The impact of chlortetracycline on *Drosophila melanogaster* and *Aedes aegypti*

Emma Victoria Ridley

Ph.D.

University of York
Department of Biology

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Abstract

Microbial symbionts of insects have been demonstrated to play an important role in the nutrition and protection of the host; these include aphids and tsetse. Studies often use antibiotics to eliminate the symbionts but the deleterious impact of using these antibiotics is not commonly addressed. The impact of chlortetracycline treatment on *Aedes aegypti* and *Drosophila melanogaster* was investigated by assessing life-span, fecundity, development time, survival, nutrition and metabolism. The impact on microbial numbers and diversity was also determined.

With *Drosophila,* treatment with 50 μg ml^{-1} and above showed a significant extension in development time and life-span, reduction in fecundity and change in nutritional content. Microbial numbers were significantly reduced at 50 μg ml^{-1} and above. Culturable techniques and 454 pyrosequencing, demonstrated that the microbial diversity of *Drosophila* was predominantly *Acetobacter*. Bacterial elimination through egg dechorionation yielded some similar results to chlortetracycline treatment. However, fecundity and life-span was not significantly affected. Microarray analysis established a significant reduction in the abundance of transcripts associated with immunity, particularly antimicrobial peptides.

With *Aedes aegypti*, treatment significantly reduced the survival and also affected the life-span and nutrition of the insect. Microbial numbers of mosquito larvae were reduced at 30 and 100 μg ml^{-1}. Colonies grew on plates supplemented with 50 μg ml^{-1} of chlortetracycline, indicating that the larvae bore chlortetracycline-resistant bacteria. 454 pyrosequencing demonstrated a change in diversity of bacteria found in mosquitoes +/- chlortetracycline, switching from *Elizabethkingia meningoseptica* to *Raoultella sp* with chlortetracycline.

It is concluded that chlortetracycline significantly impacts the performance of the 2 insects through bacterial depletion, changes to bacterial diversity and toxicity. Nevertheless, different responses were observed with *Aedes aegypti* and *Drosophila melanogaster*. Moreover, experiments with *Drosophila* using egg dechorionation, emphasised the toxic impact of using antibiotics to eliminate microbes in the insect host.

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Declaration

This thesis is a presentation of my research. Whenever contributions of others were involved, acknowledgement of collaborative research has been indicated.

Chapter 1: Introduction

The role of commensal and mutualistic bacteria have recently become appreciated within the scientific community with growing interest in the role of bacteria in humans, with models such as mice and *Drosophila melanogaster* being used (Moran, 2006). This interest has also become widespread within the insect community with research on ants, aphids and disease vectors such as *Anopheles gambiae* (Moran, 2006; Dong *et al*., 2009). Why has there been an increase in interest in bacteria found within these organisms? In this chapter I will outline why the relationship between bacteria and the host are important to the host performance in vertebrates but mainly in insect hosts.

1.1 Commensal bacteria in vertebrate hosts

The main interest in commensal bacteria in humans is to determine their role in human health and disease. The two groups of bacteria that are dominant in the human gut are the Bacteroidetes and Firmicutes (Turnbaugh *et al*., 2006) and the estimated number of bacterial cells within the host outnumbers the cells of the host (Savage, 1977).

The major question is how do bacteria play a role in protecting the host against immune disorders and pathogens? Several experiments have suggested that the bacteria protect the host from allergies and from these results the Hygiene Hypothesis was made. Where it was hypothesised that the presence of bacteria during childhood could educate the immune system and prevent the development of an over-active immune system which could lead to illnesses such as asthma and hay fever (Lui and Murphy, 2003). It has also been suggested that in children, the exposure to bacterial endotoxins could reduce the chance of developing asthma (Lui and Murphy, 2003; Lui, 2002). Further experiments with bacteria have also highlighted the role bacteria can play in the prevention of intestinal diseases (Table 1.1).

Table: 1.1. Bacteria found to promote health and protection against intestinal diseases.

Bacteria Name	Role in disease protection	How does the bacterium protect?	Animal	Key reference(s)
Bifidobacterium longum	Protection against Enterohaemorrhagic *E.coli*	Production of acetate.	Mice	Fukuda *et al*., 2011
Bifidobacterium bifidum	Improves intestinal integrity and protects against enterocolitis	Reduces inflammation, regulates the main components of the mucous layer and improves intestinal integrity.	Rat	Khailova *et al*., 2009
Bifidobacterium Infantis	Enhances intestinal epithelial cell barrier function	Peptide bioactive factors from this bacterium retains biological function, normalizes gut permeability and improves the disease colitis. Changes in MAPK and tight junction proteins.	Mice	Ewaschuk *et al*., 2008
Lactobacillus acidiphilus	Inhibits murine Citrobacter rodentum colitis	Increase TGFβ, IL-10 and decrease in TNF-α, IL-6 and IL-12. S-layer Protein A of *L.plantarum* NCFM regulates immature dendritic cells and T Cell function.	Mice, human	Chen *et al*., 2005 Konstantinov *et al*., 2008
Lactobacillus rhamnosus	Suppressed barrier impairment (Caco-2 cells) and recovered colon length (in mice with colitis)	IL-8 secretion in Caco-2 cells. In mice, increased Zolula occludens-1 and myosin light chain kinase.	Mice and human cells	Miyauchi *et al*., 2009
Lactobacillus reuteri	Inhibits colitis, through anti-inflammatory activity	Increased NGF levels, inhibits NF-Kb translocation to the nucleus.	Human cells	Ma *et al*., 2004
Lactobacillus fermentum	Anti-inflammatory effects in Colitis-induced rats	Decrease in TNFα, colonic myeloperoxidase activity, cyclooxygenase 2 expression and an induction of NO (Nitric oxide) synthase and increase in SCFA (Short-chain fatty acids).	Rats	Peran *et al*., 2007
Bacillus polyfermenticus	Increase in survival and decrease in disease severity of colitis in mice	Decrease in chemokine ligand, Intercellular adhesion molecule and TNFα. An increase of IL-10 and suppression of apoptosis and promoted cell proliferation by PI3K and Akt pathway.	Mice	Im *et al*., 2009
Lactobacillus casei	Inhibits *E.coli* isolated from Crohn's disease from invading intestinal epithelial cells.	Inhibits the interaction of adherent-invasive *E.coli* with intestinal epithelial cells.	Human cells	Ingrassia *et al*.,2005
Bifidobacterium lactis	Improved abdominal girth and gastrointestinal transit with decrease in IBS symptoms	Not available.	Human	Agrawal *et al*., 2008
Bacteroides fragilis	Protects from colitis induced by helicobacter hepaticus	Activity of polysaccharide A (PSA).	Mice	Mazmanian *et al*., 2008

Faecalibacterium prausnitzii	A decrease in this bacterium led to a higher risk of post-operative reoccurrence of ileal Crohn's disease.	Decrease in IL-12, IFN-γ and IL-10. Blocks NF-κB activation and IL-8 production.	Human cells and mice	Sokol *et al*., 2008

Symbiotic bacteria have been suggested to play an important role in preventing pathogens establishing within the host by competing with bacteria that can cause disease (Guarner and Malagelada, 2003) (Table: 1.1). The species, *Bacteroides fragilis* has been shown by Mazmanian *et al* (2008) to protect animals from colitis induced by *Helicobacter hepaticus* (an opportunistic pathogen) through the expression of Polysaccharide A (PSA). Furthermore, a recent paper in 2011 demonstrated that Bifidobacteria protect the human gut against the shiga toxin (produced by *Escherichia coli*) by the production of acetic acid, which has been suggested to improve intestinal defence (Fukuda *et al*., 2011). Gut bacteria have been shown to aid digestion by degrading nutrients such as fibre (Hooper, 2009; Savage, 1986). One example is the bacterium *Bacteroides thetaiotaomicron*, this bacterium has a large number of genes associated with polysaccharide utilisation compared with other micro-organisms found within the human gut, suggesting that this bacterium aids plant carbohydrate digestion (Hooper *et al*., 2009; Xu *et al*., 2003). Gut symbionts are well adapted at utilising the nutrients within the gut, however, pathogenic bacteria tend not to be as efficient as symbiotic bacteria and therefore invade host tissue to obtain nutrients (Hooper *et al*., 2009; Stecher *et al*., 2005; Stecher *et al*., 2007).

The balance between the two dominant groups of bacteria in the human gut, play a key role in disease (Ley *et al*., 2005). In the Western World obesity is fast becoming an epidemic in areas including North America and the United Kingdom (Seidell, 2000). Research into the cause and differences between obese and lean individuals using mice as the model organism have demonstrated the role of gut bacteria in obesity (Turnbaugh *et al*., 2006). A study using obese mice showed that the population of Bacteroidetes was reduced and the Firmicutes was increased (Ley *et al*., 2005). Firmicutes have the ability to breakdown complex polysaccharides intractable to human digestive enzymes and make this available as an energy source for the host (Ley *et al*., 2005). If there are excessive amounts of this group of bacteria, more energy is made available which could potentially be stored as fat. Furthermore, in one particular study Firmicutes were transferred to lean

mice and a weight gain was observed (Ley *et al*., 2005, Turnbaugh *et al*., 2006). This research does suggest that the gut bacteria play a role in obesity but this may not be the major cause of obesity. Obese individuals may have a different gut microbiota due to the diet consumed such as a high fat diet and/or may have a higher population of Firmicutes to deal with the influx of such large amounts of certain nutrients (Ley *et al*., 2005). What this study shows is the importance of gut bacteria, therefore what effects would happen if they were eliminated? One obvious effect would be the loss in ability to gain calories from indigestible food, this may be detrimental to individuals experiencing famine but not necessarily those that have food aplenty. Furthermore, the balance of the bacterial populations of Firmicutes and Bacteroidetes is not only involved in obesity but has been shown to be involved in the autoimmune condition Crohn's disease (Sokol *et al*., 2008). A decrease in the population of Firmicutes has been shown to be associated with individuals who have Crohn's disease (Sokol *et al*., 2008).

1.2 Role of bacterial symbionts in insects

1.2.1 Role of bacteria in insect nutrition and insect performance

Many publications have concentrated on the importance of the microbes of insects on the impact of immunity and protection, but how do these bacteria promote insect performance and nutrition? In this section I will discuss several examples of experiments which have shown the importance of bacteria in the performance of a wide range of insects.

Two of the most famous examples of symbiosis are the aphid-*Buchnera aphidicola* and the tsetse-*Wigglesworthia glossinidia* interactions. Aphids contain the obligate symbiont *Buchnera aphidicola* which is found within specialised cells in the aphid known as bacteriocytes (Buchner, 1965). If this particular symbiont is eliminated from the aphid, the aphids have a reduction in fecundity and are significantly smaller in size (Houk and Griffiths, 1980; Mittler, 1971; Sasaki *et al*., 1991). Dietary experiments have shown that the *Buchnera* provide the aphid host with essential amino acids (Douglas, 1998; Sasaki *et al*., 1991). In tsetse flies, the elimination of the *Wigglesworthia glossinidia* through the treatment with antibiotics affected the performance of the tsetse fly by reducing the fecundity (Nogge, 1976.) This deleterious impact on the fecundity could be reversed by

supplementing the diet with Vitamin B, suggesting that this symbiont provides the insect host with this vitamin (Nogge and Gerresheim, 1982). Studies of the genome of the insect host and bacteria also demonstrate that the symbiont provides the host with essential nutrients (Akman *et al*., 2002; Snyder *et al*., 2010).

Several papers have been published regarding the role of the gut microbiota in the locust, *Schistocerca gregaria*. One publication by Charnley *et al* (1985) did demonstrate that although the elimination of bacteria in the locust does not have a major impact on the nutrition of the insect, bacteria-free insects did have a higher lipid and lower carbohydrate concentration in the hemolymph compared with control insects.

In Western Flower Thrips, the bacterium *Erwinia* is obtained through feeding. The production of aposymbiotic insects had a different performance compared with control insects (De Vries *et al*., 2004). In control insects, the time to maturity was significantly shorter and had higher oviposition rates compared with aposymbiotic insects (De Vries *et al*., 2004). An elimination of the gut microbiota of *Tenebrio molitor* also led to an impact on the performance of the insect where bacteria-free larvae experienced a reduction in mass and premature pupation of more than half of the larvae (Genta *et al*., 2006). Furthermore, a diet effect was also observed; a combined effect of the presence of antibiotic (ampicillin) and saligenin (the aglycone of the plant glucoside salicin) led to even greater reduction in larval mass, premature pupation and even mortality (Genta *et al*., 2006). This combination effect of the antibiotic and saligenin does suggest that the gut microbes aid the digestion of secondary plant productions within this host-symbiosis system (Genta *et al*., 2006).

In termites (*Reticulitermes flavipes*), synthesis of uric acid occurs via purine-nucleoside phosphorylase and xanthine dehydrogenase (Potrikus and Breznak, 1981). However, these insects lack uricase, the uric acid degrading enzyme (Potrikus and Breznak, 1981). Symbiotic bacteria have been shown to recycle the uric acid nitrogen which has been suggested to be important in nitrogen conservation in oligonitrotrophic insects which feed on food with a limited amount of nitrogen (Potrikus and Breznak, 1981). As demonstrated with vertebrates, acetate production by symbiotic bacteria also played a

role in termite guts but by providing an oxidizable energy source (Breznak and Switzer, 1986).

1.2.2 Bacterial symbionts and insect immunity and protection

The major benefit of the presence of microbes in humans is the protection against pathogens and the education of the immune system (Section: 1.1). Here I will describe how this has also been observed in insects and the changes that occur when the microbes are removed.

