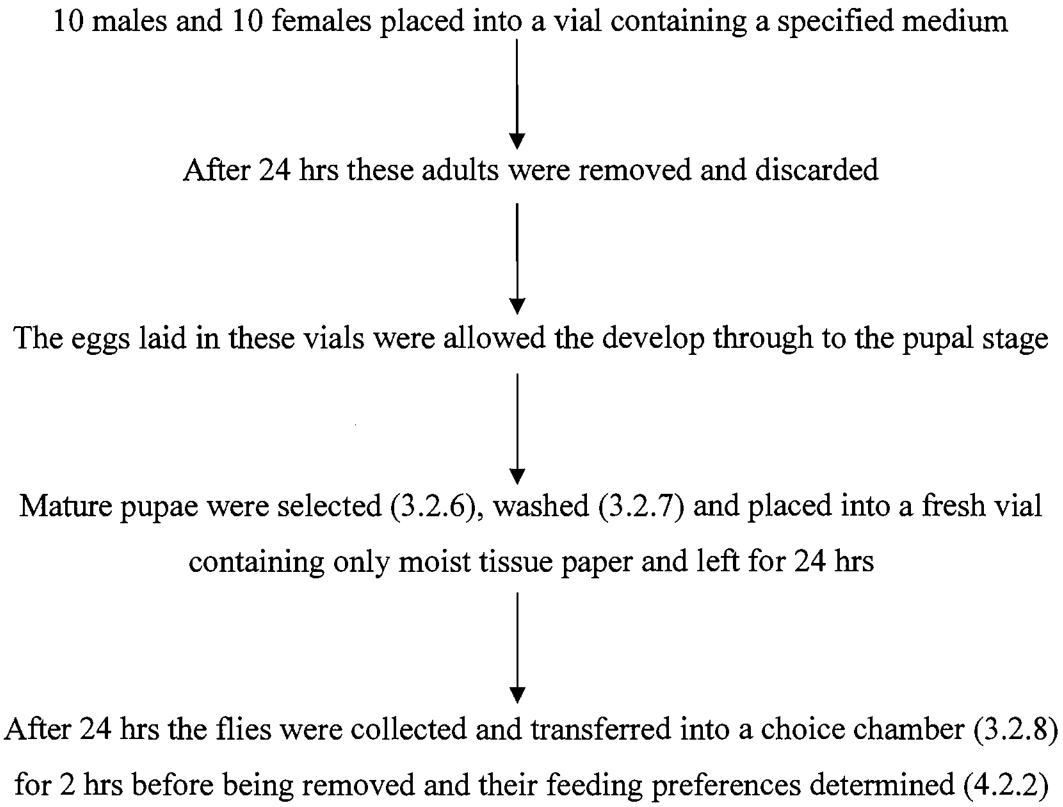
Figure 4.2: The method used to determine adult feeding preferences. When a fly feeds on a food source containing food dye, this dye is visible within the abdomen (Tanimura et al, *1982). The fly on the left has fed on a medium containing blue food dye, the fly in the middle red dye and the fly on the right has not fed on either.*

4.2.3 How Long Does the Food Take to Pass Through the Gut?

As the feeding preferences of the flies was only measured at one time point (3d of age) the time taken for the food to pass through the gut was determined to find out what time frame the results from these experiments represent. To do this, newly eclosed flies were placed into vials containing only damp tissue paper to avoid desiccation. This was done to ensure that when later provided with food they would feed upon it. After 12 hrs, these flies were placed into vials containing food with either red (0.75 ml/50 ml food) or blue (1 ml/50 ml food) food dye. The flies were left on these diets for 2 hrs to feed before being collected and separated into vials, 10 per vial, each vial again only containing damp tissue paper. Every 30 mins one vial containing flies fed on red dye and one containing flies fed on blue dye were collected and the number of flies with colour still visible in the abdomen were counted. This was repeated every 30 mins until 2 hrs had passed with no food being visible in the gut of the flies.

4.2.4 Does Larval Diet Influence the Initial Feeding Preferences of the Adults?

To also determine whether the first food choice that the fly makes is the same as at the 3 d point, the initial feeding preferences of the flies was also determined. The method used to do this involved repeating the method described in Section 4.2.1, but instead of leaving the flies in the choice chamber for 3 d they were left for 3 hrs. This, however, proved unsuccessful with no flies having any colour visible in their abdomens. This is presumably due to too little food being ingested for it to be visible in the gut of the flies. A modification in the method was therefore required. An outline of the modified method can be seen in Fig. 4.3, followed by a more detailed description below.



10 males and 10 females placed into a vial containing a specified medium

After 24 hrs these adults were removed and discarded

The eggs laid in these vials were allowed the develop through to the pupal stage

Mature pupae were selected (3.2.6), washed (3.2.7) and placed into a fresh vial containing only moist tissue paper and left for 24 hrs

After 24 hrs the flies were collected and transferred into a choice chamber (3.2.8) for 2 hrs before being removed and their feeding preferences determined (4.2.2)

Figure 4.3: A flow diagram showing an outline of the method used to determine the initial feeding preferences of the flies.

10 males and 10 females were placed into a vial containing a specified medium. 10 vials were set up per food type. These adults were left in these vials for 24 hrs before being removed and discarded. The eggs laid by these adults were allowed to develop through to the pupal stage. At this point, 200 mature pupae per food type were selected from the vials, washed (see Sections 3.2.6 and 3.2.7 respectively) and transferred into 10 fresh vials containing only moist tissue paper, 20 pupae per vial. The moist tissue paper provided the flies with water to prevent desiccation. These vials were then checked ever hour and any eclosed flies collected using a pooter and transferred into another vial again only containing moist tissue paper. The hourly collection allowed for the starvation period to be known to within 1 hr. After 23 hrs in these vials the flies were collected using a pooter and placed into a choice chamber (see Section 3.2.8). The choice chamber contained two food sources, one was the diet upon which they were raised and the other a standard medium. These individuals were left in these chambers for 2 hrs to feed on the diet of their choice.

After 2 hrs the adults were removed from these chambers and their feeding preferences determined (see Section 4.2.2). Running in parallel with these experiments, larvae were also raised on a standard diet and then as adults offered the same choices. This was done with 0% and 25% sucrose along with their 100% controls.

As there was a change in the method used (i.e. the flies were starved for 24 hrs before their feeding preferences were determined) this experiment was also repeated leaving the flies in the choice chamber for 2 d to ensure that the change in methodology had no affect on the feeding preferences at 3 d age. In addition, some flies were left in the choice chambers for 5 d so their feeding preferences at 6 d of age could be determined. The egg laying preferences of these starved flies was also determined at 4 and 7 d of age to determine whether this starvation period had any affect on egg laying preferences (see Chapter 3).

4.2.5 Filming of Initial Feeding Preferences

The method used to try to determine the initial feeding preferences of the flies (see Section 4.2.4) led to several unexpected results (see Section 4.3.3). A different approach was required. The new method involved filming the behaviour of the flies for the first two hours after eclosion. As before 10 males and 10 females were placed into vials containing a specified medium, 10 vials per food type. After 24 hrs the adults were removed and discarded. The eggs laid by these adults were allowed to develop through to the pupal stage. At this point, 50 mature pupae per food type were selected from these vials, washed (see Sections 3.2.6 and 3.2.7 respectively) and transferred into 5 fresh vials containing only moist tissue paper, 10 pupae per vial. These vials were then checked hourly until an adult had eclosed. This adult was collected using a pooter. Any other flies that had also eclosed were discarded so that when further flies were collected they would not have been eclosed for longer than 1 hr. The selected fly was placed into a choice chamber (see Section 3.2.8) although in this case the food media did not contain any food dye. This was necessary as the fly was not visible when on the food if it contained dye when filming. The choice chamber containing the fly was then placed under a video recorder (JVC TK-C1380) in a temperature controlled room (22°C). The choice chamber was placed on top of a white sheet of paper with an outline of the choice chamber drawn on it in black pen (Fig. 4.4). This was necessary to make the food patches clearly visible. The fly was allowed 5 mins to acclimatise to the choice chamber and then recording started and lasted for 2 hrs. The recordings were then analysed to determine the time spent on each food patch. In addition, a line was drawn on the sheet of paper directly through the centre of the choice chamber dividing it into two halves (Fig. 4.4). This allowed for the time spent in each half of the choice chamber to be determined. This was done with 0% and 25% sucrose along with their 100% controls.

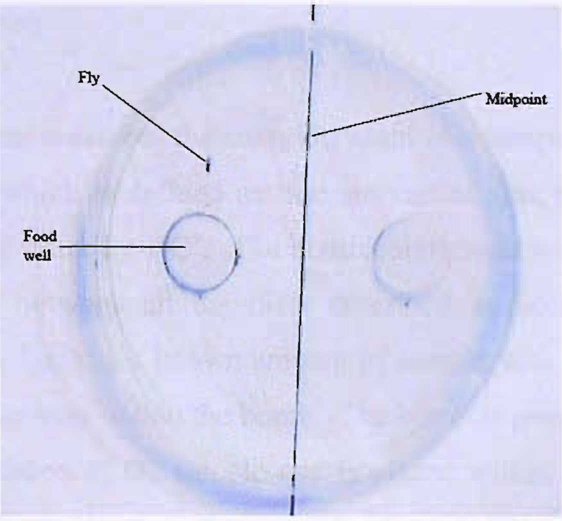


Figure 4.4: The modified choice chamber used to film the behaviour of the adults for the first two hours immediately after eclosion. The chamber was placed on top of a piece of paper with an outline of the chamber drawn onto it so that the food patches were clearly visible. In addition a line was drawn to split the chamber in half between the two food patches.

4.2.6 Does Diet Over Several Generations Affect Adult Feeding Preferences?

In addition to analysing the effect of larval diet on feeding preferences in one generation, the effect of diet on feeding preferences over several generations was determined. This was done by placing 10 males and 10 females into a vial containing a specified food medium. The adults were left for 24 hrs so that eggs would be laid. The adults were then discarded and the eggs allowed to develop through to the adult stage. The adults that eclosed were then collected, and 10 males and 10 females were again placed into fresh vials of the same medium and allowed to lay eggs. After 24 hrs these adults were again removed and the eggs allowed to develop through to the adult stage. This was repeated until the flies had gone through three generations on each food type. At generation 3 the feeding preferences of the adults were determined using as described in Section 4.2.1. This was done with 0% and 25% sucrose, along with their 100% controls.

4.2.7 Bomb Calorimetry

A bomb calorimeter measures the energy content of a sample. The basic unit of energy is a calorie, which is defined as 'the amount of heat required to raise the temperature of 1 g of water by 1°C'. The bomb calorimeter was used to define the calorific differences between all the diets described in Section 2.5. A bomb calorimeter works by igniting a known amount of sample with an electrical current running through a fuse wire within the bomb. The bomb is pressurised with oxygen to ensure full combustion of the sample and is placed within a known volume of water. As the sample combusts so the oxygen expands. The oxygen is allowed to escape from the bomb through a copper tube which feeds out of the calorimeter. As the oxygen escapes so the copper tube will also heat up and heat up the water surrounding it. The increase in temperature of the water allows for the calorific value of the sample to be calculated.

To use the calorimeter, firstly the bucket was filled with two litres of distilled water and placed into the bomb calorimeter. The sample was then weighed out, being as close to 1 g as possible, and placed into the crucible. 10 cm of fuse wire was connected to the arms of the bomb and the wire bent down so it just touched the food in the crucible. The seal of the bomb was then moistened and the lid screwed on. The bomb was then filled with oxygen to a pressure of 450 psi. Electrodes were attached to the top of the bomb before lowering it into the water bucket. The combustion was then initiated. Once completed the calorimeter converted the calorific value of the food into mega joules per kilogram, the currently accepted measure of energy (1 calorie is equal to 4.2 joules). To ensure measurements were accurate, a benzoic acid control was used every 5th run. Benzoic acid should have a calorific value of 26.45 MJ/kg.

4.2.8 Statistical Analysis

Statistical analyses compared the feeding preferences of flies that had been raised on the experimental media with those that had been raised on a standard medium and subsequently given the same choices. The statistical method used involved arranging the data into a multi-way table using crosstabs and then analysing it using the Chi Squared test (Agresti, 1996). For the video recording results and the egg laying analysis, the data was converted to the proportions. This was then transformed using the arcsine transformation, tested for a normal distribution using the Kolmogorov-Smirnov test and finally tested for homogeneity of variance using the Levene's test before a conducting a one-way ANOVA to test for significant differences. All analyses were conducted using SPSS Version 14.0 for Windows.

4.3 Results

4.3.1 Control Experiments

Initial experiments were conducted where the two food sources offered to the flies contained a standard diet. This was done to determine whether the colour of the food dye had any effect on adult feeding preferences. Initially the two colours used were red and green, however, with this colour combination the flies always fed on the red food medium. This was repeated with many different combinations of red and green dye concentrations but in all cases the majority of flies still fed on the red food medium (Fig. 4.5). Only one example is shown here, where the red dye is at a concentration of 250 μl/50 ml food and the green 450 μl/50 ml food ($t = 4.703$, $P < 0.01$) (Fig. 4.5). In this case 81.1% of the adults fed on the red food medium, 2.3% of the adults fed on the green food medium and 16.7% on a mixture of the two (n = 222).

A different colour combination therefore had to be found where the flies fed equally on both food wells independent of food colour. Red and blue food dye was therefore tested at several different concentrations. The concentrations found to be optimal for observing feeding preferences were 0.75 ml dye/50 ml food for the red dye and 1 ml dye/50 ml food for the blue dye. Results showed no significant preference for the red or blue food media as a food source at these concentrations ($t = - 0.71$; $P = 0.944$) (Fig. 4.6). 42.0% of adults fed on the red food medium, 43.5% on the blue food medium and 14.5% on a mixture of the two (n = 247).

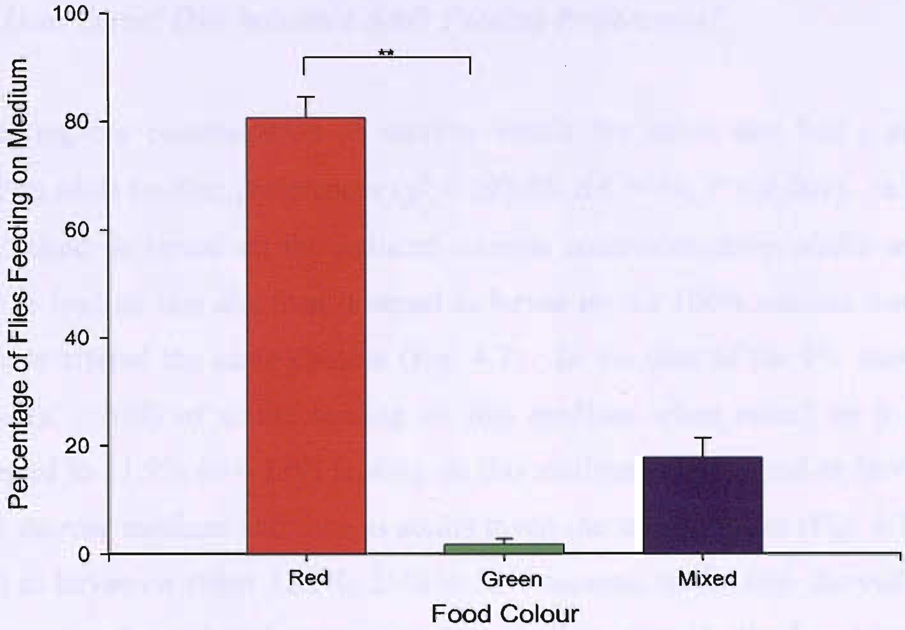


Figure 4.5: The effect of red and green food dye on feeding preferences. Results of a control experiment where adult Drosophila *were offered the choice of two standard diets to feed on. One of the choices contained red food dye and the other green food dye. ** indicates a significant difference at the* P < *0.01 level. Flies showed a significant preference for the red food medium as a food source (t =* 4.703, *P* < 0.01*).*

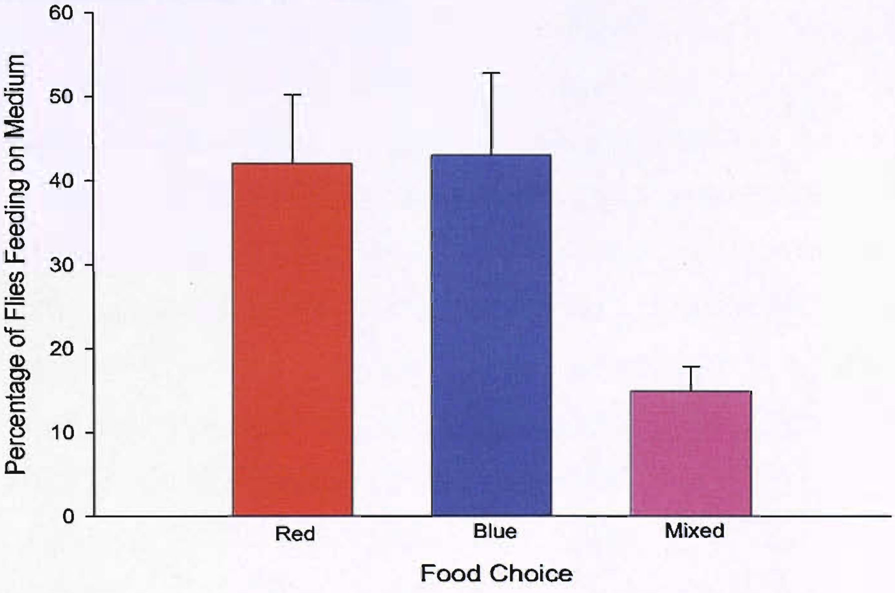


Figure 4.6: The effect of red and blue dye on feeding preferences. Results of a control experiment where adult Drosophila *were offered the choice of two standard diets to feed upon. One of the choices contained red food dye and the other contained blue food dye. Results showed that the colour of the food dye had no subsequent affect on feeding preferences (*t = *- 0.71;* P = *0.944).*

4.3.2 Does Larval Diet Influence Adult Feeding Preferences?

Varying the concentration of sucrose within the larval diet had a significant affect on adult feeding preferences ($\chi^2 = 293.55$, d.f. = 14, $P < 0.001$). In all cases, when raised as larvae on the reduced sucrose content medium adults were more likely to feed on this diet than if raised as larvae on the 100% sucrose medium and as adults offered the same choices (Fig. 4.7). In the case of the 0% sucrose data, 66.5% (n = 339) of adults feeding on this medium when raised on it as larvae compared to 11.9% (n = 339) feeding on this medium when raised as larvae on the 100% sucrose medium and then as adults given the same choices (Fig. 4.7). When raised as larvae on either 12.5%, 25% or 50% sucrose, adults also showed a greater preference to the reduced sucrose content medium than if raised as larvae on the 100% sucrose diet and then as adults given the same choices, although the difference between the experimental and the control data is not as pronounced as for the 0% sucrose experiments (Fig. 4.7). For example, when flies were raised as larvae on 25% sucrose, 29.6% (n = 330) of adults fed on this medium whereas when raised as larvae on the 100% sucrose medium 17.3% of the adults chose to feed on the 25% sucrose medium (n = 285).

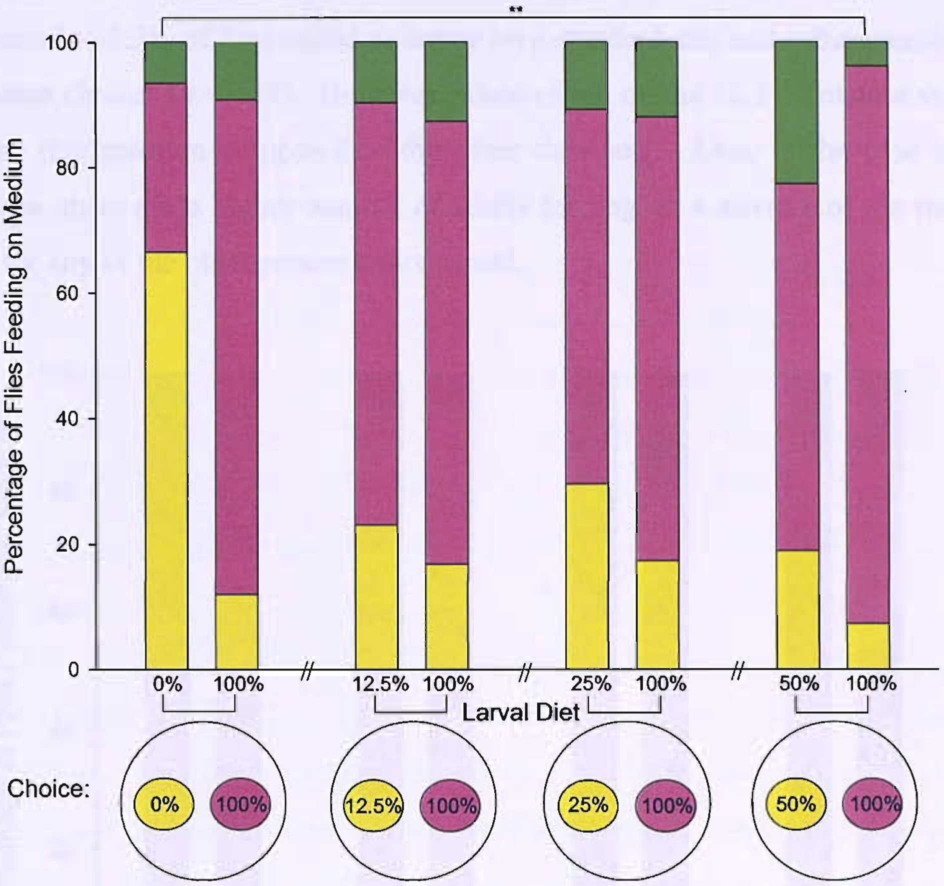


*Figure 4.7: The affect of varying sucrose concentration in the larval diet on adult feeding preferences. Yellow bars show the percentage of flies feeding on the experimental medium, pink those feeding on the 100% sucrose medium and green those feeding on a mixture of the two. ** indicates a significant difference at the* P *< 0.01 level. Results show that varying sucrose concentration in the larval diet has a significant affect on adult feeding preferences. When raised as larvae on a reduced sucrose content medium adults showed a preference to feed on this medium whereas if raised on 100% sucrose and then given the same choices adults show a preference for the 100% sucrose medium (n = 2633).*

Varying the concentration of fructose in the larval diet also had a subsequent affect on adult feeding preferences ($\chi^2 = 76.02$, d.f. = 14, P = 0.01, n = 1140). In this case, there does not appear to be an effect of larval diet on adult preference for the concentrations separately but a difference between the concentrations. in all cases, adults had a preference to feed on the 100% fructose medium (Fig. 4.8). For example, 5% of flies raised as larvae on 0% fructose (n = 187) fed on this medium compared to 5.7% of flies raised as larvae on a standard diet and subsequently given the same choices (n = 158). However, when raised on the 12.5% fructose very few fed on this medium compared to the other data sets. Also, in the case of 25% fructose, there are a higher number of adults feeding on a mixture of the two diets than for any of the other concentrations used.

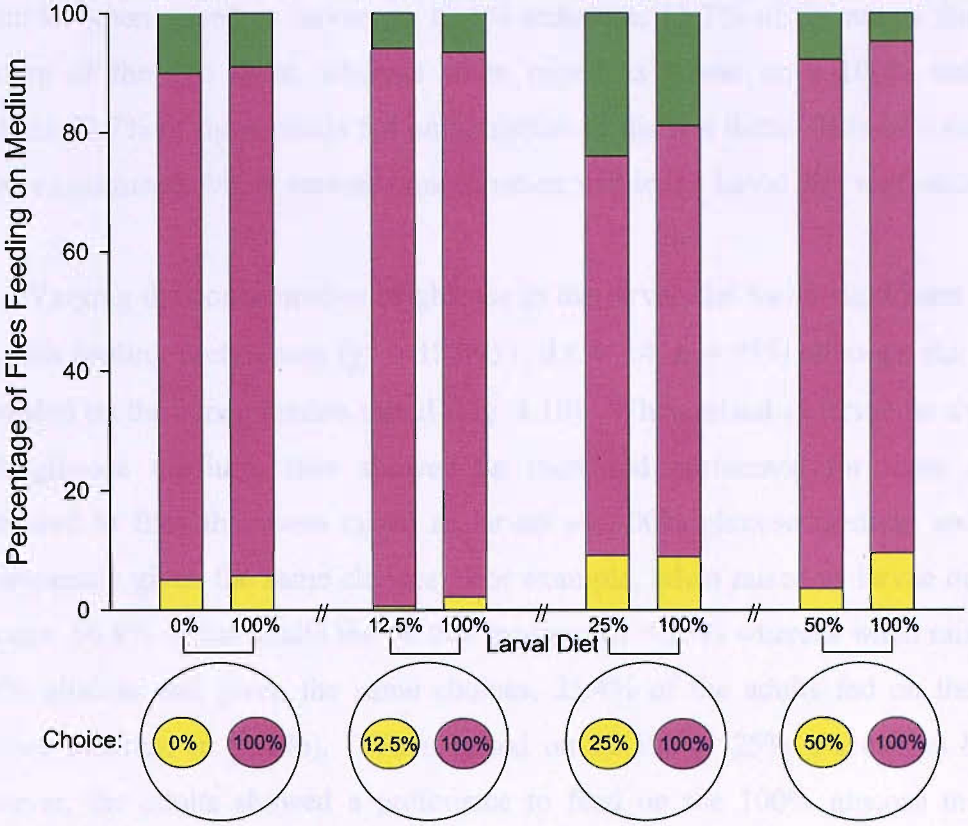


Figure 4.8: The affect of varying fructose concentration in the larval diet on adult feeding preferences. Yellow bars show the percentage of flies feeding on the experimental medium, pink those feeding on the 100% fructose medium and green those feeding on a mixture of the two. Larval diet had no subsequent affect on adult feeding preferences at any of the concentrations tested (n (0% fructose) = 345; n (12.5% fructose) = 373; n (25% fructose) = 377; n (50% fructose) = 357).

Varying the concentration of trehalose in the larval diet had a significant affect on adult feeding preferences ($\chi^2 = 187.191$, d.f. = 14, $P < 0.001$, n = 1185). When raised on reduced concentrations of trehalose adults showed a preference to feed on the 100% trehalose medium. When raised on the 100% trehalose medium, however, the flies were much more likely to feed on the reduced trehalose content medium (Fig. 4.9). For example, 8.6% of flies raised as larvae on the 12.5% trehalose medium (n = 195) chose to feed on this medium compared to 26.9% (n = 187) when raised on the 100% trehalose medium. The flies were also more likely to feed on a mixture of the two media types if they were raised on the 100% trehalose medium as larvae in the 12.5%, 25% and 50% trehalose experiments (Fig. 4.5). For example, when raised as larvae on 12.5% trehalose, 15.2% of the adults fed on a mixture of the two diets, whereas when raised as larvae on a 100% trehalose medium 23.7% of these adults fed on a mixture of the two diets. This is in contrast to the experiments where sucrose concentration within the larval diet was varied.

Varying the concentration of glucose in the larval diet had a significant affect on adult feeding preferences ($\chi^2 = 185.051$, d.f. = 14, n = 955) although the effect depended on the concentration tested (Fig. 4.10). When raised as larvae on a 0% or 50% glucose medium, flies showed an increased preference for these media compared to flies that were raised as larvae on 100% glucose medium and then subsequently given the same choices. For example, when raised as larvae on 50% glucose, 56.8% of the adults fed on this medium (n = 139) whereas when raised on 100% glucose and given the same choices, 25.4% of the adults fed on the 50% glucose medium (n = 146). When raised on 12.5% or 25% glucose as larvae, however, the adults showed a preference to feed on the 100% glucose medium irrespective of larval (Fig. 4.10). For example, 5.6% of flies raised as larvae on 12.5% glucose chose to feed on this medium (n = 185) compared to 10.8% when raised as larvae on a 100% glucose medium (n = 187).

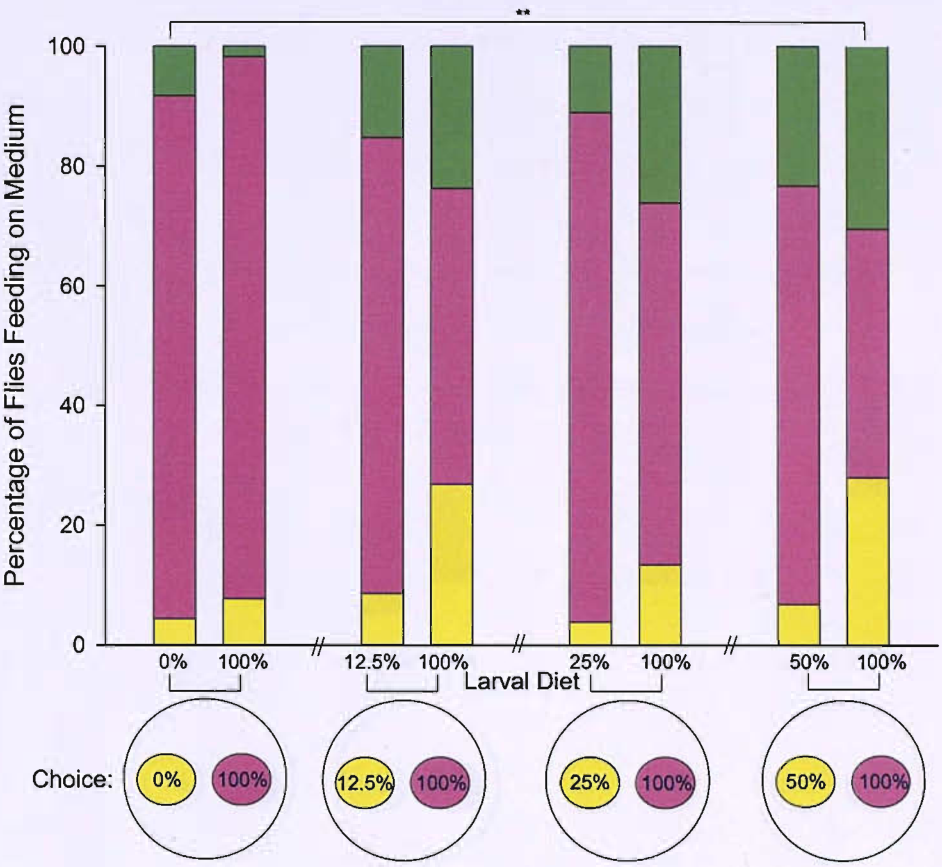


*Figure 4.9: The affect of varying trehalose concentration in the larval diet on adult feeding preferences. Yellow bars show the percentage of flies feeding on the experimental medium, pink those feeding on the 100% sucrose medium and green those feeding on a mixture of the two. ** indicates a significant difference at the* P *< 0.01 level. When raised as larvae on a reduced trehalose content medium adults were less likely to feed on this medium than if raised on 100% trehalose as larvae and as adults given the same choices (n (0% trehalose) = 372); n (12.5% trehalose) = 382); n (25% trehalose) = 362; n (50% trehalose) = 321).*

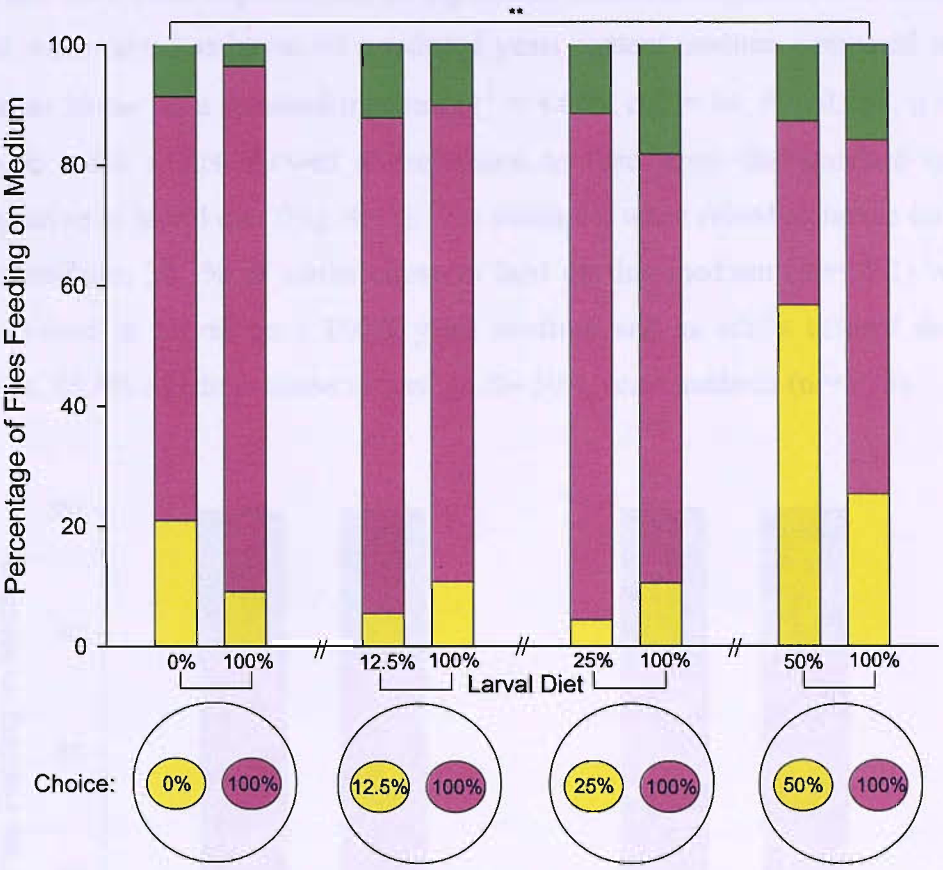


*Figure 4.10: The affect of varying glucose concentration in the larval diet on adult feeding preferences. Yellow bars show the percentage of flies feeding on the experimental medium, pink those feeding on the 100% glucose medium and green those feeding on a mixture of the two. ** indicates a significant difference at the* P *< 0.01 level. When raised as larvae on a 0% or 50% glucose medium adults showed an increased preference to feed on these media compared to those raised as larvae on a 100% glucose medium and as adults given the same choices (n (0% glucose) = 359; n (50% glucose) = 285). When raised as larvae on 12.5% (n = 372) or 25% (n = 277) glucose adults showed a preference for the 100% glucose medium irrespective of larval diet.*

Flies were also raised as larvae on diets varying in yeast concentration (0%, 12.5%, 25% or 50% of the standard concentration). Yeast is the main source of protein in the medium. In the case of 0% and 12.5% yeast, however, very few pupae developed and therefore these experiments could not be conducted. For the

25% and 50% yeast experiments, no significant difference in adult food choice was found when raised as larvae on a reduced yeast content medium compared to those raised as larvae on a standard medium ($\chi^2 = 4.065$, d.f. = 14, $P = 0.668$, n = 622). In both cases adults showed a preference to feed upon the standard medium irrespective of larval diet (Fig. 4.11). For example, when raised as larvae on a 50% yeast medium, 30.1% of adults chose to feed on this medium (n = 251) whereas when raised as larvae on a 100% yeast medium and as adults offered the same choices, 23.4% of adults chose to feed on the 50% yeast medium (n = 283).

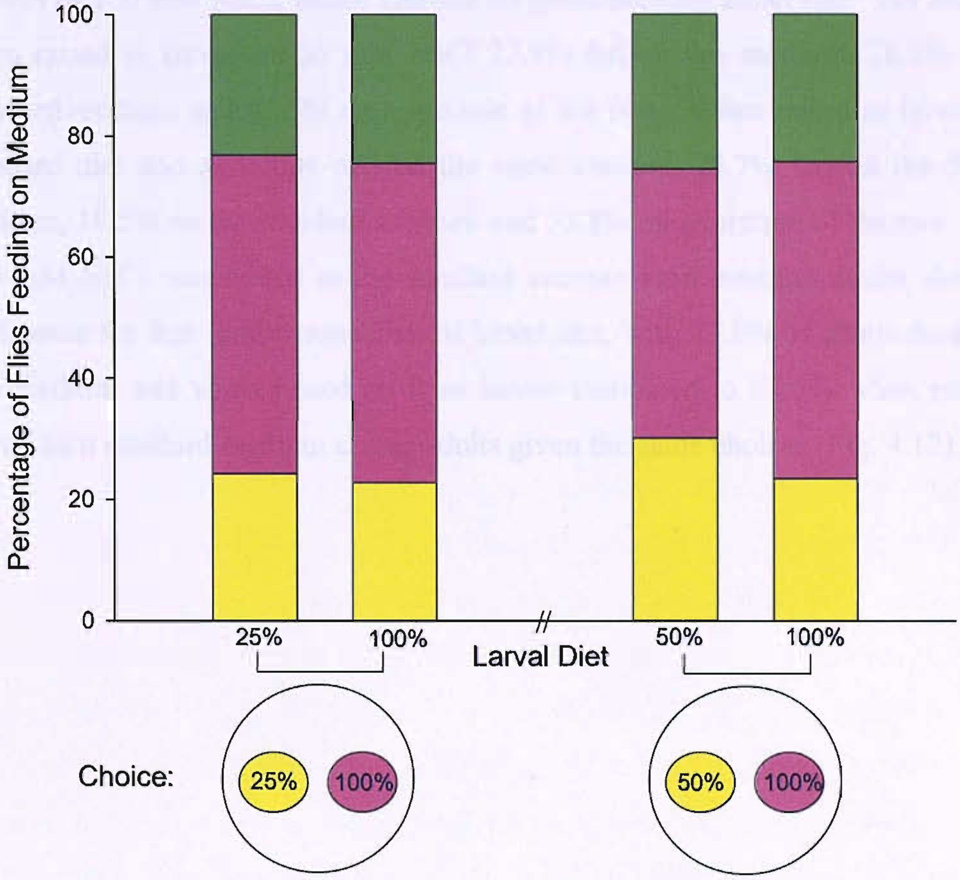


Figure 4.11: The affect of varying yeast concentration in the larval diet on adult feeding preferences. Yellow bars show the percentage of flies feeding on the experimental medium, pink those feeding on the 100% yeast medium and green those feeding on a mixture of the two. Larval diet had no subsequent affect on adult feeding preferences (n (25% yeast) = 582; n (50% yeast) = 534). In both cases adults showed a preference to feed on the 100% yeast diet irrespective of larval diet.

Varying the concentration of NaCl in the larval diet had several effects on adult feeding preferences (χ^2 = 388.16, d.f. = 14, P < 0.001, n = 2451) (Fig. 4.12) although there does not appear to be any particular trend across the concentrations. When raised on a 13 mM NaCl medium as larvae (n = 392), adults were more likely to accept this medium compared to flies raised as larvae on a standard medium (n = 235) and subsequently given the same choices (Fig. 4.12). When raised as larvae on 25 mM NaCl (n = 379) adults were less likely to feed on this medium but more likely to feed on a mixture of the two than when raised on the standard medium (n = 306) and subsequently given the same choices (Fig. 4.12). When raised as larvae on 50 mM or 100 mM NaCl, adults showed no preference for either diet. For example, when raised as larvae on 50 mM NaCl 27.9% fed on this medium, 28.6% on the standard medium and 43.5% on a mixture of the two. When raised as larvae on a standard diet and as adults offered the same choices, 25.7% fed on the 50 mM medium, 38.5% on the standard medium and 35.8% on a mixture of the two. When 200 mM NaCl was added to the standard sucrose food medium adults showed a preference for that food irrespective of larval diet, with 73.0% of adults feeding on this medium and when raised on it as larvae compared to 67.5% when raised as larvae on a standard medium and as adults given the same choices (Fig. 4.12).

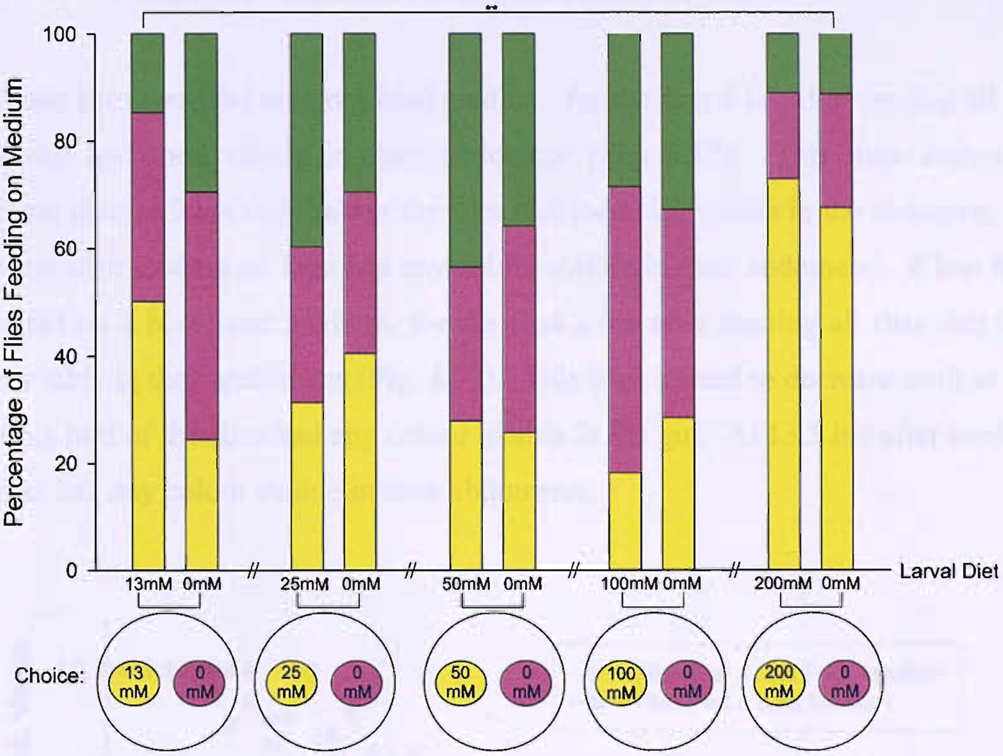


*Figure 4.12: The affect of varying NaCl concentration in the larval diet on adult feeding preferences. Yellow bars show the percentage of flies feeding on the experimental medium, pink those feeding on the standard medium and green those feeding on a mixture of the two. ** indicates a significant difference at the* $P < 0.01$ *level. When raised as larvae on a 13 mM NaCl medium adults were more likely to feed on this medium than if raised on a standard diet and subsequently given the same choice (n = 627). When raised as larvae on 25 mM NaCl adults were less likely to feed on this medium but more likely to feed on a mixture of the two than those raised on a standard diet and subsequently given the same choices (n = 685). When offered the choice of standard or 50 mM/100 mM adults showed no preference for either diet and there is no affect of larval diet (n = (50 mM) = 657; n (100 mM) = 586). Finally, when raised on 200 mM NaCl adults showed a preference for the 200 mM diet irrespective of larval diet (n = 593).*

4.3.3 How Long Does the Food Take to Pass Through the Gut?

When flies were fed on a red food medium, for the first 4 hrs after feeding all the flies still had food visible in their abdomens (Fig. 4.13). This then started to decrease until at 9 hrs only half of the flies had food still visible in the abdomen. At 17.5 hrs after feeding no flies had any colour visible in their abdomens. When flies were fed on a blue food medium, for the first 2 hrs after feeding all flies still had food visible in their abdomens (Fig. 4.13). This then started to decrease until at 7.3 hrs only half of the flies had any colour visible in the gut. At 13.5 hrs after feeding no flies had any colour visible in their abdomens.