In aphids, facultative bacterial symbionts have been demonstrated to protect their host from the development of the parasitoid, *Aphidius ervi* (Oliver *et al*., 2003). It was initially thought that the facultative symbionts conferred resistance to the parasitoid ovipositing in the aphid; however experiments showed that the symbionts caused high mortality of the parasitoid larvae (Oliver *et al*., 2003). This result also suggests that the facultative symbionts protect the host from mortality to ensure the spread and persistence of the symbiont within populations (Oliver *et al*., 2003).

The European Beewolf hunting wasps have a symbiotic relationship with the bacterium *Streptomyces* (Kaltenpoth *et al*., 2005). This bacterium has been shown in experiments conducted by Kaltenpoth *et al* (2005) to enhance larval survival and protect the cocoons from fungal infections.

One of the most famous endosymbiont is the *Wolbachia* species of bacteria which infects 20% of insects (Klasson *et al*., 2009; Welchman *et al*., 2009). This bacterium is famous for being a parasitic organism that manipulates the reproduction of the insect host to promote transmission (Welchman *et al*., 2009). *Wolbachia* infections in *Drosophila* are maternally transmitted (mother to offspring) (Werren, 1997; Hoffman *et al*., 1990). Infected females that mate with uninfected males produce offspring; however, uninfected females that mate with infected males produce non-viable eggs (O'Neil and Karr, 1990; Yen and Barr, 1971; Werren, 1997; Hoffman *et al*., 1986). This process is known as cytoplasmic incompatability and drives the infection quickly through the insect population (O'Neil and Karr, 1990; Yen and Barr, 1971; Werren, 1997; Hoffman *et al*., 1986; Turelli and Hoffman, 1991). Interestingly, this bacterium has been shown to protect *Drosophila* from RNA viruses (*Drosophila* C virus, Cricket paralysis virus, Nora virus

and Flock house virus), with *Wolbachia* infected flies having greater survival when infected with RNA viruses compared with flies without *Wolbachia* (Hedges *et al*., 2008; Teixiera., 2008).

In *Aedes aegypti* substantial progress has been made to infect the mosquito with *Wolbachia*, a bacterium not found naturally in *Aedes aegypti*. *Aedes aegypti* mosquitoes have been infected experimentally with W*olbachia*, resulting in infected mosquitoes having a shorter life-span when compared with uninfected individuals (McMeniman *et al*., 2009). This introduction of the virulent form of *Wolbachia* into the mosquito population could prove successful as cytoplasmic incompatability will ensure spread within the population and the life-span shortening will prevent the maturation of the dengue virus in the mosquito vector, preventing spread to humans (McMeniman *et al*., 2009). Furthermore, *Wolbachia* infection has the potential to inhibit replication of the dengue virus through the stimulation of the immune system of the insect (Bian *et al*., 2010).

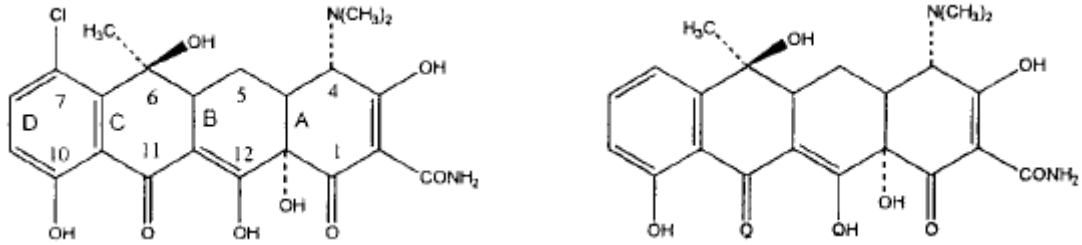
Not only have introduced bacteria proven to provide a strategy for controlling pathogen transmission but the endogenous gut microbes within *Aedes aegypti* has been proven to have effects on the dengue virus (Xi *et al*., 2008). Experiments using aseptic and control mosquitoes demonstrated two-times greater viral titre in aseptic mosquitoes in comparison with control mosquitoes (Xi *et al*., 2008). The process of reducing the viral titre was via the stimulation of the insect immune system rather than a direct effect on the virus (Xi *et al*., 2008).

In the malaria vector *Anopheles*, studies have also emphasized the role of gut bacteria with pathogen transmission. Dong *et al* (2009) revealed that the gut microbes had an anti-plasmodium effect by stimulating the insect immune system and that the removal of the bacteria resulted in an increase in parasite numbers within the insect. Specifically, gram negative bacteria inhibit the sporogonic-stage of the development of *Plasmodium falciparum* and reduce oocyst densities (Pumpuni *et al*., 1993; Pumpuni *et al*., 1996; Gonzalez-Ceron *et al.,* 2003).

1.3 Impact of tetracycline on microbes in insects

Antibiotics are commonly used to eliminate endosymbionts and symbionts found in insects to determine the impact of these bacteria on the insect host (Table: 1.2). However, many have not considered the deleterious effects of using such antibiotics on the insect performance, therefore creating a result which is the impact of the antibiotic and not the result of the depletion of bacteria.

One antibiotic which has commonly been used to eliminate the symbionts is tetracycline with varying concentrations and in a wide range of insects (Table: 1.2). Tetracycline (Figure: 1.1) kills bacteria by inhibiting protein synthesis (Chopra and Roberts, 2001; Speer *et al*., 1992; Goldman *et al*., 1983). Specifically, it prevents the attachment of aminoacyl-tRNA to the ribosomal acceptor site (Chopra and Roberts, 2001). This antibiotic targets both Gram-positive and Gram-negative bacteria (Chopra and Roberts, 2001). Mitochondrial ribosomes are very similar to those found in bacteria (Alberts *et al*., 2002, p769-828). Therefore, at high dosage tetracycline may target the mitochondrial ribosomes found in animal cells.



Chlortetracycline Tetracycline

Figure: 1.1. Structure of chlortetracycline and tetracycline. The difference between the 2 molecules is the extra Cl group found on chlortetracycline. Image taken from Chopra and Roberts (2002).

This antibiotic is commonly used to eliminate the bacterium, *Wolbachia* from several insects including *Aedes albopictus*, *Drosophila melanogaster* and *Drosophila simulans* and is commonly used as a repressor molecule for expression systems used in insects as a method of controlling the insect population (Table: 1.2). Furthermore, only a small number of papers have identified the impacts of tetracycline on the insect performance. Thompson and Sikorowski (1984) investigated the effects of tetracycline hydrochloride on

the performance of *Heliothis virescens* larvae. During this study tetracycline hydrochloride reduced the larval weight, fatty acid and glycogen content of the insect with increasing antibiotic treatment of 0.05-0.4 mg per 100 g of diet (Thompson and Sikorowski, 1984). The protein content of the insects showed a linear decrease with increasing antibiotic concentration (Thompson and Sikorowski, 1984). Larval feeding was also found to be lower at 3 hours of feeding with increasing concentration of chlortetracycline, but there was no difference after 24 hours (Thompson and Sikorowski, 1984).

Table: 1.2. Studies conducted using tetracycline, chlortetracycline and tetracycline hydrochloride with different species of insects.

Insect Name	Antibiotic	Concentration	Reference
Aedes albopictus	Tetracycline	0.33 mg ml^{-1}	Kambhampati *et al*., 1993
Aedes aegypti	Tetracycline/Chlortetracycline	30 µg ml^{-1}	Phuc *et al*.,2007
Drosophila simulans	Tetracycline hydrochloride Tetracycline	0.250 mg ml^{-1} 0.3 mg ml^{-1}	O'Neil and Karr, 1990 Ballard and Melvin, 2007
Drosophila melanogaster	Tetracycline	250 µg ml^{-1} 0.1-20 µg ml^{-1}	Mair *et al*., 2005 Thomas *et al*., 2000
Thermobia domestica	Tetracycline	100-1000 µg ml^{-1}	Treves and Martin, 1994
Sitophilus oryzae	Tetracycline	1 mg per g of flour	Heddi *et al*., 1999
Ostrinia scapulalis	Tetracycline	0.06-600 mg kg^{-1} and 2.4 mg ml^{-1}	Kageyama *et al*., 2003
Anticarsia gemmatalis	Tetracycline	32.25-75.27 µg ml^{-1}	Visôtto *et al*., 2009
Cadra cautella	Tetracycline	400 µg ml^{-1}	Sasaki *et al*., 2002
Ephestia keuhniella	Tetracycline	400 µg ml^{-1}	Sasaki *et al*., 2002
Glossina morsitans morsitans	Tetracycline	25 µg ml^{-1}	Dale and Welburn, 2001
Ostrinia furnacalis	Tetracycline hydrochloride	0.6 mg g^{-1}	Kageyama *et al*., 1998
Myzus persicae	Chlortetracycline	10-1000 µg ml^{-1}	Douglas, 1988
Acyrthosiphon pisum	Chlortetracycline	50 µg ml^{-1}	Prosser and Douglas, 1991
Sitophilus oryzae	Chlortetracycline	100-500 µg ml^{-1}	Baker and Lum, 1973

1.4 Antibiotic resistance

The widespread use of chlortetracycline has meant that tetracycline resistant bacteria are readily found in the environment and within animals (Kümmerer *et al*., 2004). This resistance in bacteria is mainly due to the over-use of this antibiotic to treat illnesses in humans and livestock, where these antibiotics are released non-metabolised in sewage and subsequently found in aquatic environments (Kümmerer *et al*., 2004). Antibiotics are also widely used to treat fruit, bee-keeping and fish farming, therefore it is not surprising that the residues of antibiotics are readily found in waste especially sewage waste with

E.coli being most resistant to the antibiotic, tetracycline (Kümmerer *et al*., 2004; Reinthaler *et al*., 2003). Furthermore, an experiment conducted by Nygaard *et al* (1992) has demonstrated that the addition of oxytetracycline to sediments tripled the number of bacteria found to be resistant to the antibiotic. This provides evidence that the addition of these antibiotics into the environment results in the development of antibiotic resistance.

Tetracycline resistance can be developed using three different methods (Figure: 1.2): 1) preventing access of tetracycline to the ribosome thus preventing attachment and protein synthesis inhibition 2) altering the ribosome structure preventing tetracycline binding and 3) producing tetracycline-inactivating enzymes (Speer *et al*., 1992; Salyers *et al*., 1990).

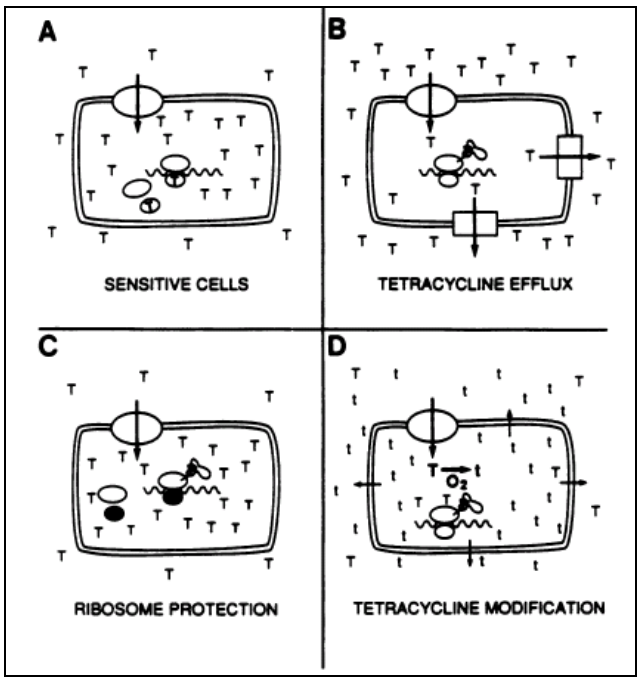


Figure: 1.2. The impact of tetracycline on tetracycline-sensitive cells and mechanisms of resistance. A) The binding of tetracycline to the ribosome preventing protein synthesis. B) A resistance method, a cytoplasmic protein pumping tetracycline out of the cell, preventing the accumulation of high concentrations of tetracycline within the cell. C) The presence of a cytoplasmic protein that has the ability to protect the ribosome from tetracycline binding. D) An enzyme is produced by the bacterium which has the ability to modify and inactivate tetracycline. (Speer *et al*., 1992; Salyers *et al*., 1990). Image taken from Speer *et al* (1992) and Salyers *et al* (1990).

1.5 RIDL® sterile insect technique – Controlling insect pests

One technique used to control insect pests is the RIDL® technique, which uses tetracycline to control the genetic expression system. Here I will describe the technique and the insects used with the technique.

The traditional sterile insect technique (SIT) controls insect pests through the sterilisation of males by ionizing radiation resulting in chromosomal fragmentation (Horn and Wimmer, 2003). This ionization can have a negative impact on sperm viability (Horn and Wimmer, 2003; Mayer *et al.*, 1998). SIT is effective with insects that mate synchronously and in isolated locations without immigration of untreated insects, e.g. the island of Zanzibar where the tsetse fly was eradicated in 1997 (Vreysen *et al*., 2000). There can be problems with SIT. The first is that the irradiated males suffer a general malaise and do not mate readily with the wild females (Horn and Wimmer, 2003). The second is that if the sexing of the insects is inefficient, females are released potentially increasing the pest load in the environment.

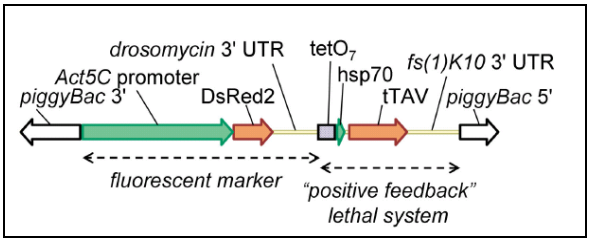
RIDL® sterile insect technique (Release of insects with dominant lethal gene) can improve SIT in two ways: to eliminate females from the release population if the tetracycline-repressible expression system is female-specific and secondly, sterilise male insects if the tetracycline-repressible expression system is specific to early development resulting in the death of progeny (Alphey, 2002). Therefore, when tetracycline is removed from the diets, female insects die and only males remain (Alphey, 2002). RIDL® males that are released into the wild, mate with wild females and produce offspring that die during development (Phuc *et al*., 2007; Alphey, 2002; Thomas *et al*., 2000).

The RIDL® technique is used with insects including Mediterranean fruit fly and *Aedes aegypti* (Gong *et al*., 2005; Phuc *et al*., 2007). Aedes *aegypti* is the vector for Dengue fever, a major disease burden with 2.5 billion people at risk worldwide. (http://www.who.int/mediacentre/factsheets/fs117/en/). The only control methods currently available are; the removal of oviposition sites and the use of insecticides (fenitrothion, malathion, deltamethrin, tetramethrin, permethrin (used in thermal fogging) (Paeporn *et al*., 2004) and DDT (dichlorodiphenyltrichloroethane) (Inwang *et al.,* 1967). However, the unrestricted use of these insecticides has enabled the mosquito to

develop resistance (Gilkes *et al.,* 1956; Porter *et al*., 1961; Sautet *et al*., 1958; Inwang *et al.,* 1967; Paeporn *et al*., 2004). Therefore, with no vaccines and emerging resistance to insecticides, *Aedes aegypti* appears to be good candidate for the use of the RIDL® technique.

An example of the RIDL® system with *Aedes aegypti* is LA513, a late-acting dominant lethal genetic system which causes the death of both male and female mosquitoes at L4-pupal stage (Phuc *et al*., 2007, Figure: 1.3). Released LA513A males into the wild mate with wild females and any offspring produced will die at L4-pupae stage. The release of enough males over a long enough period of time will result in population reduction. The expression system of tTAV is tetracycline repressible; therefore the RIDL insects are reared in water supplemented with tetracycline. Oxitec Ltd currently rear LA513A in water supplemented with 30 µg ml^{-1} of chlortetracycline to suppress the expression of tTav (Figure: 1.3).

a)



(b)

(c)

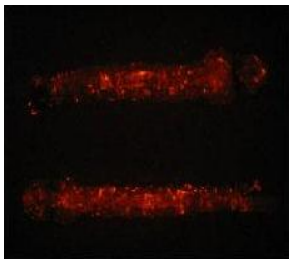


Figure: 1.3. (a) The structure of the LA513 gene inserted into *Aedes aegypti*. LA513 uses a non-autonomous *piggyBac*-based transposon system to integrate the RIDL system. Transgenic mosquitoes are identified by red fluorescence (DsRed2) expression driven by Actin5C which gives an all over spotty red fluorescence (c). tTAV is a tetracycline-repressible transcriptional activator that binds to tetO. In LA513A in the absence of tetracycline small amounts of tTAV expressed from the minimal hsp70 promoter bind to the tetO sites and enhance expression of more tTAV, this forms a positive feedback loop that produces large amounts of tTAV that builds up and eventually damages the cell. In the presence of tetracycline, the tTAV binds to tetracycline making it unable to bind to tetO, this prevents the positive feedback loop and the build-up of tTAV. *Aedes aegypti* larvae reared with no tetracycline under normal light (b) and the same larvae (c) viewed using the filters for red fluorescence (excitation 510-550, emission 590LP), both larvae show the expression of DsRed (Phuc *et al*., 2007).

An example of RIDL® female-specific lethality is OX3604, a repressible female specific flightless phenotype (Fu *et al.,* 2010, Figure: 1.4,). This concept was based on the female-specific indirect flight muscle Actin4, in which the promoter for this gene was used to sex-specifically control the expression of tTAV (Fu *et al.,* 2010). The expression of tTAV in the absence of tetracycline resulted in a flightless phenotype in female mosquitoes (Fu *et al.,* 2010).

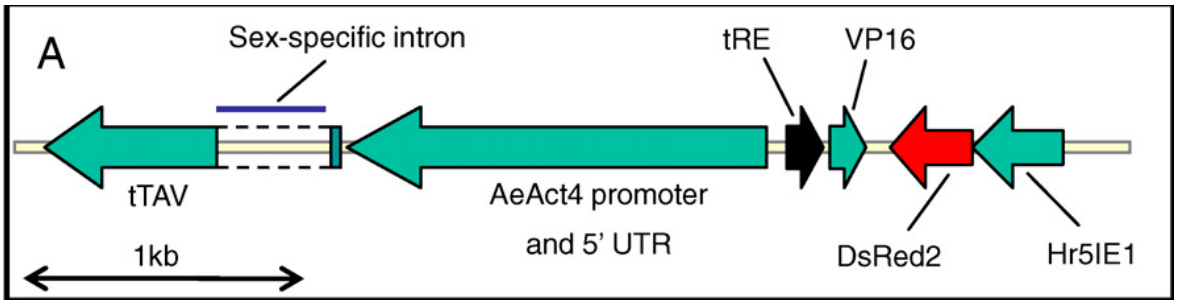


Figure: 1.4. Structure of the insertion of OX3604 into *Aedes aegypti.* Transgenic mosquitoes are identified by red fluorescence (DsRed2) expression driven by HR5IE1. tTAV is a tetracycline-repressible transcriptional activator which is used as the effector molecule (Fu *et al.,* 2010). In the absence of tetracycline tTAV is expressed from the AeAct4 promoter, binds to the tetO sites (found within tRE) and enhance expression of more tTAV (Fu *et al.,* 2010). In the presence of tetracycline, the tTAV binds to tetracycline making it unable to bind to tetO, this prevents the positive feedback loop and the build-up of tTAV (Phuc *et al*., 2007; Fu *et al.,* 2010). Image taken from Fu *et al* (2010).

The potential problem with RIDL® is the possibility that the tetracycline (30-100 µg ml^{-1}) used to repress the lethal genes might also eliminate symbiotic bacteria, resulting in reduced insect vigour (and hence mating success) and increased susceptibility to pathogens in the field.

1.6 Bacteria in *Drosophila melanogaster and Aedes aegypti* and the role in insect performance

The RIDL® technique was first developed using *Drosophila melanogaster* (Thomas *et al*., 2000) and is used for controlling one major pest, *Aedes aegypti* (Phuc *et al*., 2007). Therefore, I will discuss the bacterial diversity in *Drosophila melanogaster* and *Aedes aegypti* and the implications of removing bacteria on the insect host.

1.6.1 Bacterial diversity in *Drosophila melanogaster*

One particular investigation into the diversity of bacteria in natural populations of *Drosophila melanogaster* detected members of the phyla Proteobacteria, Bacteroidetes and Firmicutes (Corby-Harris *et al*., 2007). In laboratory *Drosophila* (strain Oregon-R), Ren *et al* (2007) discovered both cultured bacteria and non-culturable bacteria (Table: 1.3). The bacteria found within lab strains of *Drosophila melanogaster* were predominantly *Lactobacillus* and *Acetobacter* (Ren *et al.,* 2007).

Acetobacter are well adapted in sugar and ethanol rich environments (Crotti *et al*., 2010). This genus of bacteria has been found within *Drosophila* and on the exterior of the fly showing the ability to withstand different conditions (Ren *et al.,* 2007) (Table 1.3). *Lactobacillus* is lactic acid producing bacterium which was also found on the interior and exterior of the fly by Ren *et al* (2007). Both of these bacterial species were again identified by Corby-Harris *et al* (2007).

Table 1.3: Bacterial species identified in *Drosophila melanogaster* (Ren *et al.,* 2007)

Area of fly	Cultured Species	Identified by PCR
Fly Surface	*Acetobacter aceti*	*Lactobacillus homohiochii*
	Acetobacter tropicalis	*Acetobacter aceti*
	Acetobacter pasteurianus	*Lactobacillus fructivorans*
	Lactobacillus plantarum	
Fly Interior	*Acetobacter pasteurianus*	*Acetobacter tropicalis*
	Lactobacillus sp MR-2	*Lactobacillus brevis*
	Acetobacter aceti	*Lactobacillus plantarum*
	Lactobacillus plantarum	*Acetobacter pasteurianus*
	Clidosporium sphaerospermum	*Acetobacter aceti*

1.6.2 Role of commensal bacteria in *Drosophila melanogaster*

The two main methods of eliminating bacteria from *Drosophila* are: egg dechorionation/washing eggs with alcohol and rearing the insects in a sterile environment and the second method is to treat the insects with antibiotics.

As a model organism for humans, there is increasing interest in the interactions between *Drosophila melanogaster* and its resident microbiota, which have been found to enhance

life-span (Brummel *et al*., 2004) (Table: 1.4). In the experiment conducted by Brummel *et al* (2004), three different antibiotics (ampicillin, tetracycline and rifamycin) were administered to the flies to remove the bacteria (Brummel *et al*., 2004). The presence of bacteria in *Drosophila melanogaster* enhanced life-span during the first week of adult life but could have the potential to reduce life-span later in adulthood (Brummel *et al*., 2004) (Table: 1.4). An early study conducted by Bakula (1969) identified how *Drosophila melanogaster* become infected with the bacterial symbionts and how the elimination of this infection route affects the performance of the fly. In this study, it was demonstrated that *Drosophila melanogaster* gain the symbionts through the larvae consuming the egg chorion which contains the bacteria (Bakula, 1969). Axenic flies were created by washing the eggs with White's solution (1.8 mM $HgCl_2$, 0.1 M NaCl in 0.05 N HCl solution) to surface sterilise the eggs, the resulting flies had an extended development time compared with control flies (Bakula, 1969) (Table 1.4). Recent experiments have further demonstrated the role of microbes in *Drosophila melanogaster*, the removal of the symbionts through antibiotic treatment abolished mating preference (Sharon *et al*., 2010) (Table: 1.4). Reintroduction of these bacterial symbionts through injection preserved mating preferences in the flies (Sharon *et al*., 2010).

Table: 1.4. The impacts of bacterial depletion in *Drosophila melanogaster*

Performance Parameter	Result	Reference
Development time	Extension	Bakula, 1969
Life-span	Reduction No effect	Brummel *et al*., 2004 Ren *et al*., 2007
Immune response	Decrease in Anti-microbial peptides (AMPs)	Ren *et al*., 2007
Mating	Abolishes mating preferences	Sharon *et al*., 2010

One major factor that is involved in the establishment of bacteria is the diet consumed by the organism. As nutrition plays an important role in survival, reproduction and fitness of *Drosophila melanogaster*, this would also suggest a link between the diet, bacteria and performance of organisms. Therefore, bacteria could actually promote the performance of insects on suboptimal diets and a different response could also be observed with axenic flies reared on different diets.

Studies have been conducted which have highlighted the effects of nutrition on *Drosophila melanogaster.* Calorie restriction has been shown to increase life-span, reduce reproduction and increase fat content (Piper *et al*., 2005). This supports the theory that at times of plenty *Drosophila* investment of energy into reproduction is prioritised and during periods of low food availability, energy is invested into preservation and survival (Shanley and Kirkwood, 2000; Piper *et al*., 2005). However, one paper suggested that the protein: carbohydrate ratio within the diet played an important role in extending life-span of *Drosophila melanogaster* rather than the dilution of diets (calorie restriction) (Lee *et al*., 2008). A high protein diet with low carbohydrate content reduced life-span, suggesting that protein at high levels can be toxic and a diet consisting of 1:2 and 1:1.6 protein-to-carbohydrate ratio was shown to optimize egg laying rate and life-span, respectively (Lee *et al*., 2008). This result was also supported by a paper published in 2009 by Ja *et al* where it was suggested that providing water with the diet abolishes the life-span extension with dietary restricted media.

1.6.3 Bacterial diversity in mosquitoes

Investigations into the bacterial diversity of mosquitoes have mainly focused on the mosquito, *Anopheles gambiae* to identify the potential use of commensal bacteria in controlling the vector of malaria. Bacteria identified in *Anopheles gambiae* included; *Enterobacter asburiae*, *Microbacterium* sp., *Sphingomonas* sp*., Serratia* sp. and *Chryseobacterium meningosepticum* (Dong *et al*., 2009). Another paper where bacterial species were identified in *Aedes triseriatus, Culex pipiens* and *Psorophora columbiae* discovered there was a huge increase in bacterial numbers in the midgut between larval and pupal stage and also in adults after blood-feeding (Demaio *et al*., 1996). The most common bacterial species identified were *Serratia marcescens, Klebsiella ozanae*, *Enterobacter agglomerans* and *Pseudomonas aeruginosa* (Demaio *et al*., 1996).

Little is known about the importance of the bacterial species present in *Aedes aegypti* and their role in insect performance. One paper had described how female *Aedes aegypti* preferred to oviposit in water that had bacteria present and that these micro-organisms produced oviposition-stimulating kairomones (Ponnusamy *et al*., 2008). An early paper also supported this, with egg hatching being greater in water with bacteria when compared with sterile water (Rozeboom, 1934).

Within *Aedes aegypti*, the bacterial diversity has been identified in gut diverticulum through 16S rRNA gene analysis, this included *Bacillus cereus, Bacillus subtilis and Serratia sp* (Gusmão *et al*., 2007). The *Serratia* species identified by Gusmão *et al* (2007) had been suggested to play an important role in the metabolism of sugars; therefore it would be interesting to discover if bacterial depletion reduces the ability of the mosquito to metabolise sugars. Culture-dependent and culture-independent techniques identified the genera *Asaia, Bacillus, Enterobacter, Klebsiella* and *Serratia* to be dominant in the mid-gut of *Aedes aegypti* (Gusmão *et al*., 2010). The number of CFUs (colony forming units) increased from 210 colonies to 2.3 x 10^7 after 67 hours of being blood-fed (Gusmão *et al*., 2010) indicating the role of these bacteria in metabolism of a blood-meal.