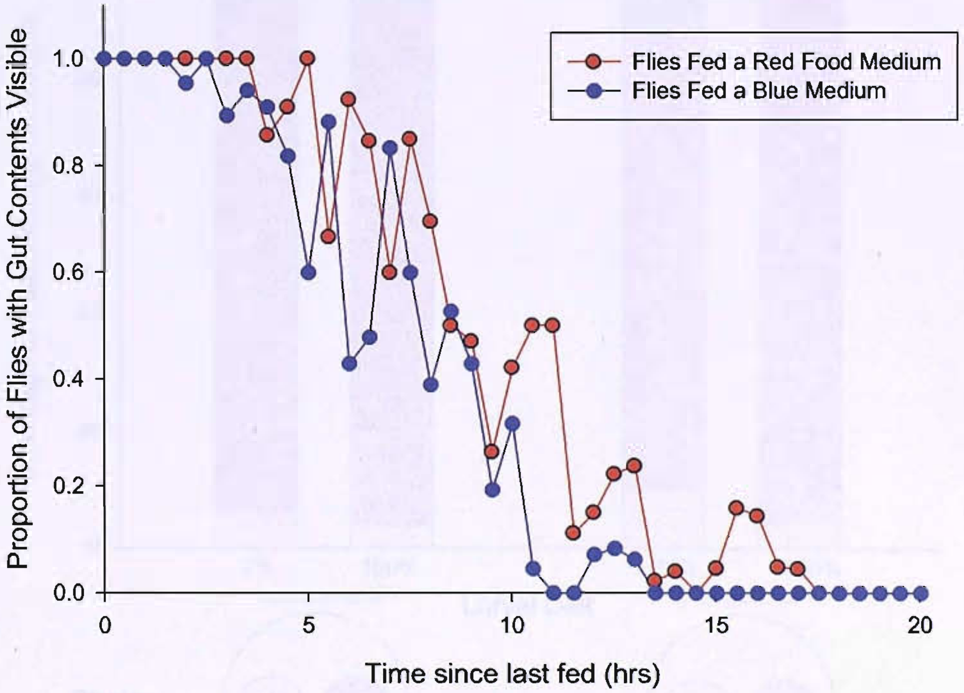


Figure 4.13: The time taken for food to pass through the gut. The blue line represents the time taken for the blue medium to pass through the gut, the red the time taken for the red food to pass through the gut.

4.3.4 Does Larval Diet Influence the Initial Feeding Preferences of the Adults?

Varying the sucrose concentration in the larval diet had no subsequent affect on the initial feeding preferences of the adults when starved for 24 hrs immediately after eclosion ($\chi^2 = 8.783$, d.f. = 6, $P = 0.186$, n = 589). In all cases adults showed a preference to feed on the 100% sucrose medium irrespective of larval diet (Fig. 4.14). For example, when raised as larvae on 0% sucrose (n = 219) as larvae 6.2% fed on this medium as adults and when raised on 100% sucrose (n = 170) as larvae and as adults offered the same choices 4% fed on the 0% sucrose medium (Fig. 4.14).

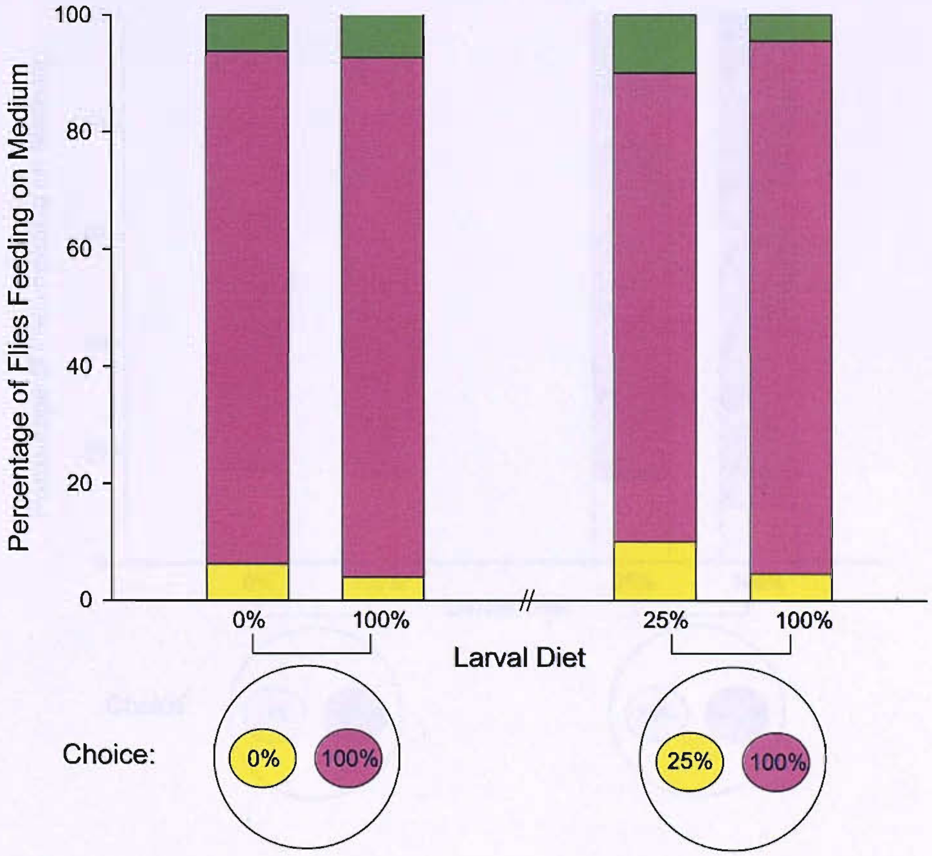


Figure 4.14: The effect of varying sucrose concentration in the larval diet upon the initial feeding preferences of the flies when starved immediately after eclosion. Yellow bars show the percentage of flies feeding on the experimental medium, pink those feeding on the standard sucrose medium and green those feeding on a mixture of the two. Varying sucrose concentration in the larval diet has no subsequent affect on the initial feeding preferences of the adults when firstly starved (n (0% sucrose) = 389; n (25% sucrose) = 379).

The initial feeding preferences of the flies as described above are in contrast to the feeding preferences observed at 3 d (see Section 4.3.2). The feeding preferences at 3 d when initially starved were also therefore analysed. The results of this experiment showed that there is no significant affect of larval diet upon adult feeding preferences at 3 d of age when starved for 24 hrs immediately after eclosion ($\chi^2 = 7.966$, d.f. = 6, $P = 0.241$, n = 607). In all cases adults showed a preference to feed on the 100% sucrose medium irrespective of larval diet. For example, when raised on 0% sucrose (n = 214) as larvae 16.8% fed on this medium as adults and when raised on 100% sucrose (n = 201) as larvae and as adults offered the same choices, 15.7% fed on the 0% sucrose medium (Fig. 4.15).

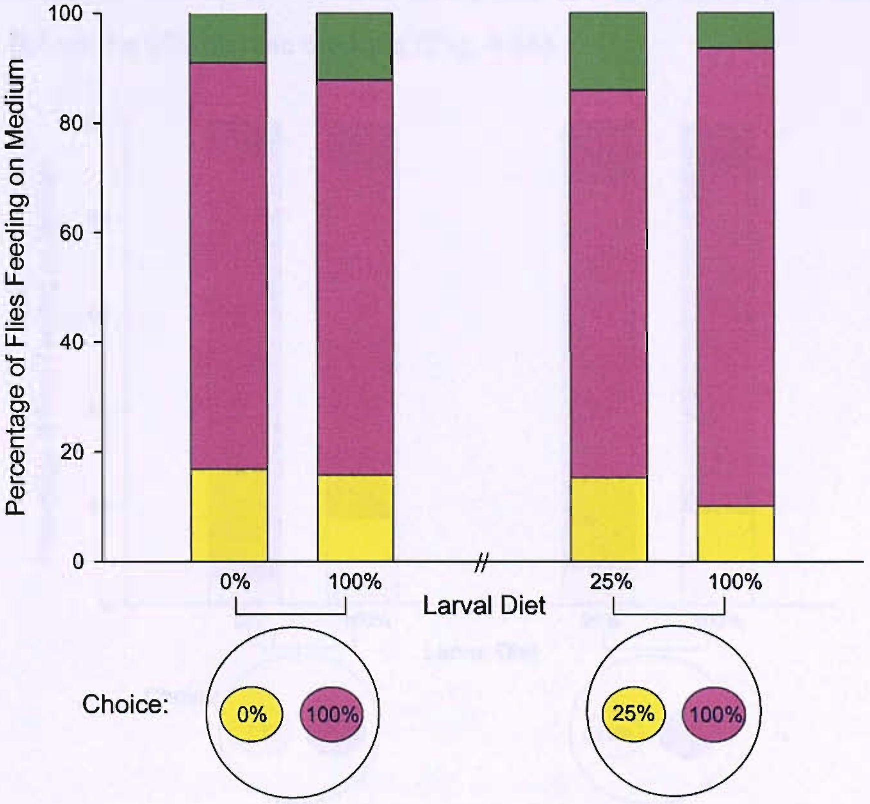


Figure 4.15: The effect of varying sucrose concentration in the larval diet upon the feeding preferences of the flies at 3 d of age when starved immediately after eclosion. Yellow bars show the percentage of flies feeding on the experimental medium, pink those feeding on the standard sucrose medium and green those feeding on a mixture of the two. Varying sucrose concentration in the larval diet has no subsequent affect on the feeding preferences of the adults at 3 d of age when initially starved (n (0% sucrose) = 415; n (25% sucrose) = 385).

The feeding preferences of the flies at 3 d of age were also therefore in contrast to the results found when tested at 3 d without being previously starved (see Section 4.3.2). To determine whether the preferences returned back to those expected at the 3 d stage if left for a longer time period, flies initially starved were left to feed in the choice chamber for 6 d and then their feeding preferences observed. The results of this experiment show that when starved immediately after eclosion for 24 hrs there is no significant affect of larval diet upon adult feeding preferences at 6 d of age ($\chi^2 = 9.467$, d.f. = 6, $P = 0.149$, n = 628. In all cases adults showed a preference to feed on the 100% sucrose medium irrespective of larval diet. For example, when raised on 0% sucrose (n = 191) as larvae 11.2% fed on this medium as adults and when raised on 100% sucrose (n = 190) as larvae and as adults offered the same choices, 16.1% fed on the 0% sucrose medium (Fig. 4.16).

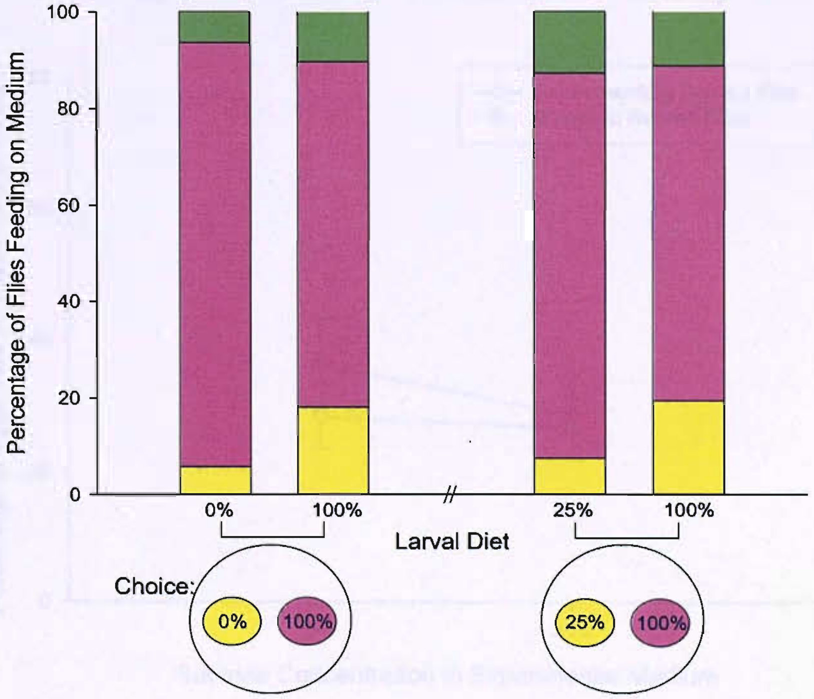


Figure 4.16: The effect of varying sucrose concentration in the larval diet upon the feeding preferences of the flies at 6 d of age when starved immediately after eclosion. Yellow bars show the percentage of flies feeding on the experimental medium, pink those feeding on the standard sucrose medium and green those feeding on a mixture of the two. Varying sucrose concentration in the larval diet has no subsequent affect on the feeding preferences of the adults at 6 d of age when initially starved (n (0% sucrose) = 381; n (25% sucrose) = 350).

The results of the feeding analysis when flies were initially starved are all therefore in contrast to those observed when the flies were not starved (see Section 4.3.2). in order to determine whether this is also the case for the egg laying preferences (see Chapter 3) the flies were again starved immediately after eclosion and then their egg laying preferences observed at 4 and 7 d of age. Results show in all cases larval diet had no subsequent affect on adult egg laying preferences when initially starved (4 d 0% (n = 451): $F_{1, 18} = 0.535$, $P = 0.474$; 4 d 25% (n = 915): $F_{1, 18} = 0.38$, $P = 0.848$; 7 d 0% (n = 404): $F_{1, 18} = 1.405$, $P = 0.251$; 7 d 25% (n = 752): $F_{1, 18} = 0.091$, $P = 0.737$). In all cases the adults showed a preference to lay their eggs on the 100% sucrose medium irrespective of larval diet (Figs. 4.17 and 4.18). For example, when raised as larvae on 0% sucrose 28.8% of eggs were laid on this medium at 4 d. When raised on 100% sucrose and as adults offered the same choices 37.2% of eggs were laid on the 0% sucrose medium (Fig. 4.17).

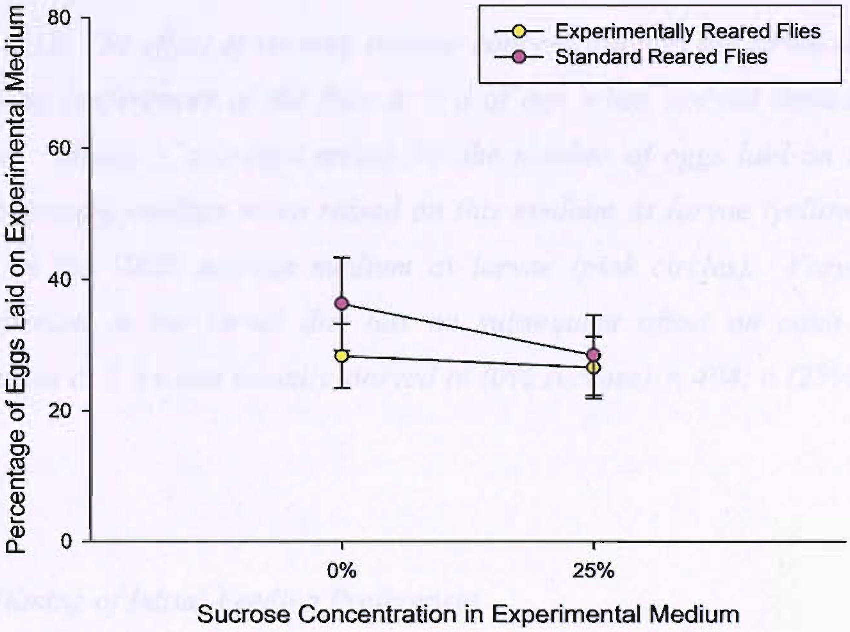


Figure 4.17: The effect of varying sucrose concentration in the larval diet upon the egg laying preferences of the flies at 4 d of age when starved immediately after eclosion. Means ± standard errors for the number of eggs laid on the reduced sucrose content medium when raised on this medium as larvae (yellow circles) or raised on the 100% sucrose medium as larvae (pink circles). Varying sucrose concentration in the larval diet has no subsequent affect on adult egg laying preferences at 4 d when initially starved (n (0% sucrose) = 451; n (25% sucrose) = 915).

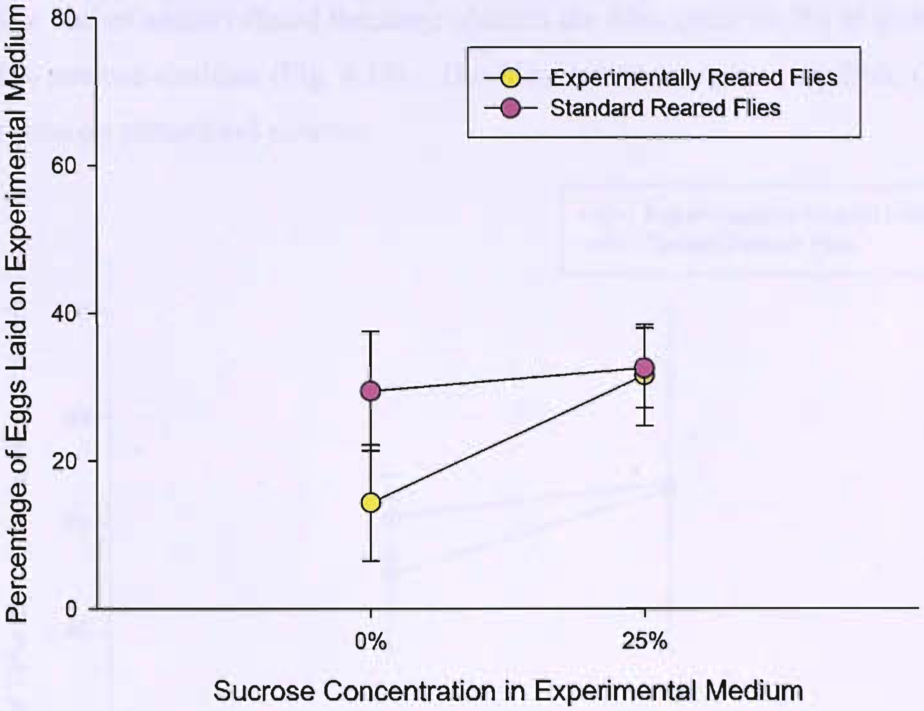


Figure 4.18: The effect of varying sucrose concentration in the larval diet upon the egg laying preferences of the flies at 7 d of age when starved immediately after eclosion. Means ± standard errors for the number of eggs laid on the reduced sucrose content medium when raised on this medium as larvae (yellow circles) or raised on the 100% sucrose medium as larvae (pink circles). Varying sucrose concentration in the larval diet has no subsequent affect on adult egg laying preferences at 7 d when initially starved (n (0% sucrose) = 404; n (25% sucrose) = 752).

4.3.5 Filming of Initial Feeding Preferences

When initial feeding preferences were observed by recording the behaviour of the flies for the first 2 hrs after eclosion results showed that larval diet had no subsequent affect on adult food preference for both 0% and 25% sucrose (0% (n = 10): $F_{1, 10} = 0.222$, $P = 0.648$; 25% (n = 8): $F_{1, 10} = 0.008$, $P = 0.931$). In both cases adults showed no preference for either the reduced sucrose content or the 100% sucrose media (Fig. 4.19). For example, when raised as larvae on 0% sucrose the flies spent 61.2% of their time on this medium. When raised as larvae on 100%

sucrose and as adults offered the same choices the flies spent 50.7% of their time on the 0% sucrose medium (Fig. 4.19). The flies, however, spent very little (> 5%) of their time on either food sources.

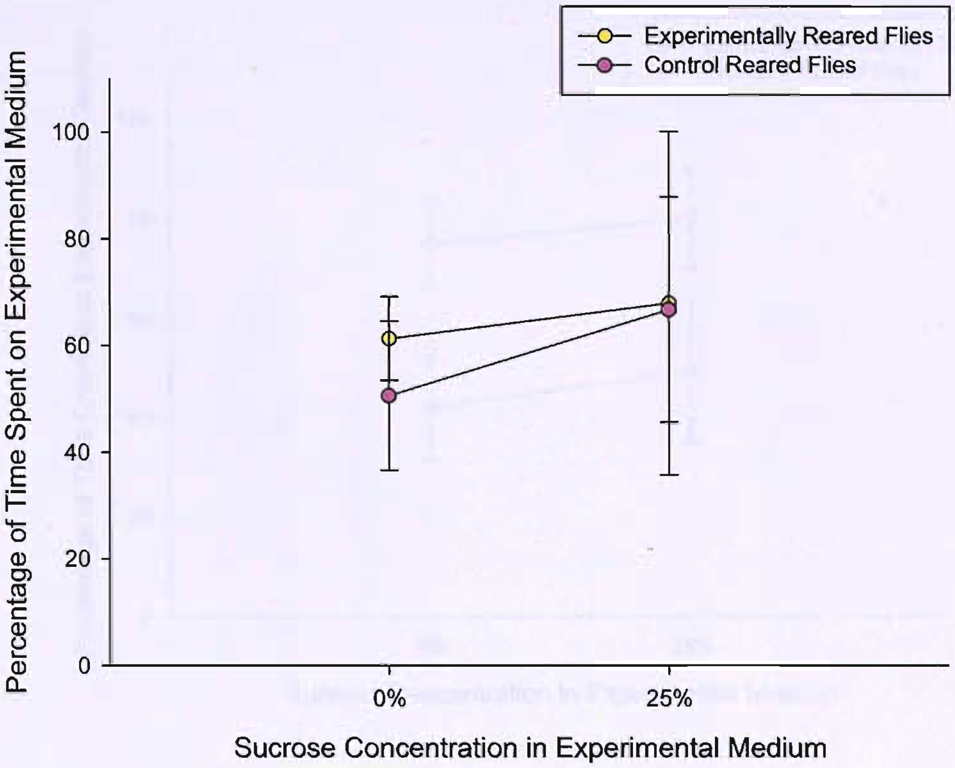


Figure 4.19: The effect of varying sucrose concentration in the larval diet upon the time spent on the reduced sucrose content medium by the adults. Means ± standard errors for the time spent on the reduced sucrose content medium when raised on it larvae (yellow circles) or raised on the 100% sucrose medium (pink circles). Varying sucrose concentration in the larval diet has no subsequent affect on time spent on the experimental medium as adults (n (0% sucrose) = 10; n (25% sucrose) = 8).

The time spent in each half of the choice chamber was also analysed. In this case there was a significant affect of larval diet on time spent near each food patch (0% (n = 20): $F_{1,18} = 4.492$, $P < 0.05$; 25% (n = 18): $F_{1,18} = 5.206$, $P < 0.05$). Flies showed a preference to spend more of their time near to the diet that they had been raised on (Fig. 4.20). For example, when raised as larvae on 0% sucrose the adults spent 74.9% of their time near to this food source, however when raised as larvae on

100% sucrose and as adults given the same choices the adults chose to spend 41.6% of their time near to the 0% sucrose medium (Fig. 4.20).

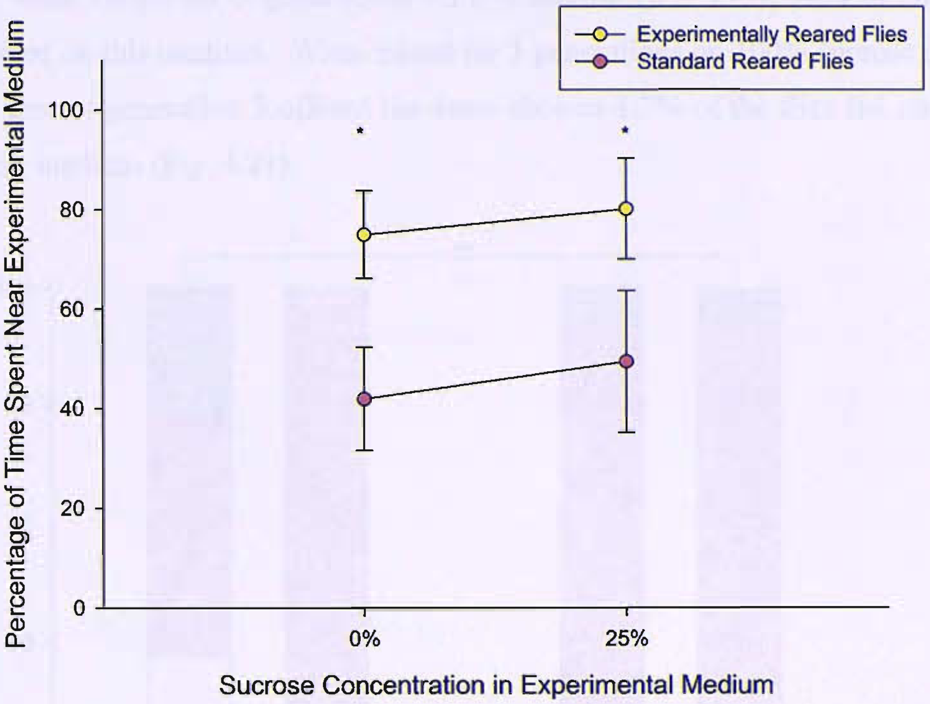


*Figure 4.20: The effect of varying sucrose concentration in the larval diet upon the time spent near the reduced sucrose content medium by the adults. Means ± standard errors for the time spent near the reduced sucrose content medium when raised on it larvae (yellow circles) or raised on the 100% sucrose medium (pink circles). * indicate a significant difference at the* $P < 0.05$ *level. Varying sucrose concentration in the larval diet had a significant affect on time spent near the experimental medium as adults for both concentrations tested (n (0% sucrose) = 20; n (25% sucrose) = 18), with adults showing a significant preference to spend time near the diet upon which they were raised.*

4.3.6 Does Diet Over Several Generations Affect Feeding Preferences?

When flies were raised for several generations on either 0% or 25% sucrose and then as adults their feeding preferences tested at generation 3, results showed that larval diet had a significant affect on adult feeding preferences ($\chi^2 = 165.879$, d.f. =

6, $P < 0.01$, n = 510). In both cases when raised for 3 generations on a reduced sucrose content medium flies were more likely to feed on this medium that if raised as larvae on 100% sucrose and as adults offered the same choices (Fig. 4.21). For example, when raised for 3 generations on 0% sucrose (n = 173), 38% of adults chose to feed on this medium. When raised for 3 generations on 100% sucrose (n = 184) and then at generation 3 offered the same choices 1.7% of the flies fed on the 0% sucrose medium (Fig. 4.21).

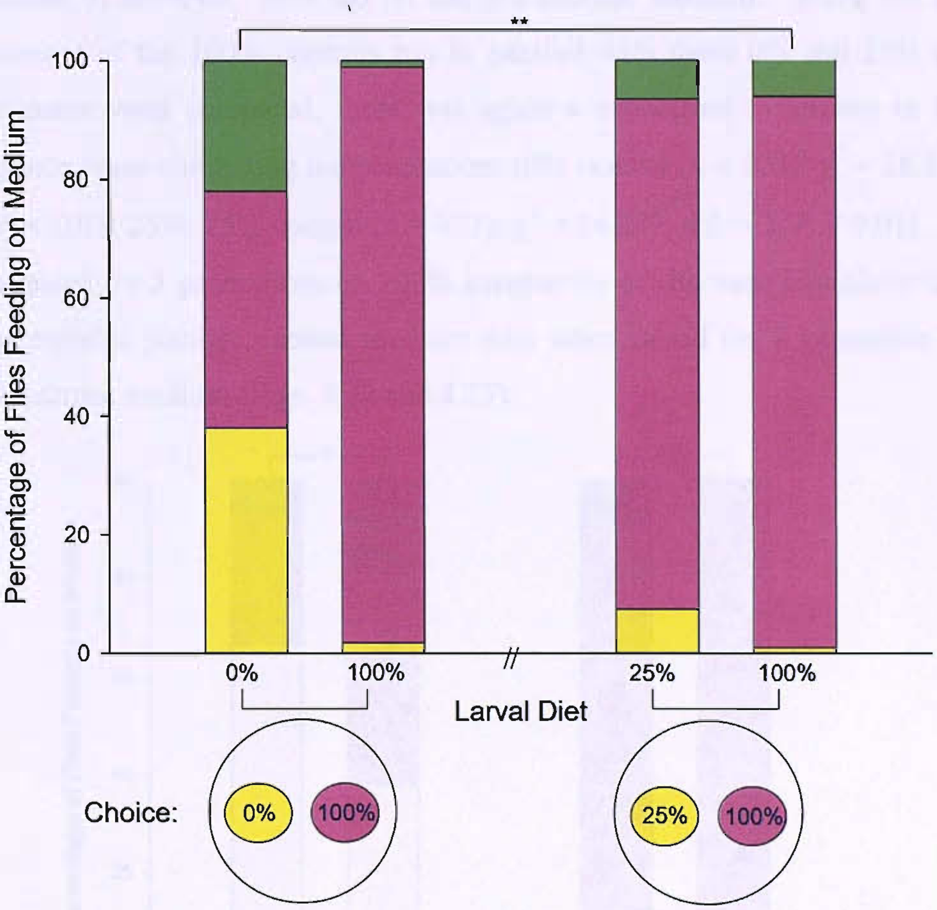


*Figure 4.21: The affect of varying sucrose concentration for 3 generations on adult feeding preferences. Yellow bars show the percentage of flies feeding on the experimental medium, pink those feeding on the standard medium and green those feeding on a mixture of the two. * indicates a significant difference at the* P < *0.01 level. Varying sucrose concentration for 3 generations increased the adults preference for the diet upon which they were raised (n (0% sucrose) = 381; n (25% sucrose) = 350).*

Comparing the generation 1 and generation 3 data showed that there was a significant difference in the feeding preferences of the adults between generations 1 and 3 (0% (n = 512): $\chi^2 = 25.490$, d.f. = 2, $P < 0.01$; 25% (n = 517): $\chi^2 = 27.665$, d.f. = 2, $P < 0.01$). When raised for 3 generations on a reduced sucrose content medium flies were less likely to feed on this diet than at generation 1 (Fig. 4.22 and 4.23). For example, when raised on 0% sucrose in generation 1 66.5% of adults chose to feed on this medium. When the feeding preferences were tested at generation 3, however, 38% fed on the 0% sucrose medium. When the feeding preferences of the 100% controls run in parallel with these 0% and 25% sucrose experiments were compared, there was again a significant difference in feeding preference when comparing the generations (0% control (n = 523): $\chi^2 = 28.857$, d.f. = 2, $P < 0.01$; 25%: 25% control (n = 472): $\chi^2 = 24.247$, d.f. = 2, $P < 0.01$). Again, when raised for 3 generations on 100% sucrose the adults were less likely too feed on the reduced sucrose content medium than when raised for 1 generation on the 100% sucrose medium (Figs. 4.22 and 4.23).

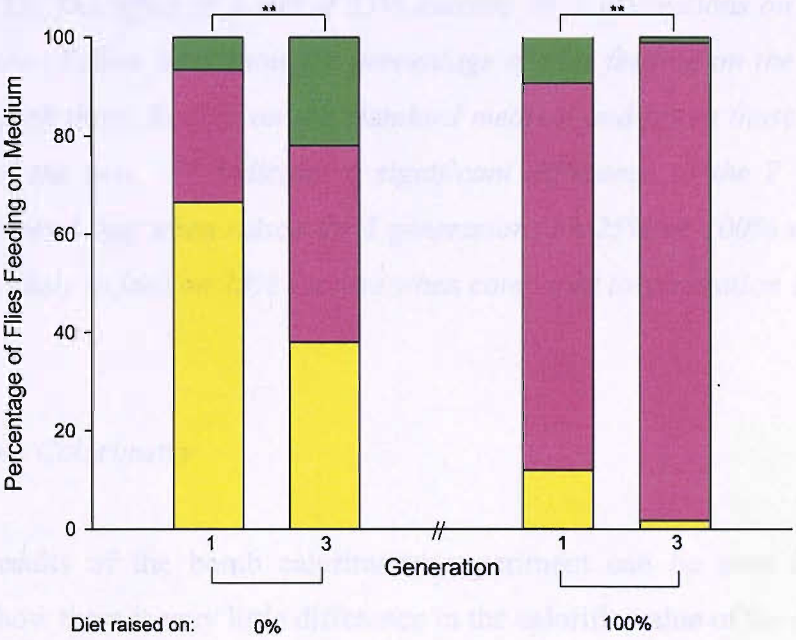


*Figure 4.22: The affect of a diet of 0% sucrose for 3 generations on adult feeding preferences. Yellow bars show the percentage of flies feeding on the experimental medium, pink those feeding on the standard medium and green those feeding on a mixture of the two. ** indicates a significant difference at the* P < *0.01 level. Results showed that when raised for 3 generations on 0% and 100% sucrose adults were less likely to feed on 0% sucrose when compared to generation 1.*

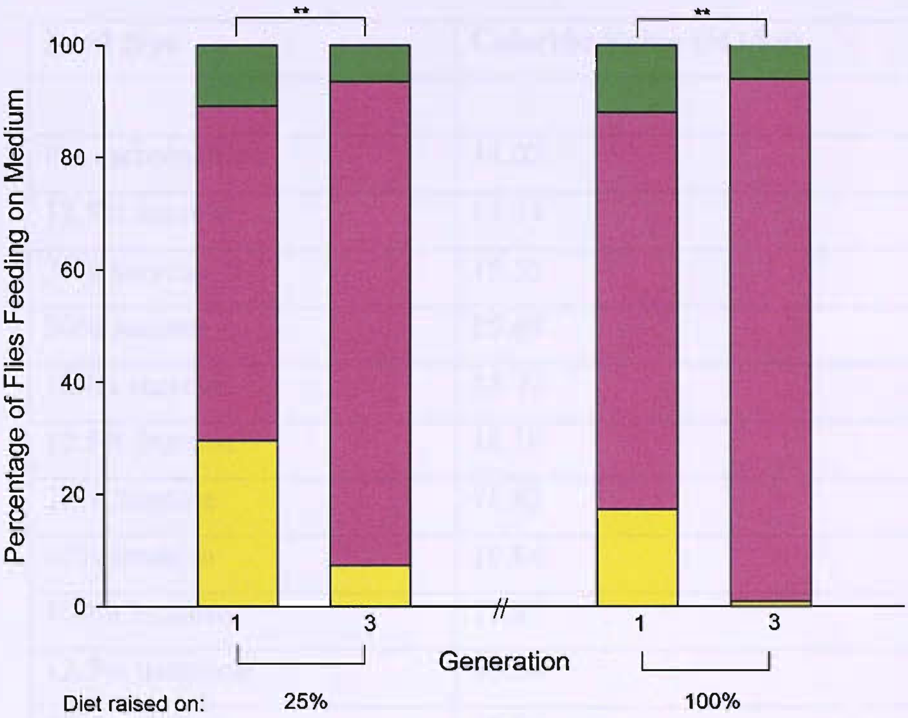


*Figure 4.23: The affect of a diet of 25% sucrose for 3 generations on adult feeding preferences. Yellow bars show the percentage of flies feeding on the experimental medium, pink those feeding on the standard medium and green those feeding on a mixture of the two. ** indicates a significant difference at the* P < *0.01 level. Results showed that when raised for 3 generations on 25% or 100% sucrose adults were less likely to feed on 25% sucrose when compared to generation 1.*

4.3.7 Bomb Calorimetry

The results of the bomb calorimetry experiment can be seen in Table 4.1. Results show there is very little difference in the calorific value of the different food types. The benzoic acid controls varied between 26.27 MJ/kg and 26.85 MJ/kg (n = 5) suggesting all results are accurate with 0.31 MJ/kg.

Food type	**Calorific Value (MJ/kg)**
0% carbohydrate	14.07
12.5% sucrose	15.34
25% sucrose	15.55
50% sucrose	15.69
100% sucrose	15.72
12.5% fructose	16.10
25% fructose	16.42
50% fructose	16.84
100% fructose	17.87
12.5% trehalose	15.64
25% trehalose	15.95
50% trehalose	16.93
100% trehalose	17.07
12.5% glucose	15.55
25% glucose	15.67
50% glucose	16.10
100% glucose	16.24
25% yeast	15.41
50% yeast	16.16
13 mM NaCl	15.59
25 mM NaCl	15.61
50 mM NaCl	15.70
100 mM NaCl	15.65
200 mM NaCl	15.66

Table 4.1: The calorific value of the food media. Results show very little difference in the calorific value of the different food sources.

4.4 Discussion

4.4.1 Does Larval Diet Influence Adult Food Choice? - Varying Carbohydrate Type and Concentration in the Larval Diet

This study clearly shows that varying carbohydrate concentrations in the larval diet can have a significant affect on adult feeding preferences. This affect, however, depends on the carbohydrate type and concentration used. At certain concentrations varying the sucrose and glucose in larval diet increased the adult's preference to feed on these diets compared to those raised on a standard diet as larvae and as adults offered the same choices. In contrast to this, varying the trehalose concentration in the larval diet reduced the adult's preference for this diet. When fructose concentrations within the larval diet were varied this had no effect on subsequent adult feeding preferences, with adults always choosing to feed on the 100% fructose medium irrespective of larval diet. These results therefore show that feeding preferences can indeed be affected by larval diet but the affect is dependent on the carbohydrate and concentration in question.

The difference in the response depending on the carbohydrate type being varied may be due to their concentrations in the flies' natural diet. In their natural habitat, *Drosophila* feed on rotting fruits such as grapes, bananas and plums (Demerec and Kaufmann, 1964). Most fruit contains high concentrations of fructose and glucose. Sucrose is also present in fruit, although generally at lower concentrations (Adao and Gloria, 2005; Li *et al*, 2001; Ordonez *et al*, 2005). As the fruit ripens so the concentrations of fructose and glucose increase (Adao and Gloria, 2005; Ordonez *et al*, 2005). In addition trehalose, which is present in yeast, will increase in concentration as the concentration of yeast increases (Chyb *et al*, 2003). The fact, therefore, that flies always chose to feed on a high concentration fructose diet as adults, irrespective of larval diet, suggests that they may be using this component of the fruit as an indicator that it is a suitable food source. This would be advantageous to the fly as fructose is always in high concentrations in fruit (Adao and Gloria, 2005; Li *et al*, 2001; Ordonez *et al*, 2005).

Varying sucrose and glucose concentrations in the larval diet can have an affect on adult food choice at specific concentrations, with their preference for the reduced concentration medium increasing if raised on it as larvae. One possible explanation for may be that the flies lack the enzymes required for the breakdown of these carbohydrates if not exposed to them. The induction of enzymes by exposure to specific chemicals in the diet has been previously described for several enzymes (Kang *et al*, 1999, Lissemore *et al*, 1990; Pecsenye *et al*, 1996). This, however, seems an unlikely reason for the change in preference as flies that have been previously starved show a preference for the 100% carbohydrate medium even if raised on the low carbohydrate content medium as larvae (see Section 4.4.5).

A much more likely possibility is that the larvae are using the concentrations of sucrose and glucose in the larval diet as an indicator of resource availability so as adults they can adapt to their environment. As already mentioned, ripe fruit contains high concentrations of glucose and a low concentration of sucrose (Adao and Gloria, 2005; Li *et al*, 2001; Ordonez *et al*, 2005). Thus, if raised on a diet with low concentrations of sucrose this would be indicative of a resource rich environment and hence the flies have a preference for low sucrose concentrations. However, if raised on a diet containing high sucrose concentrations this would be indicative of a resource limiting environment and hence flies will become more accepting of the high sucrose concentration. In the case of glucose the opposite response would be likely. In this way, flies are able to adapt to their environment and learn to accept food sources that they may otherwise reject and hence reduce time spent searching for a suitable food source. Other studies have also shown that habitat preference can be altered in response to resource availability (Oskos *et al*, 2004). The fact that fructose preferences are innate allows for individuals to utilize rotting fruit if resource availability increases.

Adult feeding preference when varying the concentration of trehalose in the larval diet can not be explained by trehalose concentrations in the natural diet of *Drosophila*. In this case, when raised as larvae on a low concentration of trehalose, adults show a stronger preference for the high concentration trehalose medium than when raised on high concentration trehalose medium and subsequently given the same choices. As trehalose concentrations increase as the fruit rots (Chyb *et al*, 2003) these results do not fit with the expected behaviour if they were learning information about their larval diet to prepare them for the availability of resources in adulthood. Another factor must therefore be driving the change of preference with larval diet.

Trehalose is the principal sugar found in the haemolymph of insects. The concentration of trehalose in the haemolymph is known to be controlled non-homeostatically by dietary self selection (Thompson, 2003) (see Section 4.1.3). Dietary self selection involves the selection of a food type dependent on the nutritional status of the individual. For example, when *Spodoptera littoralis* larvae were raised on a carbohydrate deficient diet and then given a choice of a carbohydrate or a protein rich diet, they showed a preference for the carbohydrate rich food (Simmonds *et al*, 1992). The data from this study is therefore the same as that described by Simmonds *et al* (1992). When the flies were raised on a diet containing reduced levels of trehalose, the concentration of trehalose in the haemolymph would also be low. The flies therefore select the high trehalose content diet as a food medium. When raised on the 100% trehalose medium, however, the trehalose levels within the haemolymph would be higher and so their need to find a food source rich in trehalose, and so fewer flies feed on the 100% trehalose medium. It is also known that *Drosophila* have a taste receptor gene specific for detection of trehalose (Chyb *et al*, 2003). It would appear, therefore, that the results of this part of the experiment are due to dietary self selection.

4.4.2 Does Larval Diet Influence Adult Food Choice? - Varying the Concentration of Yeast in the Larval Diet

A lack of yeast is known to arrest reproduction in *Drosophila* (Good and Tatar, 2001), and the lack of pupae in the 0% and 12.5% yeast experiments is therefore probably due to very few eggs being laid by the adults at the beginning of the experiment.

When larvae were raised on either 25% or 50% yeast, adult feeding preferences was not significantly different from those raised as larvae on a 100% yeast medium and subsequently offered the same choices. In all cases adults showed a preference to feed on the 100% yeast medium. Yeast is used in the medium as a source of protein and like fructose is an important component of the natural diet of *Drosophila*, being present in fermenting fruit (Demerec and Kaufmann, 1964). It is therefore likely that yeast is such an important component of the adult diet that a preference for a high yeast medium is innate and cannot be affected by previous experience.

4.4.3 Varying the Concentration of NaCl in the Larval Diet

The results of this study clearly show that varying NaCl concentration in the larval diet can have a significant affect on subsequent adult feeding preferences. This affect, however, depends on the concentration used. A significant affect of larval diet was observed for 13 mM. At all of the other concentrations tested there was clear affect of larval diet on adult feeding preferences. Previous studies in rats have also shown that varying the concentration of NaCl in the maternal diet affects the subsequent feeding choices of the offspring (Curtis *et al*, 2004).

NaCl is an important component of the diet in both mammals (Contreras and Lundy, 2000) and insects (Liu *et al*, 2003). In *Drosophila*, concentrations below 200 mM have been shown to be attractive to flies, whereas concentrations over this amount are deterrent (Liu *et al*, 2003). Therefore all the concentrations in this study should be attractive to the flies. This is reflective in the fact that a high proportion of the flies fed on either the NaCl supplemented medium or a mixture of the two diets in all cases. The reason for the significant differences between the feeding preferences of the adults when raised as larvae on 13 mM compared to those raised on a standard diet is unclear. At 13 mM NaCl it is possible that the NaCl is only just on the threshold of detectability and therefore after prolonged exposure to these low concentrations the flies become more sensitive to it and hence more of them feed on it.

4.4.4 The Affect of Starvation on Food Choice

By assessing the time taken for the food to pass through the gut we can see that the food choices made by the fly could be representative of a choice made up to 17.5 hours beforehand. The food preferences as measured at 3 d were therefore not representative of the initial food choice that the fly makes. In order to determine the initial food preferences of the flies one of the methods used was to starve the flies for 23 hrs immediately after eclosion. Results showed that flies always had a preference to both feed and lay their eggs on the 100% sucrose medium when initially starved, which is in contrast to the results obtained when not initially starved (see Section 4.4.1).

It is possible that the preferences observed when the flies are initially starved are reflective of their true preferences and the results obtained when preferences were measured at 3 d are due to prolonged exposure to the 0% sucrose in the adult stages. Further experiments, however, show that this is not the case (see Section 4.4.5). The results of these experiments therefore suggest that starvation in the first 24 hrs after eclosion can permanently change the feeding preferences of the adults. There are two possible explanations for this. The first is that hunger affects the choices that the flies make. One theory aiming to predict the food choices of an individual is called the "energy budget rule" (Stephens, 1981). This rule states that if an animal has sufficient energy reserves it will avoid a potentially risky food source. If the animal does not have sufficient energy reserves, however, it will be more prone to selecting a potentially risky food source (Bateson, 2002). For example, a study using *Drosophila* larvae showed that the larvae become more likely to feed on noxious foods as the duration of food deprivation increases (Wu *et al*, 2005). Therefore, in this study it is possible that information is learnt about the larval diet and is used by the adult to select the most suitable food source. When the flies are starved, however, they are more likely to feed on an unknown food source.

If the change in adult food preference with larval diet seen in the unstarved flies is due to learning and memory, another possible reason for this change in preference when starved is that the memory is initially only short term and needs to be reinforced early in adult life in order for it to become long term. Early adult experience is known to be important in both egg laying and feeding preference (see Section 3.1.4 and 4.1.4 respectively) (Cadieu *et al*, 2000; Hoffman, 1985; Jaenike, 1982 and 1983; Mery and Kawecki, 2002; Prokopy *et al*, 1982). It is therefore possible that exposure to the diet on which they were raised is required early in the adult life in order to reinforce the preferences learnt during the larval stages. The 24 hr period in which the flies were starved could therefore be sufficiently long for this information to be "forgotten" by the fly.

The fact that this study has shown that starvation can affect the food preferences of the flies will have major impact on other work in the field. Starvation prior to the testing of feeding preferences is a widely used method (Balakrishnan and Rodrigues, 1991; D'Souza *et al*, 1999; Meunier *et al*, 2003; Orgad *et al*, 1998; Rodrigues *et al*, 1995; Tanimura *et al*, 1982). However, as the results of this study show, it may lead to the flies showing a preference for a food type that if not starved they would not choose. Starving flies should therefore be used very carefully in future research.

4.4.5 Does Larval Diet Influence the Initial Feeding Preferences of the Adults?

To determine the initial feeding preferences of the flies, their behaviour in the first 2 hrs after eclosion was recorded. Results showed that the flies spent very little of their time in the first 2 hrs of adulthood on either food patch. Of the time they did spend on the food patches there was no significant difference in the time spent on the experimental medium dependent on larval diet. There was, however, a significant difference in time spent in each half of the chamber dependent on larval diet. When raised as larvae on the reduced sucrose content medium adults were more likely to spend their time perched near to this medium than if raised as larvae on a 100% sucrose medium and then as adults offered the same choices. This would therefore suggest that in the first two hours of adulthood although the flies spend very little time actually on their food of choice they have decided what food source they prefer and they perch near to it ready for when they wish to feed. This data therefore supports the feeding preferences as measured at 3 d and shows that adults have a preference for the diet upon which they were raised as larvae. This therefore supports the theory that larval experience can influence adult behaviour (Hershberger and Smith, 1967; Manning, 1967; Rietdorf and Steidle, 2002; Tully *et al*, 1994b).

4.4.6 Does Diet Over Several Generations Affect Feeding Preferences?

In addition to analysing the effect of larval diet on adult feeding preference in one generation, feeding preferences when raised on these diets for 3 generations was also analysed. Results reflect those of generation 1 (see Section 4.4.1). In addition, when the results of generation 1 and generation 3 were compared, adults were less likely to feed on the experimental medium at generation 3 than at generation 1. This was the case for both the experimentally reared flies and the control reared flies. This was an unexpected result and there is no clear reason for the change in preference over the generations when raised on 100% sucrose. These results do, however, suggest that the increased preference for the reduced sucrose content medium when raised on it as larvae will not continue through the generations as there is a decrease in the preference for that medium over the generations. This means that a change in adult feeding preference with larval diet is unlikely to have any long term affects on food choice in the flies. As discussed in Section 4.1.6, the change in preference with larval diet could, however, have a long term affect on several life history traits over the following generations. This is further investigated in Chapter 6.

4.4.7 Learning and Memory through Metamorphosis

The results of this study show that larval diet can indeed affect adult feeding preferences, but the effect is dependent on the chemical and concentration in question. This may explain the variable results of similar studies where in some cases information about the larval environment has been shown to affect adult behaviour Alloway, 1972; Borsellino *et al*, 1970; Gandolfi *et al*, 2003; Hershberger and Smith, 1967; Manning, 1967; Ray, 1999; Rietdorf and Steidle, 2002; Somberg *et al*, 1970; Thorpe, 1939; Tully *et al*, 1994b) and in other cases it has not (Jaenike, 1982 and 1983; Monteith, 1962; van Emden *et al*, 1996; Wiklund, 1974).

The results of the experiments where sucrose and glucose concentrations in the larval diet were varied suggest that the larvae may be learning information about their environment and retaining this information through metamorphosis and into adulthood. This would be advantageous because it would help identify suitable food sources in a highly complex environment. It is also possible, however, that minute traces of the larval diet remained on the pupal cases and the adults therefore came into contact with it during emergence and learn their feeding preferences at this point (Corbet, 1985). In this study this is unlikely as the pupae were thoroughly washed before the adults emerged. Nevertheless, it is still possible that residues of the larval diet may have remained inside the pupal case which could have an affect on subsequent adult behaviour (Corbet, 1985).

4.4.8 The Affect of Calorific Value on Food Preference

The results of the bomb calorimetry analysis showed very little difference in the calorific value of the food between the different food types. Similar results were found by Mair *et al* (2005). Mair *et al* (2005) also found that is it the calorific value contained within the yeast that is of much greater importance to the fly than the calorific value of the carbohydrate. This may explain why the flies survived on a 0% sucrose medium but did not on a 0% yeast medium. It has also been found that different food types induce different levels of food intake in *Drosophila* (Min *et al*, 2007), and it may therefore be the amount of food ingested rather than the calorific value of the food that is affecting subsequent adult food preferences in this study.

4.4.9 Summary

The results from this study clearly show that varying larval diet can affect adult feeding preferences, although this effect is dependent on the chemical type and concentration used. For some of the chemicals used, this effect appears to be due to dietary self selection (see Section 4.1.3). In other cases the modification of feeding preferences may be due to learning. Varying the concentration of other constituents of the larval diet (fructose and yeast) had no subsequent affect on adult feeding preferences suggesting that the feeding preferences for these chemicals may be innately controlled. The fact that some varying some chemicals in the larval diet can affect adult feeding preferences but others do not may explain the conflicting results of similar studies found in the literature.

When the sucrose concentration in the larval diet was varied and then the very first food choices of the adult's analysed results suggest that, as with the analysis at 3 d, flies show a preference for the diet upon which they were raised. This preference is reflective, however, in the time spent near to the food source rather than actually on the food source itself. This would suggest that immediately after eclosion when the flies disperse to find a suitable habitat they may not utilise the resource immediately but perch nearby so that the resources are easily accessible when required. The results from this Chapter also show that starvation immediately after eclosion can have a profound affect on the food preferences of the fly. Using starvation as a means of making the fly feed when required should therefore be used with great caution.

When the flies were raised for 3 generations on a reduced sucrose content media results showed that these adults still had a preference to feed on the reduced sucrose content medium when compared to those raised for 3 generations on the 100% sucrose medium and as adults offered the same choices. This effect of diet upon adult feeding preferences decreases between generation 1 and generation 3, suggesting that the effect of larval diet on adult feeding preference will eventually disappear.

Chapter 5: Does Learning and Memory in The Larval Stages Underlie the Change in Adult Food Preferences?

5.1 Introduction

The results from the previous two chapters (Chapters 3 and 4) have shown that larval diet can have an effect on subsequent adult food preferences. One possible explanation for this is that the larvae are learning information about their diet, retaining this information through metamorphosis and using it to help to select an appropriate food source as an adult. Great advances have been made in recent years into the understanding of the underlying mechanisms involved in learning and memory. The key question therefore arises as to whether a similar mechanism underlies the possible learning events described in the previous two chapters.

5.1.1 The Neuroanatomy of Learning and Memory

The central structures thought to be involved in olfactory learning and memory in insects are the mushroom bodies (de Belle and Heisenberg, 1994; Lessing and Carlson, 1999; Rausher, 1978). These are present in both the adults and the larvae, although in larvae the number of neurons is much reduced (Balling *et al*, 1987). Selective chemical ablation of the mushroom bodies using the toxin hydroxyurea (HU) leaves the insect unable to learn in associative olfactory tasks even though they are still able to detect the stimuli (de Belle and Heisenberg, 1994; Komischke *et al*, 2005).

The mushroom bodies are paired structures consisting of approximately 2,200 neurons in each hemisphere in adult *Drosophila* (Levin *et al*, 1992), although this varies depending on previous experience (Balling *et al*, 1987). They are found in the dorsal/posterior region of the central brain (Dubnau and Tully, 1998). The mushroom bodies consist of a calyx, columnar stalk and lobes (Levin *et al*, 1992). The calyx is thought to be the main input site, the stalk where the intrinsic neurons of the mushroom bodies (the Kenyon cells) communicate, and the lobes the main output site (Crittenden *et al*, 1998). The Kenyon cells are divided into three classes which further divides the lobes into three distinct areas; the α / ß, α' / ß' and γ lobes (Crittenden *et al*, 1998). It is the Kenyon cells within the mushroom bodies that are thought to be involved in learning and memory (Isabel *et al*, 2004; Pascual and Preat, 2001; Zars *et al*, 2000).

Olfactory information is detected by sensilla found mainly on the antennae (see Section 1.1.2). Each chemosensory neuron within these sensilla expresses one olfactory receptor gene (Heimbeck *et al*, 1999; Vosshall *et al*, 2000). All the chemosensory neurons that express that olfactory receptor project to a specific glomerulus within the antennal lobe (Dunipace *et al*, 2001; Python and Stocker, 2002; Scott *et al*, 2001; Vosshall *et al*, 2000). This produces a spatial map of olfactory input in the antennal lobe (see Section 1.1.3) (Dunipace *et al*, 2001; Python and Stocker, 2002; Scott *et al*, 2001; Vosshall *et al*, 2000). Projection neurons from these glomeruli relay olfactory information to the mushroom body calyx where it is transformed into an activation pattern in the Kenyon cells (Jefferis *et al*, 2001; Marin *et al*, 2002; Stocker *et al*, 1997; Tanaka *et al*, 2004; Wang *et al*, 2004; Wong *et al*, 2002). There is therefore clear evidence that there are connections between the antennal lobes and the mushroom bodies, allowing for olfactory learning and memory. In the case of gustatory learning and memory, the connection is less clear. In honeybees, the VUMmx1 neuron is known to connect the suboesophageal ganglion with the mushroom bodies (Mobbs, 1982). This neuron is also known to be involved in appetitive learning and memory (Hammer, 1993). No such connection, however, has been described in the fly. Nevertheless, studies have shown that associative learning between an olfactory and a gustatory cue is localised to the mushroom bodies in *Drosophila* (Hammer and Menzel, 1998; Schwaerzel *et al*, 2003). Gustatory information must therefore be reaching the mushroom bodies of the flies via some as yet unidentified pathway.

5.1.2 Synaptic Plasticity is thought to Underlie Learning and Memory

To perceive and respond to sensory stimuli, the brain must be able to internally represent it in some form. The brain must also be able integrate different types of sensory stimuli (Marin *et al*, 2002) which allows the insect to discriminate between complex sensory information and then respond to it (Wong *et al*, 2002). Many studies have shown that insects are also capable of learning information about sensory stimuli and using that information to adapt their responses to it in the future (Blaney and Simmonds, 1985; Lee and Bernays, 1990; Mery and Kawecki, 2002; Quinn *et al*, 1974; Szentesi and Bernays, 1984). For example, grasshoppers (*Schistocerca americana*) are capable of learning to associate a novel food type with the negative effects induced by the injection of a plant secondary compound (Lee and Bernays, 1990).

The ability to learn is thought to be due to synaptic plasticity. Synaptic plasticity is achieved by varying the strength of a signal transmitted through the synapse (Castellucci *et al*, 1978). Experience may strengthen these connections by increasing the amount of neurotransmitter released at the synapse (Balling *et al*, 1987; Klein and Kandel, 1980). In the case of habituation, the amount of neurotransmitter released is decreased (Castellucci and Kandel, 1974). It is thought that plasticity in the Kenyon cells is necessary for learning and memory (Balling *et al*, 1987), although definitive evidence that neuronal plasticity occurs *in vivo* in these neurons is still lacking (Gerber *et al*, 2004b). Several mutants known to be defective in learning and memory (see Section 5.1.3) have also been shown to be defective in synaptic plasticity (Davis *et al*, 1996; Zhong *et al*, 1992). Thus, there is strong evidence that synaptic plasticity underlies learning and memory, but how is synaptic plasticity achieved?

5.1.3 The Underlying Molecular Mechanisms of Learning and Memory

Much work has tried to elucidate the underlying mechanisms of learning and memory in insects. *Drosophila* is a particularly attractive model for this as the entire genome has been sequenced (Adams *et al*, 2000) and molecular genetic tools are available. Hence much of the work in this area has been conducting using *Drosophila* as a model (Davis, 2005). Studies have shown that several factors are involved in learning and memory, including gene regulation, protein phosphorylation and the firing patterns of neurons (Roman and Davis, 2001).

Several genes have been described which are known to be involved in learning and memory. The first to be discovered was the associative learning and memory mutant, *dunce* (Aceves-Pina and Quinn, 1979; Dudai *et al*, 1976; Smith *et al*, 1979a; Tully and Quinn, 1985). This was then followed by the discovery of other mutants including *rutabaga* (Mao *et al*, 2004; McGuire *et al*, 2003; Tully and Quinn, 1985), *amnesiac* (Quinn *et al*, 1979; Tully and Quinn, 1985), *cabbage* (Aceves – Pina and Quinn, 1979; Smith *et al*, 1979a) and *turnip* (Aceves – Pina and Quinn, 1979; Smith *et al*, 1979a). In addition, gene mutations that lead to anatomical defects in the adult brain have also been shown to be defective in learning and memory, for example *mushroom body miniature* and *mushroom body deranged* (de Belle and Heisenberg, 1996; Heisenberg *et al*, 1985).

dunce and *rutabaga* are both highly expressed in the mushroom bodies (Han *et al*, 1992; Nighorn *et al*, 1991) and are both involved in the cAMP signalling cascade. The *dunce* gene encodes a cAMP phosphodiesterase (Chen *et al*, 1986; Kauvar, 1982) and the *rutabaga* gene a type I adenylyl cyclase (Levin *et al*, 1992; Livingstone *et al*, 1984). Mutations in these genes lead to impairment in the associative learning ability of the flies (Dudai *et al*, 1976; Smith *et al*, 1979a; Tully and Quinn, 1985). For example, in a study by Tully and Quinn (1985), adult flies were exposed to two odours, one of which was paired with an electric shock. When the flies were then placed into a T-maze, one arm containing the odour originally paired with the electric shock and the other arm containing the odour which was not associated with any other stimuli, more than 95% of the wild type flies chose the odour not associated with an electric shock. *dunce* and *rutabaga* flies, however, were much less likely to choose the correct arm (Tully and Quinn, 1985). Transgenic expression of these genes in the mushroom bodies can rescue the learning ability of these flies (Dauwalder and Davis, 1995; Mao *et al*, 2004; McGuire *et al*, 2003; Schwaerzel *et al*, 2003; Zars *et al*, 2000). Furthermore, in a study by Tully *et al* (1994b) larvae were trained to avoid an odour paired with an electric shock. When these individuals were then tested as adults, wild type flies showed an aversion to that odour. These adults are therefore exhibiting the retention of memory through metamorphosis. When this experiment was conducted with *dunce* and *amnesiac* mutants, however, the adults showed no aversion to this odour (Tully *et al*, 1994b). Studies on all of these mutants therefore provide strong evidence that the cAMP pathway is involved in learning and memory. The cAMP pathway is also known to be involved in learning and memory in other invertebrates (Antonov *et al*, 2003; Kandel *et al*, 1983) and mammals (Villacres *et al*, 1998; Wu *et al*, 1995). The mutations do not, however, completely extinguish learning ability in the flies (Duerr and Quinn, 1982; Tully and Quinn, 1985), suggesting that other pathways may also be involved (Dubnau and Tully, 1998).

All of this work has led to a model for the molecular basis for learning and memory (Davis, 2004 and 2005; Dubnau and Tully, 1998) (Fig. 5.1). The model suggests that in the case of associative learning both the conditioned and the unconditioned stimulus converge on the mushroom bodies. This then activates adenylyl cyclase, encoded by *rutabaga,* which elevates cAMP levels (Kandel *et al*, 1983) that in turn activates protein kinase A (PKA) causing the catalytic subunit to become dissociated from the regulatory subunits. This allows the active PKA to phosphorylate cytoplasmic targets, causing short term affects on synaptic function. PKA can also phosphorylate other targets, such as cAMP response element binding protein (CREB) which is a transcription factor that can alter protein synthesis hence producing longer-lasting modifications at the synapse (Yin *et al*, 1994; Yin *et al*, 1995).

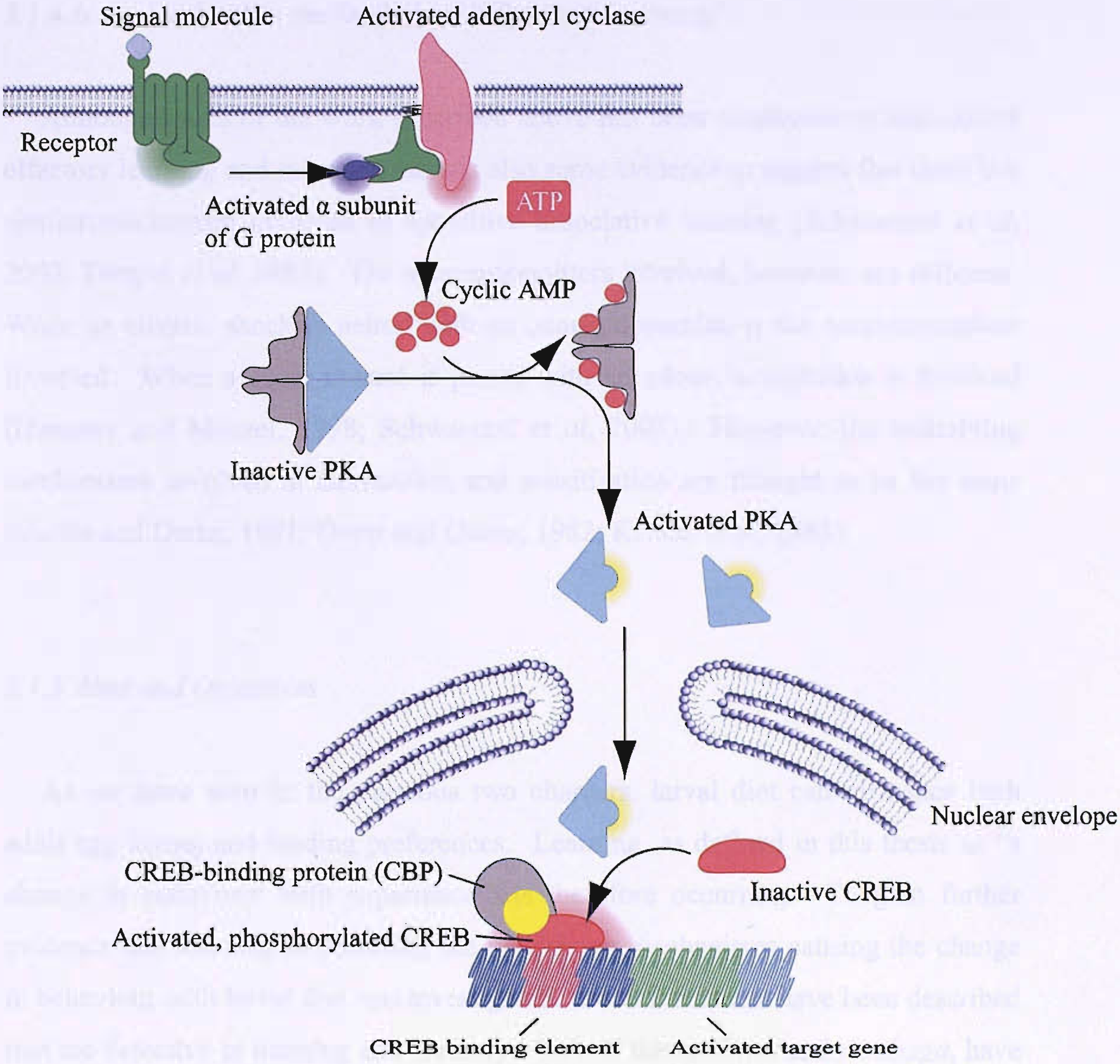


Figure 5.1: The proposed model for the molecular mechanism underlying associative learning and memory. The two stimuli converge on the mushroom body neurons. This activates the rutabaga *adenylyl cyclase which leads to an increase in cAMP levels. The increase in cAMP levels causes the catalytic subunit of PKA to become dissociated allowing it to then phosphorylate cytoplasmic targets causing a short term modification in synaptic function. The activated PKA can also translocate into the nucleus to phosphorylate nuclear targets inducing changes in gene expression and therefore affecting the response to the stimuli on a longer term basis (adapted from Dubnau and Tully, 1998).*

5.1.4 Is the Mechanism the Same for All Types of Learning?

Although much of the work described above has been conducted on associative olfactory learning and memory, there is also some evidence to suggest that there is a similar mechanism involved in appetitive associative learning (Schwaerzel *et al*, 2003; Tempel *et al*, 1983). The neurotransmitters involved, however, are different. When an electric shock is paired with an odour, dopamine is the neurotransmitter involved. When a sugar reward is paired with an odour, octopamine is involved (Hammer and Menzel, 1998; Schwaerzel *et al*, 2003). However, the underlying mechanisms involved in habituation and sensitisation are thought to be the same (Corfas and Dudai, 1991; Duerr and Quinn, 1982; Kandel *et al*, 1983).

5.1.5 Aims and Objectives

As we have seen in the previous two chapters, larval diet can influence both adult egg laying and feeding preferences. Learning, as defined in this thesis as "a change in behaviour with experience", is therefore occurring. To gain further evidence that learning is occurring the underlying mechanisms causing the change in behaviour with larval diet was investigated. Several mutants have been described that are defective in learning and memory. Two of these, *dunce* and *rutabaga*, have been extensively studied (Aceves – Pina and Quinn, 1979; Chen *et al*, 1986; Dudai *et al*, 1976; Kauvar, 1982; Levin *et al*, 1992; Livinigstone *et al*; 1984; Mao *et al*, 2004; McGuire *et al*, 2003; Smith *et al*, 1979b; Tully and Quinn, 1985) and neither are known to affect the chemosensory abilities of the flies (Aceves-Pina and Quinn, 1979; Motosaka *et al*, 2007). In addition, in studies looking at the retention of associative learning through metamorphosis, the *dunce* mutation has been shown to eliminate this ability in the flies (Tully *et al*, 1994b). These are therefore good candidates for determining the underlying mechanisms involved in the learning and memory phenomena described in the previous two chapters. In addition to using mutants, another method involving chemical ablation of the mushroom bodies will also be used to further investigate the hypothesis that the same mechanisms and structures are involved.

In addition, another interesting question that can be asked is how the flies will respond if they are unable to taste sugars. One mutant, *malvolio*, is known to affect sugar perception (D'Souza *et al*, 1999; Orgad *et al*, 1998; Rodrigues *et al*, 1995). This mutant will also therefore be tested for the effects of larval diet on adult food preference.

5.2 Methods

5.2.1. Determining Whether Learning and Memory or Gustatory Mutants Affect Adult Feeding and Egg Laying Preferences

To determine whether the changes in adult food preference with larval diet described in Chapters 3 and 4 were due to learning and memory, two *Drosophila* mutants known to be deficient in learning and memory were used. These mutants were *dunce* (dnc^1) (Dudai *et al*, 1976) and *rutabaga* (rut^1) (Tully and Quinn, 1985) and were sourced from the Bloomington Stock Centre. The dnc^1 flies were homozygous for the mutation and could therefore be used immediately. The *rutabaga* flies were balanced with *C(1)DX* chromosome in which the two X chromosomes in the female are fused to form a single genetic unit. The genotype of the females is therefore $C(1)DX, y^1 f^1 / Y$. *C(1)DX* carries the alleles y^1 and f^1, y^1 being body colour defective and f^1 having forked bristles. Males have the genotype $sc^e\ rut^1\ f^5\ Sh^{14} / Y$. Consequently only males in this stock show the rut^1 phenotype. In order to produce homozygous females, males would have to be crossed with X chromosome balancer females. As the aim of the experiment is simply to determine whether the same mechanism is involved, and as the *dnc* and mushroom body ablation are also being used, it was decided to only use males in the *rutabaga* experiment.

In addition to these learning and memory mutants, a gustatory mutant was also used which is known to be defective in its ability to taste several carbohydrates, including sucrose (Orgad *et al*, 1998; Rodrigues *et al*, 1995). The *malvolio* (w^{1118}; $P\{lacW\}Mvl^{97f}$) stock was again sourced from the Bloomington Stock Centre. The *mvl* mutation is caused by the insertion of a transposable element, is homozygous for *mvl* mutation and ready to be used immediately. This gustatory mutant was chosen because there have been many studies describing the inability of these flies to taste but no evidence that the mutation affects the flies ability to learn.

The same protocols as described in Chapters 3 and 4 were used to conduct these experiments (see Sections 3.2.2 and 4.2.1 respectively). Briefly, larvae were raised on diets in which the concentration of sucrose was varied. The concentrations used were 0%, 12.5%, 25% or 50% of the standard concentrations (see Section 3.2.5). As pupae, they were removed from these diets (see Section 3.2.6), washed (see Section 3.2.7) and placed into a choice chamber (see Section 3.2.8). 10 choice chambers were set up per concentration with 20 pupae in each. They were left in these chambers for 3 days before being removed and their feeding preferences analysed (see Section 4.2.2). They were then placed into a fresh choice chamber for a further 24 hrs and then the number of eggs laid on each medium was counted. Running in parallel with these experiments, flies were also raised as larvae on a 100% sucrose medium and then as adults offered the same choices. The egg count was not conducted on the *rutabaga* mutants as the females do not express the *rut*^{1} mutation and therefore for the *rutabaga* mutants only the feeding preferences of the males were analysed.

5.2.2 Ablation of the Mushroom Bodies Using HU

To ablate the mushroom bodies HU was used. HU inhibits DNA synthesis and kills dividing cells (Timson, 1975). In the first 8 hours after the larva hatches from the egg, only the neuroblasts that form the mushroom bodies (the mushroom body neuroblasts - MBNbs) and one other neuroblast (the lateral neurobalst – LNb) are proliferating (Ito and Hotta, 1992; Prokop and Technau, 1991). Feeding the larvae HU during this period therefore kills MBNbs and all Kenyon cell lineages (de Belle and Heisenberg, 1994) with the exception of the 40 – 300 cells per hemisphere that developed during embyrogenesis (de Belle and Heisenberg, 1994; Ito and Hotta, 1992). By using HU, the mushroom bodies can be ablated to determine whether it is indeed these structures that are involved in the affects of larvae diet on adult feeding preferences as described in Chapters 3 and 4. Figure 5.2 gives an outline of the method used and is followed by a more detailed description below.

10 5 d old males and 20 5 d old females were collected and placed onto an agar plate containing a thin layer of a specified medium

↓

After 2 hrs these adults were removed and discarded

↓

The eggs laid on these plates were continuously monitored and newly hatched larvae collected hourly

↓

These larvae were placed into a fresh agar plate containing a specified medium mixed with HU (5.2.3)

↓

After 3 hrs of feeding the larvae were collected, washed and placed into a vial containing a specified medium

↓

Pupae were collected from these vials, washed, and placed into a choice chamber (3.2.8)

↓

Adults were allowed to feed for 3 d before their feeding preferences were tested (4.2.2)

↓

Adults were then placed into a fresh choice chamber for 24 hrs

↓

Flies were removed and discarded and the number of eggs laid on each patch counted

Figure 5.2: A flow diagram giving an outline of the method used to ablate the mushroom bodies and then determine the effects of larval diet on adult food choice.

20 female and 10 males were placed into vials containing a standard medium. The adults were then removed after 24 hrs and discarded. The eggs that were laid were allowed to develop through to the adult stage. Adults were collected daily and transferred into fresh vials containing standard medium. In this way the age of the adults was known. Once these adults were 5 d old, 20 females and 10 males were

placed onto an agar plate with a thin layer of a specified food medium on top of it. This food medium was either 0%, 25% or 100% sucrose. The adults were left on these plates for 2 hrs to lay eggs. The adult were then removed and discarded the plates checked on an hourly basis to check for newly hatched larvae. Any larvae that had hatched were collected and placed onto a fresh plate with a thin layer of the same food medium but with HU added to it (see Section 5.2.3). The larvae were left to feed on this medium for 3 hrs before being removed and washed in distilled water. The larvae were then placed into a fresh vial containing the medium they had been feeding on previously. For example, if immediately after hatching the larvae were fed on HU mixed with a 0% sucrose medium the vial would also contain this 0% sucrose medium, but without the addition of the HU. In this way, the larvae only ever fed on one food type. 50 larvae were placed into each vial. These larvae were then allowed to develop through to the pupal stage. At this point, 200 mature pupae per food type were selected from the vials, washed (see Sections 3.2.6 and 3.2.7 respectively) and transferred into 10 choice chambers (see Section 3.2.8), 20 pupae per chamber. The choice chamber contained two food sources, one the diet upon which they were raised and the other a standard medium. For example, if raised on a 0% sucrose medium as larvae one of the wells would contain this 0% sucrose medium and the other a standard 100% sucrose medium. These individuals were left in these chambers for three days to feed on the diet of their choice. After 3 days the adults were removed from these chambers and examined under a microscope to determine their feeding preferences (see Section 4.2.2). The adults were then placed into fresh choice chambers and left for a further 24 hrs before being removed and discarded and the number of eggs on each food patch counted (see Section 3.2.2). Running in parallel with these experiments, larvae were also raised on a standard diet and then as adults offered the same choices. For example, in parallel with the 0% sucrose experiments, larvae were raised on 100% sucrose and then as adults offered the choice of 0% or 100% sucrose to feed upon. These experiments were conducted with 0% and 25% sucrose along with their 100% controls.

5.2.3 Addition of HU to the Food Medium

The addition of HU to the food was conducted in a fume cupboard. HU was added to the food just as the food was about to set as in preliminary experiments it was found that the HU seemed to become inactivated when added to the food when it was still hot. In addition, the food containing the HU was made up just before the larvae were placed onto it as it seemed to degrade if left for too long before the larvae were exposed to it. The food had 50 mg HU / 1 ml food and was mixed well before being poured onto the plate.

5.2.4 Statistical Analysis of the Data

Statistical analysis of the data was as previously described (see Sections 3.2.9 and 4.2.7). Briefly, for the feeding data the Chi Squared Test of Association was used, and for the egg laying data a one – way ANOVA was used.

5.3 Results

5.3.1 The Effect of Varying Sucrose Concentrations in the Larval Diet on the Adult Feeding Preferences of dnc^1 *Flies*

Varying the concentration of sucrose in the larval diet had no subsequent effect on adult feeding preferences at any of the concentrations tested with the learning and memory mutant *dunce* (0% (n = 336): $\chi^2 = 1.163$, d.f. = 2, $P = 0.559$; 12.5% (n = 336): $\chi^2 = 2.241$, d.f. = 2, $P = 0.326$; 25% (n = 355): $\chi^2 = 0.241$, d.f. = 2, $P = 0.886$; 50% (n = 323): $\chi^2 = 0.344$, d.f. = 2, $P = 0.842$). In all cases, regardless of larval diet, dnc^1 adults had a preference to feed on the 100% sucrose medium (Fig. 5.3). For example, when dnc^1 flies were raised as larvae on 0% sucrose, as adults 76.3% (n = 182) chose to feed on the standard medium. When individuals were raised as larvae on the 100% sucrose and as adults given the same choices 71.3% (n = 184) fed on the 100% sucrose medium. Thus the feeding preferences of the wild type flies were not shown in the dnc^1 flies (see Chapter 4).

5.3.2 The Effect of Varying Sucrose Concentration on the Egg Laying Preferences of dunce *Flies*

Varying the concentration of sucrose in the larval diet had no subsequent effect on adult egg laying preferences at any of the concentrations tested with the learning and memory mutant *dunce* (0% (n = 668): $F_{1, 18} = 0.128$, $P = 0.725$; 12.5% (n = 325): $F_{1, 18} = 0.808$, $P = 0.381$; 25% (n = 1572): $F_{1, 18} = 1.670$, $P = 0.213$; 50% (n = 1160): $F_{1, 18} = 0.538$, $P = 0.473$). In all cases, regardless of larval diet, dnc^1 adults had a preference to lay their eggs on the 100% sucrose medium (Fig. 5.4). For example, when raised as larvae on 0% sucrose 29.0% of the total eggs were laid on this medium (n = 290) compared to 33.1% when raised as larvae on the 100% sucrose medium (n = 378) and as adults offered the same choices. Thus the egg laying preferences of the wild type flies were not shown in dnc^1 flies (see Chapter 3).

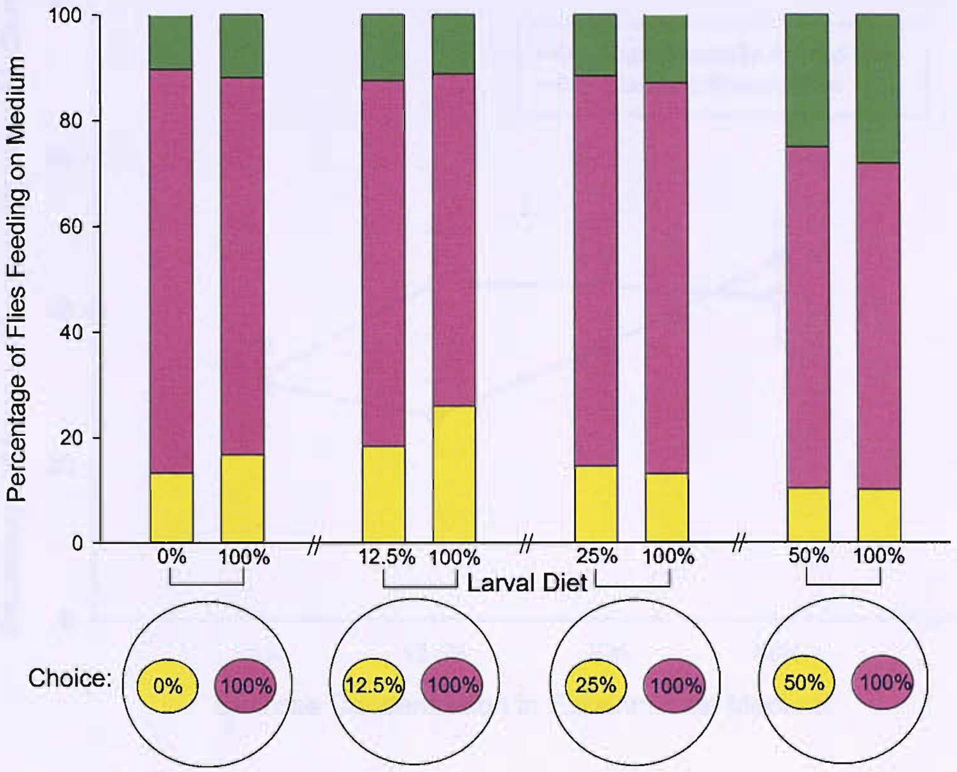


Figure 5.3: The effect of varying sucrose concentration in the larval diet on the feeding preferences of adult dnc^{1} *flies. Yellow bars show the percentage of flies feeding on the experimental medium, pink those feeding on the 100% sucrose medium and green those feeding on a mixture of the two. Larval diet had no subsequent affect on adult feeding preferences at any of the concentrations tested (n (0% sucrose) = 336; n (12.5% sucrose) = 336; n (25% sucrose) = 355; n (50% sucrose) = 323).*

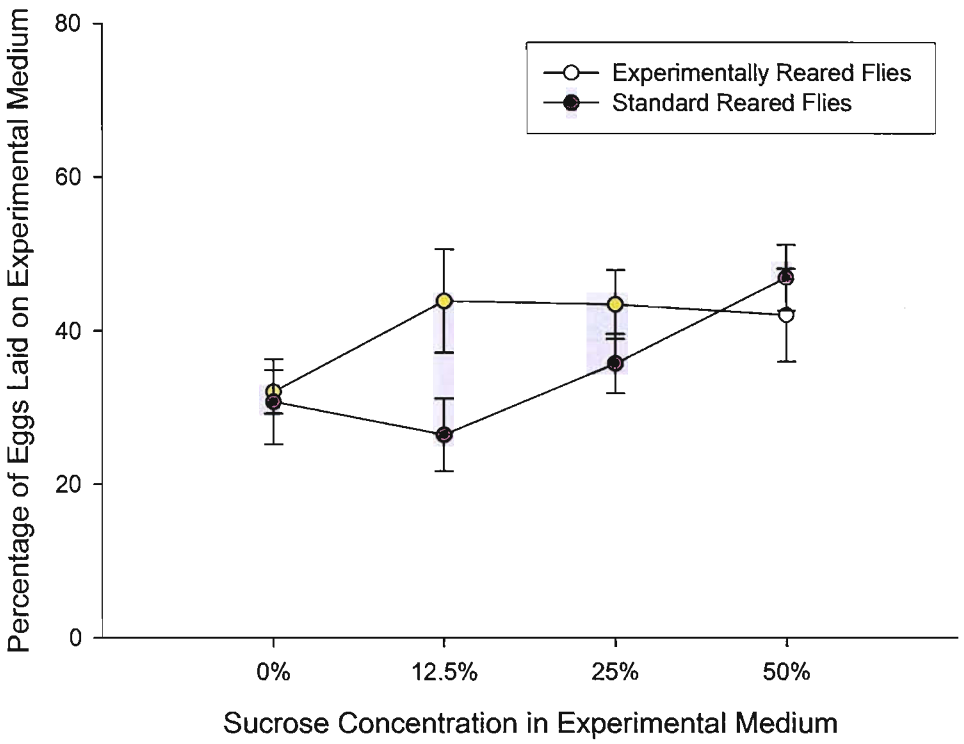


Figure 5.4: The effect of varying sucrose concentration in the larval diet upon egg laying preferences in dnc^1 *flies. Means ± standard errors for the number of eggs laid on the reduced sucrose content medium when raised as larvae on that reduced sucrose content medium (yellow circles) or the standard, 100% sucrose, medium (pink circles). Varying the sucrose concentration in the larval diet of* dnc^1 *flies had no subsequent affect on adult egg laying preference at any of the concentrations tested (n (0% sucrose) = 668; n (12.5% sucrose) = 325; n (25% sucrose) = 1572; n (50% sucrose) = 1160).*

5.3.3 The Effect of Varying Sucrose Concentrations in the Larval Diet on the Feeding Preferences of rut^1 *Males*

Varying the concentration of sucrose in the larval diet had no subsequent effect on male adult feeding preferences at any of the concentrations tested with the learning and memory mutant, *rutabaga* (0% (n =178) : χ^2 = 1.615, d.f. = 2, P = 0.446; 12.5% (n = 163): χ^2 = 3.361, d.f. = 2, P = 0.186; 25% (n = 269): χ^2 = 0.318, d.f. = 2, P = 0.853; 50% (n = 277): χ^2 = 0.894, d.f. = 2, P = 0.639). In all cases, regardless of larval diet, *rut^1* adults had a preference to feed on the 100% sucrose medium (Fig. 5.5). For example, when raised as larvae on 25% sucrose 76.9% (n = 132) of the adults fed on the 100% sucrose medium compared to 74.1% (n = 137) when raised as larvae on 100% sucrose. Thus the preference of wild type flies was abolished in *rut*^{1} flies (see Chapter 4).

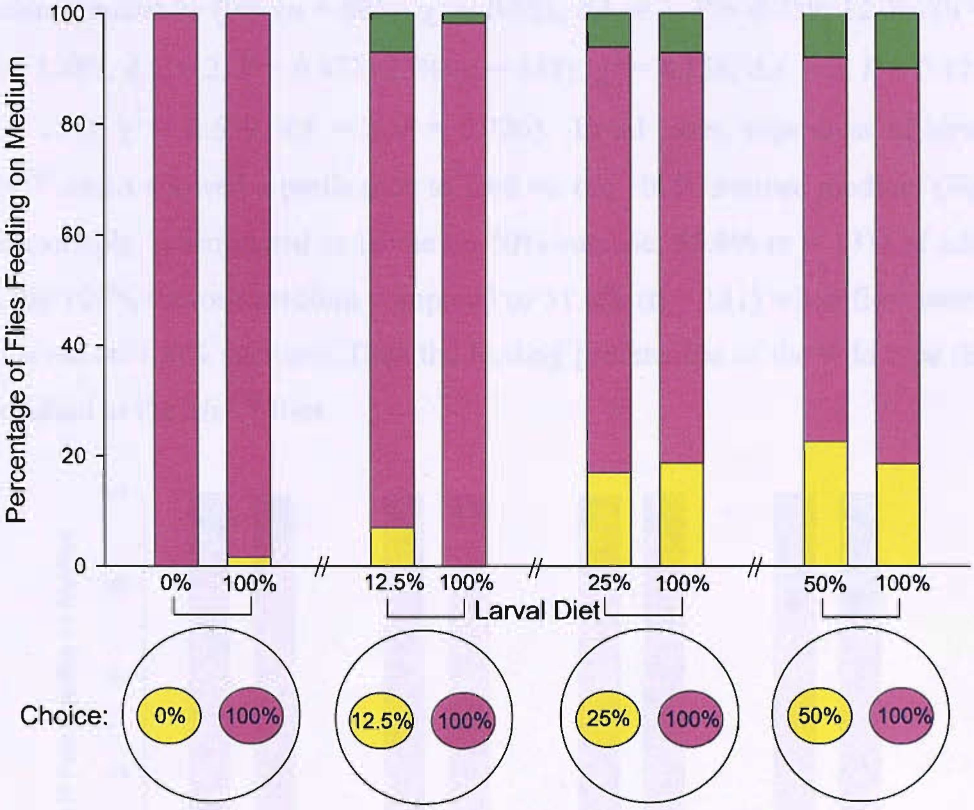


Figure 5.5: The effect of varying sucrose concentration in the larval diet on the feeding preferences of rutabaga *flies. Yellow bars show the percentage of flies feeding on the experimental medium, pink those feeding on the 100% sucrose medium and green those feeding on a mixture of the two. Larval diet had no subsequent affect on adult feeding preferences at any of the concentrations tested (n (0% sucrose) = 178; n (12.5% sucrose) = 163; n (25% sucrose) = 269; n (50% sucrose) = 277).*

5.3.4 The Effect of Varying Sucrose Concentrations in the Larval Diet on the Feeding Preferences of Mvl^{97f} *Flies*

Varying the concentration of sucrose in the larval diet had no subsequent effect on adult feeding preferences at any of the concentrations tested with the taste mutants, *malvolio* (0% (n = 585): $\chi^2 = 0.551$, d.f. = 2, $P = 0.759$; 12.5% (n = 229): $\chi^2 = 1.481$, d.f. = 2, $P = 0.477$; 25% (n = 438): $\chi^2 = 4.156$, d.f. = 2, $P = 0.125$; 50% (n = 656): $\chi^2 = 0.639$, d.f. = 2, $P = 0.726$). In all cases, regardless of larval diet, *Mvl*^{97f} adults showed a preference to feed on the 100% sucrose medium (Fig. 5.6). For example, when raised as larvae on 50% sucrose, 55.8% (n = 131) of adults fed on the 100% sucrose medium compared to 51.8% (n = 181) when flies were raised as larvae on 100% sucrose. Thus the feeding preferences of the wild type flies was abolished in the *Mvl*^{97f} flies.