1.6.4 Role of commensal bacteria in *Aedes aegypti*

As with *Drosophila melanogaster* few experiments have been conducted with *Aedes aegypti* and the cost of removing commensal bacteria from the host. Lang *et al* (1972) conducted experiments which showed no alteration in growth rates, survival and protein content but did observe a change in life-span, lipid and weight. Research in this area has mainly concentrated on the role of bacteria in dengue virus transmission which is described in Section: 1.2.2.

1.7 Thesis Objectives

To determine the deleterious impact of using chlortetracycline to deplete bacteria and during the RIDL® technique, 2 insect systems were assessed; *Drosophila melanogaster* and *Aedes aegypti*. *Drosophila melanogaster* was chosen as an ideal system due to the low cost, low bacterial diversity and the ability to use egg dechorionation as an alternative method to deplete bacteria. RIDL® is used with *Aedes aegypti* and was chosen to address the impact of chlortetracycline on an insect system which is used for RIDL®. Lastly, to gain a greater understanding of the creation of transgenic insects, a female-specific marker for *Aedes aegypti* was proposed to provide a method of genetically sexing the insects during mass-rearing.

The three major objectives were:

1. The impact of chlortetracycline on *Drosophila melanogaster* and *Aedes aegypti* (Chapter 3 and 5).

2. The impact of the depletion of bacteria via egg dechorionation on *Drosophila melanogaster* (Chapter 3 and 4).
3. The production of a female-specific marker in *Aedes aegypti* for sex sorting during mass rearing (Appendix: 7.6).

Chapter 2: Material and Methods

2.1 Materials

Unless otherwise stated, chemicals and reagents were supplied by the following companies: Sigma Aldrich® U.K and U.S.A (organic compounds, enzymatic assay kits); Bio-Rad Laboratories (qRT-PCR reagents and protein quantification assay); Fisher (organic compounds); Invitrogen™ (PCR reagents).

2.2 Insect Culture and maintenance

2.2.1 Drosophila *melanogaster* rearing and maintenance

Five strains of *Drosophila melanogaster* were used: Oregon-RS (Bloomington Stock Centre; Dec 2007), Oregon-RC (Bloomington Stock Centre; Dec 2007), Oregon-RP2 (Bloomington Stock Centre; Dec 2007), Isogenic (provided by Sean Sweeney, University of York, UK; Dec 2007) and Canton-S (provided by Mariana Wolfner Laboratory Department of Molecular Biology and Genetics, Cornell University, Ithaca NY; September 2009).

Drosophila were transferred to fresh diet at least once a week and reared at 25 °C, with a 12 hour light/dark cycle.

In preliminary experiments, the performance of the strains was investigated on 5 different diets: Semi-defined medium; Cormeal, dextrose and yeast medium; General media; Ren *et al* (2007) medium; University of York medium (Table: 2.1). Of these, the University of York diet yielded the most reliable performance, therefore stock flies and experiments were conducted using this diet. The glucose to yeast ratio in the University of York diet was 2:1.

For all experiments the diet was autoclaved and upon cooling to 50 °C, mixed with antifungal agents (Nipagin M and CBZ) and chlortetracycline at 0-500 µg ml^{-1} final concentration, prior to transfer to autoclaved vials.

Table: 2.1: Components of *Drosophila* diets

	Weight (g) per litre						
Ingredients	Semi-defined (flystocks.bio.indiana.edu/)	Cornmeal, dextrose and yeast medium (flystocks.bio.indiana.edu/)	General medium (Ashburner *et al*., 2005)	Ren *et al* (2007) medium	University of York medium	Cornell (High nutrient) medium	Low nutrient medium
Cornmeal (Tesco Supermarket)		90.9	97.14	50			
Sucrose	30						
Dextrose		147.4	85.71	105			
Glucose (Fisher Scientific, Sigma)	60				92	80	20
Yeast (Genesee Scientific, Sigma)	80		17.14	26	46	80	20
Yeast Extract – yeast cell contents (Oxoid)	20						
Peptone	20						
Agar (No.3) (Genesee Scientific)	10	5	10.28	7.5	13.79	10.685	10.685
Magnesium sulphate	0.5						
Calcium Chloride	0.5				0.46		
Ferrous sulphate					0.46		
Manganous chloride					0.46		
Sodium chloride					0.46		
Potassium sodium tartrate					7.35		
Tegosept				8.5			
Nipagin M *	10 ml	16.23 ml	28.6 ml		7.35 ml		
CBZ *					27.57		
Benzyl benzoate in ethanol		22.73					
Propionic acid	6 ml			1.9 ml		3.42 ml	3.42 ml
Phosphoric acid						0.34 ml	0.34 ml

*Stock solutions of Carbendazim (CBZ) and Nipagin M were made up of 200 mg l^{-1} and 100 g l^{-1} respectively, 27.57 ml of CBZ and 7.35 ml of Nipagin M were added to 1 litre of medium.

2.2.2 *Aedes aegypti* rearing and maintenance

Asian *Aedes aegypti* (isolated from Kuala Lumpar, Malaysia in 1975) eggs were hatched under a vacuum for 20-60 minutes in distilled water and reared at 28 °C with 60-80% humidity. Larvae were fed with TetraMin tropical fish food (Aquatics Warehouse, UK). Emerging pupae were picked daily using a 3ml plastic Pasteur pipette (Scientific Lab Supplies) into a small sized weigh boat (7 ml; Fisher Scientific, UK) and placed into a cage (15cm x 15 cm x 15 cm) (Talking Plastics Fabs, UK). Adult mosquitoes were fed on defibrinated horse blood (TCS Biosciences, UK) in Hemotek® membrane feeding system (Discovery Workshop, UK) for egg laying and fed daily with 10% sugar (Tate and Lyle, UK) water plus 14 U ml^{-1} Penicillin and 14 µg ml^{-1} streptomycin and filtered through 0.22 µM bottle top filters (Corning Inc, U.S.A and VWR, UK).

2.3 Insect Performance Experiments

2.3.1 Performance of *Drosophila melanogaster* Oregon-RS

Adult females of *Drosophila* were allowed to oviposit on the University of York medium (Table: 2.1) over 24 hours. The eggs were collected and transferred to the test diets under sterile conditions using a fine paintbrush. The experiment comprised of 10 replicate vials with 10 eggs on 0, 1, 10, 50, 100, 300, 500 µg ml^{-1} of chlortetracycline supplemented diet.

The time to the emergence of pupae and flies were recorded. Once the flies had emerged, they were collected, sexed, placed in liquid nitrogen and stored at -80 °C prior to subsequent analysis.

In a supplementary experiment, the performance was assayed on diets with different agar concentrations ranging from 0.9% to 2.5%. The time to the emergence of pupae and flies were recorded.

2.3.2 Performance of *Drosophila melanogaster* Canton-S

2.3.2.1 Development time and survival experiment with chlortetracycline and dechorionation on the high and low nutrient diets.

Adult female *Drosophila* were allowed to oviposit over 24 hours. The eggs were collected and transferred to the test diets under sterile conditions using a fine paintbrush. The experiment comprised of 12 replicate vials with 10 eggs with 0, 1, 10, 50, 100, 300, 500 µg ml^{-1} of chlortetracycline on the low and high nutrient diet (Table: 2.1). One extra treatment "dechorionation" was conducted alongside the chlortetracycline treatments. Egg dechorionation was conducted by washing in sterile water twice, treating with 10% bleach for 5 minutes and rinsing with sterile water 3 times. The time to the emergence of pupae and flies were recorded.

2.3.2.2 Performance experiments with chlortetracycline and dechorionation on the York diet

Experiments were conducted using glass vials with lids or Falcon tubes, both methods of rearing created consistent results. Adult females were allowed to oviposit on the York diet over 24 hours. The eggs were collected and transferred to treatments under sterile conditions. The experiment comprised of 15 glass vials with 10 "untreated" eggs on the diet with 0, 50, 300 µg $ml^{-1}$of chlortetracycline and 15 glass vials with 10 "treated" eggs on the diet with 0, 50, 300 µg ml^{-1} of chlortetracycline. The treated eggs were dechorionated by washing in sterile water twice, treating with 10% bleach for 5 minutes and rinsing with sterile water 3 times.

The development and emergence of pupae and flies were recorded. Once the flies had emerged they were transferred to fresh diet and allowed to mate for 48-72 hours before being sexed. After mating, male flies were used to determine life-span and females were used for bacterial counts and quantification of fecundity.

Ten mated males were placed individually into the same treatment as for rearing (see above). Fresh diet was provided every 3/4 days and every vial was monitored daily until the fly died.

Ten mated females were randomly selected for fecundity experiments, individual females were placed individually in tubes (Corning Inc), with each diet within the lid to allow for egg laying and feeding (Figure: 2.1). Tubes were placed upside down, lids with fresh diet were replaced and eggs were counted daily for 7 days.

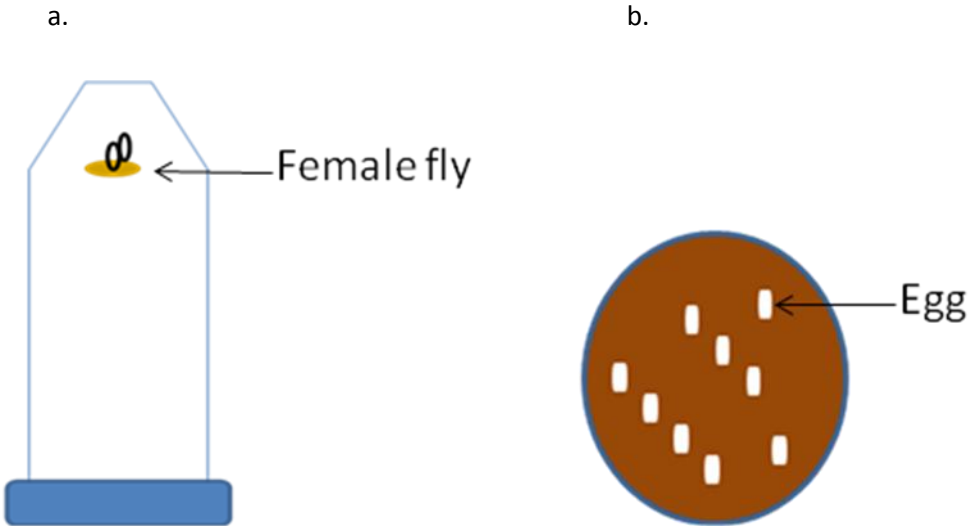


Figure 2.1: Diagram of the fecundity experiment: a) a female in the Falcon tube and b) eggs laid on the food in the lid of the tube.

In a supplementary experiment, egg hatching rates were assayed on diets with 0, 50, 300µg ml^{-1} of chlortetracycline. A further egg hatching experiment was conducted with dechorionated and control eggs where the diet was supplemented for both treatments with amaranth dye (300 µg ml^{-1}) to aid the identification of dechorionated eggs which had hatched.

2.3.3 Performance experiments on *Aedes aegypti*

Cages of male and female mosquitoes were blood fed and eggs were collected four days later. Approximately 500 eggs were hatched in a vacuum for one hour in 300 ml at 0, 0.1, 0.5, 1.0, 10, 30, 100 µg ml^{-1} of chlortetracycline hydrochloride (BioGene, UK) water. Hatched eggs were left overnight. The following day, 7 replicates of 300 larvae were transferred into 500 ml plastic pots (Scientific and Medical Products Ltd, UK) with 150 ml of each concentration of chlortetracycline, fed according to Table: 2.2 and maintained at 28 °C with 80% humidity. The time to pupation and the number of male and female pupae were recorded. Pupae were transferred to small plastic cages (15cm x 15 cm x 15

cm) (Talking Plastics Fabs, UK) and each day the number of emerged adults were recorded, collected and stored at -80 °C or used for life-span studies as below.

Five replicates of 30 male and female pupae were placed separately into cages (Scientific and Medical Products Ltd, UK). The mosquitoes were fed with 0.2 µm filtered 10% sugar (Tate and Lyle, UK) supplemented with 0, 0.1, 0.5, 1.0, 10, 30, 100 µg ml^{-1} of chlortetracycline. Life-span was monitored and fed daily for 50 days.

The effects of chlortetracycline treatment on Mexican *Aedes aegypti* (Laura Harrington, Cornell University, Ithaca, NY) was tested as above, except that 6 replicates of 150 larvae were used and life-span was monitored on 6 replicates of 20 male and female mosquitoes until all were dead, Tetramin Fish Food was obtained from Walmart and sugar from Wegman, Ithaca, NY. Mexican *Aedes aegypti* originated from the Tapachula area (14° 45'N, 92° 15'W) and had been in the colony since 2006, the strain was augmented yearly with wild mosquitoes from the collection site.

The response of transgenic Asian *Aedes aegypti* to 0-30 µg ml^{-1} of chlortetracycline was also tested as above except that 6 replicates of 150 larvae were conducted and life-span was monitored on 6 replicates of 20 male and female mosquitoes until all were dead and fed with 10% sugar water only.

Table: 2.2. Feeding regime for Asian and Mexican *Aedes aegypti* fed on tropical fish food (Tetramin) (Determined by Irka Bargielowski).