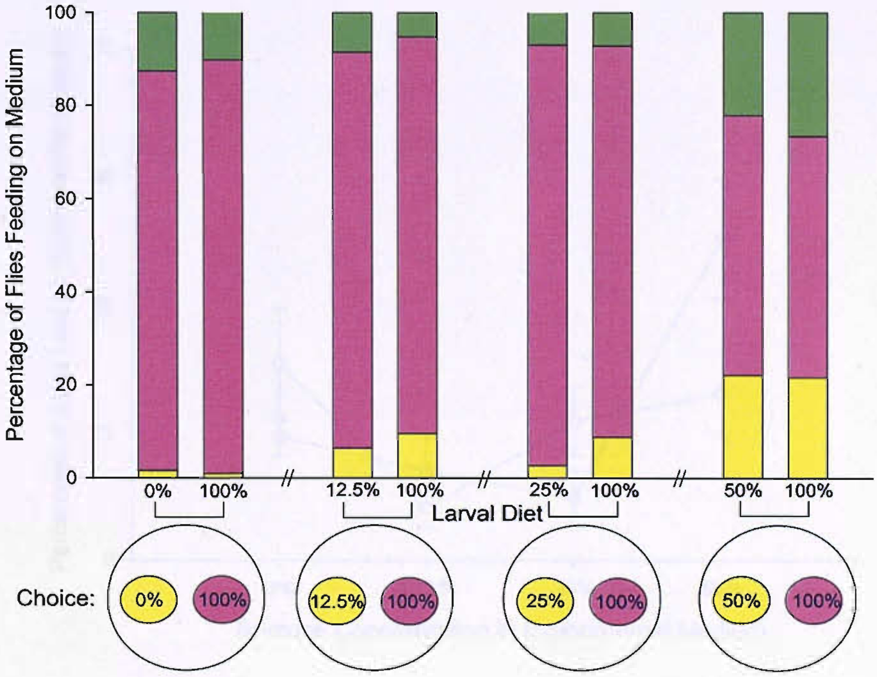


Figure 5.6: The effect of varying sucrose concentrations in the larval diet on the feeding preferences of Mvl^{97f} *flies. Yellow bars show the percentage of flies feeding on the experimental medium, pink those feeding on the 100% sucrose medium and green those feeding on a mixture of the two. Larval diet had no subsequent affect on feeding preferences of the adults at any of the concentrations tested (n (0% sucrose) = 585; n (12.5% sucrose) = 229; n (25% sucrose) = 438; n (50% sucrose) = 656).*

5.3.5 The Effect of Varying Sucrose Concentrations in the Larval Diet on the Egg Laying Preferences of Mvl^{97f} *Flies*

Varying the concentration of sucrose in the larval diet had no subsequent effect on adult egg laying preferences at any of the concentrations tested with the taste mutant *malvolio* (0% (n = 585): $F_{1,18} = 1.548$, $P = 0.229$; 12.5% (n = 229): $F_{1,18} = 0.344$, $P = 0.565$; 25% (n = 438): $F_{1,18} = 0.743$, $P = 0.400$; 50% (n = 475): $F_{1,18} = 2.284$, $P = 0.148$). In all cases, regardless of larval diet, *Mvl*^{97f} adults had a preference to lay their eggs on the 100% sucrose medium (Fig. 5.7). For example, when raised as larvae on 0% sucrose, 30.4% of the total eggs laid were laid on the 0% sucrose medium (n = 254) compared to 20.2% of the total eggs when raised as larvae on the 100% sucrose medium and as adults offered the same choices (n = 331). Thus the egg laying preferences seen in the wild type flies are abolished in the *Mvl*^{97f} flies.

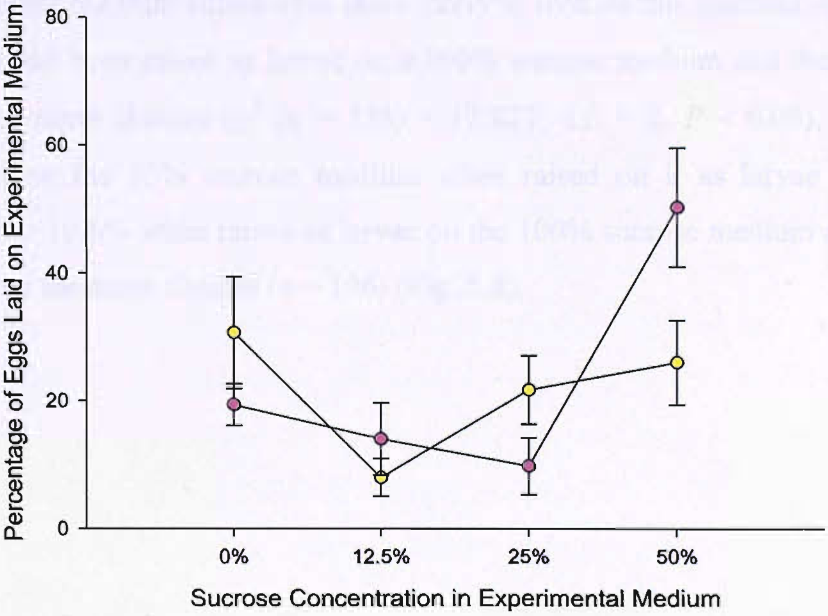


Figure 5.7: The effect of varying sucrose concentration in the larval diet upon egg laying preferences in Mvl^{97f} *flies. Means* ± *standard errors for the number of eggs laid on the reduced sucrose content medium when raised as larvae on that reduced sucrose content medium (yellow circles) or the standard, 100% sucrose, medium (pink circles). Varying the sucrose concentration in the larval diet of* Mvl^{97f} *flies had no subsequent affect on adult egg laying preference at any of the concentrations tested (n (0% sucrose) = 585; n (12.5% sucrose) = 229; n (25% sucrose) = 438; n (50% sucrose) = 475).*

5.3.6 The Effect of Ablating the Mushroom Bodies Using Hydroxyurea on Adult Food Preferences

To ensure that the protocol used did not affect the feeding preferences of the flies, some individuals underwent the same procedure as the HU ablated flies, but no HU was added to the larval food. Results showed that in this case larval diet had a significant affect on both adult feeding and egg laying preference (Figs. 5.8 and 5.9 respectively). For the feeding analysis, results showed that when raised as larvae on a 0% sucrose medium adults showed a preference to feed on this medium when compared to those that had been raised as larvae on a 100% sucrose medium and then as adults offered the same choices (χ^2 (n = 381) = 97.45, d.f. = 2, $P <$ 0.01). 61.7% of adults fed on the 0% sucrose medium when raised on it as larvae (n = 190) compared to 10.4% when raised as larvae on the 100% sucrose medium and then as adults given the same choices (n = 191) (Fig. 5.8). When raised as larvae on a 25% sucrose medium adults were more likely to feed on this medium compared to those that had been raised as larvae on a 100% sucrose medium and then as adults offered the same choices (χ^2 (n = 376) = 12.823, d.f. = 2, $P < 0.05$). 36.8% of adults fed on the 25% sucrose medium when raised on it as larvae (n = 180) compared to 19.6% when raised as larvae on the 100% sucrose medium and then as adults given the same choices (n = 196) (Fig. 5.8).

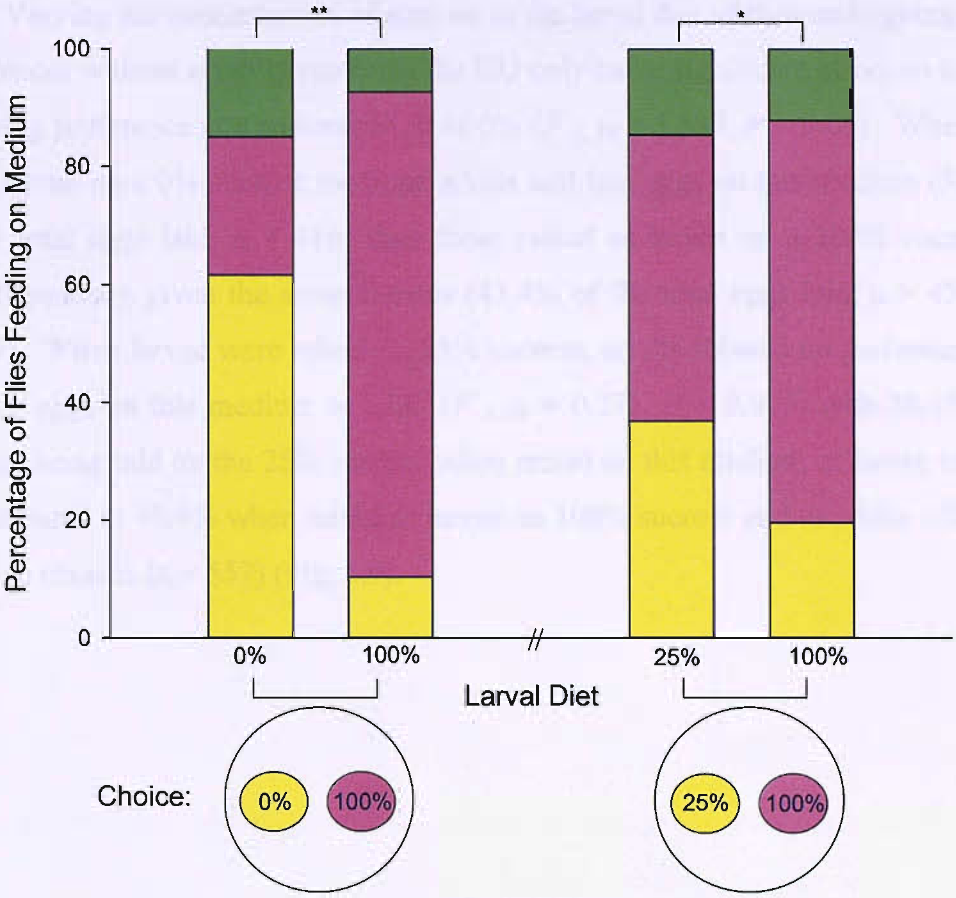


*Figure 5.8: The affect of varying sucrose concentration in the larval diet on adult feeding preferences when following the HU ablation protocol but without using HU. Yellow bars show the percentage of flies feeding on the experimental medium, pink those feeding on the 100% sucrose medium and green those feeding on a mixture of the two. * indicates a significant difference at the* $P < 0.05$ *level and ** at the* $P < 0.01$ *level. When raised as larvae on a 0% sucrose medium adults showed a preference to feed on this medium whereas if raised on 100% sucrose and then given the same choices adults show a preference for the 100% sucrose medium (n =381). When raised as larvae on 25% sucrose adults were more likely to feed on this diet than those raised as larvae on 100% sucrose and subsequently given the same choices (n =376).*

Varying the concentration of sucrose in the larval diet of flies undergoing the HU protocol without actually receiving the HU only had a significant affect on adult egg laying preference at a concentration of 0% ($F_{1, 18} = 5.587$, $P < 0.05$). When raised as larvae on a 0% sucrose medium, adults laid less eggs on this medium (35.8% of the total eggs laid; n = 416) than those raised as larvae on a 100% sucrose and subsequently given the same choices (43.4% of the total eggs laid; n = 472) (Fig. 5.9). When larvae were raised on 25% sucrose, adults showed no preference to lay their eggs on this medium as adult ($F_{1, 18} = 0.271$, $P = 0.609$) with 38.4% of the eggs being laid on the 25% sucrose when raised on this medium as larvae (n = 534) compared to 40.9% when raised as larvae on 100% sucrose and as adults offered the same choices (n = 557) (Fig. 5.9).

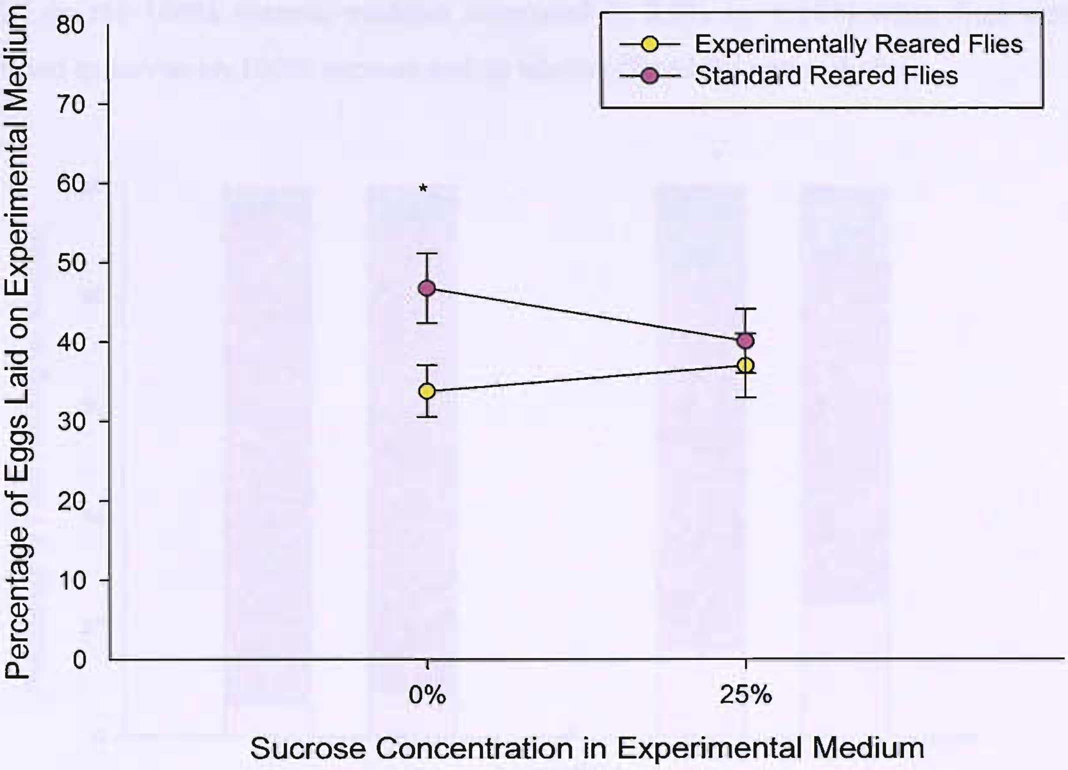


*Figure 5.9: The affect of varying sucrose concentration in the larval diet on adult egg laying preferences when following the HU ablation protocol but without using HU. Means ± standard errors for the number of eggs laid on the reduced sucrose content medium when individuals were raised via the HU protocol without being exposed to HU. Yellow circles indicate those raised as larvae on the reduced sucrose content medium and pink those raised as larvae on the standard, 100% sucrose, medium. * indicates a significant difference at the* P < *0.05 level. When raised as larvae on 0% sucrose adults were less likely to lay their eggs on this medium than those raised as larvae on 100% sucrose medium and given the same choices (n = 888). At 25% sucrose larval diet had no subsequent affect on adult egg laying preference (n = 1091).*

Varying the concentration of sucrose in the larval diet of Oregon R flies with their mushroom bodies ablated had no subsequent effect on adult feeding preferences at any of the concentrations tested (0% (n = 325): $\chi^2 = 1.990$, d.f. = 2, $P = 0.370$; 25% (n = 309): $\chi^2 = 3.828$, d.f. = 2, $P = 0.147$). In all cases, regardless of larval diet, adults showed a preference to feed on the 100% sucrose medium (Fig. 5.10). For example, when raised as larvae on 0% sucrose, 6.1% (n = 185) of adults fed on the 100% sucrose medium compared to 8.0% (n = 189) when flies were raised as larvae on 100% sucrose and as adults offered the same choices.

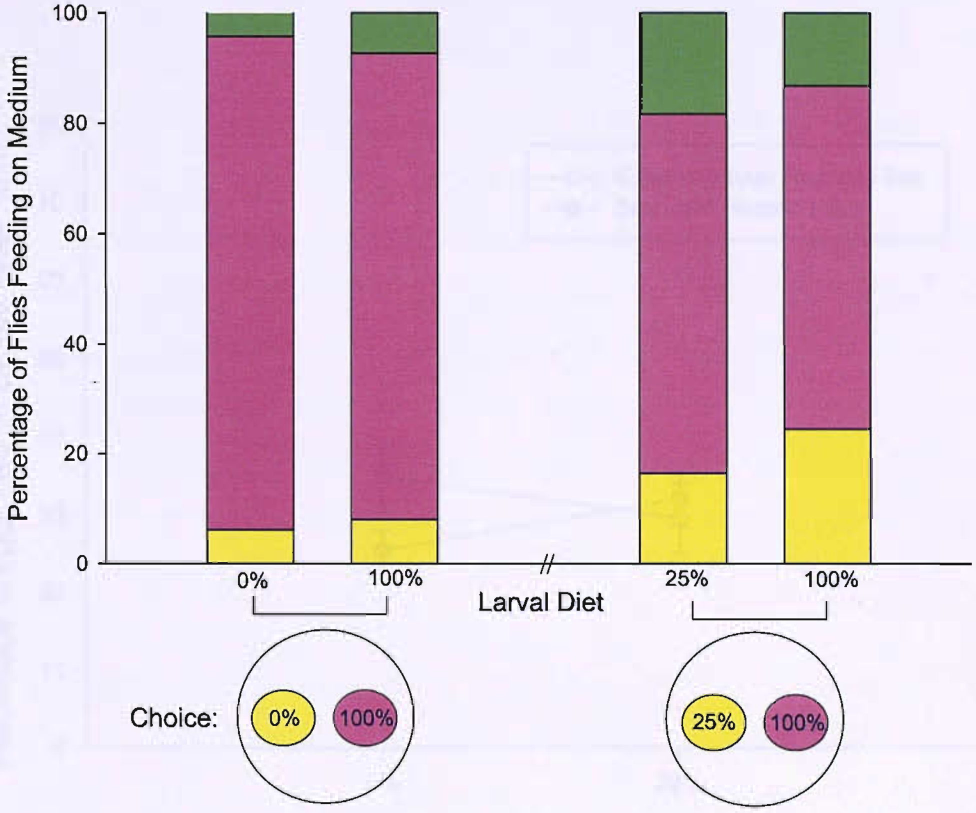


Figure 5.10: The effect of varying sucrose concentration in the larval diet on the feeding preferences of mushroom body ablated flies. Yellow bars show the percentage of flies feeding on the experimental medium, pink those feeding on the 100% sucrose medium and green those feeding on a mixture of the two. Larval diet had no subsequent affect on adult feeding preferences at any of the concentrations tested (n (0% sucrose) = 325; n (25% sucrose) = 309).

Varying the concentration of sucrose in the larval diet of Oregon R flies with their mushroom bodies ablated had no subsequent effect on adult egg laying preferences at any of the concentrations tested (0% (n = 1599): $F_{1, 18} = 3.175$, $P = 0.092$; 25% (n = 1959): $F_{1, 18} = 0.222$, $P = 0.643$). In all cases, regardless of larval diet, adults had a preference to lay their eggs on the 100% sucrose medium (Fig. 5.11). For example, when raised as larvae on 0% sucrose, 34.9% of the total eggs laid by the adults were laid on the 0% sucrose medium (n = 718) compared to 26.8% of the total eggs when raised as larvae on the 100% sucrose medium (n = 818).

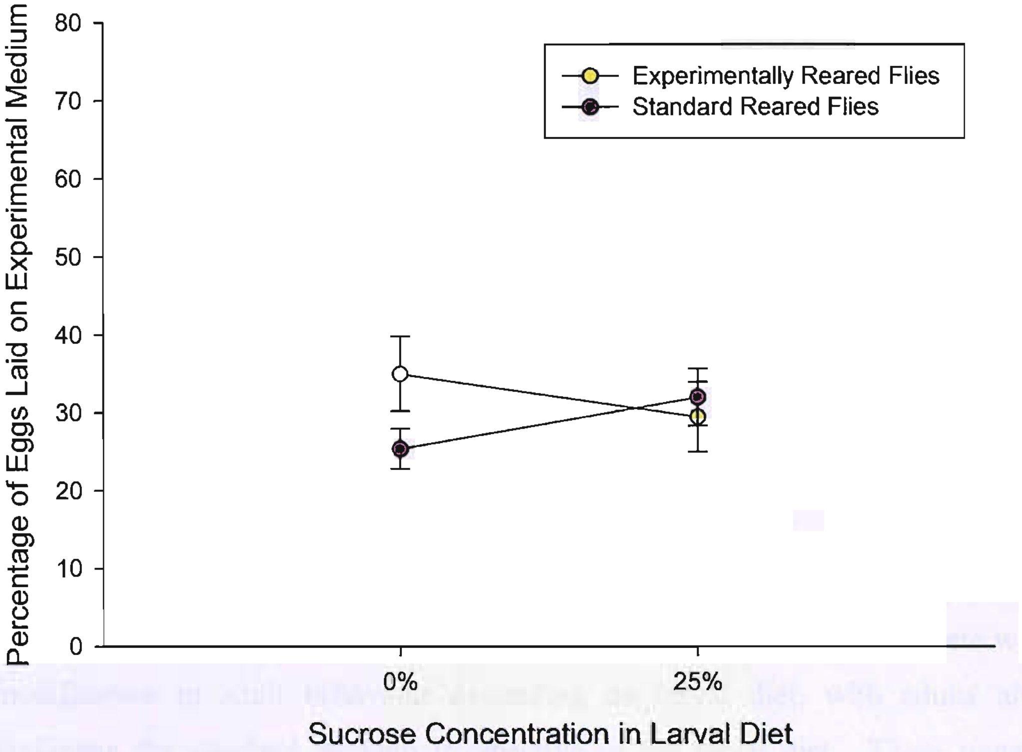


Figure 5.11: The effect of varying sucrose concentration in the larval diet upon egg laying preferences in mushroom body ablated flies. Means ± standard errors for the number of eggs laid on the reduced sucrose content medium when raised as larvae on that reduced sucrose content medium (yellow circles) or the standard, 100% sucrose, medium (pink circles). Varying the sucrose concentration in the larval diet had no subsequent affect on adult egg laying preference at any of the concentrations tested (n (0% sucrose) = 1599; n (25% sucrose) = 1959).

5.4 Discussion

5.4.1 Is Larval Diet Learnt and This Information Retained Through Metamorphosis?

When wild type *Drosophila* were raised as larvae on diets varying in sucrose concentration, this was shown to affect both adult feeding and egg laying preferences (see Chapters 3 and 4). In the case of feeding preference, when raised as larvae on either 0%, 25% or 50% sucrose the adults showed an increased preference to feed on these media compared to those raised as larvae on a 100% sucrose medium and subsequently given the same choices. For the egg laying preferences, when raised as larvae on 0% sucrose, adults were less likely to lay their eggs on this medium than if raised on a 100% sucrose diet and subsequently given the same choices.

When the learning and memory mutants *dunce* and *rutabaga* were used in these experiments, an entirely different result was observed. In these cases, there was no modification in adult behaviour depending on larval diet, with adults always preferring the standard medium irrespective of the larval diet. These mutations therefore in some way impair the flies' ability to modify their adult behaviour depending on larval diet. The *dunce* gene encodes for a cAMP phosphodiesterase, and the *rutabaga* gene a type I adenylyl cyclase (Chen *et al*, 1986; Kauvar, 1982; Levin *et al*, 1992; Livingstone *et al*, 1984). This would therefore suggest that the cAMP pathway is very important in retaining the effects of larval diet on adult feeding preferences. The cAMP signalling cascade is thought to underlie synaptic plasticity (see Section 5.1.3) and so one possible reason for the modification in adult egg laying and feeding preferences with larval diet may too be due to learning and memory. Both *dunce* and *rutabaga* are highly expressed in the MBs, the structure in the brains of insects that are thought to be involved in learning and memory (de Belle and Heisenberg, 1994; Lessing and Carlson, 1999; Rausher, 1978). It is therefore possible that it is learning and memory that is causing this modification in adult behaviour. However, the cAMP pathway is also involved in many other functions, including tasting of carbohydrates at the sensillar level (Liscia *et al*, 1989). Therefore although this data suggests that cAMP is involved in someway in the effects of larval diet on adult food choice, it does not give any indication of how it is doing this.

To further investigate the effects of larval diet on adult food preferences, the MBs were also ablated using HU. HU inhibits DNA synthesis and kills dividing cells (Timson, 1975). In the first 8 hours after larval hatching, only the MBNbs and the LNb are proliferating (Ito and Hotta, 1992; Prokop and Technau, 1991). Feeding the larvae HU during this period therefore kills MBNbs and all Kenyon cell lineages (de Belle and Heisenberg, 1994) with the exception of the 40 – 300 cells per hemisphere that developed during embyrogenesis (de Belle and Heisenberg, 1994; Ito and Hotta, 1992). By using HU, the mushroom bodies can therefore be ablated to determine whether it is these structures that are involved in the affects of larvae diet on adult feeding preferences as described in Chapters 3 and 4.

When the mushroom bodies were ablated no effect of larval diet on adult feeding or egg laying preferences were observed. To ensure that the change in protocol required in order to ablate the MBs did not affect adult behaviour, wild type flies were also treated to the same methodology as those that had the mushroom bodies ablated, but in this case the food medium did not containing HU. The results of these experiments were as found in Chapters 3 and 4, showing that the change in adult behaviour observed in the MB ablated flies must be due to the ablation and not simply due to the change in protocol used. HU has been shown to completely ablate MBs in 93.5% of flies (de Belle and Heisenberg, 1994) and the data from this chapter therefore provides strong evidence that ablation of the MBs erases the flies ability to alter adult behaviour with larvae diet. This, along with the *dunce* and *rutabaga* data therefore provides strong evidence that the change in adult food preference with larval diet is due to a change in the cAMP pathway in the mushroom bodies. It could, however, be that the disruption of the cAMP pathway in the mutants and the ablation of the mushroom bodies are acting separately but lead to the same affect on behaviour.

5.4.2 How Does the Taste Mutant malvolio *Affect the Feeding and Egg Laying Preferences of the Flies?*

When *malvolio* flies, which lack the ability to taste carbohydrates, were raised as larvae on diets varying in sucrose concentration there was no modification in adult behaviour with varying larval diet. In all cases, the adults chose to feed and lay their eggs on the 100% sucrose medium irrespective of larval diet. This is an interesting result considering the flies are unable to taste the difference in the sucrose contents of the two media. There are two possible explanations for this. Firstly, the mutants are not impaired in their ability to learn. It is therefore possible that the choice they make is due to internal feedback mechanisms. If they feed on the diet containing low sucrose concentrations there will be low concentrations of carbohydrate in their haemolymph. If they feed on the 100% sucrose medium, however, the concentration of carbohydrate in the haemolymph will increase (see Section 4.1.3). It is therefore possible that the flies are learning through a feedback mechanism which of the two diets contains the best balance of nutrients. Similar feedback mechanisms have been described in several insect species (Abisgold and Simpson, 1987; Amakawa, 2001, Friedman *et al*, 1991).

Another possible reason for these results maybe that the *malvolio* flies can taste the sucrose in the 100% sucrose medium due to its high concentration. In a study by Rodrigues *et al* (1995) the responses of adult *malvolio* flies to various carbohydrates, including sucrose, were investigated. The results showed that these flies are indeed defective in their ability to taste carbohydrates. This ability was not, however, completely lost with more than 85% of the flies extending their proboscises in response to tarsal stimulation of 100 mM sucrose. In addition, when offered a choice of agar alone or agar plus sucrose at a concentration 100 mM, 100% of the flies choose to feed on the sucrose medium. Therefore, although the *malvolio* mutation reduces the flies ability to taste sucrose, it does not completely erase it and at high enough concentrations the flies are still able to detect the sucrose. In this thesis, when the concentration of sucrose in the diet was reduced, the *malvolio* flies would be unable to detect it according to the results of Rodrigues *et al* (1995). When offered the choice of this diet or a standard diet, however, they would be able to detect the sucrose in the standard medium (which is at a concentration of 257 mM) and hence always chose to feed on that medium irrespective of larval diet. This would also be true of the egg laying data. The flies would be able to detect the sucrose in the 100% sucrose medium and would therefore lay their eggs on the reduced sucrose content medium as is seen in the wild type flies (see Chapter 3).

5.4.3 Summary

The results of this study show that the cAMP mutants *dunce* and *rutabaga* do not have the same feeding and egg laying preferences as those of wild type flies when sucrose concentration in larval diet was varied. This therefore suggests that cAMP is involved in the modification in adult food preference with larval diet. cAMP is, however, involved in many processes, including learning and memory and taste transduction. Where the affect of the defective cAMP pathway is affecting the ability of the flies to modify their adult behaviour depending on larval diet therefore remains unclear. When the MBs were ablated the effect of larval diet on adult food preference was again extinguished, suggesting that the mushroom bodies play an important role in the transfer of dietary information from thee larva to the adult. The mushroom bodies are known to be involved in learning and memory, suggesting this may be one possible mechanism that may explain the change in behaviour with larval diet.

When the concentration of sucrose was varied in the diet of the gustatory mutant *malvolio*, adults always showed a preference for the 100% sucrose medium irrespective of larval diet. This is probably due to the flies being able to detect the high concentrations of sucrose in the 100% sucrose medium but not being able to detect the sucrose in the lower concentration media.

6. Does Diet Affect Life History Traits in *Drosophila melanogaster*?

6.1 Introduction

6.1.1 The Foetal Origins of Adult Disease (FOAD)

The phenotype of an individual is determined by both genetically and environmentally. The environmental affects upon the phenotype begin very early in life with the intrauterine environment (Aerts and Van Assche, 2006). This is called programming, which can be defined as 'a process whereby a stimulus or insult, at a critical or sensitive period of development, has lasting or lifelong significance' (Lucas, 1991). For example, studies have shown that birth weight can be maternally determined by nutritional supply, with genetics playing a relatively small role (Carr-Hill *et al*, 1987). When maternal nutrition is reduced, the foetus is able to reduce its growth and development to adapt to reduced maternal nutrition (Widdowson and McCance, 1975). This allows for a means of survival under nutritionally adverse conditions (Desai and Hales, 1997). For example, for 7 months during the winter of 1944-1945 many areas of the Netherlands experienced a severe famine due to a German blockade on rail transport. This meant that no food could be bought into the Western part of the Netherlands by train and a particularly harsh winter meant that all the canals froze and so no food could be bought in by barge either. This led to official rations being imposed of less then 1000 calories per person. The famine was then abruptly ended in May 1945 when the Allied forces liberated the Netherlands and food relief was distributed. Records of birth weight from this period are available from Midwifery schools and studies of this data clearly show that malnutrition during pregnancy leads to reduced weight at birth (Stein *et al*, 1995; Stein *et al*, 2004; Stein and Susser, 1975). The effect of maternal diet on birth weight has also been described in other human populations (Andreasyan *et al,* 2007) and in other mammals (Campbell *et al*, 2005; Lister and McCance, 1965; Stewart *et al*, 1975).

It has been hypothesised that programming during foetal development, such as lowered birth rate due to malnutrition, can lead to disease in later life. This is known as the 'Foetal Origins of Adult Disease' (FOAD) or the 'Developmental Origins of Health and Disease' (DOHaD) (Aerts and Van Assche, 2006). Many studies have linked low birth weight with disease in later life, however disease can also occur due to malnutrition with no effect on birth weight (Lumey, 1998; Ravelli *et al*, 1999). Some of the adult diseases that have been associated with foetal malnutrition include diabetes mellitus and metabolic disorder (Dahri *et al*, 1995; de Rooij *et al*, 2006), cardiovascular disease (Painter *et al*, 2006; Roseboom *et al*, 2000), hypertension (Stein *et al*, 2006) and obesity (Ravelli *et al*, 1976; Ravelli *et al*, 1999; Stanner *et al*, 1997; Stein *et al*, 2007). In addition, psychological disorders have been associated with malnutrition during pregnancy such as affective disorder (Brown *et al*, 2000), antisocial personality disorder (Neugebauer *et al*, 2004) and schizophrenia (Hoek *et al*, 1998; St Clair *et al*, 2005).

6.1.2 The 'Thrifty Phenotype' and 'Predictive Adaptive Response' Hypotheses

The 'thrifty phenotype' hypothesis was suggested to try to explain the link between poor foetal growth and the increased risk of glucose intolerance and metabolic syndrome in the adult (de Rooij *et al*, 2006; Hales and Barker, 1992). The central point of the theory is that poor nutrition *in utero* and in early life leads to permanent changes which can lead to disease in later life (Hales and Barker, 1992). The theory accepts that genetics also plays a role, but proposes that the environmental factors are the dominant cause of adult disease (Hales and Barker, 2001). These permanent changes are thought to be due to selective distribution of nutrients under poor nutritional conditions so that the development of essential organs such as the brain is conserved at the cost of peripheral organs such as the liver and pancreas (Hales and Barker, 1992; Hales and Barker, 2001; Hinchliffe *et al*, 1992, Merlet – Bénichou *et al*, 1994). Reduction in the growth of certain organs leads to permanent changes in their metabolism, structure or both (Desai and Hales, 1997). There are several pieces of evidence to support this theory (Barker *et al*, 1993; Curhan *et al*, 1996; Flanagan *et al*, 2000; Hales *et al*, 1991; Hofman *et al*, 1997; McKeigue *et al*, 1998). For example, in Hertfordshire the birth weights of all babies have been measured since 1911. Follow up studies of these individuals show reduced growth in early life is strongly linked with an increased risk of developing impaired glucose tolerance and metabolic syndrome in adult life (Barker *et al*, 1993; Hales *et al*, 1991).

The thrifty phenotype hypothesis explains changes caused by under nutrition but not over nutrition (Armitage *et al*, 2005). The thrifty phenotype hypothesis was then extended by Gluckman and Hanson (2004). Their theory suggested that the foetus uses maternal nutritional status to predict the postnatal environment and therefore the changes *in utero* adapt the offspring to the external environment. This is known as a 'predictive adaptive response' (Gluckman and Hanson, 2004), and the Thrifty Phenotype Hypothesis has since been extended to include this (Hales and Barker, 2001). If this prediction is correct the phenotype is normal, however, if the prediction is wrong disease may occurs (Gluckman and Hanson, 2004). Therefore if an individual received poor nutrition *in utero* and then when born remains in an environment of poor nutrition there will be no adverse effects. Once born, however, if the individual is in an environment of increased nutrition disease is likely to occur (Gluckman *et al*, 2005). There are several pieces of evidence to support this theory. Studies into coronary heart disease (Forsen *et al*, 1999) and type 2 diabetes (Forsen *et al*, 2000; Crowther *et al*, 1998) have shown that those that were small at birth and then showed rapid catch-up growth throughout childhood had the greatest risk of developing disease in later life. It has also been argued that this theory can explain the rapid increase in diseases such as metabolic syndrome in societies undergoing rapid economic and nutritional transitions (Gluckman *et al*, 2005). In rat models, foetal under nutrition followed by postnatal over nutrition amplified the likelihood of developing metabolic and cardiovascular abnormalities in the adults (Khan *et al*, 2004; Vickers *et al*, 2000). In addition recent evidence has suggested that prenatal undernutrition can reduce voluntary locomotor behaviour and increase levels of food intake in rats (Bellinger *et al*, 2006; Vickers *et al*, 2003). This could further exacerbate the effects of maternal nutrition on disease in later life (Bellinger *et al*, 2006; Vickers *et al*, 2003).

6.1.3 The Effect of Diet on Future Generations

There is growing amounts of evidence to suggest that the changes induced in the offspring due to nutritional availability can continue on through subsequent generations (Kaati *et al*, 2002; Pembrey *et al*, 2006; Stein and Lumley, 2000; Stewart *et al*, 1975; Torrens *et al*, 2003; Zambrano *et al*, 2005). For example in a study looking at the effect of a low protein diet in rats over 12 generations, results showed that the offspring of these rats had significantly lower birth weights and when these rats were then placed onto a normal diet it took 3 generations before the birth weights of the offspring returned to normal levels again (Stewart *et al*, 1975). These low protein fed rats also showed slow growth, delayed sexual maturity, smaller adult size and increased exploratory behaviour (Stewart *et al*, 1975).

This intergenerational affect of diet upon phenotype is also apparent in the affect upon the health of the offspring (Torrens *et al*, 2003; Zambrano *et al*, 2005), including cardiovascular disease (Kaati *et al*, 2002) and diabetes mellitus (Boloker *et al*, 2002; Kaati *et al*, 2002). For example in a study using rats, females were fed either a normal or protein restricted diet during pregnancy and lactation. After weaning, these first generation (F_1) offspring received a normal diet. From these F_1 *in utero* protein restricted rats, females were selected and mated. The pregnant F_1 females were fed a normal diet throughout pregnancy and lactation. The offspring of these F_1 females (F_2) were also fed a normal diet post weaning and were again allowed to breed and the F_3 offspring again kept on a normal diet. Therefore all individuals received a normal diet apart from the F_0 females during pregnancy, hence the F_1 females were subject to protein restriction *in utero* and throughout lactation. Each generation was tested for measurements relating to glucose metabolism and results showed that all generations of rats tested where the original females were fed on a protein restricted diet had an altered glucose homeostasis. This suggest that even though all the subsequent generations received a normal diet, the protein restriction suffered by the grandmother (F_0) whilst pregnant had a lasting affect on the glucose metabolism of subsequent generations (Benyshek *et al*, 2006). In humans it is more difficult to determine the intergenerational impact of nutrition *in utero* because of highly variable genetic and environmental factors (Aerts and Van Assche, 2006). However, there is some evidence to suggest that the effect of early nutrition can continue through generations (Kaati *et al*, 2002; Pembrey *et al*, 2006; Stein and Lumley, 2000). For example, research has shown that susceptibility to diabetes is higher in offspring that have descended from grandfathers exposed to an excess of food in early life (Kaati *et al*, 2002). Similar results have been found for the effects of the grandmother's early food supply on offspring mortality (Pembrey *et al*, 2006). In addition, in Pima Indians if the mother did not suffer from gestational diabetes in her first pregnancy but did in later pregnancies then these offspring had a much higher prevalence of diabetes as adults than the first born (Pettitt, 1996).

6.1.4 What is the Underlying Mechanism Involved in Developmental Plasticity?

The results described above show that variables in the prenatal environment, such as maternal nutrition, can modify the phenotype of the offspring, and these changes can contribute to the development of disease in later life. This suggests that *in utero* nutrition alters the transcription levels of specific genes which affect metabolic pathways (Burdge *et al*, 2007a). The mechanism underlying this phenotypic plasticity is thought to involve epigenetic regulation of gene expression (Erhuma *et al*, 2007). This epigenetic regulation can be achieved by DNA methylation or covalent modifications to histones (Burdge *et al*, 2007a). Silencing of genes by DNA methylation has been well described (Bird, 2002; Weaver *et al*, 2004). DNA methylation is catalysed and maintained by DNA methyltransferases (Dnmt) (Bird, 2002) and a protein restricted diet has been shown to decrease Dnmt1 expression (Lillycrop *et al*, 2007). This provides a possible mechanism for the effect of maternal diet on the phenotype of the offspring via epigenetic regulation.

Nutrition has also been shown to affect methylation levels of several other genes (Doherty *et al*, 2000; Khosla *et al*, 2001; Lillycrop *et al*, 2005; 2007; Waterland and Jirtle, 2003). For example, rats fed a low protein diet were shown to have decreased methylation and increased expression of peroxisomal proliforator - activated receptor

(PPAR) alpha and gluoccorticoid receptor (GR) in the liver (Lillycrop *et al*, 2005). The expression of the PPARα target genes (acyl CoA oxidase (ACOX)) also increased in these protein restricted rats (Lillycrop *et al*, 2005). This supports the idea that epigenetic regulation of transcription factors can modify the activity of important metabolic pathways (Lillycrop *et al*, 2005; 2007). Altered methylation of PPARα and GR promotors induced by a protein restricted diet in early life has also been shown to continue through to the F_2 generation even when the mothers exposed to protein restriction during development received a normal diet as an adult and throughout pregnancy (Burdge *et al*, 2007b). This suggests altered methylation may also be the mechanism involved in the affect of nutrition on phenotype across generations (Burdge *et al*, 2007b).

6.1.5 What Genes are Involved in Developmental Plasticity?

Recently much work has concentrated on determining which genes are involved in the effects of *in utero* nutrition on adult disease. The work has generally been based upon a candidate gene approach, concentrating on genes known to be involved in carbohydrate and lipid metabolism and corticosteroid activity (Burdge *et al*, 2007a). There are many genes that have been implicated in the effects of maternal diet on adult health and disease. These including PPARα (Erhuma *et al*, 2007; Lillycrop *et al*, 2005), PPARγ (Erhuma *et al*, 2007), leptin (Bruce *et al*, 2006), adiponectin (Bruce *et al*, 2006; Sellayah *et al*, 2006), GR (Bertram *et al*, 2001; Lillycrop *et al*, 2005), PEPCK (Burdge *et al*, 2007b), ACOX (Lillycrop *et al*, 2005), Dnmt (Lillycrop *et al*, 2007), lipoprotein lipase (LPL) (Lillycrop *et al*, 2006), carnitine: palmitoyl transferase (CPT) (Lillycrop *et al*, 2006), nitric oxide synthase (NOS) (Casanello and Sobrevia, 2002; Khorrham *et al*, 2006), 11 β hydroxysteroid dehydrogenase (11βHSD) (Bertram *et al*, 2001), insulin receptor (IR) (Barry *et al*, 2006), insulin-like growth factors (IGF) (Regnault *et al*, 2006; Tosh *et al*, 2006), tumour necrosis factor alpha (TNFα) and glucose transporter 4 (GLUT4) (Barry *et al*, 2006; Wyrwoll *et al*, 2006).

6.1.6 The Effect of Diet on Life History Traits in Insects

Much of the data so far described has looked at the effect of maternal diet on health and development in mammalian model organisms. This is because data in humans is only available when people suffer famine. It may however be possible that invertebrates such as *Drosophila* could provide a good model system for trying to gain a further understanding of the effect of diet upon future health. All animals obtain energy and nutrients through their food and this is a key factor affecting life history components (Taylor *et al*, 2005) and therefore insects may also provide a suitable model for looking at the effects of diet upon life history traits.

This has been studied to some degree in relation to pest control and evolution but has never been looked at in relation to human health. In relation to pest control, this has been an important area of research as it allows for better understanding of host suitability of pest species (Azidah and Sofian-Azirun, 2006). In the pest control studies, there has been shown to be relationships between diet and measures such as starvation resistance (Burger *et al*, 2007; Chippindale *et al*, 1997), fecundity (Burger *et al*, 2007; Butler and O'Neil, 2007; Kyneb and Toft, 2004; Naya *et al*, 2007; Roff and Gelinas, 2003), development time (Azidah and Sofian-Azirun, 2006; Butler and O'Neil, 2007; Kyneb and Toft, 2004), mortality/survival (Butler and O'Neil, 2007; Kyneb and Toft, 2004) and life span (Burger *et al*, 2007; Butler and O'Neil, 2007; Chippindale *et al*, 1997). However, in most of these studies the different diets have not been of a defined composition but rather have been food types found in the insects natural environment (Butler and O'Neil, 2007; Kyneb and Toft, 2004; Roff and Gelinas, 2003). Some have, however, looked at the effect of specific components of the diet on life history traits. For example, a protein rich diet has been shown to increase fecundity, whereas a carbohydrate rich diet decreases fecundity in the harvestman (Naya *et al*, 2007). In addition, dietary restriction by reduction in yeast and sucrose concentrations increases starvation resistance in early life but decreases it in later life (Burger *et al*, 2007). Interestingly, there is also some evidence that diet can affect life history characteristics over several generations (Amarillo–Suarez and Fox, 2006; Fox *et al*, 1995; Spitzer, 2004). For example, in the seed-feeding beetle (*Stator limbatus*) offspring matured at a faster rate when the mothers had been reared on *Pseudosamanea guachapele* compared to mothers that had been reared on *Acacia greggii,* irrespective of offspring diet (Amarillo–Suarez and Fox, 2006). The fact that diet clearly affects many traits in insects means that invertebrates may provide a good model for gaining a further understanding of the effects of diet upon the health of future offspring.

6.1.7 Aims and Objectives

The aim of this Chapter was to determine whether diet can affect factors associated with the health and development of *Drosophila.* As already discussed, maternal diet can have a profound affect on the future health of offspring in mammals and these experiments aim to determine if the same is true in *Drosophila.* In order to do this, several measurements will be made throughout the life cycle of the flies raised on varying diets. These measurements include locomotion, development time, fecundity, survival and starvation resistance. The concentration of either sucrose or yeast was varied for these experiments, the concentrations used being 0%, 12.5%, 50% or 100% of the concentrations found in the standard diet.

6.2 Methods

6.2.1 Outline of Methods Used to Determine the Affect of Diet on Life History Traits in Drosophila

40 females and 20 males placed into egg laying pots for 24 hrs (6.2.2)

↓

Adults removed and number of eggs counted (6.2.2)

↓

80 eggs collected and placed into a fresh vial (6.2.2)

↓

One L3 larvae per vial was collected and its fitness measured (6.2.3)

↓

Number of pupae developing per day counted (6.2.4)

↓

Mature pupae removed and starvation resistance of these adults tested (6.2.5)

↓

Number of adults eclosing per day counted and collected (6.2.4)

↓

These adults were then used for egg laying, starting from the beginning again for the next generation (6.2.4)

Figure 6.1: An outline of the method used to determine the effect of diet upon several indicators of health in flies.