Day	Food (µg) per larva
1	30
2	-
3	40
4	80
5	160
6	320
7	320
8	320

2.4 Microbial Culture

2.4.1 Culturing microbes in *Drosophila melanogaster*

To determine the effect of chlortetracycline on culturable bacteria from Oregon-RS, three larvae grown on each concentration of chlortetracycline (0-500 µg ml^{-1}) were sampled, surface sterilised with 70% ethanol and homogenised in 0.5 ml of 1X sterile phosphate buffer saline solution (pH 7.4). A sample of the homogenate (0.1 ml) was plated onto nutrient agar (Oxoid – 28 g l^{-1}) and plates were incubated for 7 days at 37 °C when the number of colonies was recorded.

To check for culturable bacteria in Canton-S culture medium derived from dechorionated eggs (Section 2.3.2.2), food was sampled on Day 14 (after most of adults had emerged) and plated onto nutrient agar plates. These plates were grown at 25 °C for 7 days and growth was monitored on Day 1, 3 and 7. Bacterial counts on females were conducted on 7-10 day old flies. Five female flies were randomly selected from each treatment and individually homogenised in 250 µl of sterile 1X phosphate buffer saline solution (pH 7.4) using a small pestle until pieces of tissue were no longer visible (Ren *et al*., 2007). Samples (100 µl) of the homogenate at X 1, X 1/10, X 1/100 and X1/1000 dilutions were plated onto nutrient agar plates (Oxoid – 28gl^{-1}) with +/- 50 µg ml^{-1} chlortetracycline (shown to eliminate chlortetracycline sensitive *E.coli* cells) using sterile techniques. The homogenate from flies derived from dechorionated eggs were not diluted.

2.4.2 Culturing microbes in *Aedes aegypti*

To determine whether the treatment of mosquitoes with 0 to 100 µg ml^{-1} of chlortetracycline had an effect on the culturable bacteria found in mosquitoes, mosquito larvae were grown on each concentration of chlortetracycline (0-100 µg ml^{-1}) and sampled on Day 7. Three larvae were then surface sterilised with 70% ethanol, homogenised in 0.5 ml of sterile 1X phosphate buffer saline solution (pH 7.4) and 0.1 ml of the homogenate was plated nutrient agar (Oxoid – 28 g l^{-1}) plates with and without 50 µg ml^{-1} chlortetracycline for the identification of chlortetracycline resistant bacteria. The agar plates were incubated for 7 days at 28 ± 1 °C (temperature used for *Aedes aegypti* culture) and the number of colonies was recorded.

Five (Asian) and six (Mexican) colonies from each plate were picked at random to inoculate 5 ml nutrient broth and placed in a shaking incubator at 28 ± 1 °C overnight. DNA was extracted from 1 ml samples in Section 2.8.1.

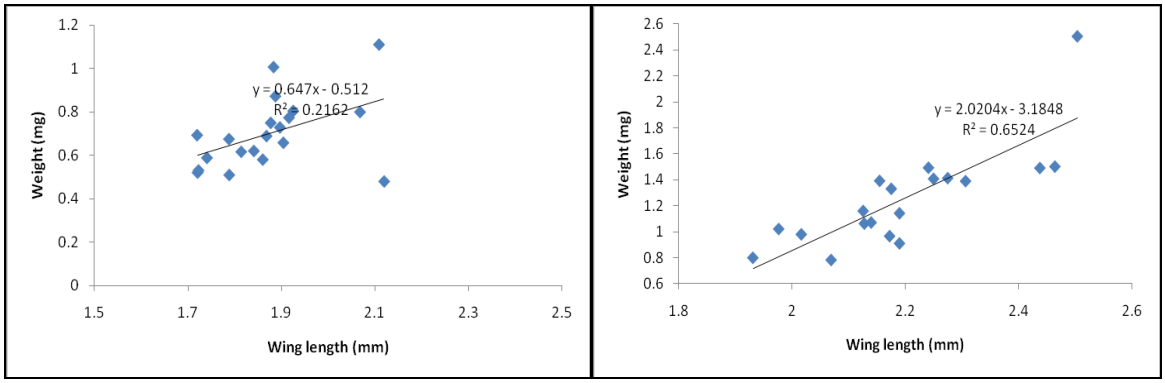
2.5 Nutrition Assays

2.5.1 Wing length and area measurements

Due to experimental constraints the weight of the insect could not be measured in some experiments therefore, the size of the insects was estimated from wing length (mosquitoes) or area (*Drosophila)*. *Drosophila* wing area was chosen as a suitable measure of size according to Shingleton *et al*., 2005 and wing length (from wing notch to tip, Nasci 1990) was measured as a measure of size for mosquitoes (Figure: 2.3). Wings of mosquitoes and *Drosophila* were removed using scissors and forceps and placed onto a glass slide. The wings were either digitally scanned (Canon LiDE 200 colour image scanner) or photographed using the Olympus SZX9 stereomicroscope. Wing length and wing area was measured using ImageJ (http://rsbweb.nih.gov/ij/) or using a leaf-area measurement machine (LI-COR Portable area meter model: LI-3000A) (Figure: 2.2).

Figure: 2.2. a) Mosquito wing length from wing notch to wing tip excluding hairs and b) *Drosophila* wing area measurements indicated with the dashed lines.

1a) 1b)



2a) 2b)

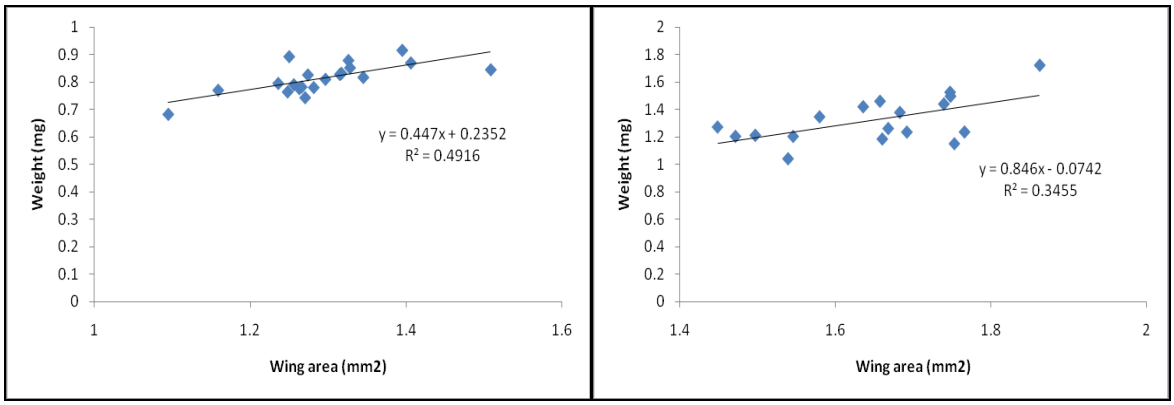


Figure 2.3: 1) Wing length and weight measurements were made with 20 males (a) and females (b) *Aedes aegypti*. The linear regression of the weight and wing length was calculated. Regression significantly departed from zero in males ($F_{1, 18} = 4.965$, $p<0.05$) and females ($F_{1, 17} = 31.912$, $p<0.001$). These results suggest that wing length is a suitable measure of size. 2) Wing area measurements were made with 20 male (a) and female (b) *Drosophila*. The linear regression of the weight and wing area was measured. Regression significantly departed from zero in males ($F_{1, 18} = 17.408$, $p<0.01$) and females ($F_{1,16} = 8.448$, $p<0.05$).

2.5.2 Tissue Preparation

Individual *Drosophila* and mosquitoes were homogenised in 80 µl and 60 µl of extraction buffer, respectively in Tris-EDTA buffer (10mM Tris, 1 mM EDTA, pH 8.0, with 0.1% (v/v) Triton-X-100 (Dionne *et al.*, 2006). Samples were centrifuged for one minute at 17,949 g and 4°C and the supernatant was removed and placed in new tube. Samples were kept on ice while assays were conducted to prevent degradation of nutrients.

For *Drosophila* Oregon-RS sample flies were homogenised in 200 µl of Lysis Buffer (150 mM NaCl, 20 mM Tris (pH 8.0), 2 mM EDTA, 0.5% nonylphenoxypolyethoxylethanol (NP-40) and protease inhibitors – Roche tablets (Cat no: 1 836 153 1 tablet/10 ml) and incubated on ice for 15 minutes. Samples were then centrifuged at 4 °C at 17,949 rpm for 10 minutes and the supernatant was placed in a new tube and analysed immediately.

2.5.3 Protein Assays

Two assays were used, Pierce for Oregon-RS and Bio-Rad for Canton-S.

For the Bio-Rad assay, samples were diluted 1 in 5 in TE buffer. Five micro-litres of the sample and Bovine Albumin Serum standards were placed into a 96 well plate. A Bio-Rad (Bio-Rad Laboratories, USA) protein assay was conducted by adding 25 µl of reagent A and 200 µl of reagent B (samples were mixed by pipetting) and allowed to incubate for 15 minutes at room temperature. The absorbance was measured at 750 nm on a Bio-Rad xMark^{TM} microplate spectrophotometer with 0-2.4 µg BSA (Bovine Serum Albumin).

For the Pierce Assay, samples were diluted in Lysis buffer. Twenty-five micro-litres of the sample and Bovine Albumin Serum standards were placed into a 96 well plate. A Pierce BCA (Thermo Scientific) protein assay was conducted by adding 200 µl of BCA reagent (made from Reagent A and B at a 50:1 ratio) to each sample/standard and mixed by pipetting. The plate was incubated for 30 minutes at 37 °C and the absorbance measured at 544 nm on a BMG Labtech POLARstar OPTIMA spectrophotometer.

2.5.4 Assay for Glucose, Glycogen and Trehalose

Five micro-litres of the samples and standards were placed into the wells of a 96 well plate. The glucose assay was conducted by adding 150 µl of enzyme cocktail containing 500 units of glucose oxidase and 100 units of peroxidase (39.2 ml of Milli-Q water to the enzyme tablet and 800 µl of o-Dianisidine reconstituted in 1 ml of Milli-Q water) to each well and mixed by pipetting. The plate was then incubated for 30 minutes at 37 °C. The reaction was terminated by adding 150 µl sulphuric acid (6 M). The absorbance was measured at 544 nm on an xMark^{TM} microplate spectrophotometer.

To assay glycogen and trehalose, samples were pre-treated with amyloglucosidase and trehalase respectively. For glycogen assays, 5 µl of 2 U ml^{-1} of amyloglucosidase in 10 mM acetate buffer was added to each sample and to one of 2 glycogen standard curves. The plate was incubated for 1 hour (*Aedes* aegypti) and overnight (advised for *Drosophila* samples) at 37 °C. Glucose quantity was then measured using the method described above.

For the trehalose assay, 2 µl of 0.2M sodium citrate and 1 mM of EDTA was added to the samples and 1 of 2 trehalose standards. The plate was incubated at 37 °C for 10 minutes, this was followed by the addition of 2.5 µl of trehalase (3.7 U/ml) (converts trehalose to glucose) to the samples and 1 of 2 standards. The plate was then incubated for 60 minutes at 37 °C and the glucose quantity then measured using the method described above.

For both the glycogen and trehalose assays, the quantity of trehalose and glycogen was calculated by subtracting the value gained from the glucose assay from the value gained from the trehalose/glycogen assay.

2.5.5 Assay for glycerol and triglyceride Assay

The glycerol and triglyceride assay were conducted using Sigma Triglyceride Assay Kit. This kit quantifies glycerol by the addition of 1.25 U ml^{-1} of glycerol kinase (converts glycerol to glycerol-1-phosphate), 2.5 U ml^{-1} of glycerol phosphate oxidase (converts glycerol-1-phosphate to hydrogen peroxide and dihydroxyacetone phosphate) and 2.5 U ml^{-1} of peroxidase (converts hydrogen peroxide, 4-aminoantipyrine and sodium N-ethyl-N-[3-sulfopropyl]m-anisidine to Quinoneimine dye). The triglyceride reagent converts triglycerides to glycerol and fatty acids by using 250 U ml^{-1} of lipoprotein lipase.

Fifteen micro-litres of the samples and 15 µl of the glycerol standards (0-6.25 ug) were placed into a 96 well plate. The assay was conducted by adding 200 µl of pre-warmed glycerol reagent (resuspended in 40 ml Milli-Q water) to each well and mixed by pipetting. The plate was then incubated for 10 minutes at 37 °C. The absorbance was measured at 540 nm using a xMark™ microplate spectrophotometer.

Following the glycerol assay, the triglyceride was quantified. Fifty micro-litres of pre-warmed triglyceride reagent (reconstituted in 10 ml of Milli-Q water) was added to the plate after the glycerol was quantified and incubated for 10 minutes at 37 °C. The absorbance was measured at 540 nm using an xMark^{TM} microplate spectrophotometer.

The triglyceride content was calculated by subtracting the value for free glycerol (first reading) from the final absorbance.

2.5.6 Total Lipid Content

Total lipid content was measured by drying individual insects (*Drosophila* and Mexican *Aedes aegypti)* or groups of 20 (Asian *Aedes aegypti* at Oxitec Ltd to weigh accurately*)* in eppendorf tubes to a constant weight at 50 °C for 48 hours. After determination of dry weight of insect on a microbalance (*Drosophila* and Mexican *Aedes aegypti;* Mettler MT5 microbalance and Asian *Aedes aegypti;* Mettler Toledo AG104 balance), 1 ml (5 ml for Asian *Aedes aegypti*) of 2:1 methanol/chloroform mix was added to each tube and incubated at room temperature for 24 hours. The methanol/chloroform mix was then removed and left in the fume hood for 24 hours to allow the evaporation of the methanol/chloroform mix. The insects were then re-weighed and the change in weight of the dried insects after methanol/chloroform treatment was calculated to produce the total lipid content per insect (Cockbain, 1962).