This chapter looks at the effects of the various diets on life history traits in *Drosophila* over five generations. Figure 6.1 shows an outline of the methods used and this is followed below by a more detailed description of each step.

6.2.2 Egg Laying and Collection

40 3 – 8 d old females and 20 3 – 8 d old males were anaesthetised and placed into an egg laying pot. A 55 mm Petri dish containing a specified diet was placed over the open end of the egg laying pot. This was repeated 10 times per food type, and the food types used were 0%, 12.5%, 25%, 50% or 100% sucrose or yeast (see Section 2.5). These egg laying pots were placed into the incubator for 24 hrs. After 24 hrs, the flies were anaesthetised, removed and discarded. The number of eggs laid on each plate was counted under the microscope. 80 eggs were then collected using a paint brush and placed into vial containing the same food type as was in the Petri dish for egg laying. 10 vials were set up per food type per generation. If there was not enough eggs were laid to set up the 10 vials from these egg lays they were repeated until enough eggs had been collected. These vials were then placed into the incubator.

6.2.3 Larval Fitness

The vials were checked daily for the emergence of pupae. The day after the first pupae had developed an L3 larva was gently taken from out of the food using a paint brush. The larval stage was determined based on size. The larva was placed into a piece of tubing with some fine mesh attached to one end. It was then gently washed with distilled water to remove any food left on the larva. Once clean, the larva was placed on a piece of tissue to dry off. The larva was then placed into the centre of a 90 mm Petri dish containing 17 ml 3% agar. Underneath this Petri dish was a grid with squares 5 mm x 5 mm. The number of squares the whole body of the larva entered over in 5 mins was counted. This was repeated 10 times per food type per generation, 1 larva taken from each vial. The Petri dish was washed thoroughly before each experiment, and the Petri dish changed every 5 tests, or sooner if required.

6.2.4 Development Time and Survival

Once the eggs had been placed into the vials as described in Section 6.2.2, the vials were checked on a daily basis and the number of pupae developing each day was counted. In order to count the pupae accurately, a mark was put on the vial by each pupa when it appeared and the total number of pupae noted. This count was conducted daily until 5 days had elapsed with no further pupae developing. In addition to this, the number of flies eclosing each day was also counted. This was done by collecting the adults each day using a pooter. These adults were then placed into fresh vials of the same food type to make sure that no additional eggs would be laid in the vials. The adults were collected on a daily basis until 5 days had passed with no more eclosing. Once these five days had passed with no adults or pupae emerging the vial was discarded. By counting the number of pupae and adults on a daily basis, the total number of pupae and adults was also established, so survival through to the pupae and adult stages was also determined.

The adults that were collected from these experiments were then used to set up the egg laying (see Section 6.2.2) for the next generation, so starting the experiment from the beginning again for the next generation. This was repeated for 5 generations for each food type. However, in some cases the flies did not survive until the 5th generation and in these cases the experiments had to be stopped earlier.

6.2.5 Starvation Resistance

20 mature male and 20 mature female pupae per food type per generation were collected (see Section 3.2.6) and washed (see Section 3.2.7). These pupae were then placed into fresh vials containing only damp kimwipe paper, 10 per vial with the sexes

separated. The males and females were kept in separate vials so that as adults they would not mate. The following day the eclosed flies were collected using a pooter and transferred into a fresh vial again containing only damp kimwipe paper. This was done to ensure that the adults did not feed on the pupal cases or any minute traces of food that may be left on them. These vials were then checked daily for dead adults and the age of each fly upon death was recorded. The flies were transferred to fresh vials every 2 days to ensure that there was always a fresh supply of water available. Any dead flies were removed to ensure that those still alive did not feed on the carcasses. The experiment continued until all flies were dead.

6.2.6 Statistical Analysis of Life History Trait Data

Where possible, a 2-way ANOVA was conducted comparing the effect of both diet and generation on each of the life history traits described above. Before the 2-way ANOVA was conducted, the data was first transformed using the arcsine transformation for proportional data or the log transformation for all other data. All data was also tested for a normal distribution using the Kolmogorov-Smirnov test and for homogeneity of variance using the Levene's test. Where the data failed to meet the assumptions of the 2-way ANOVA a nonparametric 2-way ANOVA (the Scheirer-Ray-Hare test) was used in its place. All statistics were carried out in SPSS version 14.0.

6.3 Results

6.3.1 The Effect of Varying the Diet Over 5 Generations on Fecundity

When sucrose concentration in the diet was varied over 5 generations results showed that both diet and generation had a significant effect on fecundity. In addition, there was also a significant interaction of both diet and generation (see Table 6.1).

Factor	d.f.	SS	MS	*F*-ratio	*P*-value
Diet	4	215.168	53.792	15.422	<0.01
Generation	5	817.825	163.565	46.894	<0.01
Interaction	20	256.123	12.806	3.672	<0.01
Error	252	878.970	3.488		

Table 6.1: Results of a 2-way ANOVA conducted to determine the effect of varying sucrose concentration in the diet over 5 generations on fecundity.

Post hoc tests were conducted to determine which groups significantly differed. As sucrose concentration in the diet increased so the number of eggs laid by the flies decreased (Fig. 6.1). When raised on 0% sucrose, more eggs were laid by these adults than for any other medium. When raised on 12.5% or 25% sucrose, more eggs were laid by these adults than those raised on 100% sucrose. When raised on either 50% or 100% sucrose, fewer eggs were laid than on any other medium. There was also a significant affect of generation on fecundity, with there being an initial increase in number of eggs laid from generation 0 to generation 1 followed by a slow decrease over the following generations for all food types (Fig. 6.2). The effect of diet and generation also significantly interacted, as can be seen in Figure 6.3. For all food types apart from 100% sucrose, there was an increase in fecundity between generation 0 and generation 1. This then decreased a little in generations 2-3 and levelled out in the following generations.

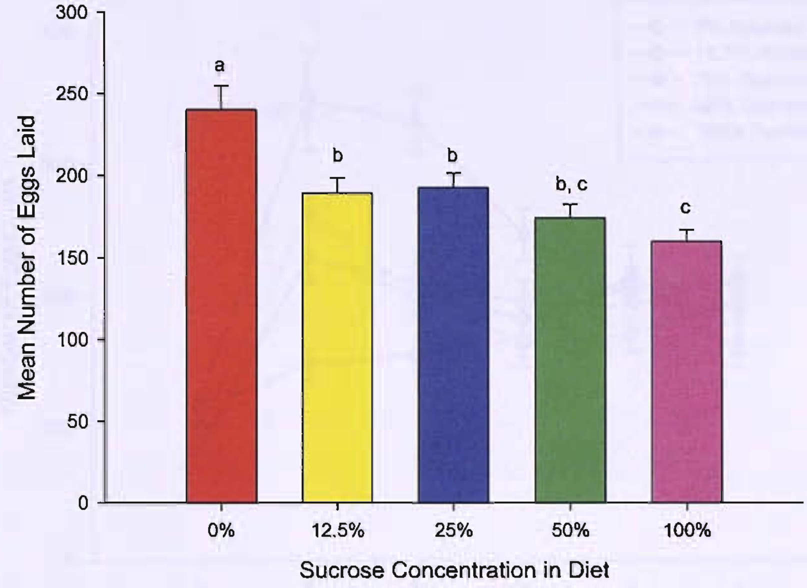


Figure 6.2: The effect of sucrose concentration in the diet on fecundity. Bars represent the mean ± standard error for the number of eggs laid. Letters above the bars indicate those that do not significantly differ. Significance is at the $P < 0.05$ *level. Results showed that as sucrose concentration in the diet increased so the number of eggs laid by the adults decreased.*

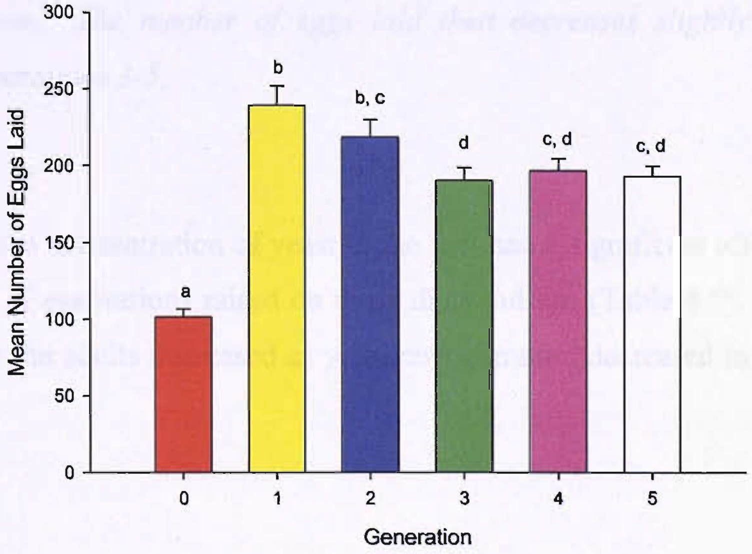


Figure 6.3: The effect of generation on fecundity when raised on varying concentrations of sucrose. Bars represent mean ± standard error for the number of eggs laid. Letters above the bars indicate no significant difference. Significance is at $P < 0.05$*. Results show that there was an initial increase in the number of eggs laid between generations 0 and 1. This then decreased slightly before levelling out in generations 3 – 5.*

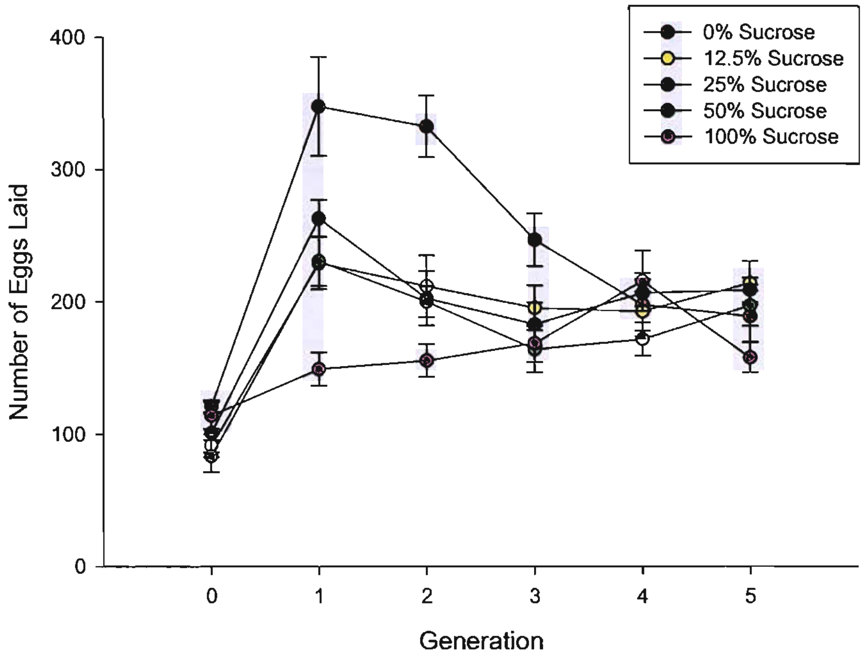


Figure 6.4: The effect of sucrose concentration in the diet on fecundity over 5 generations. Data points represent mean ± standard error for the number of eggs laid. Results show that both diet and generation have a significant effect on fecundity, with the number of eggs being laid initially increasing for all food types apart from 100% sucrose. The number of eggs laid then decreases slightly and levels out between generations 3-5.

Varying the concentration of yeast in the diet had a significant affect on fecundity but number of generations raised on these diets did not (Table 6.5). The number of eggs laid by the adults decreased as yeast concentration decreased in the diet (Figure 6.9).

	SS	SS/MS_{Total}	d.f.	*P*-value
Concentration	99335.575	18.922	2	<0.01
Generation	7226.120	1.376	5	0.93
Interaction	24781.367	4.721	9	0.86

Table 6.2: Results of a nonparametric 2-way ANOVA conducted to determine the effect of varying yeast concentration in the diet over 5 generations on fecundity.

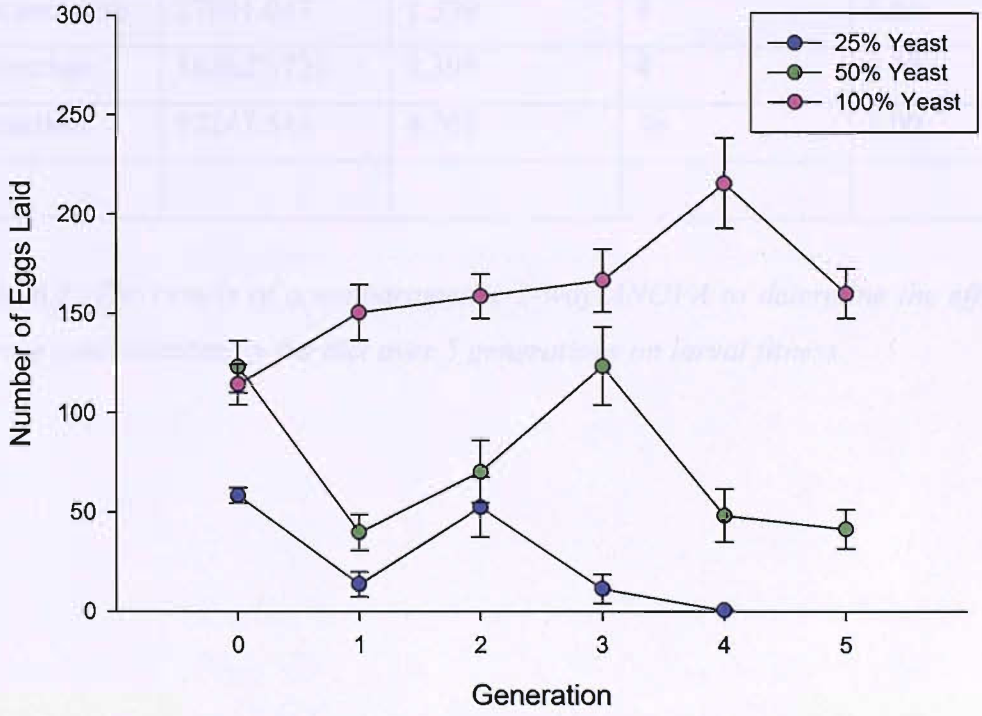


Figure 6.5: The effect of varying yeast concentration in the diet on fecundity over 5 generations. Data points represent mean ± standard error for the number of eggs laid. Results show that as yeast concentration decreased so the number of eggs laid also decreased. Generation had no effect on number of eggs laid.

6.3.2 The Effect of Diet Over 5 Generations on Larval Fitness

Varying sucrose or yeast concentration in the diet had no subsequent affects on larval fitness at any of the concentrations or generations tested (Tables 6.7 and 6.11 respectively). Although not significant, there does appear to be a trend for a decrease in larval fitness across the generations with these diets (Figs. 6.11, 6.12, 6.13, 6.14 and 6.15 respectively).

	SS	SS/MS_{Total}	d.f.	*P*-value
Concentration	27091.043	1.399	4	0.84
Generation	162627.726	8.395	4	0.08
Interaction	92247.544	4.762	16	1.00

Table 6.3: The results of a nonparametric 2-way ANOVA to determine the effects of sucrose concentration in the diet over 5 generations on larval fitness.

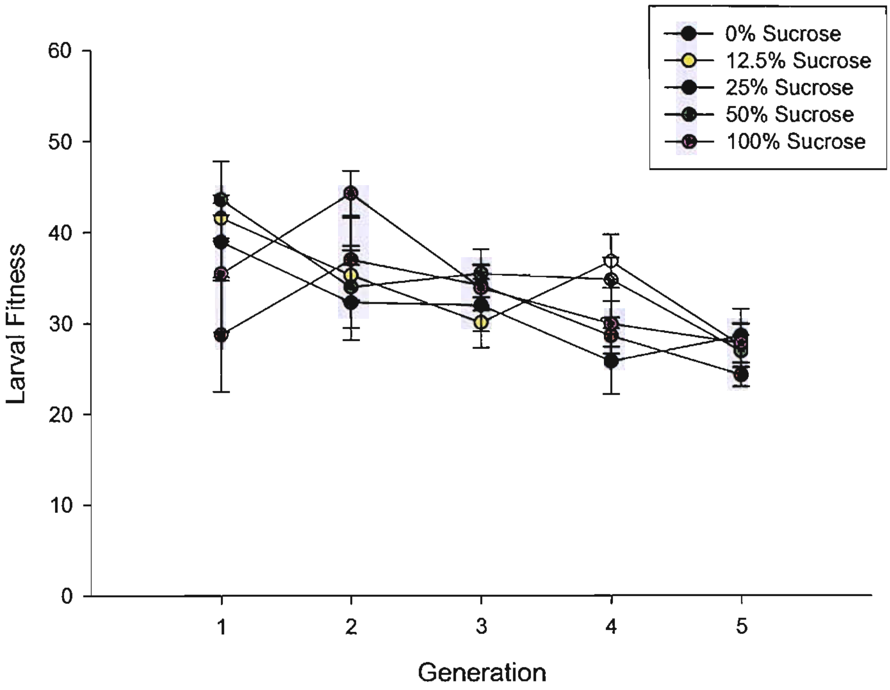


Figure 6.6: The effect of varying sucrose concentration in the diet over 5 generations on larval fitness. Results showed no significant affect of sucrose concentration in the diet on larval fitness for any of the generations.

	SS	SS/MS_{Total}	d.f.	*P*-value
Concentration	1431.957	2	2.367	0.31
Generation	3200.182	4	5.288	0.26
Interaction	1408.693	7	2.328	0.94

Table 6.4: The results of a nonparametric 2-way ANOVA to determine the effects of yeast concentration in the diet over 5 generations on larval fitness.

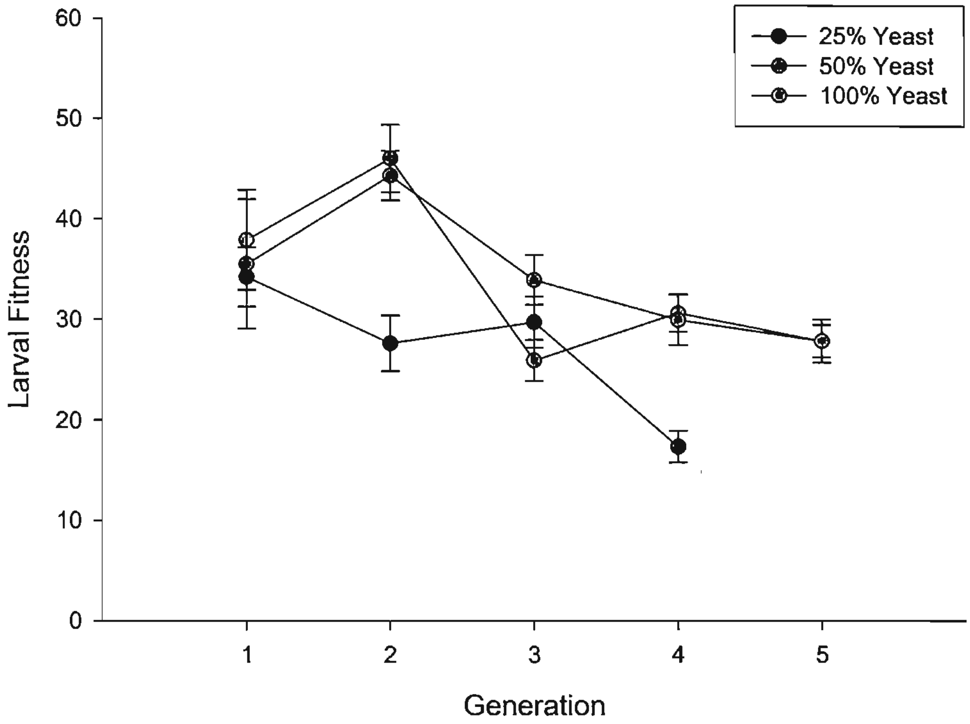


Figure 6.7: The effect of varying yeast concentration in the diet over 5 generations on larval fitness. Results showed no significant affect of yeast concentration in the diet on larval fitness for any of the generations.

6.3.3 The Effect of Diet Over 5 Generations on Development Time

Varying the concentration of sucrose in the diet had a significant affect on the development time of both the pupae and the adults (Tables 6.13 and 6.14 respectively). The time taken for both the pupae and the adults to develop was higher when raised on either a 0% or 100% sucrose diet compared to those raised on 12.5%, 25% or 50% sucrose (Figs. 6.17 and 6.18).

	SS	SS/MS_{Total}	d.f.	P-value
Concentration	634699.385	30.649	4	<0.01
Generation	42258.692	2.041	4	0.73
Interaction	116539.996	5.628	16	0.99

Table 6.5: The results of a nonparametric 2-way ANOVA to determine the effects of sucrose concentration in the diet over 5 generations on pupal development time.

	SS	SS/MS_{Total}	d.f.	P-value
Concentration	618451.716	29.857	4	<0.01
Generation	70264.029	3.392	4	0.49
Interaction	108874.799	5.256	16	0.99

Table 6.6: The results of a nonparametric 2-way ANOVA to determine the effects of sucrose concentration in the diet over 5 generations on adult development time.

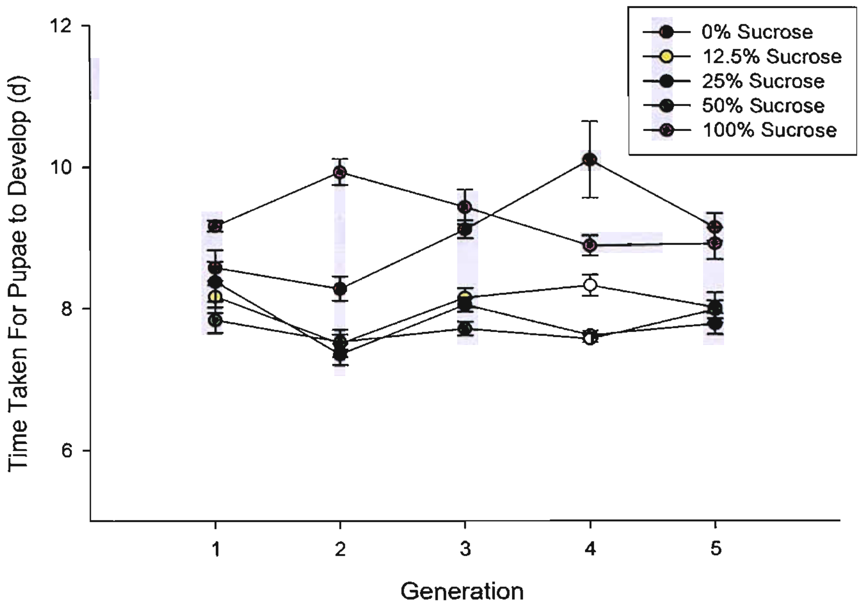


Figure 6.8: The effect of varying sucrose in the diet on the time taken for the pupae to develop over 5 generations. Data points represent means ± standard errors for number days taken for the pupae to develop. Results show that when raised on 0% or 100% sucrose pupae took longer to develop than when raised on 12.5%, 25% or 500% sucrose.

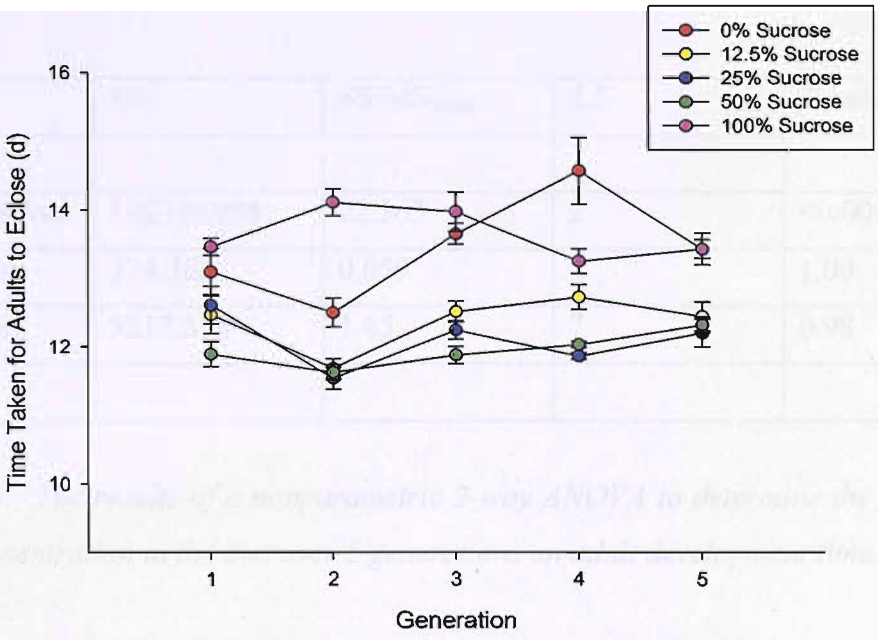


Figure 6.9: The effect of varying sucrose in the diet on the time taken for the adults to eclose over 5 generations. Data points represent means ± standard errors for number days taken for the adults to eclose. Results show that when raised on 0% or 100% sucrose adults took longer to eclose than when raised on 12.5%, 25% or 50% sucrose.

Varying the concentration of yeast in the diet had a significant affect on development time of both pupae and adults (Tables 6.21 and 6.22). In both cases as yeast concentration in the diet decreased so development time increased (Figs. 6.25 and 6.26). The number of generations the flies were kept on the diets had no effect on their development time (Tables 6.21 and 6.22).

	SS	SS/MS_{Total}	d.f.	*P*-value
Concentration	173028.556	26.827	2	<0.01
Generation	984.534	0.152	4	1.00
Interaction	7919.683	1.228	7	0.99

Table 6.7: The results of a nonparametric 2-way ANOVA to determine the effects of yeast concentration in the diet over 5 generations on pupal development time.

	SS	SS/MS_{Total}	d.f.	*P*-value
Concentration	142390.054	22.363	2	<0.001
Generation	374.362	0.059	4	1.00
Interaction	9217.558	1.45	7	0.98

Table 6.8: The results of a nonparametric 2-way ANOVA to determine the effects of yeast concentration in the diet over 5 generations on adult development time.

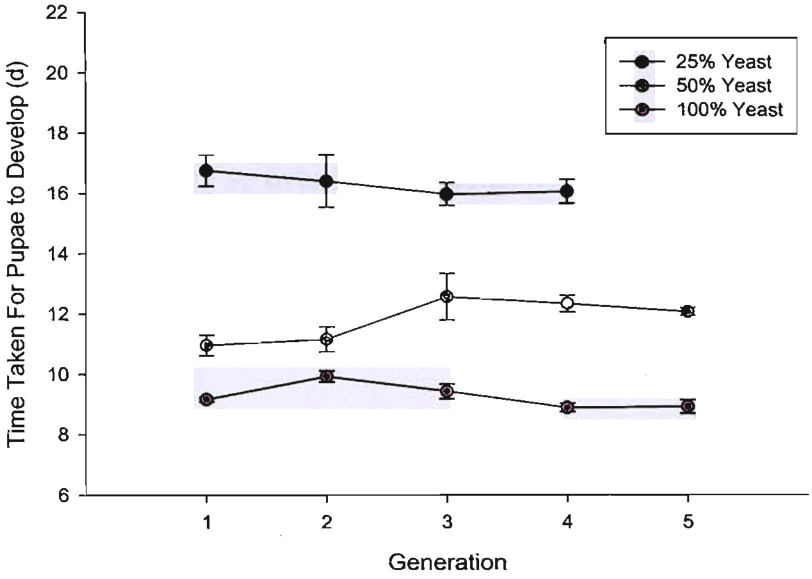


Figure 6.10: The effect of varying yeast in the diet on the time taken for the pupae to develop over 5 generations. Data points represent means ± standard errors for number days taken for the pupae to develop. Results show that as the concentration of yeast in the diet decreased so the time taken for the pupae to develop increased. Keeping the flies on these diets over 5 generations did not affect the time taken for the pupae to develop.

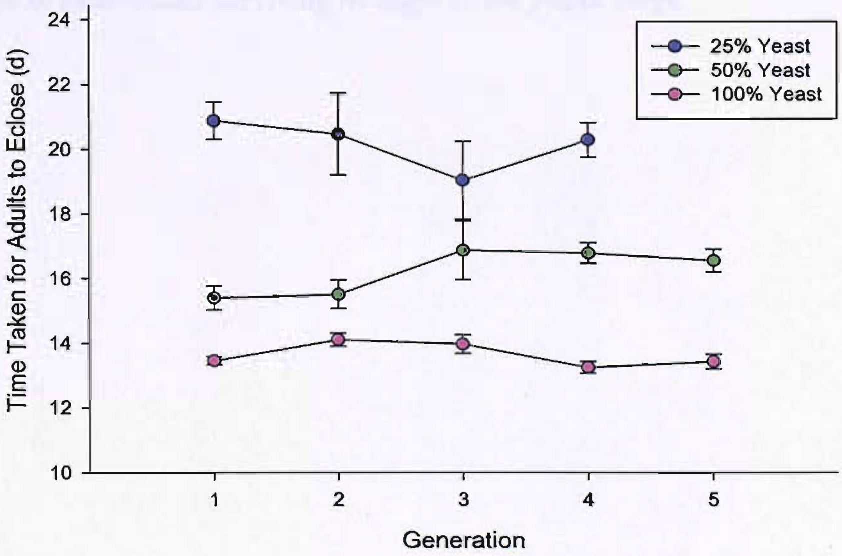


Figure 6.11: The effect of varying yeast in the diet on the time taken for the adults to develop over 5 generations. Data points represent means ± standard errors for number days taken for the adults to develop. Results show that as the concentration of yeast in the diet decreased so the time taken for the adults to develop increased. Keeping the flies on these diets over 5 generations did not affect the time taken for the adults to develop.

6.3.4 The Effect of Diet Over 5 Generations on Survival

Varying the concentration of sucrose alone had no significant affect on pupal survival (Fig 6.29). Doing so over 5 generations, however, did (Table 6.25). As the number of generations the flies were kept on the reduced sucrose content medium increased so the numbers surviving through to the pupal stage also increased for all concentrations (Fig. 6.30).

	SS	SS/MS_{Total}	d.f.	*P*-value
Concentration	27969.376	1.428	4	0.83
Generation	214253.096	10.937	4	< 0.05
Interaction	142260.344	7.262	16	0.97

Table 6.9: The results of a nonparametric two-way ANOVA conducted to determine the effect of varying sucrose concentration in the diet over 5 generations on the proportion of individuals surviving through to the pupal stage.

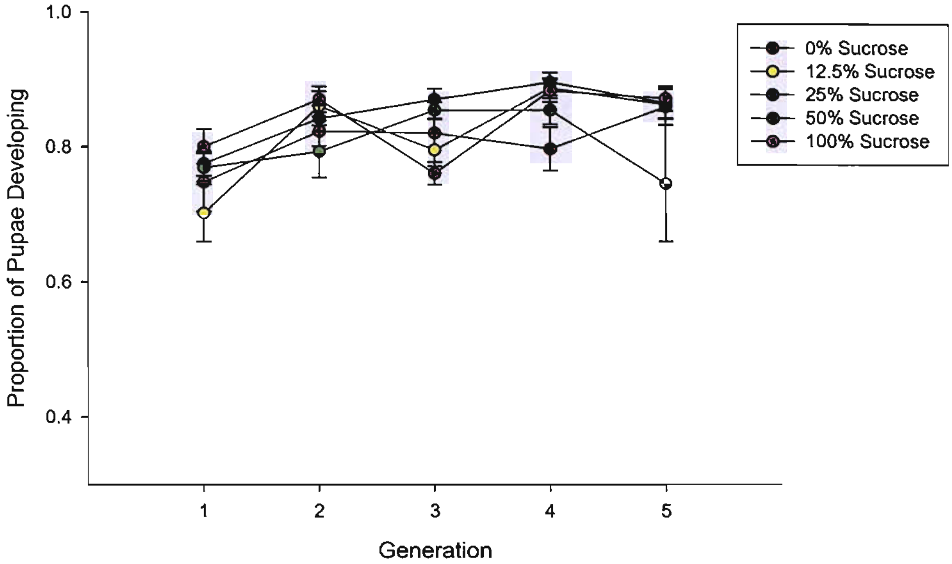


Figure 6.12: The effect of varying sucrose concentration in the diet over 5 generations on the number of pupae developing. Data points indicate mean ± standard error for the number of pupae developing from the 80 eggs transferred into each vial. Results showed that sucrose concentration in the diet had no significant affect on pupal survival. The more generations the flies were kept on the reduced sucrose content media, the more pupae survived. This can be seen more clearly in Fig. 6.30.

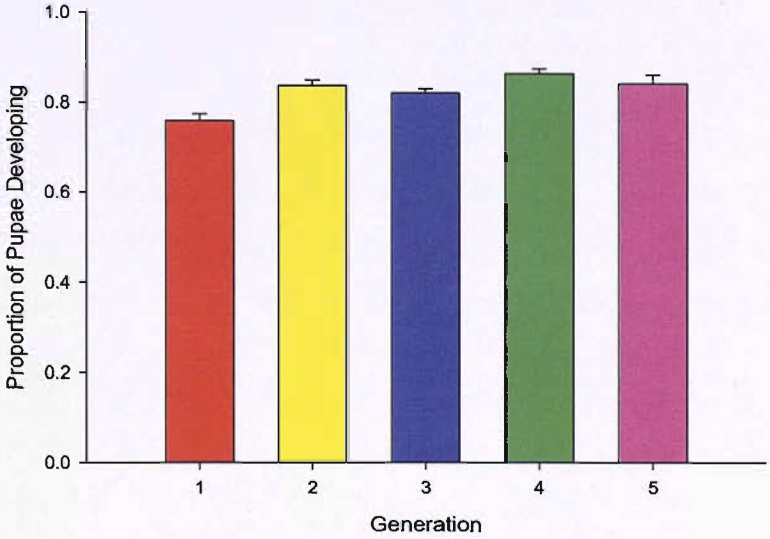


Figure 6.13: The effect of the number of generations the flies were kept on a reduced sucrose content medium on pupal survival. Bars indicate means ± standard errors for the number of pupae developing from the 80 eggs placed in to each vial. Results show as the number of generations on the reduced sucrose content medium increased so too does the number of pupae developing.

Varying the concentration of sucrose in the diet also had a significant affect on adult development (Table 6.26). The number of adults developing was highest for those raised on a 50% or 100% sucrose diet (Fig. 6.31). The number of generations the flies were kept on these diets had no significant affect on numbers of adults developing.

	SS	SS/MS_{Total}	d.f.	*P*-value
Concentration	358436.664	18.312	4	< 0.01
Generation	16481.624	0.842	4	0.93
Interaction	68159.496	3.482	16	1.00

Table 6.10: The results of a nonparametric two-way ANOVA conducted to determine the effect of varying sucrose concentration in the diet over 5 generations on the proportion of adults developing from the total number of pupae that developed.

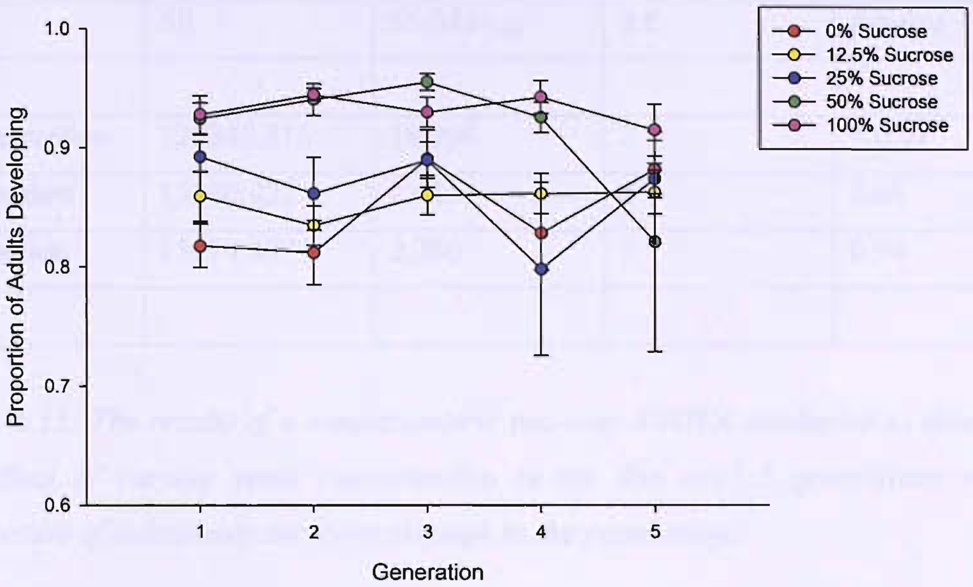


Figure 6.14: The effect of varying sucrose concentration in the diet over 5 generations on the number of adults developing. Data points indicate mean ± standard error for the number of adults developing from the number of pupae that developed. Results showed that at high concentrations of sucrose (50% and 100%) the number of adults developing is higher than for those raised on 0%, 12.5% or 35% sucrose. The number of generations the flies were kept on these diets had no effect on the number of adults developing.

When yeast concentration in the diet increased so the proportion of individuals surviving to the pupal stage increased (Table 6.33; Fig. 6.41). Varying the concentration in the diet did not, however, have any effect on the number of adults developing from these pupae (Table 6.34; Figure 6.42). Generation had no effect on either pupal or adult survival.

	SS	SS/MS$_{Total}$	d.f.	*P*-value
Concentration	120342.313	18.996	2	< 0.01
Generation	15300.422	2.415	4	0.66
Interaction	15074.857	2.380	7	0.94

Table 6.11: The results of a nonparametric two-way ANOVA conducted to determine the effect of varying yeast concentration in the diet over 5 generations on the proportion of individuals surviving through to the pupal stage.

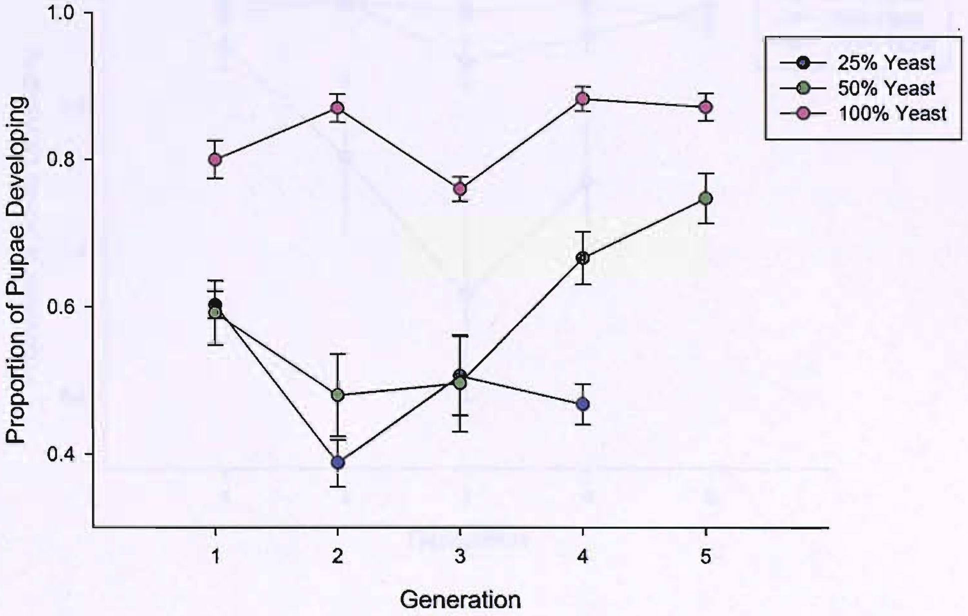


Figure 6.15: The effect of varying yeast concentration in the diet over 5 generations on the number of pupae developing. Data points indicate mean ± standard error for the number of pupae developing from the 80 eggs transferred into each vial. Results showed that as yeast concentration in the diet increased so the time taken for the pupae to develop decreased.

	SS	SS/MS$_{Total}$	d.f.	*P*-value
Concentration	4914.895	0.862	2	0.65
Generation	5868.739	1.029	4	0.91
Interaction	6142.887	1.078	7	0.99

Table 6.12: The results of a nonparametric two-way ANOVA conducted to determine the effect of varying yeast concentration in the diet over 5 generations on the proportion of adults eclosing from the total number of pupae that developed.

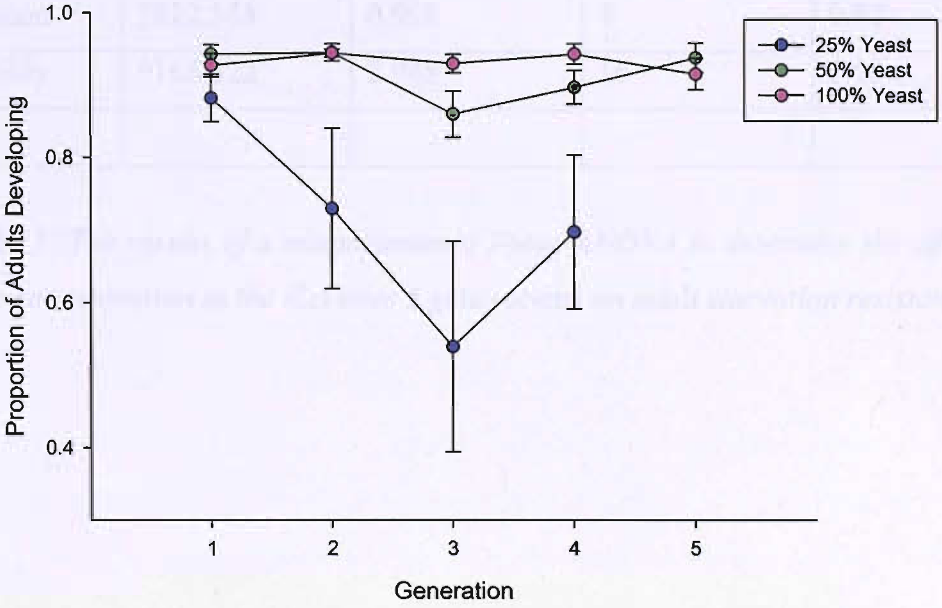


Figure 6.16: The effect of varying yeast concentration in the diet over 5 generations on the number of adults developing. Data points indicate mean ± standard error for the number of adults developing from the total pupae that developed. Results showed that yeast concentration in the diet had no effect on the number of individuals surviving to the adult stage.

6.3.5 The Effect of Diet on Starvation Resistance Over 5 Generations

Varying the concentration of sucrose or yeast in the diet had no significant effect on starvation resistance for either food type tested. Results showed that for sucrose, adults survived on average for between 3 and 5 days (Table 6.37; Fig. 6.45). When yeast concentration in the diet was varied, flies survived for 5-6 d when starved (Table 6.41; Fig. 6.49). These results therefore show that when raised on low yeast flies are able to resist starvation for longer than those on a varied sucrose diet.

	SS	SS/MS_{Total}	d.f.	*P*-value
Concentration	23776.488	7.648	4	0.11
Generation	2822.588	0.908	4	0.92
Interaction	9166.322	2.948	16	1.00

Table 6.13: The results of a nonparametric 2-way ANOVA to determine the effects of sucrose concentration in the diet over 5 generations on adult starvation resistance.

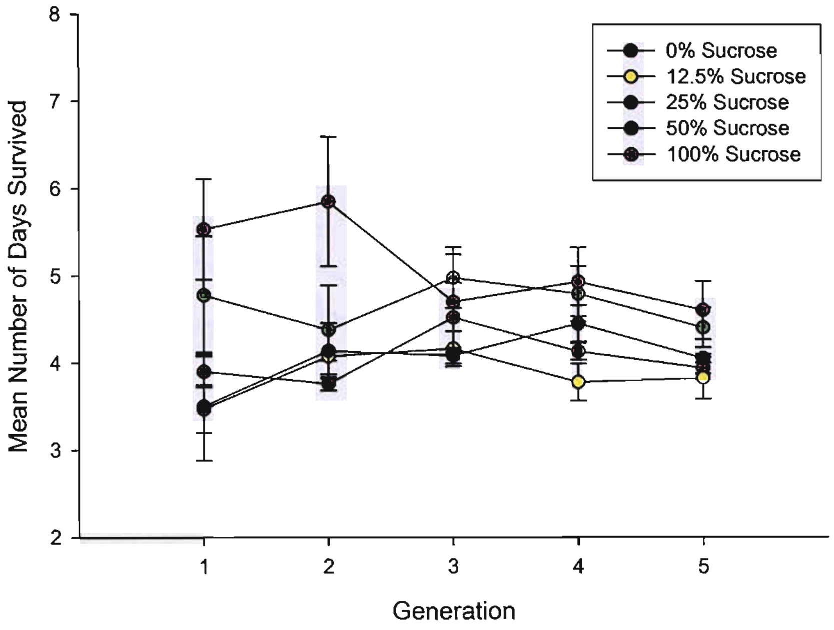


Figure 6.17: The effect of varying sucrose concentration in the diet over 5 generations on the starvation resistance of adult flies. Data points represent the mean ± *standard error for the number of days survived. Results show no significant effect of sucrose concentration in the diet on starvation resistance.*

	SS	SS/MS$_{Total}$	d.f.	*P*-value
Concentration	165.635	0.161	2	0.92
Generation	985.719	0.958	4	0.92
Interaction	487.031	0.473	7	1.00

Table 6.14: The results of a nonparametric 2-way ANOVA to determine the effects of yeast concentration in the diet over 5 generations on adult starvation resistance.

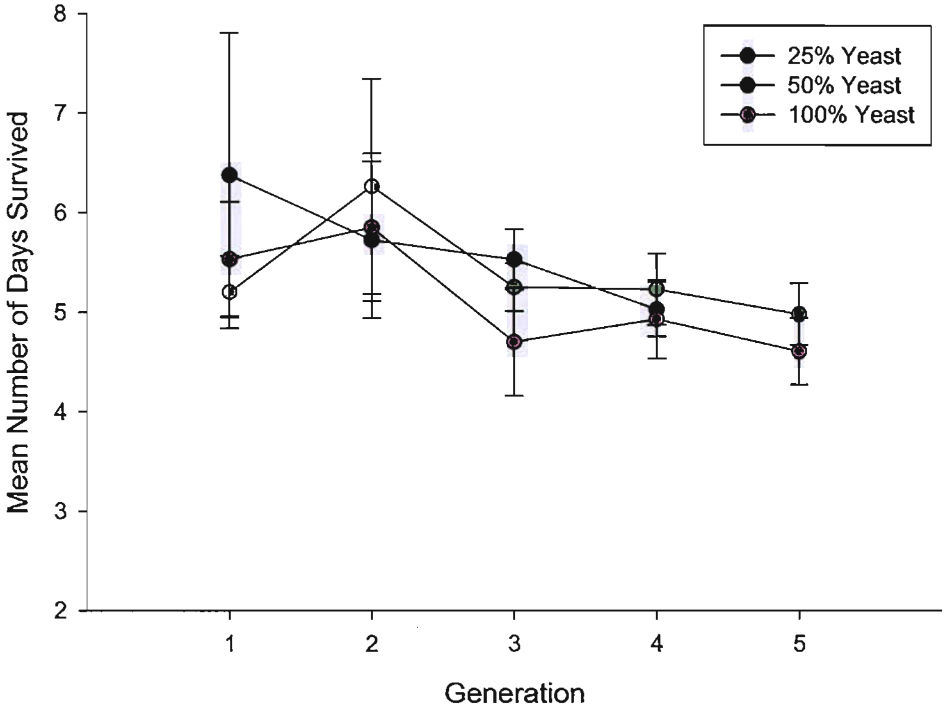


Figure 6.17: The effect of varying yeast concentration in the diet over 5 generations on the starvation resistance of adult flies. Data points represent the mean ± standard error for the number of days survived. Results show no significant effect of yeast concentration in the diet on starvation resistance.

6.4 Discussion

6.4.1 The Effect of Diet Over 5 Generations on Fecundity

Fecundity is defined as the number of offspring an individual has the capacity to produce in a life time, and is one of the major measures of fitness used in scientific research. It is controlled both genetically and environmentally. The number of offspring an individual can produce in a lifetime has been shown to be affected by many environmental factors, including food quality (Boggs and Freeman, 2005; Burger *et al*, 2007; Chippindale *et al*, 1993; Douglas *et al,* 2006; Naya *et al*, 2007 Partridge *et al*, 2005), moisture content (Ryne *et al*, 2004) and presence of inhibitory metabolites (Pompermayer *et al*, 2001).

In this thesis, the effect of yeast and sucrose concentration over 5 generations was studied. Much research has looked at the effects of diet on fecundity. For example, a carbohydrate rich diet has been shown to decrease fecundity (Douglas *et al,* 2006; Naya *et al*, 2007) and a protein rich diet has to increase fecundity in both *Drosophila* (Chippindale *et al*, 1993; Partridge *et al*, 2005) and in other insects (Boggs and Freeman, 2005; Burger *et al*, 2007). It has been argued that the decrease in fecundity is a trade off with an increased life span (Chippindale *et al*, 1993), however life span was not measured in this study so this cannot be either confirmed or rejected.

The results of this study showed that a high sucrose content diet decreased the number of eggs laid. This is therefore in support of other studies looking at the effect of carbohydrate content in the diet on fecundity (Douglas *et al* 2006; Naya *et al*, 2007). In addition, when sucrose concentration in the diet was reduced the number of eggs laid increased between generations 0 and 1 for all concentrations tested. The number of eggs laid then decreased from generations 2- 5. This may be due to the generation 0 flies all being raised on 100% sucrose and therefore when moved onto the lower sucrose content media the number of eggs laid increased. The initial increase in number of eggs laid when transferred onto a lower sucrose content diet therefore supports previous data which has shown that lower carbohydrate diets increase fecundity (Douglas *et al,* 2006; Naya *et al*, 2007). The subsequent decrease in fecundity over the following generations may be due to maternal effects. When a female lays an egg, that egg contains some energy stores provided by the mother. It is possible, therefore, that when raised on the reduced carbohydrate content media the females do not allocate as many resources to the egg due to the reduction in carbohydrate the female ingests. This may then have an effect on the fecundity of the next generation. The reduction in allocation to eggs in response to maternal diet has been described in several studies (Amarillo-Suarez and Fox, 2006; Moreau *et al*, 2006; Wallin *et al*, 1992).

When the concentration of yeast in the diet was reduced the number of eggs laid by the females was also reduced. In the case of 25% yeast, so few eggs were laid by the generation 4 flies that these experiments had to be abandoned. There was no generational effect. These results are therefore similar to previous studies which have also found that low protein in the diet leads to reduced fecundity in insects (Boggs and Freeman, 2005; Burger *et al*, 2007; Chippindale *et al*, 1993; Partridge *et al*, 2005). It has previously been shown that fecundity and starvation resistance are linked, with reduced yeast concentrations in the diet leading to a decrease in fecundity and an increase in resistance to starvation (Chippindale *et al*, 1993). Similar results have been found in this study, where a reduced yeast concentration in the diet led to an increase in starvation resistance (see Section 6.4.5) and a decrease in the number of eggs laid. This would therefore suggest that energy is either allocated to one or other of these processes (Chippindale *et al*, 1993). It has been suggested that this alternate allocation depending on resource availability may be due to changes in lipid metabolism (Zwaan *et al*, 1991). The neuroendocrine system has also been implicated, with the hormones involved in the formation of yolk proteins being reduced in nutritionally deprived flies (Bownes and Reid, 1990). Another possibility is that metabolic rates could be reduced in nutritionally deprived flies, leading to increased starvation resistance when subjected to a nutritionally poor diet due to a lower metabolic rate than. Fecundity would therefore also be lower in these nutritionally deprived flies due to resources being allocated preferentially to the fat body rather than to reproduction (Chippindale *et al*, 1993).

The results of both the sucrose and yeast experiments would therefore suggest that when faced with a low quality diet the insect responds by decreasing fecundity. This could possibly be to increase life span so that if a better quality diet is found in the future the female is able to produce eggs then and hence provide the offspring with a greater chance of survival (Chippindale *et al*, 1993).

6.4.2 The Effect of Diet Over 5 Generations on Larval Fitness

Larval fitness as described in this study is a measure of the distance moved by the larvae on a non-nutritive surface. This is in contrast to studies looking at foraging behaviour, where movement in the presence of food is measured (Pereira and Sokolowski, 1993; Sokolowski *et al*, 1997). Recent evidence suggests that prenatal under nutrition in mammals can reduce voluntary locomotor behaviour, including distance moved, in rats (Bellinger *et al*, 2004; Vickers *et al*, 2003). This is a very interesting phenomenon, as this means that not only can under nutrition *in utero* affect metabolic process which can lead to an increased risk of disease in later life, but it can also lead to changes in behaviour that can exacerbate these problems (Vickers *et al*, 2003). The underlying mechanisms involved in this are not as yet clear, although it is possible it may be due to the lack of nutrients during development leading to reduced skeletal muscle which could lead to altered locomotion in the adult (Toscano *et al*, 2008).

In the present study, the concentration of sucrose or yeast was reduced over 5 generations in *Drosophila*. In both cases there was no significant affect of diet on larval fitness for any of the food types used. This is in contrast to the studies in mammals (Vickers *et al*, 2003). However, in the mammalian experiments were locomotory behaviour was affected by nutrition *in utero* the effects were induced by an over all reduction in food availability rather than limiting specific nutrients. It may therefore be that in this study the flies locomotory behaviour was not adversely affected as they were able to ingest an increased amount of food in order to provide the nutrients necessary for larval diet to not adversely affect locomotory behaviour in the adults. In order to determine whether this is indeed the case further studies would be required where the total amount food available is reduced rather than just one specific component of the diet.

6.4.3 The Effect of Diet Over 5 Generations on Development Time

The time it takes for an organism to develop through to a sexually reproductive age will affect its fitness as the longer it takes for an organism to reach this stage the more likely it is to die before reproducing (Nylin and Gotthard, 1998). Development time is thought to be an important factor which shapes other life history traits such as adult size, and development time itself is affected by extrinsic factors such as diet (Kawecki, 1993). In addition, nutrition has also been linked to age at sexual maturity in human (Euling *et al*, 2008) making it of particular interest in this study.

The effect of diet on development time has been well described in insects (Azidah and Sofian-Azirun, 2006; Butler and O'Neil, 2007; Kyneb and Toft, 2004; Moreau *et al*, 2006). Many studies have found that as host quality decreases so development time increases (Nylin and Gotthard, 1998). For example, in *Drosophila* lower levels of yeast concentration in the diet lead to increased development time (Gebhardt and Stearns, 1993). Similar results have also been described in other insects (Fox and Dingle, 1994; Lindroth *et al*, 1991).

Some have argued that the effects of nutrition on development time are due to maternal effects (Fox and Dingle, 1994). However, the results of this thesis show no effect of the number of generations raised on these diets on development time. This would therefore suggest that it is larval diet that is affecting development time rather than maternal diet. Other studies have also found little correlation between number of generations raised on a diet and development time (Thorpe and Bennett, 2003).

In this study, the time taken for the individuals to reach both the pupal and adult stages was measured. The results showed that the development time for both the pupae and adults paralleled one anther for all food types and all generations i.e. the time between the pupae developing and the adult eclosing did not differ between food types or over generations. This would therefore suggest that diet has no effect on the time taken for the flies to undergo metamorphosis. As there is no difference in the development time of the pupae and the adults, development time will now be discussed referring to both these stages.

When carbohydrate in the diet was removed, development time increased. In addition when raised on a 100% sucrose, development time also increased. Varying the concentration of yeast in the diet also had a significant affect on the development time of the flies. As the concentration of yeast in the diet decreased so the development time increased. No generational effect of diet on development time was observed in either the sucrose or yeast data. The yeast data therefore supports previously published data looking at the effects of yeast concentration in the diet upon development time (Gebhardt and Stearns, 1993; Fox and Dingle, 1994; Lindroth *et al*, 1991). As the lowest and highest concentrations of sucrose increased the development time of the flies, this would suggest that the intermediate levels of sucrose in the diet are therefore most suitable for *Drosophila* development.

It is thought that the increase in development with decreasing quality of the diet is due to the larvae extending their period of feeding to gain enough nutrients to be able to under metamorphosis (Fox and Dingle, 1994). For example, the comma butterfly (*Polygonia c-album*) prolonged development time when reared on less suitable hosts in order to maintain adult size (Nylin, 1988). Others have found that the egg size, and hence the amount of resources given by the mother to the offspring can affect development time. For example, in ladybirds species with smaller eggs took longer to develop then those with larger eggs (Stewart, Hemptinne and Dixon, 1991). Similar results were found in the beetle *Callosobruchus maculatus* (Fox, 1993). However, in other studies egg size had little effect on development time (Nylin, 1992). The reason for these varied results may be due to the amount of nutrients that the egg can carry (Nylin and Gotthard, 1998). In this study, there was no effect of diet on development time over the generations, suggesting that in this case maternal diet does not affect the amount of nutrients allocated to the egg. Some evidence has found that the rapamycin and insulin signaling pathways may underlie the temporal control of development depending on nutritional status (Rougvie, 2005), although much research is still required to fully understand the role these pathways play.

6.4.4 The Effect of Diet Over 5 Generations on Survival

Survival in this thesis was measured in two ways. Firstly, it was measured by the total number of pupae developing from a set amount (80) of eggs. Secondly it was measured by the number of adult flies that emerged from these pupae. Many other studies have looked at the effects of diet upon survival in a wide range of insect species (Burger *et al*, 2007; Fox and Dingle, 1994). For example, the concentration of yeast in the maternal diet has been shown to affect larval survival in the beetle *Callosobruchus maculates* (Fox and Dingle, 1994).

Varying the concentration of sucrose in the diet over 5 generations had various affects on survival in *Drosophila.* When the concentration of sucrose in the diet was increased so the number of individuals surviving to the adult stage increased. This therefore supports the results of other studies (Burger *et al*, 2007; Fox and Dingle, 1994). There is also a significant effect of the number of generations the flies were kept on the reduced sucrose content medium on numbers surviving to the pupal stage, with survival increasing the more generations they were kept on each diet. This may be due to adaptation to the food type across the generations, so that in each generation more offspring will survive. It has been well described that insects are capable of adapting to new hosts in a relative short space time (Ehrlich and Murphy, 1988; Singer *et al*, 1993).

Varying the concentration of yeast in the diet had a significant affect on the number of individuals surviving through to the pupal stage, with an increasing concentration of yeast in the diet leading to more pupae developing. It did not, however, have any affect on the number of adults developing from these pupae. This would therefore suggest that the negative effects of low yeast in the diet are only detrimental to the survival of the larvae. At the lowest concentration of yeast used, no data was collected for the last generation. This was due to very few eggs being laid to set up the next generation and not due to no individuals surviving at all. Several other studies have described the detrimental effects of a low protein diet on survival (Burger *et al*, 2007; Fox and Dingle, 1994; Stamp and Harmon, 1991).

It is thought that a poor maternal diet may influence the survival of her offspring as she will not be able to supply an egg of adequate quality for the individuals to survive. This thought to be due to reduced quality of the egg yolk and reduced lipid content of the egg (Rossiter, 1991). When in a resource poor environment the mother is not able to provide as many resources to her eggs. This has been hypothesised to lead to the eggs being smaller and subsequently doing less well than the larger eggs. There is many studies that have shown that females produce smaller eggs when raised on a nutritionally poor diet (Fox and Dingle, 1994; Wallin *et al*, 1992)

6.4.5 The Effect of Diet on Starvation Resistance Over 5 Generations

Either a shortage of food or the availability of only low quality food is a major cause of environmental stress and hence an important source of natural selection in the wild for every organism (Rion and Kawecki, 2007). The underlying mechanisms involved in response to nutritional stress also appear to be conserved throughout the animal kingdom (Partridge *et al*, 2005) making starvation resistance an interesting measure in this study. Starvation resistance is also thought to be a good index of the calorific stores the fly has (Chippindale *et al*, 1997).

The effect of dietary restriction has been well studied in relation to the aging process but less so for other life history traits (Rion and Kawaecki, 2007). Some studies have, however, been conducted with some interesting results. For example, several studies have shown an increase in starvation resistance when levels of yeast and sucrose in the diet were restricted (Burger *et al,* 2007). In addition, when only yeast was restricted starvation resistance in flies was also shown to increase (Chippindale, 1993). Dietary restriction in the form of larval overcrowding has also been shown to increase starvation resistance (Baldal *et al*, 2005). So there has been an overall finding the dietary restriction leads to an increase in starvation resistance.

In this study, varying the concentration of sucrose or yeast within the diet had no subsequent affect on starvation resistance in adult flies for any of the concentrations used. There was also no affect of these diets on starvation resistance over several generations. However, when looking at the average number of days the adults survived when raised on a reduced yeast diet compared to when raised on a reduce sucrose diet there does appear to be an increase the number of days the adults survive (4-6 d) when raised on the low yeast diet compared to when raised on a reduced carbohydrate diet (3-5 d).

The results of this study therefore suggest that reducing yeast in the diet may be stressful to the fly, leading to the induction of the mechanisms required to survive in adverse conditions whereas reducing carbohydrate in the diet does not. Genetics has been indicated to play a role in the effect of diet on starvation resistance. For example, laboratory selection for starvation resistance leads to a rapid and large increase in starvation resistance over several generations (Hoffman *et al*, 2005). It is thought that dietary restriction leads to the induction of mechanisms which aid survival in adverse conditions (Burger *et al,* 2007). Three mechanisms have been suggested for how starvation resistance may be manipulated. Firstly, a reduction in food availability may lead to greater reserves being stored. Therefore, when raised on a restricted diet flies increase their storage of energy reserves to overcome periods of low resources. Hence if exposed to a poor diet they will increase their energy stores and so will under conditions of starvation live for longer. For example, several studies have shown that increased starvation resistance is linked to increased lipid storage (Borash and Ho, 2001; Hoffman *et al,* 2005; Kenny *et al*, 2008). There is also an increase in the enzymes involved in lipid synthesis (Harshman *et al*, 1999). There is some evidence to suggest that this increase in the storage of energy can occur during the larval stages and therefore larval diet can influence adult starvation resistance (Chippindale *et al*, 1996). However, not everyone has found a correlation between fat content and starvation resistance (Baldal *et al*, 2005).

The second proposed mechanism for how starvation resistance may be controlled is that under conditions of reduced food availability the metabolic rate may be reduced (Djawdan *et al*, 1997; Simmons and Bradley, 1997). Also, starvation has been shown to in some cases reduce locomotion and hence reduce energy expenditure (Williams *et al*, 2004). However, others have found that starvation can lead to an increase in locomotory behaviour (Knoppien *et al*, 2000). Finally, it is possible that the minimum amount of resources required for survival can be reduced (Rion and Kawecki, 2007), although little work has been conducted to determine whether this may indeed be the case.

6.4.7 Summary

The aim of this chapter was to determine the effects of various diets over 5 generations on several measures of health in *Drosophila*. This was done to determine whether *Drosophila* may be a good model organism for researching the effects of diet over several generations in mammals (see Section 6.1). Results showed that dietary restriction affected different traits in different ways. In some cases there was a significant affect of diet on measures of health in *Drosophila* (e.g. fecundity). In other cases, however, there was no effect of diet on the traits measured (e.g. larval fitness). The effect of diet on the traits measured depended on the food type being used. For example, when sucrose concentration in the diet was reduced the number of eggs laid increased whereas when yeast concentration in the diet was reduced the number of eggs laid decreased.

Much work is required in this area in order to determine whether *Drosophila* may be a good model organism to use to study the effects of diet on life history traits. One important factor would be to standardise the methods used so that a clear comparison can be drawn. For example, some studies define dietary restriction as a reduction in one component such as in this study. In other case, it is defined a reduction in overall food availability (Vickers *et al*, 2003). It is difficult to compare the results of these two different methods for dietary restriction as in the first method the individuals may modify the amount of food ingested in order to compensate for the lack of a specific nutrient.

One interesting observation is that individuals raised on a reduced yeast content medium took longer to develop into pupae were also able to resist starvation for a longer time period than those raised on a reduced sucrose content medium. It may be that the effect of the increased development time of those raised on the reduced yeast content medium was to allow the individuals to store greater reserves than those on the reduced sucrose content medium, which then allowed them to survive for a longer time period when starved immediately after eclosion. It would be of interest to determine the fat content of flies when raised on these differing diets to determine if this is indeed the case.

Chapter 7: General Discussion

7.1 Summary of the Data

Chapters 3 and 4 have shown that larval diet can have an affect on subsequent adult egg laying and feeding preferences. This affect, however, depends on the chemical and concentration in question. The results from Chapter 5 suggest that this may be due to learning and memory. This data therefore supports the Hopkins Host Selection Theory (see Section 3.1.4) and other evidence for the retention of memory through metamorphosis (see Section 1.3.3). Residues of food on the outside of the pupal case were eliminated by washing the pupae. Traces of food may, however, remain within the pupal case (Corbet, 1985). Adults could therefore learn about their larval environments from these traces upon eclosion. Thorpe (1939), however, found no evidence for food deposits being found in the pupal case, although some was found outside the terminal segment of the pupal cast due to the rectum being emptied just prior to pupation (Thorpe, 1939). It cannot, therefore, be completely excluded that results are due exposure of the adults to residues of the larval diet within the pupal cases but this would seem unlikely. Determining the calorific value of the food sources suggested that it is not this that is affecting the choices of the adults as there was very little difference in the calorific values of the different food types.

The results of Chapters 3 and 4 showed that the feeding and egg laying preferences of the flies are often different. For example, when flies were raised as larvae on 0% sucrose, adults showed a preference to feed on this diet but were less likely to lay their eggs on this diet than if raised on a standard diet as larvae. These results therefore suggest that adult feeding and egg laying preferences are differentially controlled. This may be because feeding preference involves an immediate requirement of the food source. Egg laying preferences, however, involve a requirement for a food source once the egg has hatched and through to pupation. Studies have shown that as a fruit ripens so the sucrose concentration decreases (Adao and Gloria, 2005). It would therefore follow that when choosing a food source to feed upon low concentration of sucrose would be appropriate. When choosing a food source to lay eggs on, however, a higher concentration of sucrose would indicate a food source which will be ready for consumption by the larva when it hatches and is not so ripe that by the time the larvae hatches the fruit will have rotted away.

Chapter 4 also showed that adult feeding and egg laying preferences can be affected by starvation. When flies were starved for 24 hrs after eclosion, all of the statistically significant differences with larval diet seen in Chapters 3 and 4 disappeared. This may be because flies become more accepting of novel food sources when hungry, or because food preferences that survive metamorphosis need to be reinforced early in adult life in order for them to be retained later in adult life. This data therefore brings into question the use of starvation when studying behaviour.

The results of Chapter 5 suggested that cAMP underlies the change in adult food preference with larval diet. Results also showed that the mushroom bodies were important in this process. As discussed in Section 1.6, the MBs are the putative centres for learning and memory in insects (de Belle and Heisenberg, 1994), and a subset of MB interneurons, called Kenyon cells, survive metamorphosis (Technau and Heisenberg, 1982). The cAMP mutants, *dunce* and *rutabaga* (the mutants used in Chapter 5) are both preferentially expressed in the mushroom bodies and are thought to be involved in learning and memory (Han *et al*, 1992; Nighorn *et al*, 1991). When the MBs were ablated or these mutants used the affect of diet upon adult food preferences disappeared, suggesting that it may be learning and memory that is leading to the affect of larval diet on adult food preferences. However, both the cAMP cascade and the mushroom bodies are also involved in many other processes, including gustation and olfaction so there are many possible explanations for how larval diet may affect adult food preference.

In the final chapter (Chapter 6), the effects of the different diet on the development and survival of *Drosophila* was determined. Diet affected several life history traits in *Drosophila*, including fecundity, development time, larval fitness and survival. The effects, however, depended on the diet type. For example, varying the concentration of NaCl in the diet significantly reduced larval fitness but for all other diet types there was no affect on larval fitness. Together, the results of this Chapter suggest that *Drosophila* may indeed be a good model organism for gaining a better understanding of the effects of maternal diet on the future health of the offspring.

7.2 Future Work

The results of Chapters 3, 4 and 5 provide some evidence to suggest that flies may be capable to learn gustatory information about their larval diet and use this information to help them to select appropriate food sources as adults. To further investigatet this possibility it would be interesting determine whether this was also the case for associative information. In this case, an olfactory stimulus could be paired with the larval food source. As adults, these individuals could be tested in a T-tube to determine whether they select the arm containing the odour they were exposed to as larvae or not. This experiment would be similar to that of Thorpe (1939) (see Section 1.3.3). However, if results suggest retention of memory through metamorphosis then the same mutants and the HU ablation procedure as described in Chapter 5 could be repeated to determine whether it is learning and memory involved and to determine whether this information is stored in the mushroom bodies.

The results of Chapter 4 showed that the calorific content of the different food sources do not significantly differ. It may therefore be that the effect of diet upon food choices may be due to differences in the amount of food ingested for the different diet types (Min and Tartar, 2006). It would therefore be interesting to determine the amount of food ingested for the different diets.

Chapter 6 showed that diet can affect certain life history traits in *Drosophila*. It would be interesting to see if other characteristics are also influenced by diet, such as fat content (Vermeulen *et al*, 2006). Another interesting trait to measure would be pupal and adult size (Ojeda-Avila *et al*, 2003). In mammals, diet has shown to affect birth weight and it would therefore be interesting to determine whether diet affects weight in *Drosophila* (Andreasyan *et al,* 2007; Campbell *et al*, 2005; Lister and McCance, 1965; Stein *et al*, 1995; Stein *et al*, 2004; Stein and Susser, 1975; Stewart *et al*, 1975).

In addition, some of the experiments conducted in Chapter 6 have not yet been conducted in mammals, e.g. the effect of NaCl in diet on locomotory behaviour. These experiments therefore need to be repeated in mammals to determine whether similar results are found. Finally, in Chapter 6 microarrays were also conducted on 0% and 100% sucrose. It would be of interest to repeat these experiments with the protein restricted flies. This is because much the literature studying the effect of maternal diet on health of the offspring in mammals has used protein restriction as a model (Benyshek *et al*, 2006; Lillycrop *et al*, 2005; Stewart *et al*, 1975).

Finally, there have been some interesting results in mammals where protein restriction that occurred in generation 1 can affect health and gene expression levels in subsequent generations even when the following generations were maintained on an unrestricted diet (Benyshek *et al,* 2006; Burdge *et al*, 2007). It would therefore be of great interest to repeat these experiments in *Drosophila* to determine whether similar effects are observed. If so, this would provide a very strong case for the use of *Drosophila* as a model for understanding the effects of diet *in utero* on adult health and disease.

7.3 Implications

Gaining an understanding of whether gustatory information about the larval diet can be learnt and remembered and this information retained through metamorphosis and into the adult fly is important for several reasons. Firstly, if insects are capable of learning information about their larval diet and this affects subsequent adult feeding and egg laying preferences this could be of relevance to the pest control industry (Prokopy *et al*, 1982; Sheeba *et al*, 1998). There is an increasing need to find methods of pest management where the use of pesticides is reduced due to their deleterious effects on the environment and as resistance to pesticides increases (Gilbert and Firestein, 2002). An understanding of how an insect selects food could therefore provide a means to help prevent the damage that they cause to crops (Newland, 1998). If individuals concentrate their search effort on hosts they have been preconditioned to through learning then this could further increase the possibility of manipulating and directing the host choice of pest species (Vinson *et al*, 1977; Wardle and Borden, 1991). For example, African arboreal ant nest site selection has been shown to be affected, at least in part, by the larval environment. The habitat preferences of these ants could therefore be manipulated so that they make nests in crop plants and hence provide them with protection against herbivores (Djieto – Lordon and Dejean, 1999).

Secondly, understanding oviposition preferences could be important for evolutionary ecology. Changes in preferences could lead to the formation of different races within a population that have preferences for different hosts. If these two populations do not mate due to this difference in host preference this could in time lead to speciation (Feder *et al*, 1990; Maynard-Smith, 1966; Olson and Andow, 2002; Prokopy *et al*, 1982). Therefore, gaining a better understanding of feeding and oviposition preferences could allow us to further understand the mechanisms involved in sympatric speciation. According to Jaenike and Papaj (1992) even modest learning can have an effect on patterns of host use, increasing the use of that host and hence accelerating rates of adaptation to that host. This could reinforce genetic differences among individuals in host preference and therefore facilitate host race formation

(Jaenike and Papaj, 1992). In addition, if natal habitat can have an effect on adult food preferences, this could also have a major impact on conservation biology. When attempts are made to reintroduce animals to habitats this is much more likely to be successful if the habitat in question is similar to the natal habitat (Davis and Stamps, 2004).

Finally, the results of this study could also have important implications for other animals, including humans. Studies in both rats and humans have shown that mammals are able to recognise tastes they encountered prenatally from the maternal diet (Gruest *et al*, 2004; Mennella *et al*, 2001). In addition, prenatal nutrition has been linked with obesity in later life, with the nutritional environment *in utero* affecting subsequent feeding preferences in rats (Bellinger *et al*, 2004). If *Drosophila* are capable of learning information about larval diet, and this information modifies subsequent adult feeding preferences, they could be used as a model system to gain a further understanding into the mechanisms involved in prenatal learning of tastes. In addition, the experiments looking at the effect of diet on life history traits over several generations may also provide an insight into the effects of diet upon health described in mammals. *Drosophila* is a suitable model organism for much scientific research because it has a short generation time, many mutants are already available and the whole genome has now been sequenced (Le Bourg, 2004).

Appendix

Appendix 1. Additional results used in Chapter 6 experiments

A1.1 The Effect of Varying the Diet Over 5 Generations on Fecundity

Varying the concentration of fructose in the diet had a significant affect across the generations (Table A1). In all cases accept 100% fructose there was an increase in the number of eggs laid between generations 0 and generation 1 (Fig. A1). This is particularly evident in the 0% fructose data. The number of eggs laid then appears to level out in the following generations.

	SS	SS/MS_{Total}	d.f.	*P*-value
Concentration	107753.420	5.252	4	0.26
Generation	226728.888	11.05	5	< 0.05
Interaction	307304.564	14.978	20	0.78

Table A1: Results of a nonparametric 2-way ANOVA conducted to determine the effect of varying fructose concentration in the diet over 5 generations on fecundity.

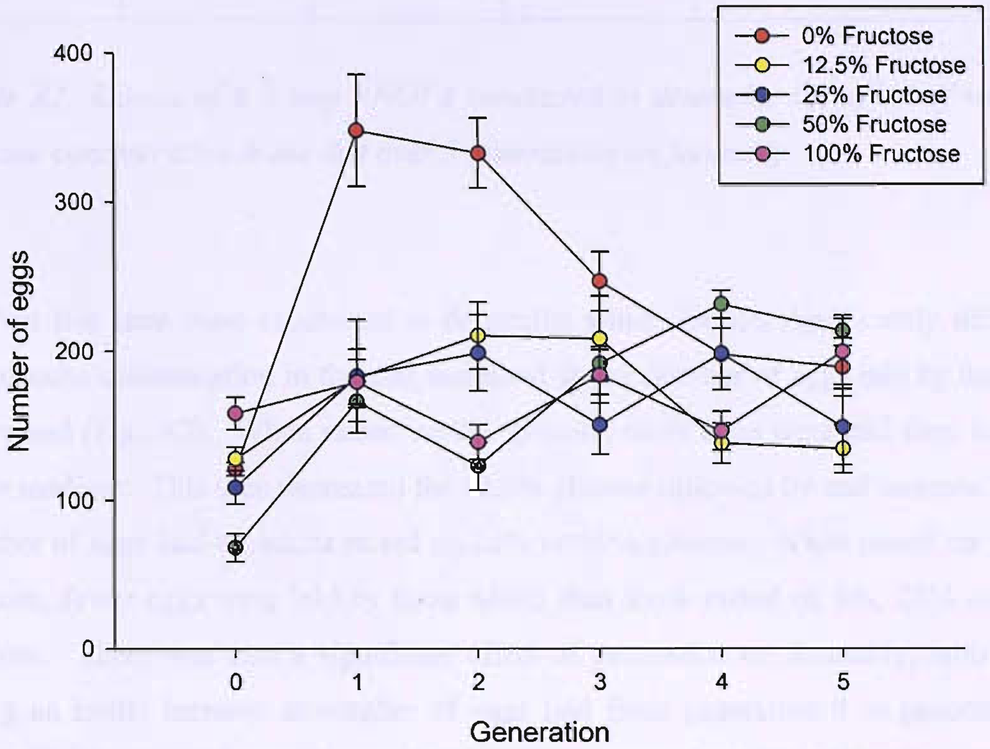


Figure A1: The effect of fructose concentration in the diet on fecundity over 5 generations. Data points represent the mean ± standard error for the number of eggs laid. Results show an initial increase in the number of eggs laid between generations 0 and 1, particularly for 0% fructose. After this the number of eggs laid does not seem to vary between the generations.

When glucose concentration in the diet was varied over 5 generations results showed that both diet and generation had a significant effect on fecundity. In addition, there was also a significant interaction of two (see Table A2).

Factor	d.f.	SS	MS	*F*-ratio	*P*-value
Diet	4	1.705	0.426	28.099	<0.01
Generation	5	2.978	0.596	39.246	<0.01
Interaction	20	1.707	0.085	5.625	<0.01
Error	203	3.080	0.015		

Table A2: Results of a 2-way ANOVA conducted to determine the effect of varying glucose concentration in the diet over 5 generations on fecundity.

Post hoc tests were conducted to determine which groups significantly differed. As glucose concentration in the diet increased so the number of eggs laid by the flies decreased (Fig. A2). When raised on 0% glucose, more eggs were laid than for any other medium. This then decreased for 12.5% glucose followed by and increase in the number of eggs laid by adults raised on 25% or 50% glucose. When raised on 100% glucose, fewer eggs were laid by these adults than those raised on 0%, 25% or 50% glucose. There was also a significant affect of generation on fecundity, with there being an initial increase in number of eggs laid from generation 0 to generation 1 followed by a slow decrease over the following generations for all food types (Fig. A3). The effect of diet and generation also significantly interacted, as can be seen in Figure A4. For all food types, there was an increase in fecundity between generation 0 and generation 1. The number of eggs laid then fluctuated between 100 – 250 eggs for all food types (Fig. A4).

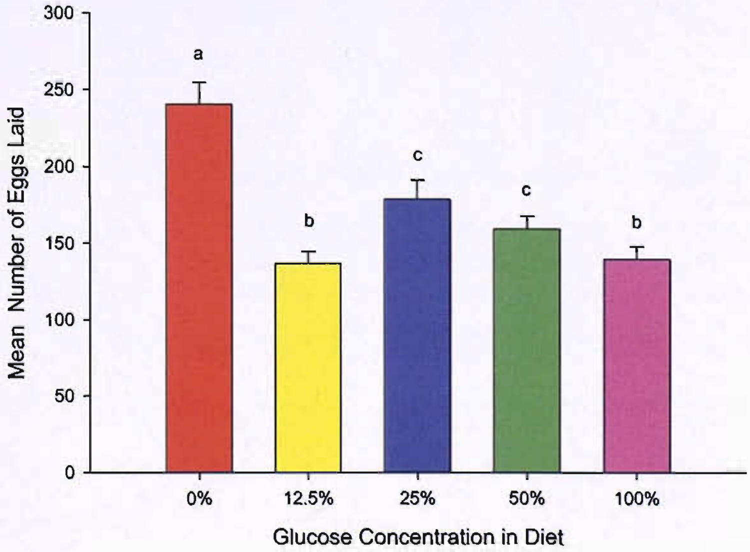


Figure A2: The effect of glucose concentration in the diet on fecundity. Bars represent mean ± standard error for the number of eggs laid. Letters above the bars indicate no significant difference. Significance is at the $P < 0.05$ *level. Results show* *that as glucose concentration in the diet increased so the number of eggs laid by the adults decreased.*

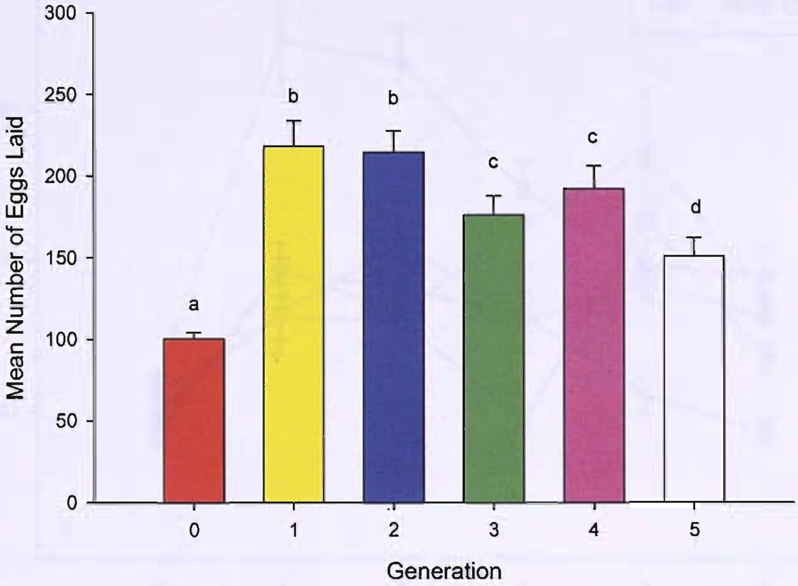


Figure A3: The effect of generation on fecundity when raised in varying concentrations of glucose. Bars represent mean ± standard error for the number of eggs laid. Letters above the bars indicate no significant difference. Significance is at the $P < 0.05$ *level. Results show that there was a big increase in the number of eggs laid between generations 0 and 1. This then decreased over the following generations.*

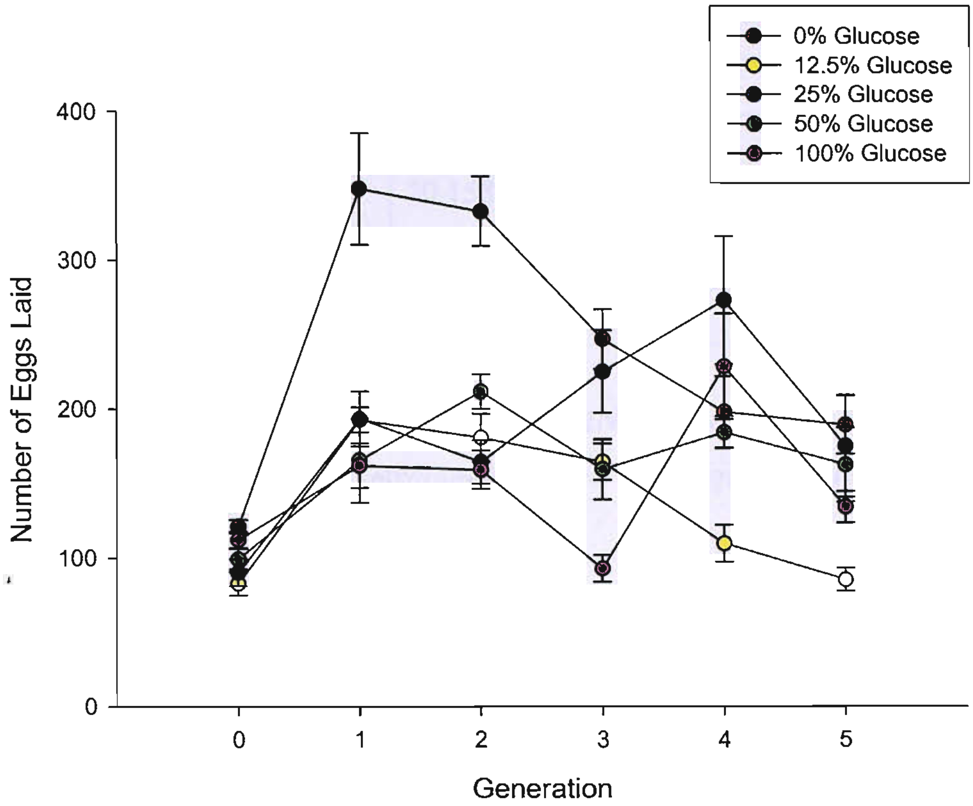


Figure A4: The effect of glucose concentration in the diet on fecundity over 5 generations. Data points represent mean ± standard error for the number of eggs laid. Results show that both diet and generation have a significant effect on fecundity, with the number of eggs being laid initially increasing for all food types. The number of eggs laid then fluctuated between 100 – 250 for all food types.

Varying the concentration of trehalose in the diet had a significant affect across the generations (Table A3). In all cases there was an increase in the number of eggs laid between generations 0 and 1 (Fig. A5). This is particularly evident in the 0% trehalose data. The number of eggs laid then decreased for 0% trehalose and levelled out for all other food types.

	SS	SS/MS_{Total}	d.f.	*P*-value
Concentration	146691.814	8.165	4	0.09
Generation	362172.408	20.158	5	<0.01
Interaction	52148.835	2.903	20	1.00

Table A3: Results of a nonparametric 2-way ANOVA conducted to determine the effect of varying trehalose concentration in the diet over 5 generations on fecundity.

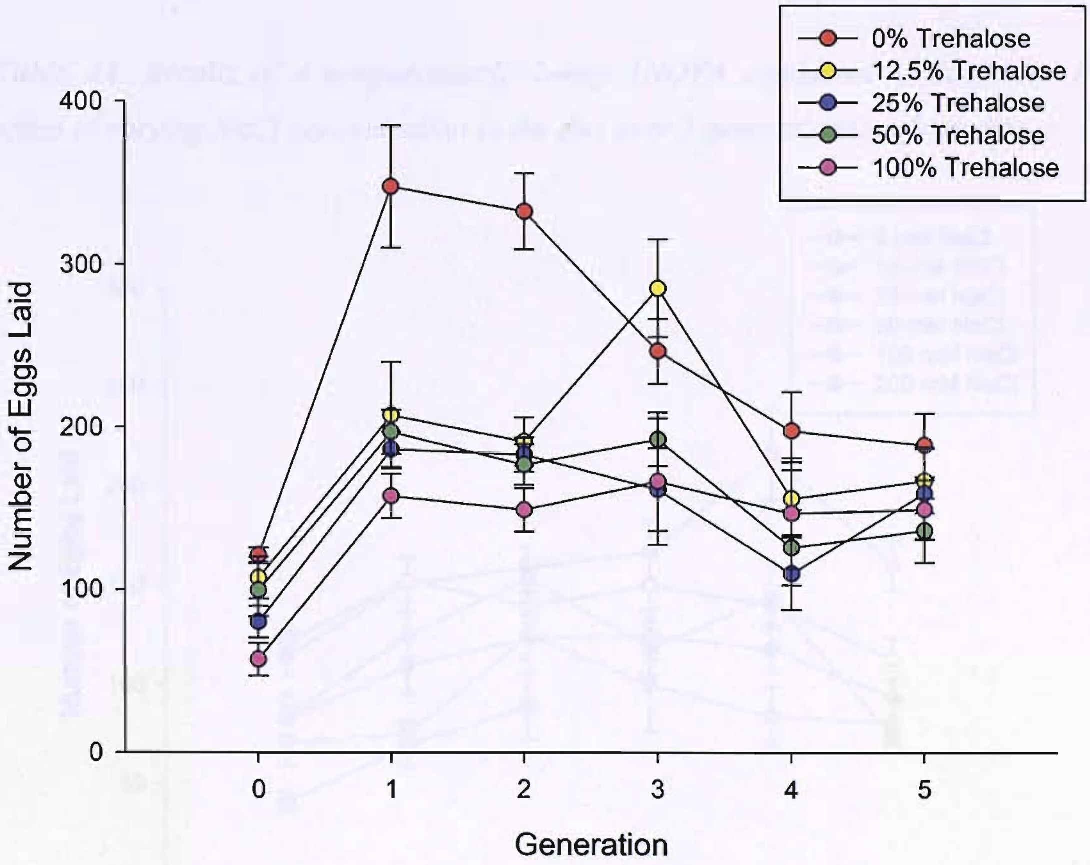


Figure A5: The effect of varying trehalose concentrations in the diet over 5 generations on fecundity. Data points represent mean ± standard error for the number of eggs laid. Results show an increase in the number of eggs laid between generations 0 and 1. For 0% trehalose, the number of eggs then decreases over the following generations and for all other food types number of eggs laid levels out.