2.6 Respirometry

The O_2 consumption and CO_2 production of male and female flies of 7-10 day old *Drosophila* Canton-S was determined by stopped-flow respirometry, with 5 flies per replicate. Flies were transferred to a 5 ml syringe and allowed to acclimate for 30 minutes prior to analysis. All experiments were conducted in dim light (0.02 µE m^{-2} s^{-1}) to minimise movement, and the time of experiment was scored. The input to the respirometer was room air with water vapour and CO_2 removed by two silica columns and one ascarite column respectively. To initiate each experiment, the air in the syringe was replaced by 3.2 ml of dried CO_2 free air, with airflow at 57 ml min^{-1}. The CO_2 and O_2 content of the syringe was determined at 30 minutes by injecting 3 ml of the syringe volume into Sable Systems SS3 Gas Analyzer Sub-sampler with an FCA-10A CO_2 analyzer

and FC-10 O_2 Analyzer (Sable systems, Nevada, USA) respectively. CO_2 and O_2 content was calibrated with 50 ppm CO_2 gas and 20.9% O_2 gas.

CO_2 and O_2 content were analysed using the Sable System data acquisition software (Expedata, Sable Systems, Nevada, USA).

2.7 *Drosophila* Gut Dissections

Adult flies were surface sterilised with three washes in 1 ml 10% sodium hypochlorite, followed by three rinses with sterile water. *Drosophila* guts were dissected in sterile Ringer's solution (3 mM $CaCl_2$, 182 mM KCl, 46 mM NaCl and Tris 10 mM, pH 7.2) using sterilised forceps and scissors.

2.8 16S rRNA Gene Analysis

2.8.1 DNA extraction

Bacteria colonies were cultured from the insects (Section 2.4).

Three different methods were used to extract DNA. For bacterial colonies obtained from Oregon-RS and for Oregon-RS larvae, DNA was extracted by using a modified method from Short Protocols in Molecular Biology 4th Edition. This involved precipitating the DNA in one volume of isopropanol, washing the DNA pellet in 500 µl of 70% ethanol and re-suspending the dried pellet in 100 µl of sterile water (Ausebel, 1999 page 2-12).

For bacterial colonies obtained from Canton-S and for Canton-S adults (Section: 2.3.2.2), DNA was extracted using a modified method from Cenis *et al* (1993). This involved a pre-incubation step in 180 µl enzymatic lysis buffer (20 mM Tris-Cl, 2 mM sodium EDTA, 1.2% Trition-X-100 and 20 mg lysozyme per ml) at 37 °C for 45 minutes. Followed by, bead-beating with 0.1 mm cell disruption beads (50% volume) using a DisrupterGenie (Scientific Industries, Inc). The samples were incubated for a further 45 minutes at 37 °C. One hundred microlitres of Extraction Buffer (200 mM Tris-HCl, pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% [W/V] SDS) and 25 µl of proteinase K (20 mg ml^{-1}) was added to each sample. The samples were mixed and incubated at 55 °C for 1 hour, followed by the addition of 150 µl of 3M NaOAc, pH 5.2. The samples were transferred to -20 °C for 10 minutes and centrifuged for 5 minutes at 17,949 g. The supernatant was transferred to a new tube

and 1 volume of 100% freezing isopropanol was added. The samples were incubated at room temperature for 30 minutes, followed by centrifugation at 17,949 g for 30 minutes. The supernatant was removed and the pellet was washed in 500 µl of ice cold 70% ethanol. The DNA samples were then vortexed, centrifuged at 17,949 g at room temperature and the supernatant was discarded. The dried DNA pellet was re-suspended in 100 µl of sterile water.

For liquid microbial cultures obtained from Asian *Aedes aegypti,* DNA was extracted by using Nucleospin® Tissue kit (Clontech) and cultures from Mexican *Aedes aegypti* DNA were extracted by the same method for Canton-S (Cenis *et al.,* 1993).

2.8.2 PCR Amplification

The bacteria were identified by 16S rRNA gene PCR using general primers 16SA1 (5' – AGA-GTT-TGA-TCM-TGG-CTC-AG-3') and 16SB1 (5' – TAC-GGY-TAC-CTT-GTT-ACG-ACT-T-3') from Fukatsu and Nikoh (1998). PCR amplification was performed in 25 µl of a sterile mix containing 1X *Taq* polymerase buffer, 0.24 mM of each deoxynucleoside triphosphate, 2 mM $MgCl_2$, 0.32 µM of each primer, 1 µL of template DNA/colony sample and 0.025 U of Platinum *Taq*. The PCR mixtures were incubated for 5 minutes at 94 °C, followed by one cycle of 1 minute at 55 °C, 72 °C for 2 minutes and 25-30 cycles of 1 minute at 94 °C, 1 minute at 55 °C and 2 minutes at 72 °C with a final incubation of 8 minutes at 72 °C. Negative controls with no DNA addition and a positive control (*Ochrobactrum anthropi* DNA/control fly DNA) were also prepared.

To certify amplicon size (1.5 kb), a 3 µl aliquot of the PCR product was run on either a 1.5% agarose gel stained with Sybr Safe (Invitrogen) (*Drosophila* and Mexican *Aedes aegypti*) or 0.8% gel stained with ethidium bromide (Asian *Aedes aegypti*) and visualized using UV. PCR samples were purified using QIAquick PCR purification kit (Qiagen, Valencia, California, USA and Qiagen, Crawley, West Sussex, UK) following the manual instructions. PCR products were then sequenced with 16SA1 and 16SB1 primers using Macrogen (Oregon-RS), GATC Biotech, Germany (Asian *Aedes aegypti*) and with 16SA1 only at Cornell Life Sciences Core Laboratory Centre, Biotechnology, Cornell University, Ithaca, NY (Canton-S and Mexican *Aedes aegypti*).

Sequences were then trimmed using Sequencher 4.10.1 and blasted using NCBI nucleotide BLAST tool to identify the closest match for each sequence. This analysis was conducted between November 2010 and February 2011.

2.8.3 *Wolbachia* identification in Drosophila melanogaster using PCR

Wolbachia was identified by 16S rRNA gene PCR using universal primers *wsp* 81F: 5' TGG TCC AAT AAG TGA TGA AGA AAC and *wsp* 691R: 5' AAA AAT TAA ACG CTA CTC CA from Zhou *et al* (1998). PCR amplification was performed in 20 µl mix containing 1X *Taq* polymerase buffer, 0.40 mM of each deoxynucleoside triphosphate, 2.5 mM $MgCl_2$, 0.32 µM of each primer, 1 µL template DNA and 0.02 U Platinum *Taq*. Negative controls with no DNA addition and a positive control (Isogenic strain) were also prepared. The PCR mixtures were incubated for 1 minute at 95 °C, followed by 30 cycles of 1 minute at 95 °C, 1 minute at 54 °C and 1 minute at 72 °C with a final incubation of 5 minutes at 72 °C. To certify amplicon size (590-632 bp), a 8 µl aliquot of the PCR product was run on a 1.5% agarose gel stained with Sybr Safe (Invitrogen)

2.9 454 pyrosequencing

2.9.1 Sample Preparation and DNA Extraction

Fifty guts were dissected from the *Drosophila* (Canton-S, male and female). For DNA extraction the guts were placed in 180 µl enzymatic lysis buffer (20 mM Tris-Cl, 2 mM sodium EDTA, 1.2% Trition-X-100 and 20 mg lysozyme per ml). Twenty male adult *Aedes aegypti* treated with 30 µg ml^{-1} of chlortetracycline, non-chlortetracycline treated male adults, non-chlortetracycline treated female adults and non-chlortetracycline treated larvae were also placed in 180 µl enzymatic lysis buffer. The samples were homogenised with a pestle and mortar, and incubated at 37 °C for 45 minutes. Then, 0.1 mm cell disruption beads (50% volume) were added to the samples and bead-beating was conducted using a DisrupterGenie (Scientific Industries, Inc). DNA extraction was continued using the DNeasy® Blood and Tissue Kit (Qiagen, Valencia, California, USA) following the protocol for Gram-Positive DNA and eluted in 30 µL of buffer EB.

2.9.2 Amplification of Variable Region 2 (V2) of bacterial 16S rRNA gene (assisted by Adam Wong)

PCR amplification of the Variable Region 2 (V2) of bacterial 16S rRNA gene was conducted using the primers 27F (5' – AGA-GTT-TGA-TCM-TGG-CTC-AG-3') and 338R (5' – TGC-TGC-CTC-CCG-TAG-GAG-T-3') with a sample-specific 27F primer with a specific multiplex identifier (MID) sequence. All 27F and 338R primers were modified with 5'-Adaptor A and 5'-Adaptor B sequences, respectively for the pyrosequencing (Roche). PCR amplification was conducted in triplicate on six DNA samples containing 1X *Taq* polymerase buffer, 0.24 mM of each deoxynucleoside triphosphate, 2 mM $MgCl_2$, 8 pM each primer, 1 µL template DNA and 0.6 U Platinum® *Taq* DNA polymerase. The 25 µl PCR mixtures were incubated for 10 minutes at 94 °C, followed by 25 cycles of 1 minute at 94 °C, 1 minute at 58 °C and 1 minute at 72 °C with a final incubation of 8 minutes at 72 °C. PCR reactions (22.5 µl) were purified using Agencourt Ampure® SPRI kit and quantified using the Quant-iT™ PicoGreen® kit. Each reaction was diluted to 1 x 10^9 molecules µl $^{-1}$. Equal volumes of the three reaction products per sample were mixed together and diluted to 1 x 10^7 molecules for emulsion PCR at one copy per bead using only "A" beads for unidirectional sequencing. Beads were subjected to one full plate of the 454 GS-FLX pyrosequencing instrument using standard Titanium chemistry (Cornell Life Sciences Core Laboratory Centre, Biotechnology, Cornell University, Ithaca, NY).

2.9.3 Analysis of 454 sequences

Sequences were checked for quality using Genome Sequencer FLX System Software Manual, version 2.3 and clustered at 97% or more identity using Pyrotagger (http://pyrotagger.jgi-psf.org/release/; Kunin and Hugenholtz, 2010) (assisted by Adam Wong). Sequences were identified using the NCBI nucleotide Basic Local Alignment Search tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the closest match for each sequence. This analysis was conducted in December 2010.

2.10 Microarray analysis

A microarray analysis of *Drosophila melanogaster* was conducted to gain a global overview of the effect of diet and bacteria depleted conditions (flies derived from dechorionated eggs) on gene expression.

2.10.1 Insect rearing

Drosophila Canton S was reared on the Cornell diet (Table: 2.1). Flies were transferred into egg-laying cages with 1% grape juice agar overnight. On Day 1, 200 control (untreated) and 200 dechorionated eggs (Section: 2.3.2.1) were transferred onto autoclaved diets: low nutrient (20 gl^{-1} of glucose and yeast) and Cornell (High nutrient) (80 gl^{-1} of glucose and yeast). Both diets were autoclaved at 121°C for 20 minutes. Emerged adults were collected and placed onto fresh diet for 3/4 days before being flash frozen with liquid nitrogen and stored at -80 °C.

2.10.2 RNA Extraction and Sample Preparation (Method from John Ramsey)

RNA extraction was conducted on 10 male flies per replicate, 3 replicates per treatment. Flies were placed into chilled tubes on dry ice with 2 metal beads per tube and ground at 1600 strokes/min, for 95 seconds (2000 Geno/Grinder). Five hundred micro-litres of Trizol® (Invitrogen) was added to each tube and homogenised with a needle and 1 ml syringe. The mix was then centifuged at 10,000 g for 10 minutes. The supernatant was transferred to a new tube and 50 µl of bromochloropropane was added to the sample, the sample was mixed by invertion and left for 5 minutes at room temperature. The samples were centrifuged for 10 minutes at 10,000 g at 4 °C, the upper aqueous layer was transferred to a new tube and 100 µl of ethanol was added to the sample. The sample was then added to a cartridge (Ambion, Ribopure™ Kit), centrifuged for 30 seconds at 10,000 g and the eluent discarded. The cartridge was washed with 500 µl wash buffer (Ambion, Ribopure™ Kit), centrifuged for 30 seconds at 10,000 g and the eluent discarded. The centrifugation step was repeated and the remaining eluent was discarded. The RNA was eluted in 50 µl RNase-free water was added to the cartridge, left for 2 minutes at room temperature and centrifuged for 1 minute at 10,000 g. The quantity of RNA was measured using a Nanodrop and stored at -80 °C.

2.10.3 Microarray

The microarray used was a *Drosophila* Single Channel Gene Expression Microarray (Agilent, California, USA) with a format of 4 x 44k and 44,000 60-mer drosophila probes. RNA sample quality and integrity was evaluated using an Agilent Bioanalyzer. The RNA sample was amplified and labelled with Cy3 Amino Allyl MessageAmp™ II aRNA Amplification Kit (Ambion/Applied Biosystems) according to instructions, hybridised to the 4 x 44k microarray and washed according to manufacturers protocols. The microarray was scanned by an Axon 4000B scanner; fluorescent signals were obtained using Agilent Feature Extraction software (Cornell Life Sciences Core Laboratory Centre, Biotechnology, Cornell University, Ithaca, NY).

2.10.4 Microarray Analysis

The data were analysed by Limma (analysis with John Ramsey) and GeneSpring®.

For the analysis using Limma, the raw data files were imported into R 2.10.1 (cran-r-project.org/) and analysed with BioConductor (www.bioconductor.com) package Limma. The median foreground probe intensities were calculated without background subtraction, as recommended by Zahurak *et al* (2007). The values were then $\log_2$ transformed and normalised using the *vsn* (Variance Stabilization and Normalization) package in R (R2.10.1, cran-r-project.org/). Variance Stabilization and Normalization method is based on the fact that variance of the microarray depends on the signal intensity

Normalisation using the *vsn* method of analysis is based on three assumptions: 1) The variance of the measurements on a probe depends on the mean intensity 2) The relationship of measurements between samples is by an affine-linear transformation and 3) The variance-stabilised intensities per spot are normally distributed (Huber *et al*., 2002). This method is used to preprocess microarray intensity data by an affine transformation of each column, followed by a variance-stabilising transformation of the whole data (Huber, 2006; Huber, 2010). The method is summarised by the equation; > e2 <-vsn(e1) where e1 is the raw intensity measurements and e2 is the calibrated and generalised log-transformed data (Huber, 2006). The generalised log-transformed data is

a function similar to natural logarithm for large values (large compared to the background noise) but is less steep (has a smaller slope) for smaller values (Huber *et al*., 2003; Huber, 2006; Huber, 2010). The difference between the transformed values is the generalised 1 log-ratio (Huber, 2006; Huber, 2010). These generalised 1 log-ratios are shrinkage estimators of the natural logarithm of the fold change where shrinkage estimators at low intensities are smaller than or equal to to the naive log-ratios and become equal at large intensities (Huber, 2006; Huber, 2010). Therefore, they are not affected by the variance divergence of the naive log-ratios at the lower intensity which allows the value to maintain a significant result when the data are negative or close to zero (Huber, 2006; Huber, 2010). This method removes the intensity dependence of the variance (Huber *et al*., 2003).

For the analysis using GeneSpring® GX Software Version 11 (Agilent), the raw data files were imported into GeneSpring® and the data was normalised using Quantile Normalisation (reduces variance between arrays). Quantile normalisation is performed by sorting the expression values of each sample into ascending order and placed next to each other. The mean of the sorted order across all samples is taken; therefore each row in the matrix has equal variance to the previous mean. The modified matrix which has been obtained is then re-arranged to have the same ordering as the input matrix (Details obtained via email contact with Agilent Technologies).

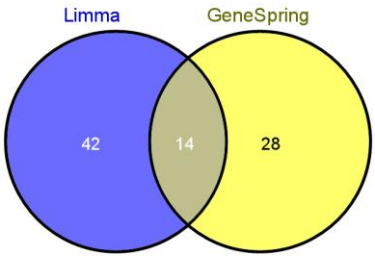
Differential Expression analysis was performed with both methods at the 0.05 level.

A comparison of the two methods of analyses (GeneSpring® and Limma) were conducted by comparing the number of genes where the expression was significantly changed ($p<0.05$) to determine which analysis was the most conservative. With the exception of the comparison of low nutrient diet dechorionation v high nutrient diet dechorionation (Figure: 2.4d), GeneSpring® was shown to be the most conservative with similar number of genes as with Limma for the High nutrient diet comparison (Figure: 2.4b) and lower genes with the low nutrient (Figure: 2.4a) and control comparison (Figure: 2.4c).

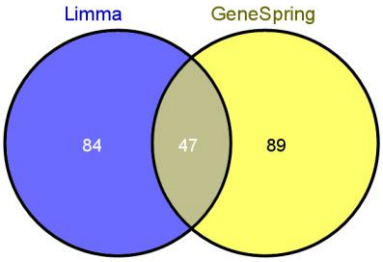
The gene ontology of each sequences was gained through the use of Blast2GO (blast2go.org) and searches using NCBI (http://www.ncbi.nlm.nih.gov/), panther

(www.pantherdb.org/), DGI TC Annotator (http://compbio.dfci.harvard.edu) and FlyBase (www.flybase.org).

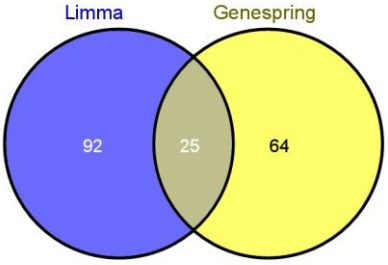
a) Low nutrient diet: Dechorionation V Control



b) High nutrient diet: Dechorionation V Control



c) Low nutrient diet control V High nutrient diet control



d) Low nutrient diet dechorionation V High nutrient diet dechorionation

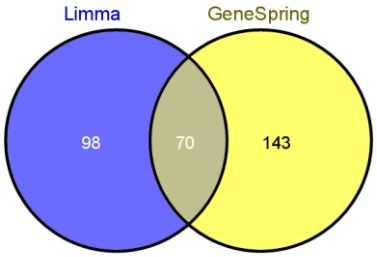


Figure 2.4: Comparison of the two different methods of analysis, GeneSpring® and Limma with the number of genes where expression is significantly altered; a) low nutrient diet: dechorionation V control b) high nutrient diet: dechorionation V control c) low nutrient diet control V high nutrient diet control d) low nutrient diet dechorionation V high nutrient diet dechorionation. Overlapping numbers represented the number of genes shared by both methods; numbers within each circle represents the number of genes found to have a significantly altered expression. The Venn diagram was created using an online source produced by Oliveros (2007).

2.10.5 Quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR) was conducted to confirm the results gained from the microarray data.

The samples used for the microarray were treated with DNase (Ambion) to remove contaminating DNA from the samples by the addition of 1X DNase I buffer and 1 µl rDNase I. The samples were incubated for 30 minutes at 37 °C and the reaction was terminated by the addition of 5 µl of inactivation reagent. The samples were then centrifuged for 1.5 minutes at 10,000g.

The cDNA was created by adding 200 ng of random primers (Fisher), 200 ng RNA, 10 pmole dNTPs and made up in sterile distilled water. The mixture was heated to 65 °C for 5 minutes and chilled on ice. 1 X First-Strand Buffer and 0.01M Dithiothreitol were added and gently mixed and incubated at 25 °C for 2 minutes. Two hundred units of SuperScript™ II RT (Invitrogen) was added and mixed by pipetting. The sample was then incubated at 25 °C for 10 minutes, followed by incubation at 42 °C for 50 minutes and heat inactivated for 15 minutes at 70 °C. The cDNA was stored at -20 °C.

Table: 2.3. Primers (designed using Primer-BLAST, NCBI) used for quantifying Diptericin, Diptericin B, Fat Body Protein 1, CG31148. Defensin and Phosophoenolpyruvate carboxykinase by qRT-PCR with the housekeeping gene RPL32 and Zwischenferment gene with no fold change expression across treatments in the microarray analysis.

Gene name	Forward sequence (5'-3')	Reverse sequence (5'-3')
DiptericinB	TTGGACTGGCTTGTGCCTTCTCG	TTGGGAGCATATGCCAGTGGTTCA
Fat Body Protein 1	GCTGCAGGCCATTAATCCATCCGT	TGCCAGTCAGATTCATGCCCATCG
CG31148	AGCTTGGGATGGACGCCACA	TGGTCGAGTGCGGTTCATCATTTT
Defensin	GAAGCGAGCCACATGCGACCT	AAACGCAGACGGCCTTGTCGT
Phosophoenolpyruvate carboxykinase	GCCAAAAACCCTTTCACGCGCA	TCCCCCATTGAATGCGTTTCGAGT
Diptericin	GCAGTTCACCATTGCCGTCGC	GCAGTTCACCATTGCCGTCGC
Zwischenferment (control)	GCAGTTCACCATTGCCGTCGC	ACCGCCGCCTCCCTGAAGAT
Ribosomal protein L32 (Housekeeping gene)	AGATCGTGAAGAAGCGCACCAAG	CACCAGGAACTTCTTGAATCCGG

The qRT-PCR reactions were performed in 96 well plates using 2 µl cDNA and 19 µl master mix, consisting of 1X Power Sybr Green PCR Master mix (Applied Biosystems) and

2.5 pM of the forward and reverse primers (Table 2.3). Two negative controls were also prepared, the reagent only negative controls which contained sterile distilled water with no DNA sample and No RT (Reverse transcriptase) control. The qRT-PCR reactions were carried out in a CFX96 Real time system on a C1000 thermal cycler (BioRad) with the following thermal profile: 2 minutes at 50 °C, 10 minutes at 95 °C followed by 40 cycles of 15 seconds at 95 °C and 1 minute at 60 °C. To ensure primer specificity to each gene, a melt curve was run for each plate from 65-95 °C with an increment of 0.5°C (Figure: 2.5).

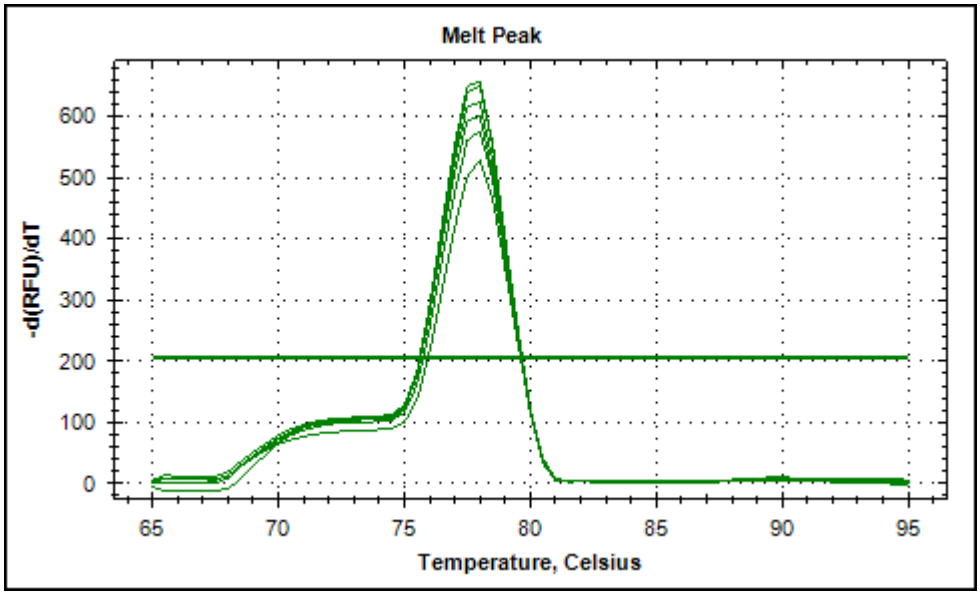


Figure: 2.5. An example of a melt-curve analysis with the primer pairs of Zwischenferment (control) with cDNA from replicate 1 of the control flies reared on a low nutrient diet.

The expression level of each gene (Table: 2.2) was determined by the comparative CT value method where the CT value obtained for each gene was normalized to the CT values obtained for the housekeeping gene RPL32.

2.11 Statistical Analysis

Statistical analyses were conducted using SPSS Inc 16.0 and 17.0 (Dytham, 2003 p66-199.). Significance was tested at 95% and above confidence level. The statistical test was selected depending on the data type and distribution.

For normally distributed and continuous data with 2 or more sample groups; an analysis of variance (ANOVA) was conducted. For data with a non-normal distribution and discontinuous data; a Mann-Whitney U test, a Kruskal-Wallis test and a Scheirer-Ray-Hare test was selected according to the number of groups and factors. Catagorical data was analysed using a Chi^2 analysis.

Table: Summary table of statistical tests used in this thesis (Dytham, 2003).

Samples/Groups	Factors	Data type	Statistical test
2	1	Catagorical	Chi^2 test
		Discontinuous	Mann-Whitney U test
		Continuous	t-test, one-way ANOVA
>2	1	Catagorical	Chi^2 test
		Discontinuous	Kruskal-Wallis test
		Continuous	One-way ANOVA
2+	>1	Discontinuous	Scheirer-Ray-Hare test
		Continuous	Two-way ANOVA, Multi-way ANOVA and Analysis of covariance (ANCOVA)

Chapter 3: Bacteria and performance of *Drosophila melanogaster*

3.1 Introduction

There is growing interest in the importance of gut microbes in animals and how the depletion of these microbes affects the health and performance of the animal. Several scientists have investigated the implications of antibiotics on the gut microbiota; in mice antibiotics have been shown to alter the diversity of bacteria found within the gut (Antonopoulos *et al*., 2009). In control mice the microbes were Firmicutes and Bacteroidetes but with antibiotic treatment the gut microbiota was predominantly Proteobacteria (Antonopoulos *et al*., 2009).

Drosophila melanogaster has been used for decades as one of the most useful model organisms when researching human diseases, and now there has been an increase in the use of *Drosophila melanogaster* to enable scientists to gain more insight into the role of gut bacteria. Sharon *et al* (2010) has shown that commensal bacteria play an important role in the mating preferences in *Drosophila melanogaster* and that diet also determines the species diversity in the insects. In this study it was shown that on a cornmeal-molasses-yeast diet, the insects have a more diverse population of bacteria in the gut compared with the insects on starch diet where only *Lactobacillus plantarum* was identified.

The importance of symbiotic bacteria to the host has also been demonstrated by several other authors (Ren, *et al*., 2007; Brummel, *et al*., 2004; Bakula, 1969) with *Drosophila melanogaster* (Chapter 1). Nevertheless, these studies have been inconsistent and in some cases not repeatable due to differences in conditions such as; the diet, the methods used to produce bacteria-free flies (use of antibiotic treatment and egg dechorionation) and how bacteria-free flies were characterised (culturable (Bakula, 1969) and 16S rRNA gene analysis (Ren, *et al*., 2007). Certain authors have used high concentrations of antibiotics to deplete the bacteria however they have not considered the implication of using such a high dose on the insect performance an example is a study by Mair *et al* (2005) and Fry and Rand (2002) both of which used 250 µg ml $^{-1}$ of tetracycline.