Varying the concentration in NaCl in the diet had a significant effect on the number of eggs laid. This effect did not change over the generations (Table A4). As NaCl concentrations in the diet increased so the number of eggs laid by the adults decreased (Figure A6).

	SS	SS/MS$_{Total}$	d.f.	P-value
Concentration	379684.490	16.639	5	<0.05
Generation	177399.965	7.774	5	0.17
Interaction	106701.298	4.676	22	1.00

Table A4: Results of a nonparametric 2-way ANOVA conducted to determine the effect of varying NaCl concentration in the diet over 5 generations on fecundity.

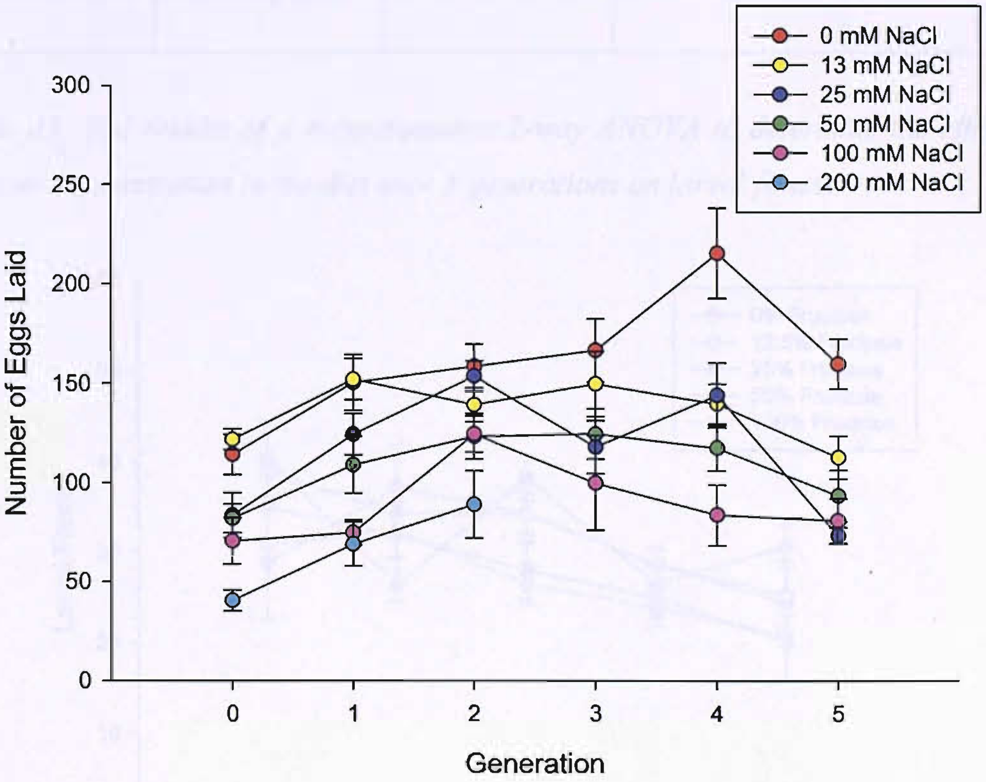


Figure A6: The effect of varying NaCl concentration in the diet on fecundity over 5 generations. Data points represent mean ± standard error for the number of eggs laid. Results show that as NaCl concentration in the diet increased so the number of eggs laid decreased. Generation had no effect on number of eggs laid.

A1.2 The Effect of Diet Over 5 Generations on Larval Fitness

Varying fructose, glucose, trehalose or NaCl concentration in the diet had no subsequent affects on larval fitness at any of the concentrations or generations tested (Tables A5, A6, A7 and A8 respectively). Although not significant, there does appear to be a trend for a decrease in larval fitness across the generations with these diets (Figs. A7, A8, A9 and A10 respectively).

	SS	SS/MS_{Total}	d.f.	*P*-value
Concentration	60997.341	3.319	4	0.51
Generation	167152.379	9.095	4	0.06
Interaction	91647.354	4.986	16	1.00

Table A5: The results of a nonparametric 2-way ANOVA to determine the effects of fructose concentration in the diet over 5 generations on larval fitness.

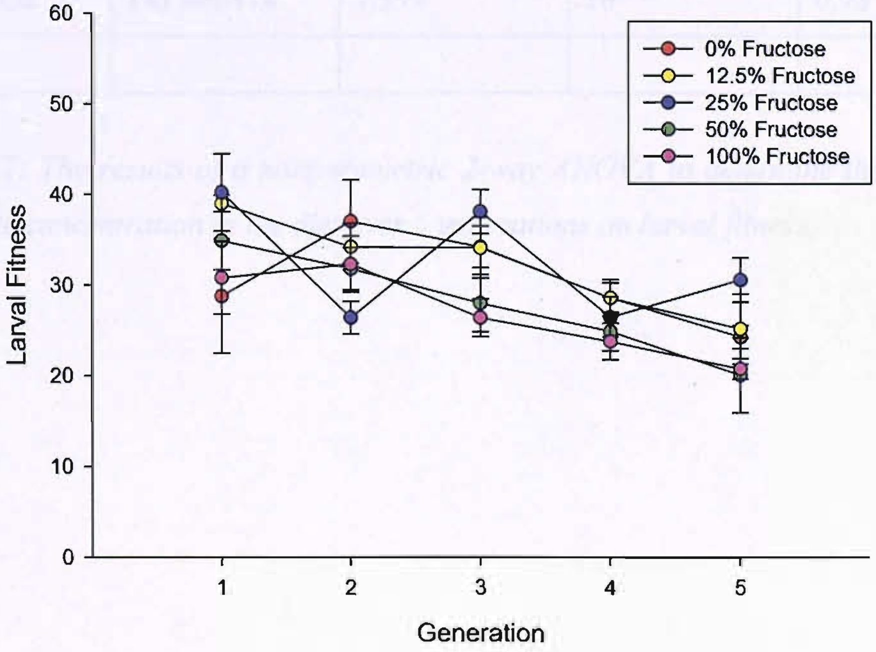


Figure A7: The effect of varying fructose concentration in the diet over 5 generations on larval fitness. Results showed no significant affect of fructose concentration in the diet on larval fitness for any of the generations.

	SS	SS/MS_{Total}	d.f.	*P*-value
Concentration	15784.916	0.943	4	0.92
Generation	96354.455	5.759	4	0.22
Interaction	79679.399	4.762	16	0.99

Table A6: The results of a nonparametric 2-way ANOVA to determine the effects of glucose concentration in the diet over 5 generations on larval fitness.

	SS	SS/MS_{Total}	d.f.	*P*-value
Concentration	11860.993	0.621	4	0.96
Generation	136002.507	7.121	4	0.13
Interaction	141308.478	7.399	16	0.96

Table A7: The results of a nonparametric 2-way ANOVA to determine the effects of trehalose concentration in the diet over 5 generations on larval fitness.

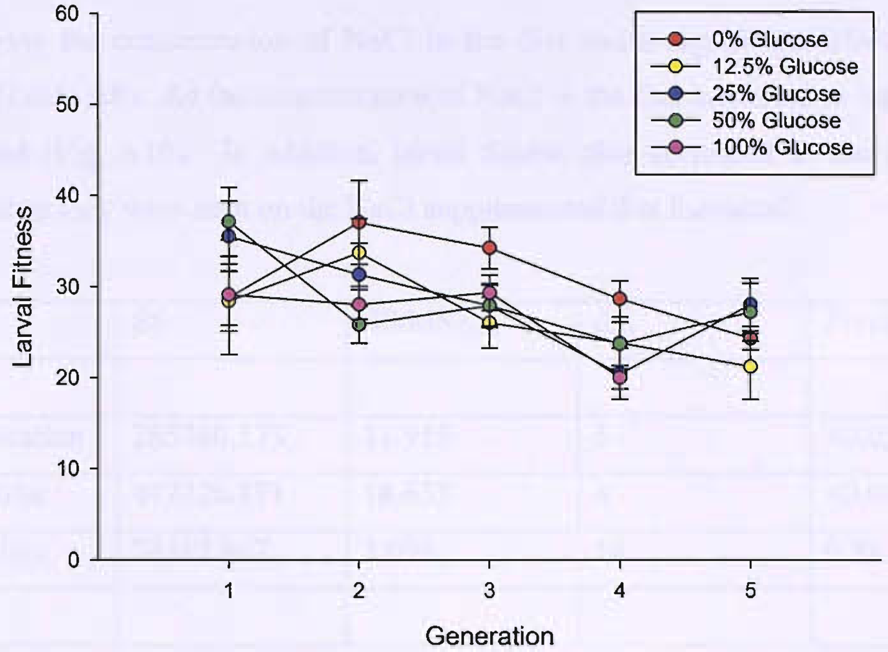


Figure A8: The effect of varying glucose concentration in the diet over 5 generations on larval fitness. Results showed no significant affect of glucose concentration in the diet on larval fitness for any of the generations.

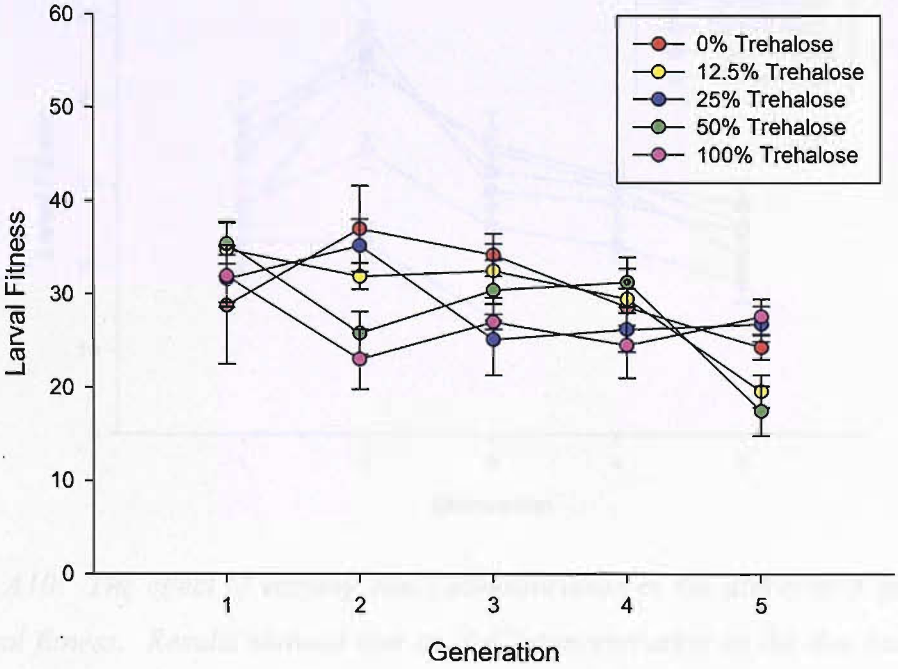


Figure A9: The effect of varying trehalose concentration in the diet over 5 generations on larval fitness. Results showed no significant affect of trehalose concentration in the diet on larval fitness for any of the generations.

Varying the concentration of NaCl in the diet had a significant affect on larval fitness (Table A8). As the concentration of NaCl in the diet increased so larval fitness decreased (Fig. A10). In addition, larval fitness also decreased as the number of generations they were kept on the NaCl supplemented diet increased.

	SS	SS/MS_{Total}	d.f.	*P*-value
Concentration	285980.573	11.915	5	<0.05
Generation	447726.891	18.653	4	<0.01
Interaction	74367.867	3.098	18	0.93

Table A8: The results of a nonparametric 2-way ANOVA to determine the effects of NaCl concentration in the diet over 5 generations on larval fitness.

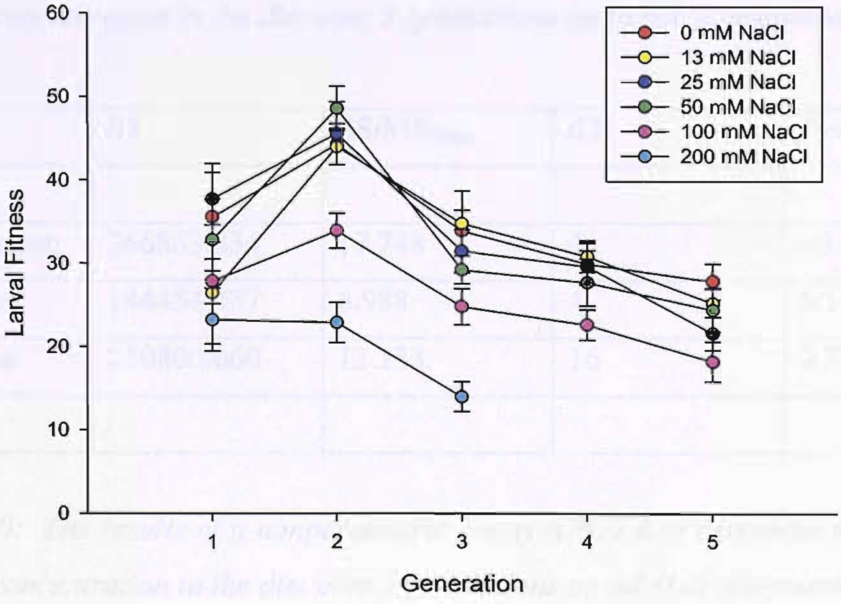


Figure A10: The effect of varying NaCl concentration in the diet over 5 generations on larval fitness. Results showed that as NaCl concentration in the diet increased so larval fitness decreased. In addition, for all food types there was a decrease in larval fitness over the generations.

A1.3 The Effect of Diet Over 5 Generations on Development Time

Varying the concentration of fructose in the diet had a significant affect on the development time of both the pupae and the adults (Tables A9 and A10 respectively). The time taken for both the pupae and the adults to develop was higher when raised on a 0% fructose diet compared to those raised on all other concentrations of fructose (Figs. A11 and A12).

	SS	SS/MS_{Total}	d.f.	*P*-value
Concentration	475067.689	22.979	4	<0.01
Generation	104178.111	5.039	4	0.28
Interaction	246028.982	11.901	16	0.75

Table A9: The results of a nonparametric 2-way ANOVA to determine the effects of fructose concentration in the diet over 5 generations on pupal development time.

	SS	SS/MS_{Total}	d.f.	*P*-value
Concentration	366863.834	17.748	4	<0.01
Generation	144454.487	6.988	4	0.14
Interaction	250800.660	12.133	16	0.73

Table A10: The results of a nonparametric 2-way ANOVA to determine the effects of fructose concentration in the diet over 5 generations on adult development time.

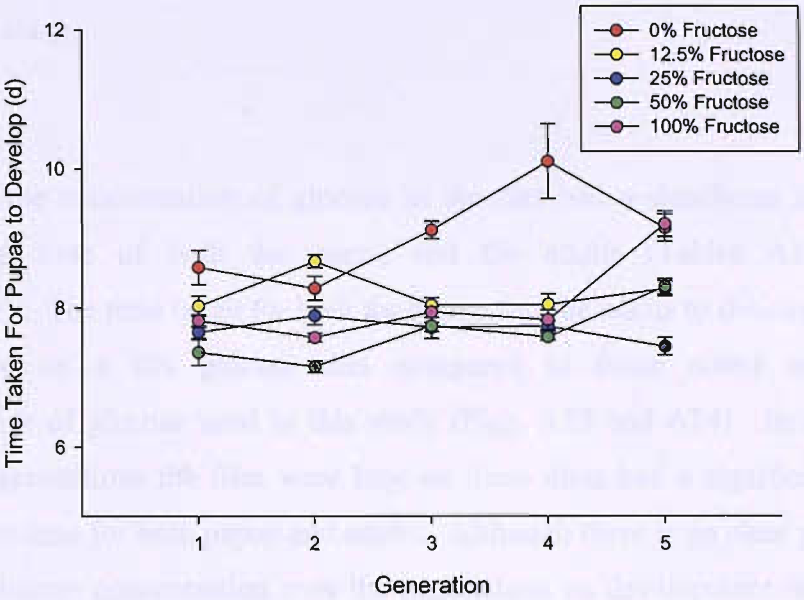


Figure A11: The effect of varying fructose in the diet on the time taken for the pupae to develop over 5 generations. Data points represent means ± standard errors for number days taken for the pupae to develop. Results show that when raised on 0% fructose pupae took longer to develop than when raised on the other concentrations used in this study.

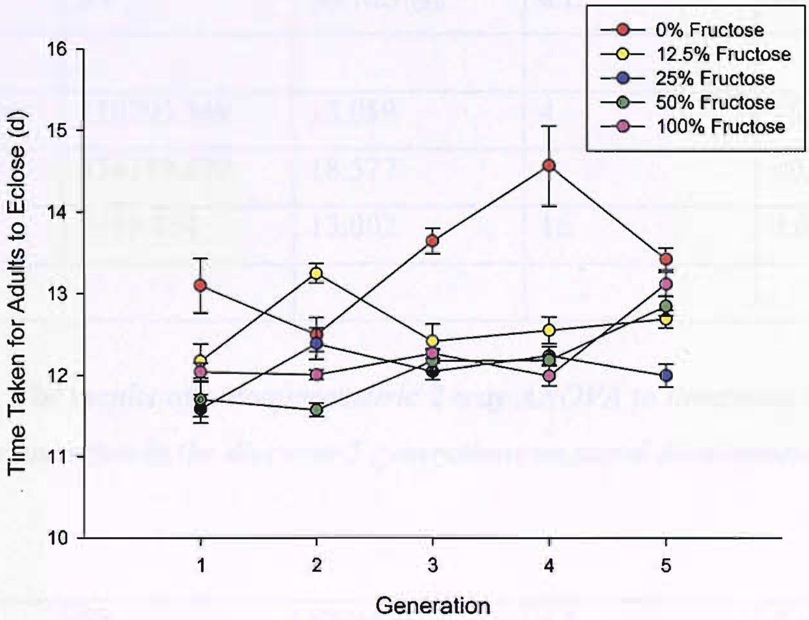


Figure A12: The effect of varying fructose in the diet on the time taken for the adults to develop over 5 generations. Data points represent means ± standard errors for number days taken for the adults to develop. Results show that when raised on 0%

fructose adults took longer to develop than when raised on the other concentrations used in this study.

Varying the concentration of glucose in the diet had a significant affect on the development time of both the pupae and the adults (Tables A11 and A12 respectively). The time taken for both the pupae and the adults to develop was higher when raised on a 0% glucose diet compared to those raised on other the concentrations of glucose used in this study (Figs. A13 and A14). In addition, the number of generations the flies were kept on these diets had a significant affect on development time for both pupae and adults. Although there is no clear pattern in the effects of glucose concentration over the generations on development time, it would appear that for all diets apart from 100% glucose (which seems to remain the same throughout the generations) there is a slow increase in development time over the generations until generation 5 where development time decreases again (Figs. A13 and A14).

	SS	SS/MS_{Total}	d.f.	*P*-value
Concentration	250795.548	13.059	4	<0.01
Generation	356759.939	18.577	4	<0.01
Interaction	2469.854	13.002	16	0.67

Table A11: The results of a nonparametric 2-way ANOVA to determine the effects of glucose concentration in the diet over 5 generations on pupal development time.

	SS	SS/MS_{Total}	d.f.	*P*-value
Concentration	218971.099	11.390	4	<0.05
Generation	3310161.544	16.133	4	<0.01

Interaction	291647.218	15.170	16	0.51

Table A12: The results of a nonparametric 2-way ANOVA to determine the effects of glucose concentration in the diet over 5 generations on adult development time.

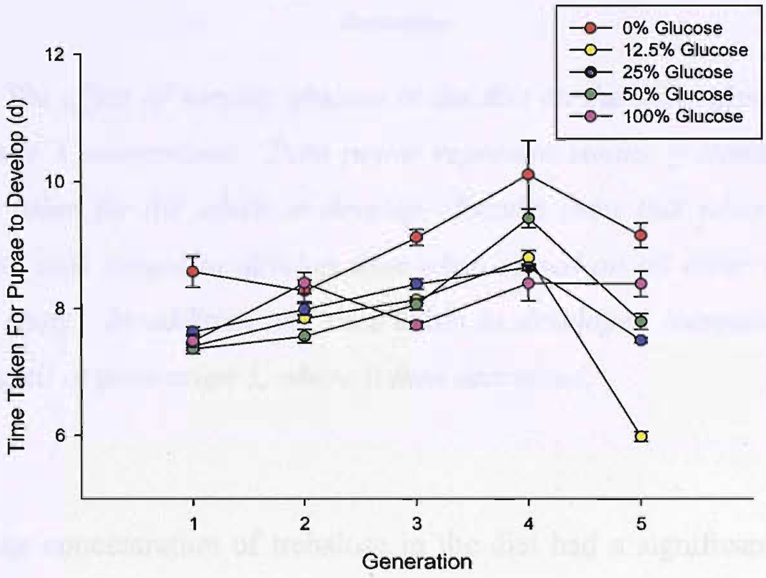


Figure A13: The effect of varying glucose in the diet on the time taken for the pupae to develop over 5 generations. Data points represent means ± standard errors for number days taken for the pupae to develop. Results show that when raised on 0% glucose pupae took longer to develop than when raised on all other concentrations used in this study. In addition, the time taken to develop increased through the generations until at generation 5, where it decreased.

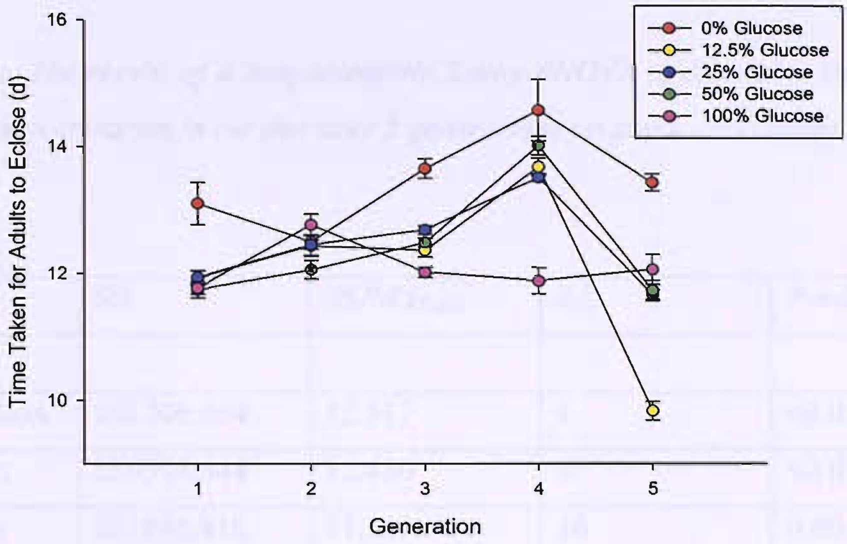


Figure A14: The effect of varying glucose in the diet on the time taken for the adults to develop over 5 generations. Data points represent means ± standard errors for number days taken for the adults to develop. Results show that when raised on 0% glucose adults took longer to develop than when raised on all other concentrations used in this study. In addition, the time taken to developed increased through the generations until at generation 5, where it then decreased.

Varying the concentration of trehalose in the diet had a significant affect on the development time of both the pupae and the adults. There was also a significant affect of generation on the development time of both the pupae and the adults (Tables A13 and A14 respectively). In the generations 1 and 2, the development time of the adults and the pupae do not appear to differ depending on trehalose concentration in the diet. In generations 3 – 5, however, the development time of the pupae and the adults increased when raised on 0% and 100% trehalose (Figs. A15 and A16).

	SS	SS/MS_{Total}	d.f.	*P*-value
Concentration	422287.256	20.247	4	<0.01
Generation	45157.256	2.165	4	<0.05
Interaction	225549.904	10.815	16	0.82

Table A13: The results of a nonparametric 2-way ANOVA to determine the effects of trehalose concentration in the diet over 5 generations on pupal development time.

	SS	SS/MS$_{Total}$	d.f.	*P*-value
Concentration	261206.664	12.517	4	<0.01
Generation	259394.344	12.430	4	<0.01
Interaction	231846.816	11.110	16	0.80

Table A14: The results of a nonparametric 2-way ANOVA to determine the effects of trehalose concentration in the diet over 5 generations on adult development time.

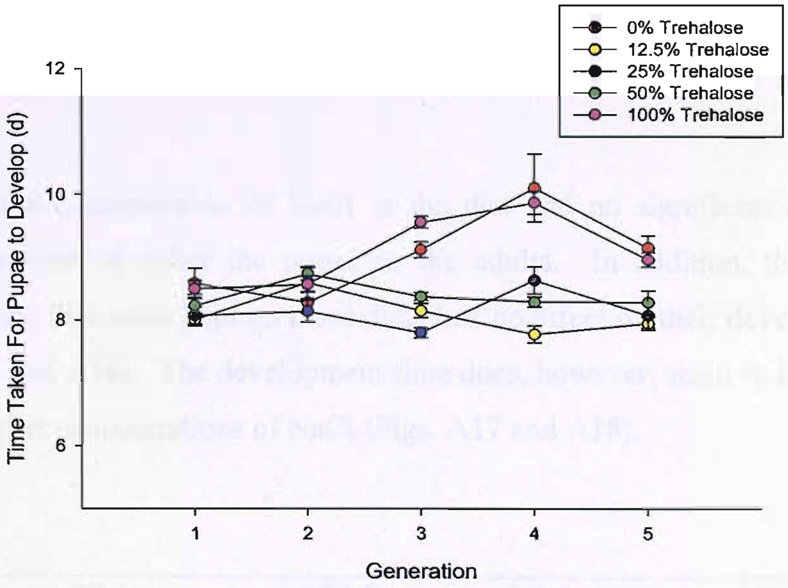


Figure A15: The effect of varying trehalose concentration in the diet on the time taken for the pupae to develop over 5 generations. Data points represent means ± standard errors for number days taken for the pupae to develop. Results show for the first two generations there was no change in development time for any of the food types. In generations 3 – 5 there was an increase in development time for both 0% and 100% trehalose.

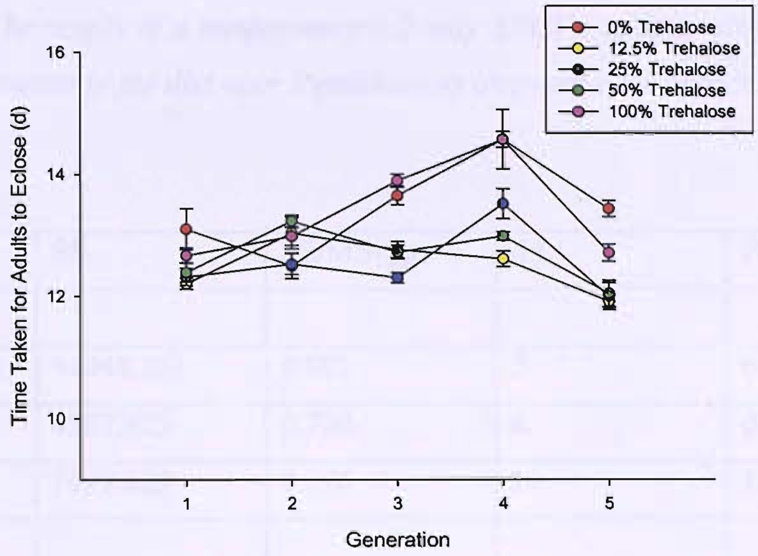


Figure A16: The effect of varying trehalose concentration in the diet on the time taken for the adults to develop over 5 generations. Data points represent means ± standard errors for number days taken for the adults to develop. Results show for the first two generations there was no change in development time for any of the food types. In generations 3 – 5 there was an increase in development time for both 0% and 100% trehalose.

Varying the concentration of NaCl in the diet had no significant affect on the development time of either the pupae or the adults. In addition, the number of generations the flies were kept on these diets had no effect on their development time (Tables A15 and A16). The development time does, however, seem to increase when raised on higher concentrations of NaCl (Figs. A17 and A18).

	SS	SS/MS$_{Total}$	d.f.	*P*-value
Concentration	26391.638	4.150	5	0.53
Generation	4460.195	0.701	4	0.95
Interaction	15343.711	2.413	12	1.00

Table A15: The results of a nonparametric 2-way ANOVA to determine the effects of NaCl concentration in the diet over 5 generations on pupal development time.

	SS	SS/MS$_{Total}$	d.f.	*P*-value
Concentration	44468.161	6.981	5	0.22
Generation	4587.925	0.720	4	0.95
Interaction	7492.629	1.176	11	1.00

Table A16: The results of a nonparametric 2-way ANOVA to determine the effects of NaCl concentration in the diet over 5 generations on adult development time.

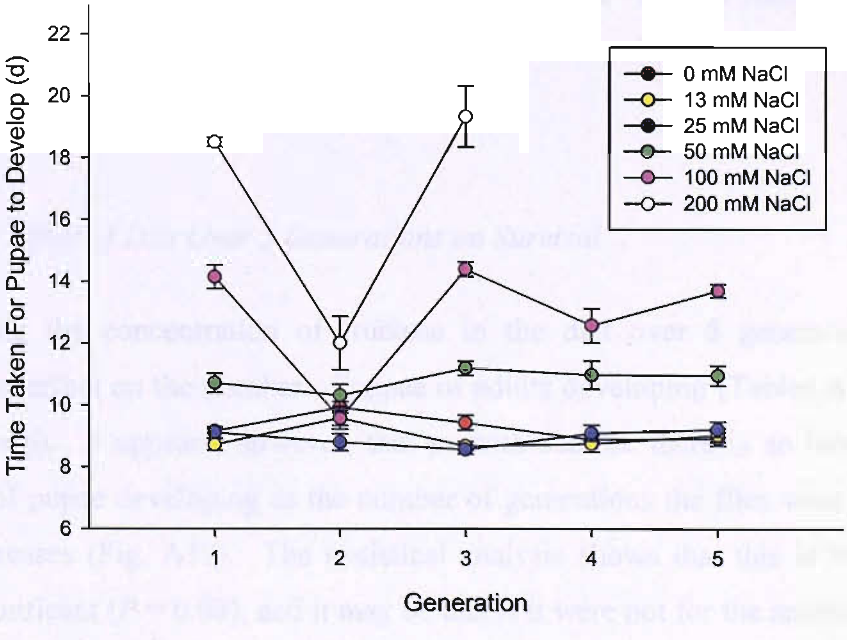


Figure A17: The effect of varying NaCl concentration in the diet on the time taken for the pupae to develop over 5 generations. Data points represent means ± standard errors for number days taken for the pupae to develop. Results show that, although not significant, increased concentration of NaCl in the diet increased the time taken for the pupae to develop.

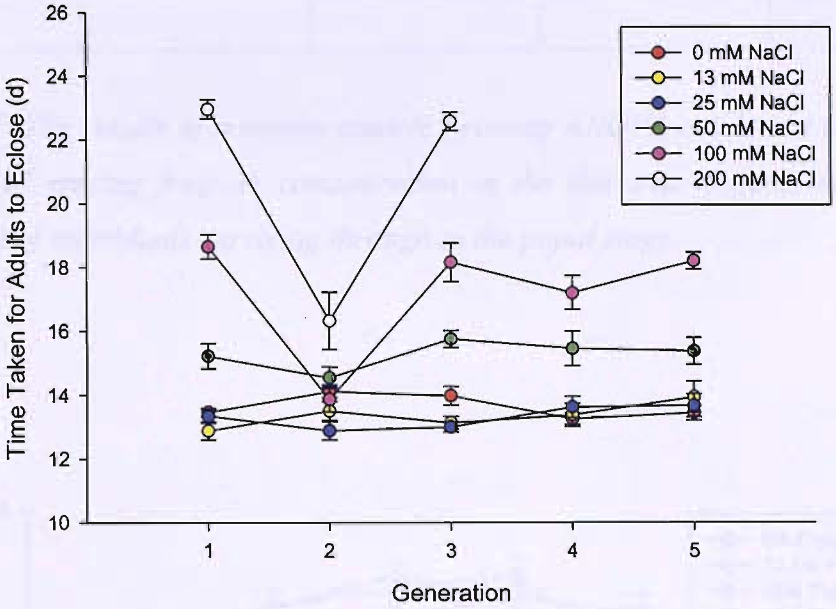


Figure A18: The effect of varying NaCl concentration in the diet on the time taken for the adults to develop over 5 generations. Data points represent means ± standard errors for number days taken for the adults to develop. Results show that, although not significant, increased concentrations of NaCl in the diet increased the time taken for the adults to develop.

A1.4 The Effect of Diet Over 5 Generations on Survival

Varying the concentration of fructose in the diet over 5 generations had no significant affect on the number of pupae or adults developing (Tables A17 and A18 respectively). It appears, however, that as with sucrose there is an increase in the number of pupae developing as the number of generations the flies were kept on the diets increases (Fig. A19). The statistical analysis shows that this is very close to being significant (*P* = 0.08), and it may be that if it were not for the anomalous results for the 100% fructose at generation 5 this may indeed have proved a significant result. This affect was not seen in the number of adults developing (Fig. A20).

	SS	SS/MS_{Total}	d.f.	*P*-value
Concentration	25693.080	1.305	4	0.86
Generation	166135.960	8.438	4	0.08
Interaction	214624.160	10.901	16	0.82

Table A17: The results of a nonparametric two-way ANOVA conducted to determine the effect of varying fructose concentration in the diet over 5 generations on the proportion of individuals surviving through to the pupal stage.

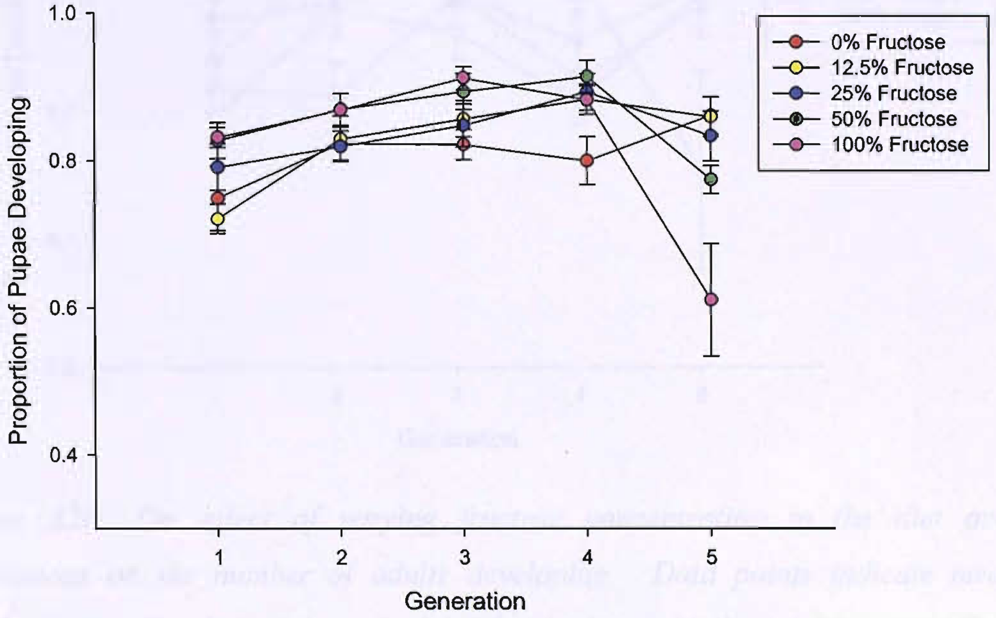


Figure A19: The effect of varying fructose concentration in the diet over 5 generations on the number of pupae developing. Data points indicate mean ± standard error for the number of pupae developing from the 80 eggs introduced into each vial. Results showed that varying fructose concentration in the diet had no significant affect on the number of pupae developing.

	SS	SS/MS_{Total}	d.f.	*P*-value
Concentration	114745.022	5.908	4	0.21
Generation	56441.385	2.905	4	0.57
Interaction	143125.818	7.369	16	0.97

Table A18: The results of a nonparametric two-way ANOVA conducted to determine the effect of varying fructose concentration in the diet over 5 generations on the proportion of adults developing from the total number of pupae that developed.

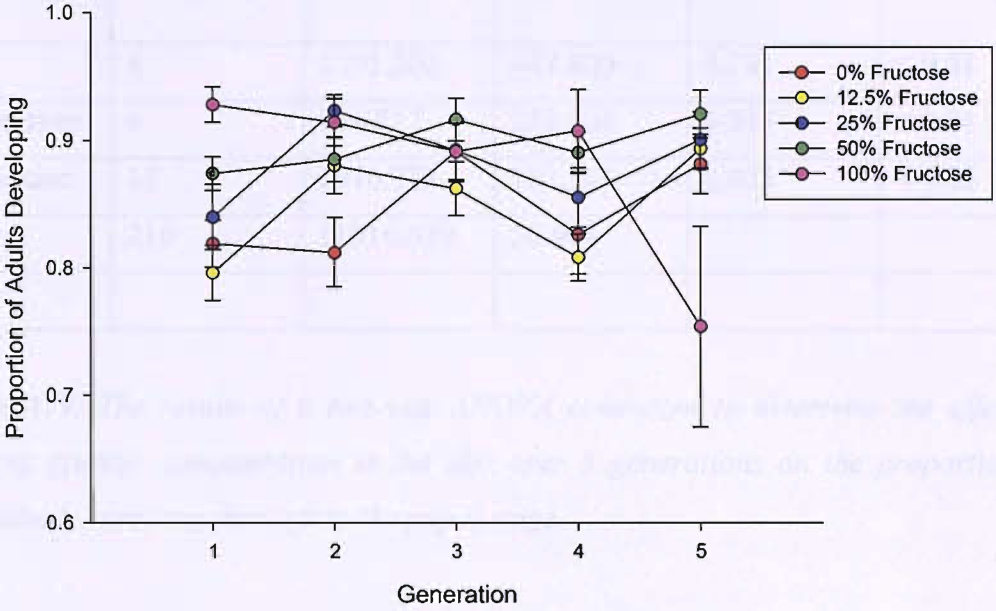


Figure A20: The effect of varying fructose concentration in the diet over 5 generations on the number of adults developing. Data points indicate mean ± standard error for the number of adults developing from the total number of pupae that developed. Results showed that varying fructose concentration in the diet had no significant affect on the number of adults developing.

Varying the concentration of glucose in the diet had a significant affect on the number of pupae developing (Table A19). Increasing the concentration of glucose in the diet up to 50% glucose led to an increase in the number of pupae developing. This then decreased for 100% glucose (Fig. A21). The number of generations these individuals were kept on these diets also had a significant affect on the number of pupae developing (Table A20). There was an initial increase in the number of pupae developing between generations 1 and 2. Generation 3 remained the same, but the number of pupae developing then decreased in generations 4 and 5 (Fig. A22). Varying the concentration of glucose in the diet also had a significant effect on adult survival (Table 6.30) with 100% glucose having the highest proportion of individuals surviving through to adulthood (Fig. 6.36).

Factor	d.f.	SS	MS	*F*-ratio	*P*-value
Diet	4	1791.202	447.800	8.781	< 0.01
Generation	4	934.817	233.704	4.583	< 0.01
Interaction	16	1976.723	123.545	2.423	< 0.05
Error	216	11016.639	50.994		

Table A19: The results of a two-way ANOVA conducted to determine the effect of varying glucose concentration in the diet over 5 generations on the proportion of individuals surviving through to the pupal stage.

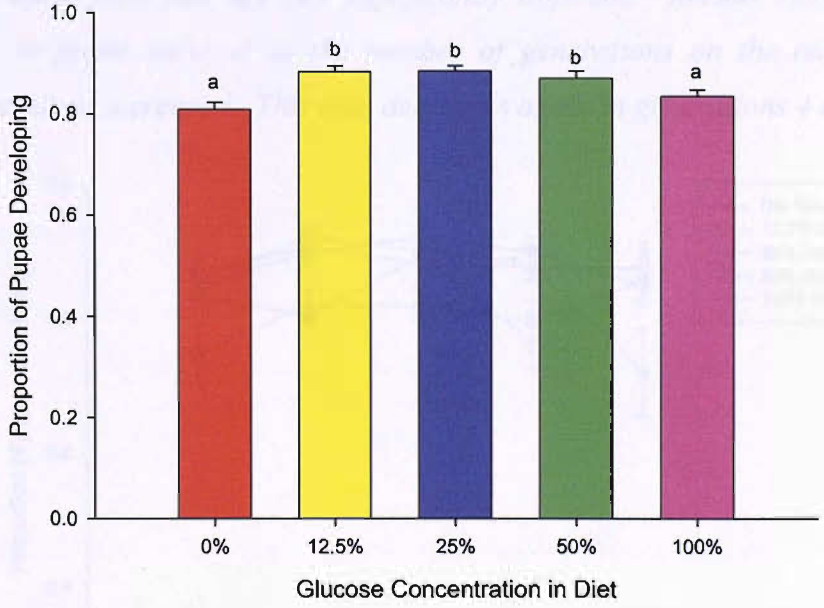


Figure A21: The effect of varying glucose concentration in the diet on the proportion of pupae developing. Bars show mean ± standard errors for the proportion of pupae developing from the 80 eggs initially introduced into each vial. Letters indicate those *bars that are not significantly different. Significance is at the* P < *0.05 level. Results show that the proportion of pupae developing initially increased as the glucose concentration in the diet increased but then decreased again for 100% glucose.*

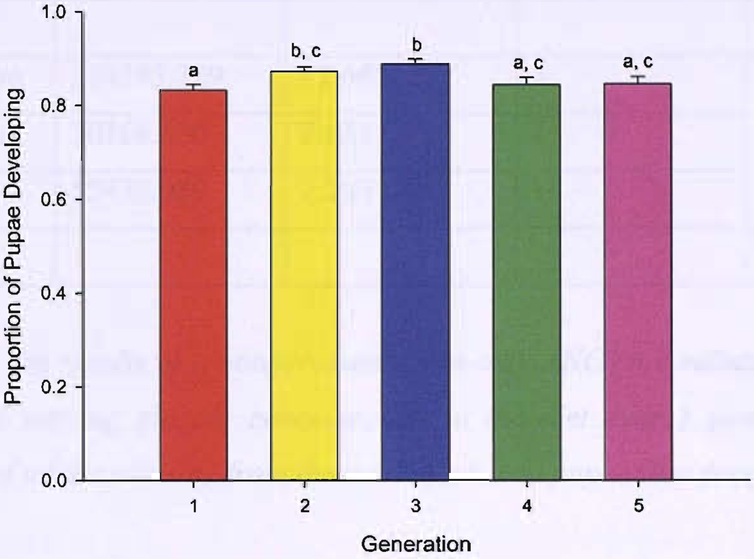


Figure 6.35: The effect of the number of generations the flies were kept on a reduced glucose content medium on pupal survival. Bars indicate means ± standard errors for the number of pupae developing from the 80 eggs placed in to each vial. Letters indicate those bars that are not significantly different. Results showed an initial increase in pupal survival as the number of generations on the reduced glucose content medium increased. This then decreases again in generations 4 and 5.

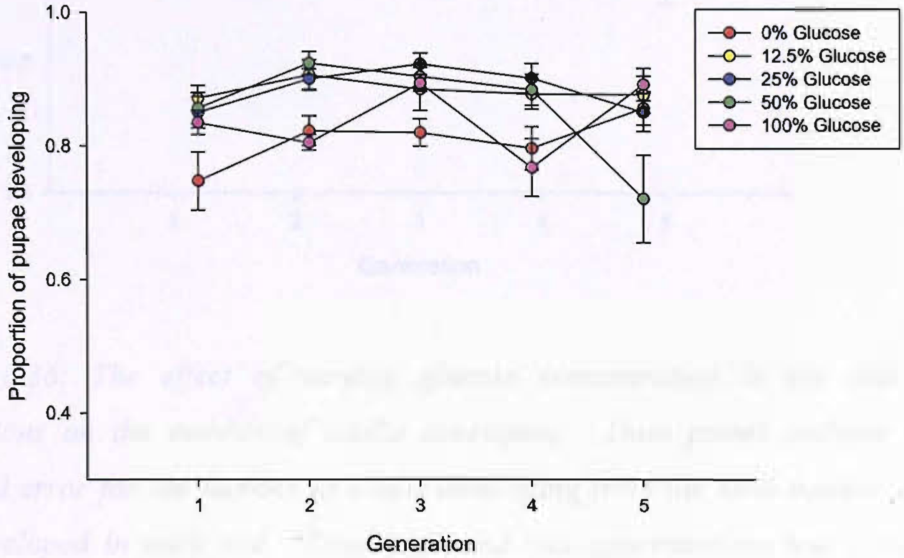


Figure 6.36: The effect of varying glucose concentration in the diet over 5 generations on the number of pupae developing. Data points indicate mean ±

standard error for the number of pupae developing from the 80 eggs that were introduced into each vial. Results showed that both concentration and generation had a significant affect on the proportion of pupae developing.

	SS	SS/MS_{Total}	d.f.	P-value
Concentration	214393.729	11.665	4	< 0.05
Generation	70366.500	3.632	4	0.46
Interaction	52433.989	2.706	16	1.00

Table 6.30: The results of a nonparametric two-way ANOVA conducted to determine the effect of varying glucose concentration in the diet over 5 generations on the proportion of adults eclosing from the total number of pupae that developed.

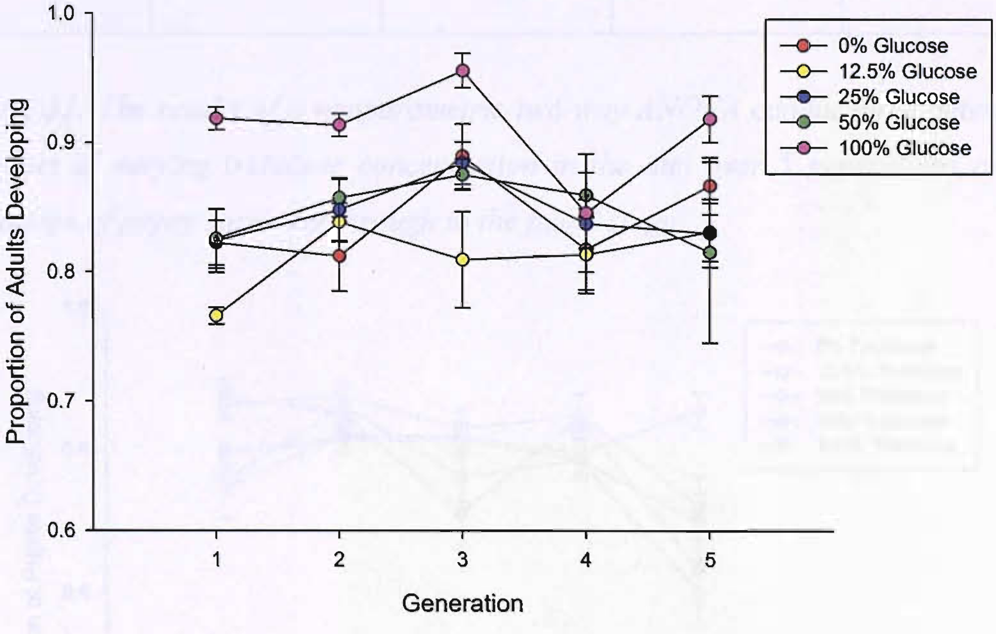


Figure 6.36: The effect of varying glucose concentration in the diet over 5 generations on the number of adults developing. Data points indicate mean ± standard error for the number of adults developing from the total number of pupae that developed in each vial. Results showed that concentration had a significant affect with 100% glucose having the highest proportion of pupae developing.

Varying the concentration of trehalose in the diet had no affect on the number of pupae developing (Table 6.31; Fig. 6.37). It did, however, have a significant affect on the number of adults developing (Table 6.32). As the trehalose concentration in the diet increased so the number of adults developing also increased (Fig. 6.38). In addition, the number of generations these individuals were kept on these diets also had a significant affect on the number of adults developing (Table 6.32). There was an increase in the number of adults developing as the number of generations the flies were kept on the diets increased (Fig. 6.39).

	SS	SS/MS_{Total}	d.f.	*P*-value
Concentration	50309.544	2.531	4	0.64
Generation	146930.304	7.393	4	0.12
Interaction	249697.696	12.563	16	0.70

Table 6.31: The results of a nonparametric two-way ANOVA conducted to determine the effect of varying trehalose concentration in the diet over 5 generations on the proportion of pupae surviving through to the pupal stage.

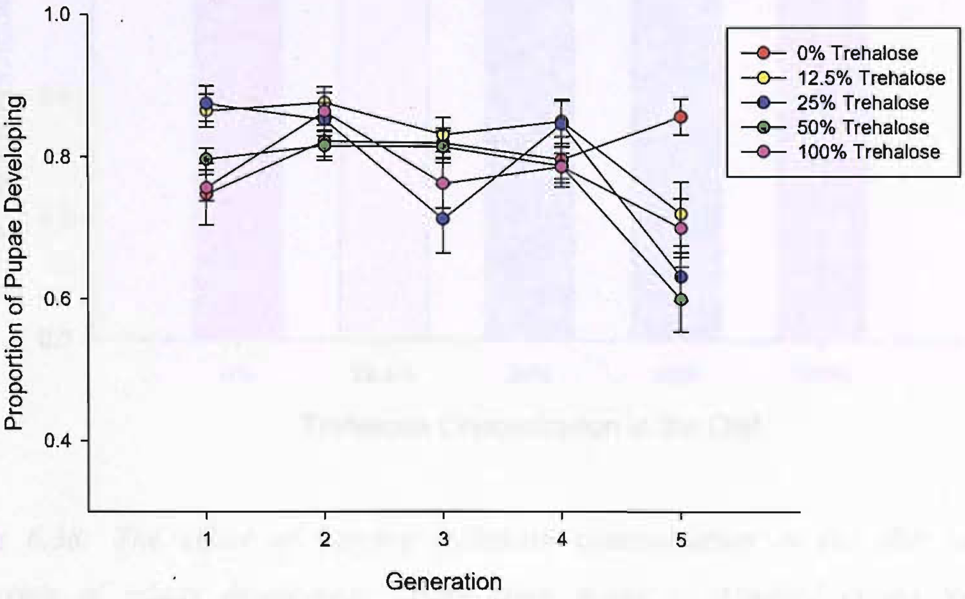


Figure 6.37: The effect of varying trehalose concentration in the diet over 5 generations on the number of pupae developing. Data points indicate mean ±

standard error for the number of pupae developing from the 80 eggs introduced into each vial. Results showed that varying trehalose concentration in the diet had no effect on the number of pupae developing.

Factor	d.f.	SS	MS	*F*-ratio	*P*-value
Diet	4	1211.955	302.989	6.007	< 0.01
Generation	4	1010.315	252.579	5.008	< 0.01
Interaction	16	1610.517	100.657	1.996	< 0.05
Error	225	11348.216	50.437		

Table 6.32: The results of a two-way ANOVA conducted to determine the effect of varying trehalose concentration in the diet over 5 generations on the proportion of adults eclosing from the total number of pupae that developed.

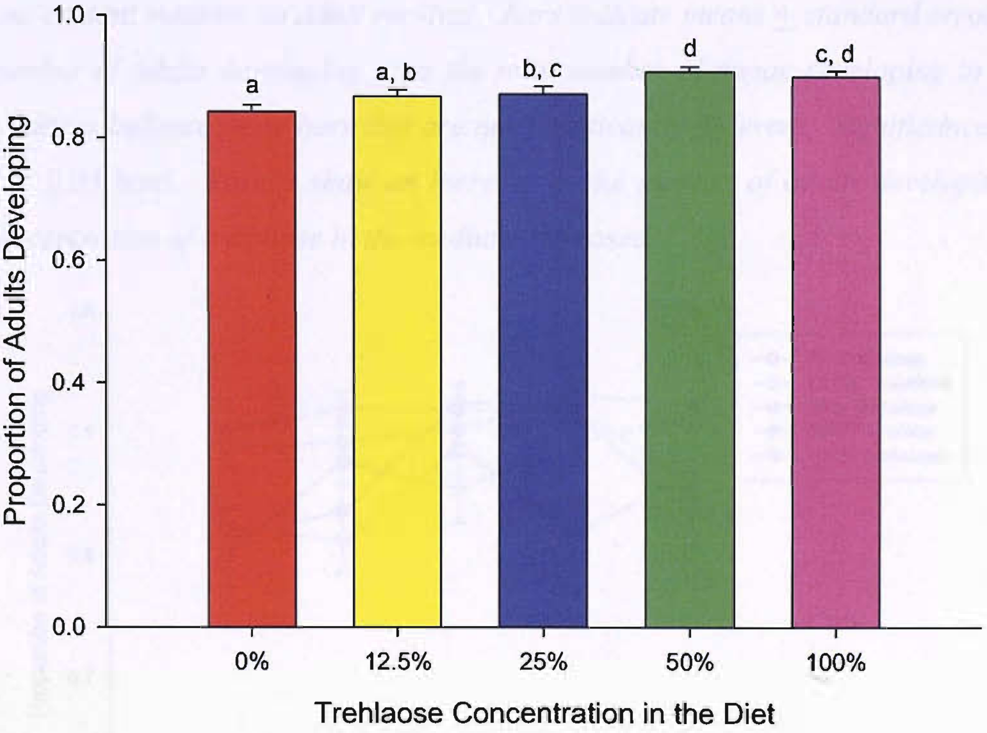


Figure 6.38: The effect of varying trehalose concentration in the diet on the proportion of adults developing. Bars show mean ± standard errors for the proportion of adults developing from the total pupae developing in each vial. Letters indicate those bars that are not significantly. Significance is at the P < *0.05 level.*

Results show that the proportion of adults developing increased with increasing trehalose concentration in the diet.

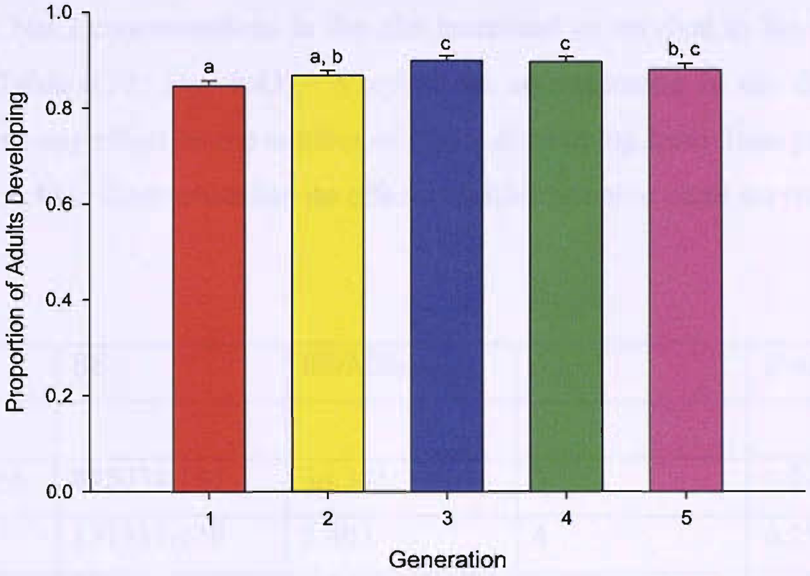


Figure 6.39: The effect of the number of generations the flies were kept on a reduced glucose content medium on adult survival. Bars indicate means ± standard errors for the number of adults developing from the total number of pupae developing in each vial. Letters indicate those bars that are not significantly different. Significance is at the P < *0.05 level. Results show an increase in the number of adults developing as the concentration of trehalose in the medium increased.*

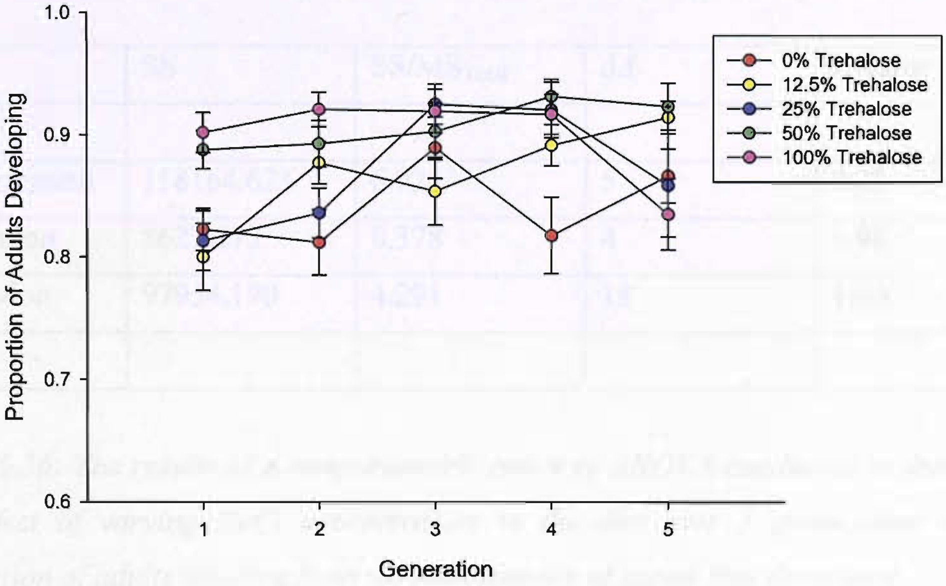


Figure 6.40: The effect of varying trehalose concentration in the diet over 5 generations on the number of adults developing. Data points indicate mean ±

standard error for the number of adults developing from the total number of pupae developing in each vial. Results showed that varying trehalose concentration in the diet had no effect on the number of adults developing.

When NaCl concentrations in the diet increased so survival to the pupal stage decreased (Table 6.35; Fig. 6.43). Varying the concentration in the diet did not, however, have any effect on the number of adults developing from these pupae (Table 6.36; Figure 6.44). Generation had no effect on either pupal or adult survival.

	SS	SS/MS_{Total}	d.f.	*P*-value
Concentration	835034.184	34.381	5	< 0.01
Generation	131331.136	5.407	4	0.25
Interaction	214987.344	8.857	18	0.96

Table 6.35: The results of a nonparametric two-way ANOVA conducted to determine the effect of varying NaCl concentration in the diet over 5 generations on the proportion of individuals surviving through to the pupal stage.

	SS	SS/MS_{Total}	d.f.	*P*-value
Concentration	158164.621	6.928	5	0.23
Generation	8627.873	0.378	4	0.98
Interaction	97954.190	4.291	18	1.00

Table 6.36: The results of a nonparametric two-way ANOVA conducted to determine the effect of varying NaCl concentration in the diet over 5 generations on the proportion of adults eclosing from the total number of pupae that developed.

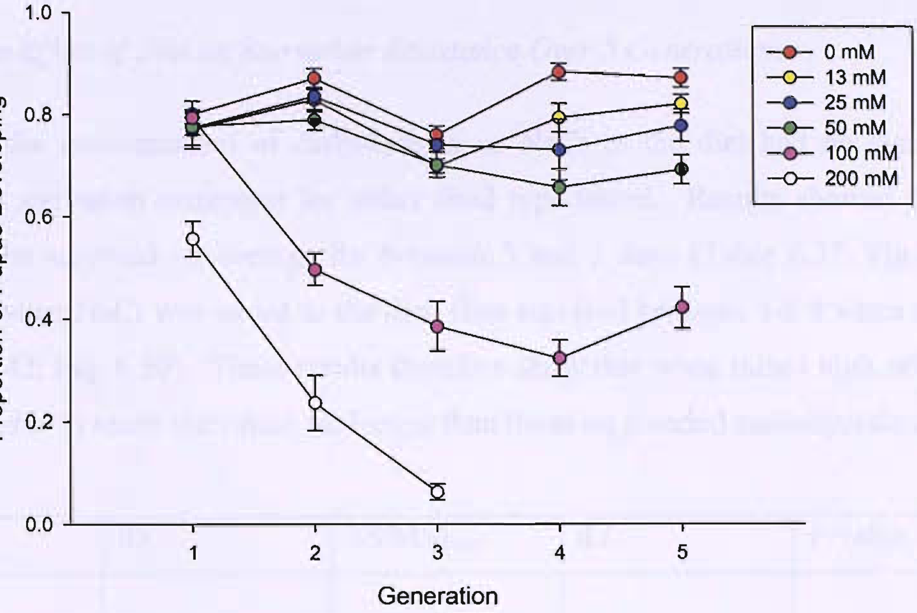


Figure 6.43: The effect of varying NaCl concentration in the diet over 5 generations on the number of pupae developing. Data points indicate mean ± standard error for the number of pupae developing from the 80 eggs transferred into each vial. Results showed that as NaCl concentration in the diet increased so the time taken for the pupae to develop also increased.

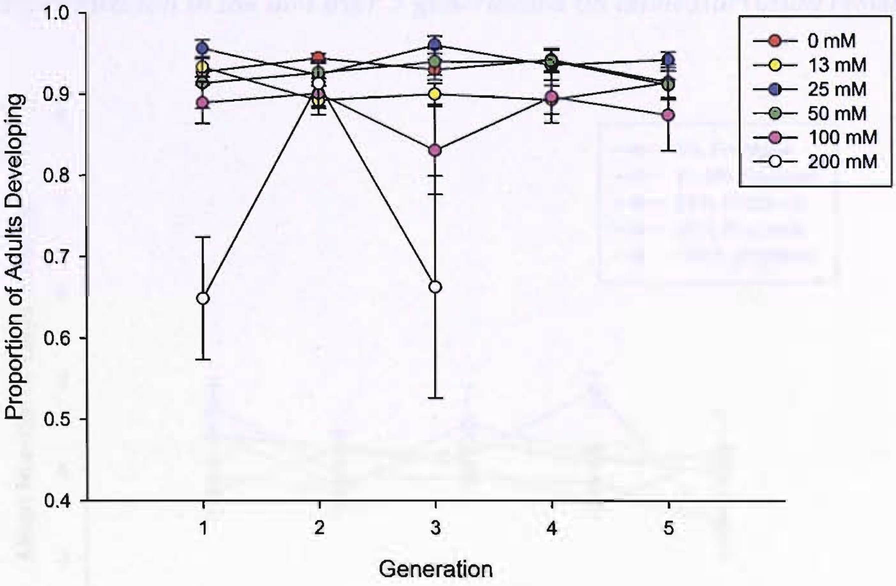


Figure 6.44: The effect of varying NaCl concentration in the diet over 5 generations on the number of adults developing. Data points indicate mean ± standard error for the number of adults developing from the total number of pupae that developed. Results showed that NaCl concentration in the diet had no effect on adult survival.

A.1.5 The Effect of Diet on Starvation Resistance Over 5 Generations

Varying the concentration of carbohydrate or NaCl in the diet had no significant effect on starvation resistance for either food type tested. Results showed that for xxx, adults survived on average for between 3 and 5 days (Table 6.37; Fig. 6.45). Finally, when NaCl was added to the diet, flies survived between 4-6 d when starved (Table 6.42; Fig. 6.50). These results therefore show that when raised high salt diets, flies are able to resist starvation for longer than those on a varied carbohydrate diet.

	SS	SS/MS_{Total}	d.f.	*P*-value
Concentration	517.3	0.161	4	1.00
Generation	5599.0	10747	4	0.78
Interaction	27906.2	8.707	16	0.92

Table 6.38: The results of a nonparametric 2-way ANOVA to determine the effects of fructose concentration in the diet over 5 generations on adult starvation resistance.

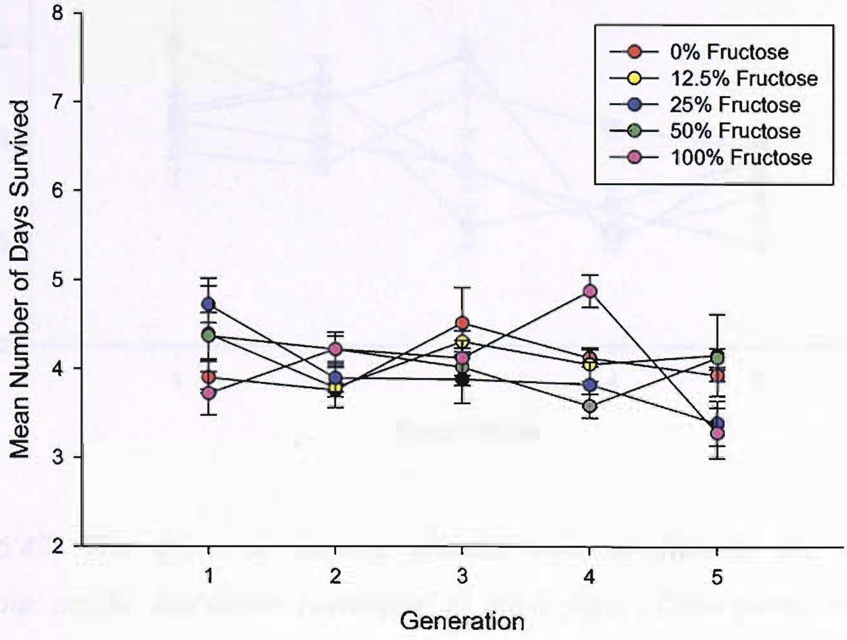


Figure 6.46: The effect of varying fructose concentration in the diet over 5 generations on the starvation resistance of adult flies. Data points represent the mean ± standard error for the number of days survived. Results show no significant effect of fructose concentration in the diet on starvation resistance.

	SS	SS/MS$_{Total}$	d.f.	*P*-value
Concentration	2526.784	1.14	4	0.89
Generation	11541.234	5.209	4	0.27
Interaction	13722.447	6.194	15	0.98

Table 6.39: The results of a nonparametric 2-way ANOVA to determine the effects of glucose concentration in the diet over 5 generations on adult starvation resistance.

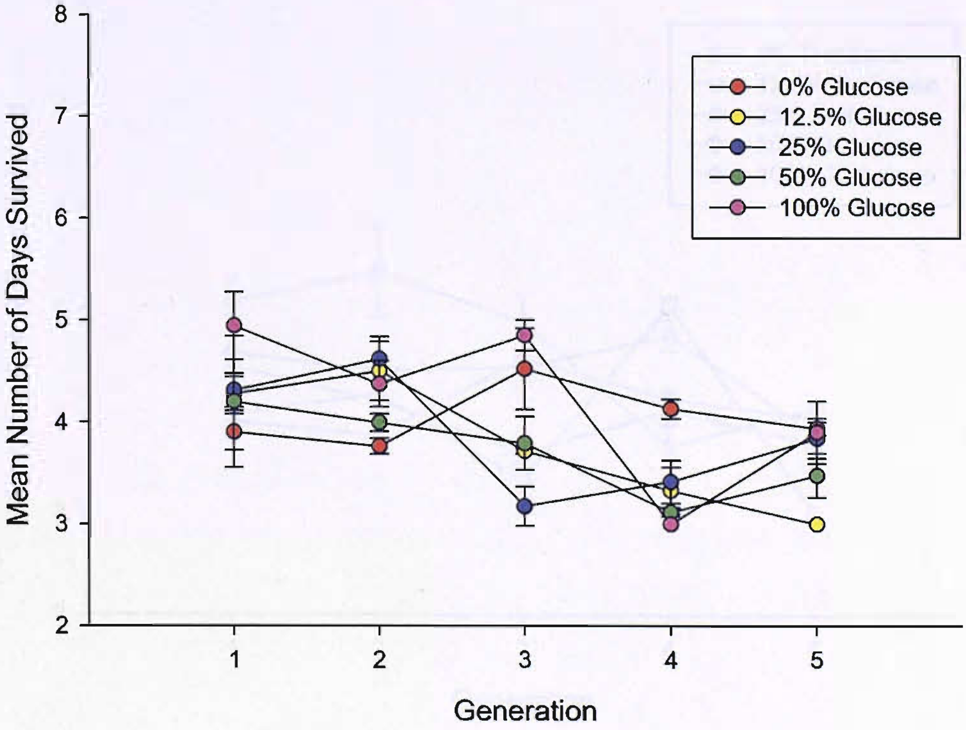


Figure 6.47: The effect of varying glucose concentration in the diet over 5 generations on the starvation resistance of adult flies. Data points represent the mean ± standard error for the number of days survived. Results show no significant effect of glucose concentration in the diet on starvation resistance.

	SS	SS/MS_{Total}	d.f.	P-value
Concentration	13047.404	4.021	4	0.40
Generation	7164.301	20208	4	0.70
Interaction	16801.709	5.178	16	0.99

Table 6.40: The results of a nonparametric 2-way ANOVA to determine the effects of trehalose concentration in the diet over 5 generations on adult starvation resistance.

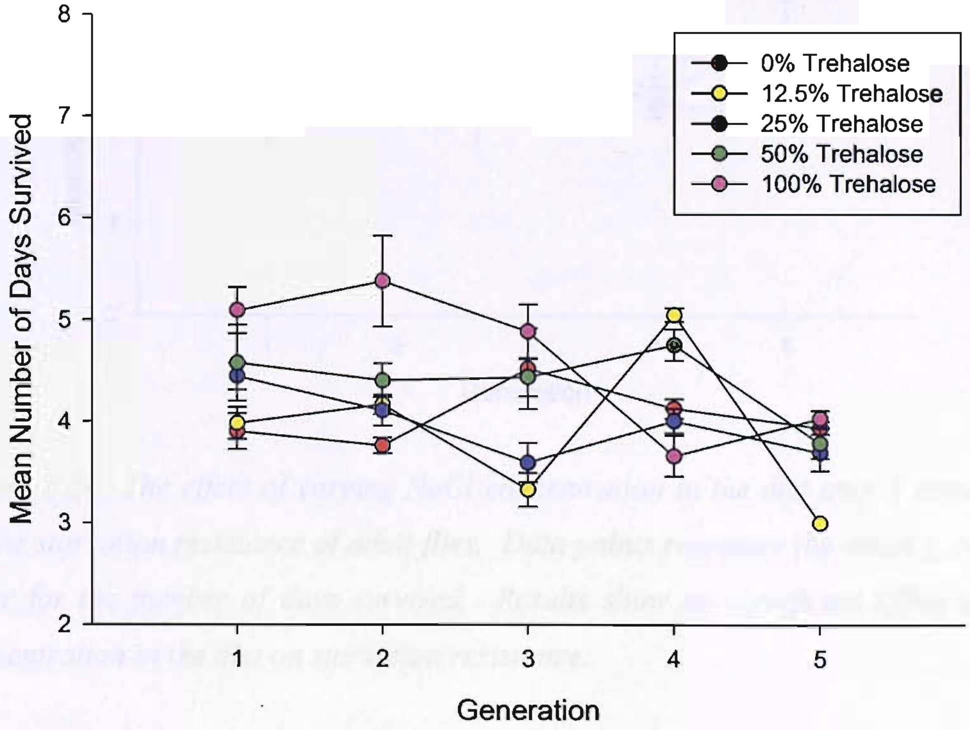


Figure 6.48: The effect of varying trehalose concentration in the diet over 5 generations on the starvation resistance of adult flies. Data points represent the mean ± standard error for the number of days survived. Results show no significant effect of trehalose concentration in the diet on starvation resistance.

	SS	SS/MS_{Total}	d.f.	P-value
Concentration	12480.627	3.325	5	0.65
Generation	10076.085	2.684	4	0.61
Interaction	14213.640	3.787	17	1.00

Table 6.42: The results of a nonparametric 2-way ANOVA to determine the effects of NaCl concentration in the diet over 5 generations on adult starvation resistance.

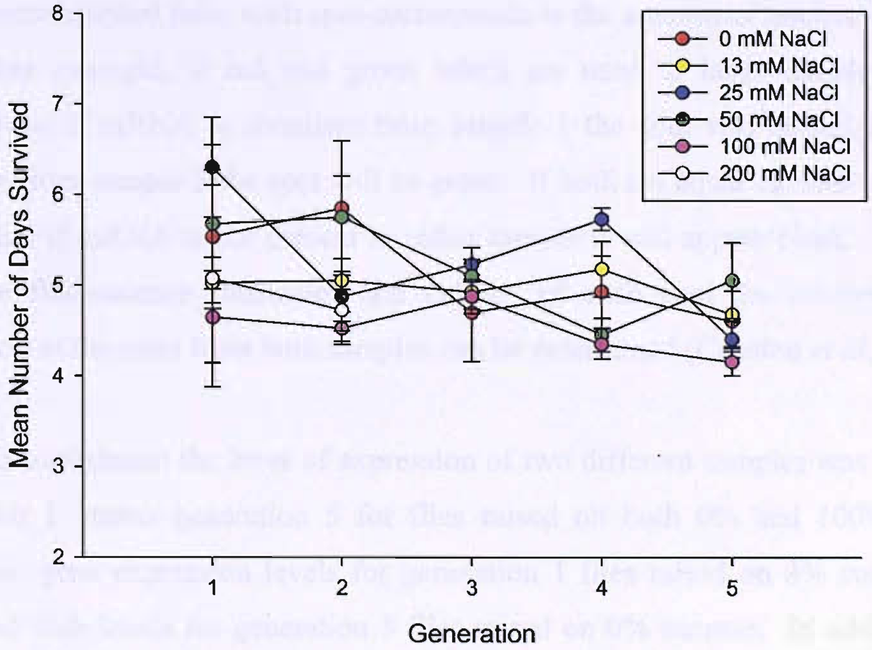


Figure 6.50: The effect of varying NaCl concentration in the diet over 5 generations on the starvation resistance of adult flies. Data points represent the mean ± standard error for the number of days survived. Results show no significant effect of NaCl concentration in the diet on starvation resistance.

A.1.6 Microarrays – An Overview

A microarray is a tool for measuring gene expression. It consists of glass slide containing samples of many genes arranged in a regular pattern. Each location where a DNA molecule is located is called a *spot*. In this experiment the gene expression levels of two samples were compared. This was done by labelling the mRNA from the two different samples with two different dyes. These samples are then hybridising with the microarray. The mRNA will hybridise with the DNA molecule on the microarray from which it originated. The microarray is then excited with a laser and scanned for wavelengths suitable for the detection of the dyes. The amount of fluorescence emitted from each spot corresponds to the amount of nucleic acid bound to it. For example, if red and green labels are used to label samples 1 and 2 respectively, if mRNA is abundant from sample 1 the spot will be red and if it is abundant from sample 2 the spot will be green. If both are equal the spot will appear yellow and if mRNA is not present in either sample it will appear black. Therefore, from the fluorescence intensities and colours of each spot the relative level of expression of the gene from both samples can be determined (Causton *et al*, 2003).

In this experiment the level of expression of two different samples was compared, generation 1 versus generation 5 for flies raised on both 0% and 100% sucrose. Therefore, gene expression levels for generation 1 flies raised on 0% sucrose were compared with levels for generation 5 flies raised on 0% sucrose. In addition, gene expression levels for generation 1 flies raised on 100% sucrose were compared with levels for generation 5 flies raised on 100% sucrose. In this study, the microarrays plus all associated protocols (apart from sample preparation) were conducted by FlyChip, Cambridge. The following description of methods (apart from sample preparation) is therefore just a brief description of the methods used by FlyChip. More detailed methodology can be found on their website (www.flychip.org.uk).

A.1.7 Sample Preparation

50 μg total RNA per slide is required. To ensure that this amount could be extracted a pilot study was conducted using from 30 adult females. These samples

were sent to FlyChip, who confirmed that enough RNA could be extracted from these samples to run the experiments. The samples were prepared for posting to FlyChip by collecting 30 3 – 10 d old adult females. These were placed into an RNase free 1.5 ml microfuge tube (Anachem). 300 μl TRIzol (Invitrogen) was added and the sample which was then homogenised for 60 secs using an RNase free 1.5 ml pellet pestle (Anachem) over ice. These were then flash frozen in liquid nitrogen and stored in a -78°C freezer until all samples had been collected. Samples were then sent on dry ice to FlyChip for further processing.

A.1.8 RNA Extraction, Labelling and Hybridisation

The method used for RNA extraction can be seen on the FlyChip website (www.flychip.org.uk). The method involved the use of TRIzol (Invitrogen) which maintains the integrity of the RNA whilst disrupting cells and dissolving cell components (Chomczynski, 1993). Once extracted, the RNA was reverse transcribed to produce cDNA. A second strand synthesis was then conducted to produce dsDNA. The samples were then differentially labelled with the florescent dyes Cy3 (green) and Cy5 (red) using Klenow labelling. The two samples were then mixed together and hybridised to the microarray. Image acquisition was then conducted using the GenePix Axon scanner. The sample labelled with Cy5 was dye swapped in one of the microarrays to control for any differences between incorporation rates of the dyes.

Microarrays can come in several different formats. In this study a long-oligonucleotide microarray was used. This is a slide on which oligonucleotide molecules are attached at fixed locations called spots. The spots are 90-120 μm in diameter. These oligonucleotides contain between 65 and 69 nucleotides (Cherbas *et al*, 2006). There were 18,240 spots on this microarray, 14,444 being transcript specific and the remainder being controls.

A.1.9 Processing and Statistical Analysis of Microarray Data

The raw data from a microarray experiment are the digital images. To transform this into levels of gene expression, the intensity of each spot is measured. This data is further processed by Flychip to normalise the data. This normalisation involves correcting for systematic differences between data sets due to differences in background fluorescence and overall brightness. It also takes into account the dye swap. The data is presented as a ratio of Cy5/Cy3. The normalised data is therefore very similar to a log[2] scale.

To analyse the microarray data Cyber-T was used (Baldi and Long, 2001). Cyber T (http://cybert.microarray.ics.uci.edu/index.shtml) is a statistical programme that is based on a *t* test to determine whether there is any significant change in expression levels between the paired data.

A.1.10 Determining Homologous Genes Using the Ensembl Database

As mentioned in Section 6.1.6, many genes have been described in mammals which are known to be involved in the effects of maternal diet on the future health of the offspring. The aim of this part of the experiment was to determine whether diet affects these genes in *Drosophila.* To do this, homologous genes in *Drosophila* first had to be identified. The online database, Ensembl (Hubbard *et al*, 2006), compares the sequences of human genes with those of *Drosophila* to provide possible orthologues. All the genes described in Section 6.1.6 were searched for in Ensembl to find those that have orthologues in *Drosophila.* A BLAST analysis was then conducted (Tatusova and Madden, 1999) to measure the similarity between the human and *Drosophila* sequences for all genes where an orthologue was found. The microarray gene expression data of these genes was then analysed (see Section 6.2.10) to determine whether there is a change in expression of these genes with diet over several generations.

A.1.11 Emsembl Analysis for Homologous Genes

The results of the Ensembl analysis to find homologous genes in *Drosophila* showed that only 5 of all the possible human genes have homologues in *Drosophila* (Table 6.43). These genes were ACOX3, CHPT1, LPL, NOS and Dnmt.

Gene	Ensemble Gene	*Drosophila* orthologue	Human Length	*Drosophila* length	Human overlap [a]	*Drosophila* overlap [a]	Identity [b]	Positive [c]	Gaps [d]
GR	NR3C1	N/A							
11βHSD	HSD11B1 HSD11B2	N/A N/A							
PEPCK	PCK1 PCK2	N/A N/A							
PPARα	PPARA	N/A							
PPARγ	PPARG	N/A							
ACOX	ACOX2	N/A							
	ACOX3	CG17544	700	693	0.988571	0.994228	0.428571	0.594286	0.027143
CPT1	CHPT1	CG6016	406	417	0.894089	0.870504	0.520661	0.716253	0
LPL	LPL	CG5966	475	540	0.675789	0.625926	0.313783	0.463343	0.067449
Leptin	LEP	N/A							
LR	LEPR	N/A							
ER	ESR2	N/A							
NOS	NOS2A	CG6713	1153	1349	0.936687	0.849518	0.438961	0.593074	0.072727
GH	GH1 GH2	N/A N/A							

IGF	IGF1 IGF2	N/A N/A							
IR	INSR	N/A							
GLUT4	SLC2A4	N/A							
Adiponectin	ADIPOR1 ADIPOR2 ADIPOQ	N/A N/A N/A							
TNFα	TNF	N/A							
HDAC	HDAC4 HDAC9	N/A N/A							
Dnmt	DNMT2	CG10692	391	345	0.951407	0.944928	0.34375	0.536458	0.182292

Table 6.43: The results of the Ensembl and BLAST analyses to determine which genes known to be involved in the effect of maternal diet on health of the offspring in mammals were also present in Drosophila. *Results show that only 5 of these genes have homologues in* Drosophila. [a] *overlap indicates the similarity between the two sequences, 1 meaning they are identical;* [b] *identity indicates the number of amino acids that are identical;* [c] *positives indicates the number of amino acids that are either exactly the same or in the same family;* [d] *the number of gaps indicates the differences between the sequences.*

A.1.12 Results of the Microarray Experiment

A section from one of the microarrays can be seen in Figure 6.51. The results of the microarray experiment showed that of the 5 genes of interest, only 2 showed a significant change in expression levels between generation 1 and generation 5 for 0% sucrose (Table 6.44). These two genes were ACOX3 and LPL. Expression levels of ACOX3 decreased 2.4 fold in generation 5 compared to generation 1. Expression levels of LPL increased 2.7 fold in generation 5 compared to generation 1 (Fig. 6.52). For 100% sucrose data, there was no significant affect of generation on expression levels of any of the genes (Table 6.45).

Figure 6.51: A section of one of the microarrays. Spots where hybridisation has occurred can be clearly seen along with the colour.

Gene	Mean Intensity	Number of Observation	*t* statistic	*P*- value
ACOX3	-1.0749	3	-9.132	<0.05
CHPT1	-0.147	3	-0.821	0.50
LPL	1.309	3	7.833	<0.05
NOS2A	0.506	3	3.799	0.06
DNMT2	-0.131	3	-0.919	0.46

Table 6.44: Results of the statistical analysis of the microarray data for the 5 genes of interest for 0% sucrose. Results show that only 2 of the genes have significantly different gene expression levels between generation 1 and generation 5.

Gene	Mean Intensity	Number of Observation	*t* statistic	*P*- value
ACOX3	-0.568	3	-3.683	0.07
CHPT1	0.021	3	0.754	0.52
LPL	0.084	3	3.756	0.07
NOS2A	0.169	3	1.828	0.21
DNMT2	0.111	3	0.869	0.48

Table 6.45: Results of the statistical analysis of the microarray data for the 5 genes of interest for 100% sucrose. Results show that none of the genes of interest have significantly different gene expression levels between generation 1 and generation 5.

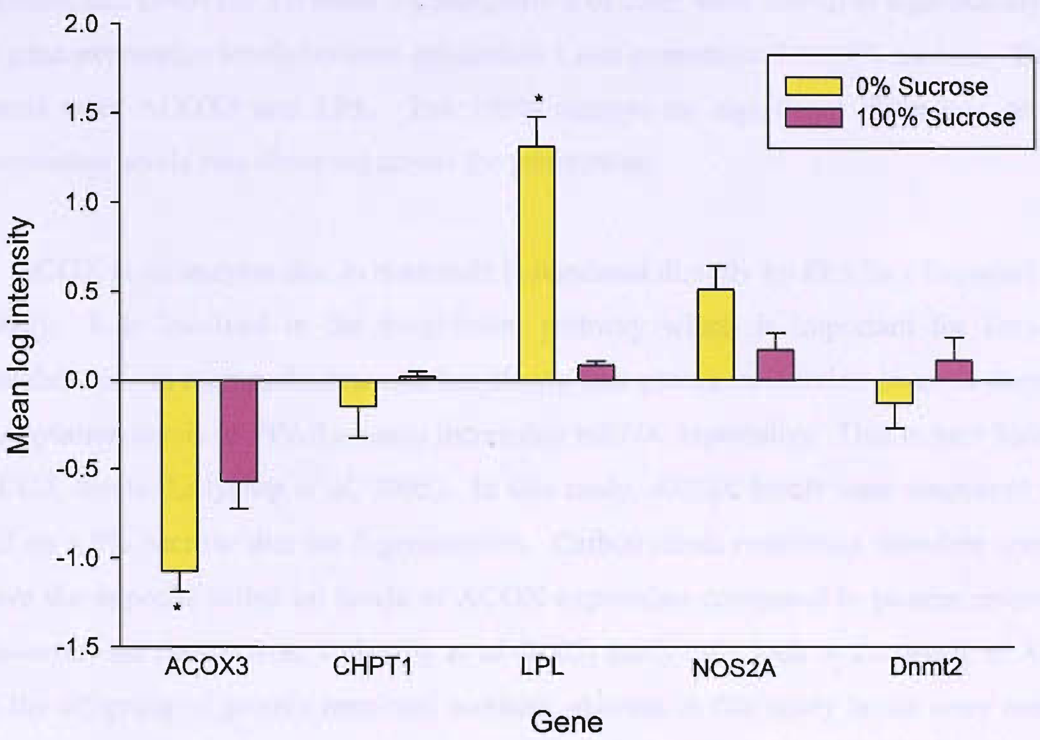


*Figure 6.52: A graph showing the change in gene expression levels of the genes of interest between generation 1 and generation 5 for 0% (yellow) and 100% (pink) sucrose. * indicates a significant difference at the* P *< 0.05 level. Results show that when raised for 5 generations on 0% sucrose the ACOX3 gene is down regulated and the LPL gene up regulated compared to generation 1.*

A.1.13 The Effect of Diet Over 5 Generations on Levels of Gene Expression

As discussed in Section 6.1.6, many genes have been implicated in the effects of *in utero* nutrition on adult health in mammals. The aim of this experiment was to determine whether the expression levels of these genes also varied with nutritional status in *Drosophila*. To do this, the first step was to determine which of the genes described in mammals have orthologues in *Drosophila*. The results of this analysis found 5 genes in *Drosophila* homologous to those in mammals. Those genes were ACOX3, CHPT1, LPL, NOS2A and DNMT2. Of those 5 genes, only 2 of them were shown to significantly vary in gene expression levels between generation 1 and generation 5 for 0% sucrose. These 2 genes were ACOX3 and LPL. For 100% sucrose no significant difference on gene expression levels was observed across the generations.

ACOX is an enzyme that in mammals is regulated directly by PPARα (Tugwood *et al,* 1992). It is involved in the β-oxidation pathway which is important for fatty acid metabolism. In mammals, research has shown that protein restriction *in utero* decreases methylation levels of PPARα hence increasing mRNA expression. This in turn increases ACOX levels (Lillycrop *et al*, 2005). In this study, ACOX levels were decreased when fed on a 0% sucrose diet for 5 generations. Carbohydrate restriction therefore seems to have the opposite effect on levels of ACOX expression compared to protein restriction. However, the results from Lillycrop *et al* (2005) study only look at the levels of ACOX in the offspring of protein restricted mothers whereas in this study levels were measure after 5 generations of a carbohydrate restricted diet. It may therefore be that in mammals if fed a carbohydrate restricted diet for several generations ACOX levels could decrease as can be seen in the results of this experiment.

LPL is an enzyme that hydrolyses lipids into fatty acids. Previous studies have shown that rats maintained on a high carbohydrate diet have elevated levels of LPL in adipose tissue (Dourmashkin *et al*, 2005). In a study by Lillycrop *et al* (2006), the effect of protein restriction *in utero* on the expression of several genes involved in lipid metabolism was investigated. The results showed no effect of protein restriction on LPL levels. However, LPL levels were shown to increase in offspring exposed to a neonatal diet containing leptin and a high fat diet post weaning. Desai *et al* (2006) also reported no effect of *in utero* protein restriction on LPL mRNA expression levels. In this study, however, 5 generations of carbohydrate restriction led to an increase in LPL expression levels compared to those of generation 1. This could be due to the prolonged exposure to a reduced carbohydrate diet inflicted in this study compared to the previous studies where the effects of a restricted diet on LPL levels was only investigated in on generation.

The aim of this experiment was to determine whether diet over several generations can affect levels of gene expression in genes known to be affected by this in mammals. The results show that although not always responding in the same manner some genes do indeed vary in their expression levels depending on length of time exposed to a carbohydrate restricted diet. This would therefore suggest that, as in mammals, dietary restriction in *Drosophila* can lead to altered metabolic pathways. *Drosophila* may therefore provide a new model system for further investigating the effects of dietary restriction on gene expression levels with the aim of gaining a further understanding of how this may go onto affect disease in later life.

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