Most papers investigating the importance of *Drosophila melanogaster* symbionts in the host performance concentrate on life-span with only Bakula (1969) measuring development time. One particular aspect which has not been investigated is the role of *Drosophila* symbionts in host nutrition. Therefore, in this chapter I determine the impact of chlortetracycline on two wild-type lab strains: Oregon-RS and Canton-S by treating the *Drosophila melanogaster* with a range of chlortetracycline concentrations (0-500 μg ml^{-1}). Survival and development to pupae/adulthood, lifespan, the nutrition and respiration of the flies were measured. The effect of the treatment with chlortetracycline on the bacterial content within larvae and adults was also investigated by culturable bacterial counts and 454 pyrosequencing to compare the bacterial diversity with chlortetracycline and non-chlortetracycline treated adult flies. Further experiments using Canton-S with bacteria depleted flies (derived from egg dechorionation) were also used as a comparison with chlortetracycline treated flies, and to determine whether the depletion of bacteria or toxicity of the chlortetracycline was responsible for the changes in performance.

Finally, survival studies with *Drosophila melanogaster* (Canton-S) using a high nutrient (Cornell diet) and low nutrient diet will establish whether different responses are observed with different diets when the bacteria are depleted in flies via treatment with chlortetracycline and egg dechorionation.

3.2 Oregon-RS survival and development

3.2.1 Oregon-RS survival and sex-ratio

Initial experiments using the lab strain, Oregon-RS were used to test whether chlortetracycline treatment had a significant impact on *Drosophila melanogaster*.

Oregon-RS eggs were transferred to the York diet containing different concentrations of chlortetracycline up to 500 µg ml^{-1}. The mean number of individuals that survived to pupation varied from 5.1-7.2 out of 10 and did not vary significantly with treatment (Kruskal-Wallis: $H_6 = 5.973$, $p>0.05$) (Figure: 3.1). Pupal mortality was also low such that 4.9-6.8 survived to adulthood, again with no significant difference between treatments (Kruskal-Wallis: $H_6 = 6.039$, $p>0.05$) (Figure: 3.1). At the lower concentrations of chlortetracycline (0-10 µg ml^{-1}) the flies had a tendency to stick to the diet upon emergence, these were still alive when the numbers were recorded. The diet appeared to be more liquid than diets with a higher concentration of chlortetracycline (100 µg ml^{-1} and above). The "sticking" to the diets could possibly be due to the growth of bacteria on the diets as this was not observed with the diets containing 100 µg ml^{-1} and higher of chlortetracycline. Therefore, the addition of antibiotics could actually prevent this process from occurring and the survival data could have been different if the flies that were "stuck" on the food had not been removed on the day of their emergence.

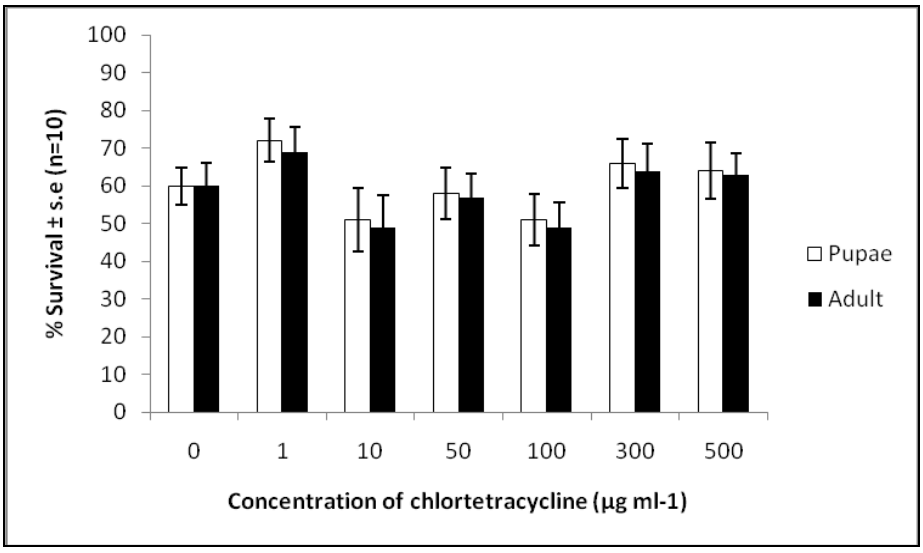


Figure: 3.1. Mean percent survival to pupae and adulthood of the 10 vials (10 eggs per vial) of each treatment of 0-500 µg ml^{-1} chlortetracycline (Methods Chapter, Section: 2.3.1, page 39).

The ratio of males and females was also analysed to determine whether chlortetracycline had an impact on the number of males and females in the population. The sex ratio did not vary significantly with increasing concentration of chlortetracycline. At 0 μg ml^{-1}, the ratio was 26:31 and for 500 μg ml^{-1} the ratio was 26:37 (males to females) (Table: 3.1). The concentration of chlortetracycline where there was quite a variation was at 1, 10 and 50 μg ml^{-1} where at 50 μg ml^{-1} there was double the number of females compared to male flies. Using a goodness of fit chi-square statistical test, the sex ratio was analysed to determine whether there is a significant effect on the sex ratio of *Drosophila* by antibiotic treatment. This analysis revealed no significant difference between the ratios of males and females ($X^2_6 = 8.321$, $p>0.05$) between the different treatments.

Table: 3.1. Sex ratio of *Drosophila* treated with 0-500 μg ml^{-1} chlortetracycline, number of replicates = 10 (Methods Chapter, Section: 2.3.1, page 39).

Concentration of chlortetracycline (μg ml^{-1})	Sex ratio Males:Females (% males)
0	26:31 (45.6%)
1	27:41(39.7%)
10	16:30 (34.8%)
50	18:38 (32.1%)
100	28:28 (50%)
300	29:29 (50%)
500	26:37 (41.3%)

3.2.2 Development time to pupae and adulthood

Development time measurements were also included in this experiment to determine whether the removal of the bacteria altered the development time. The development time of Oregon-RS to pupation ($p<0.001$, Table 3.2) and adulthood ($p<0.001$, Table: 3.2) varied significantly with chlortetracycline concentration. At 0 μg ml^{-1} of chlortetracycline, larvae started to develop into pupae by day 7. As the concentration of antibiotic increased, pupae formation was delayed in 2 steps; firstly, the median development to pupae was extended by one day at 1-10 μg ml^{-1} of chlortetracycline and by 2 days at 50-500 μg ml^{-1} of chlortetracycline. This delay in development was also observed in the emergence of adults, median development to adulthood was extended from 11 days to 12 days at 1-10 μg ml^{-1} and 13 days at 50-500 μg ml^{-1}.

Table: 3.2. The effect of chlortetracycline on the development time of *Drosophila melanogaster* (Oregon-RS) of 10 vials (10 eggs per vial) of each treatment of 0-500 μg ml^{-1} chlortetracycline (Methods Chapter, Section: 2.3.1, page 39).

	Median development time (Days)	
Concentration of chlortetracycline μg ml^{-1}	to pupae	to adulthood
0	7	11
1	8	12
10	8	12
50	9	13
100	9	13
300	9	13
500	9	13
	KW: $H_6 = 253$, $p<0.001$	KW: $H_6 = 226$, $p<0.001$

3.2.3 Behaviour of larvae on chlortetracycline

Oregon-RS larvae behaved differently on diet with high a concentration of chlortetracycline (100-500 μg ml^{-1}) compared with the larvae reared on the diet with 0-50 μg ml^{-1} of chlortetracycline. Larvae on low concentrations of chlortetracycline were able to burrow into the food, but at higher concentrations the larvae had a tendency to remain on the surface. One possible explanation to this observation is that the bacteria that grow on the diets with low concentrations of chlortetracycline are softening the diet and therefore the larvae have the ability to tunnel through the diet. At higher concentrations of chlortetracycline there is little or no bacterial growth and therefore the diet remains solid and harder for the larvae to tunnel into the food. Another possible explanation for this observation could be that the larvae treated with high concentrations of chlortetracycline are less vigorous and are not able to penetrate the food as much as the larvae treated with 0-50 μg ml^{-1} of chlortetracycline.

To test whether the extension of development time was due to a direct effect of chlortetracycline rather than a deleterious consequence of feeding on the surface, control Oregon-RS was reared on a diet with 0.9-2.5% agar. This experiment showed that there was a significant difference in the median development time ($p<0.001$. Table: 3.3). The concentration where a difference was observed was at 2% agar where the median development time was shortened from 11 days to 9 days, suggesting that this result could be an artefact rather than a real result as the reduction in development time was not observed at concentrations of agar higher than 2% and no difference was observed between the other concentrations (Table: 3.3). Larvae were also observed to persist on the top of the diet at 1.5% and at percentages greater than 1.5%, larvae were found within the cracks in the food. At 0.9-1%, larvae were able to penetrate the food and were not observed on the top of the diet.

Table: 3.3. The effect of agar percentage in the diet on the development time of control *Drosophila melanogaster* (Oregon-RS) of 10 vials (10 eggs per vial) of each treatment of 0.9-2.5% agar (Methods Chapter; Section: 2.3.1, page 39). All data are median.

Percent agar	Median development time adulthood (Days)
0.9	11
1	11
1.25	11
1.5	11
2	9
2.5	11

KW: $H_5 = 46.08$, $p<0.001$

3.3 Effects of Chlortetracycline on Oregon-RS size and nutrition

As described in the introduction, experiments have been conducted with flies which included measurements for life-span and survival. The change in the nutrition of the flies has not been considered, therefore in this section I will describe the changes which occur with flies treated with chlortetracycline.

3.3.1 Effects of chlortetracycline on Oregon-RS wing area

Size of the flies was assessed using wing area which had previously been shown by Shingleton *et al* (2005) and in Chapter 2 to be positively correlated with weight. The wing area was used as a measure of size; wing area was compared between flies treated with 0-500 µg ml^{-1}. A 2-way ANOVA was conducted to test whether chlortetracycline affected the wing area. There was no significant difference observed between the different concentrations of chlortetracycline ($F_{6, 43} = 1.889$, $p>0.05$) (Figure: 3.2). The graph (Figure 3.2) shows that the wing area did not vary dramatically between the different treatments (in males; mean value of 1.368 mm^2 at 0 µg ml^{-1} and 1.357 mm^2 at 500 µg ml^{-1} and in females; 1.8 mm^2 at 0 µg ml^{-1} and 1.55 mm^2 at 500 µg ml^{-1}). Female flies have a greater wing area than males as they were much larger than the male flies ($F_{1, 43} = 85.988$, $p<0.001$). However, there was no significant difference in the effect of chlortetracycline in male and female flies ($F_{6, 43} = 0.926$, $p>0.05$).

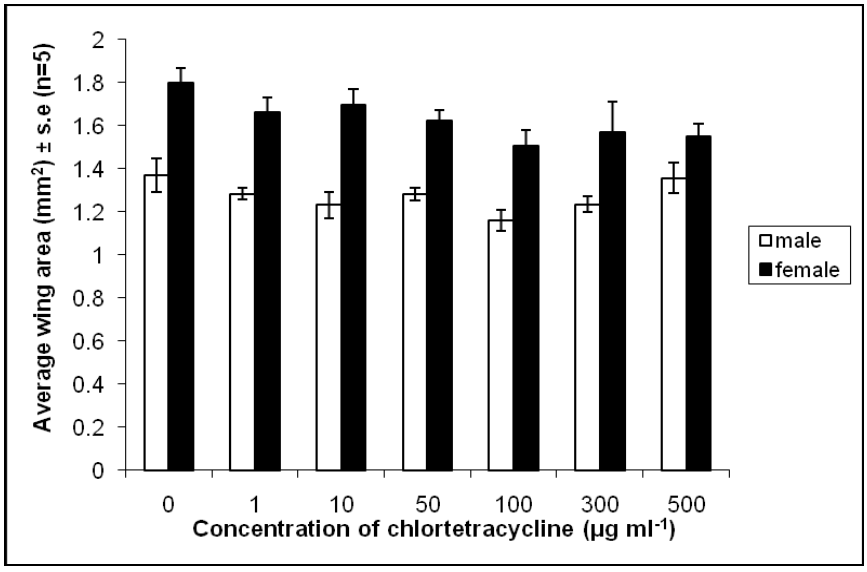


Figure: 3.2. Average wing area of male and female flies treated with 0-500 µg ml^{-1} of chlortetracycline, number of replicates per treatment = 5 (Methods Chapter; Section: 2.5.1, page 44).

3.3.2 Effects of chlortetracycline on Oregon-RS protein content

The average protein content of the flies varied from 157 to 248 µg. The average protein content of female flies was 243.3 µg of protein at 0 µg ml^{-1}, this reduced to 202.2 µg at 500 µg ml^{-1} (Figure: 3.3). In male flies the protein content varied from 157 to 190 µg.

An analysis of covariance (ANCOVA) was conducted to take into account the size of the fly using the wing area as a covariate ($F_{1, 54}$ = 0.110, p>0.05). This analysis showed that protein content varied significantly with chlortetracycline treatment ($F_{6, 54}$ = 6.598, p<0.001) and between sexes ($F_{1, 54}$ = 46.601, p<0.001). Yet, the test showed that there was a significant difference in response of male and female flies ($F_{6, 54}$ = 3.534, p<0.01). As the statistical test suggested; the response of male and female flies differed, a one-way ANOVA was conducted separately for the 2 sexes. This analysis showed wing area did not differ in male ($F_{1, 26}$ = 2.304, p>0.05) and female flies ($F_{1, 27}$ = 0.238, p>0.05). Furthermore, the protein content varied significantly with chlortetracycline treatment in females ($F_{1, 27}$ = 5.524, p<0.01) but not in males ($F_{1, 26}$ = 2.312, p>0.05).

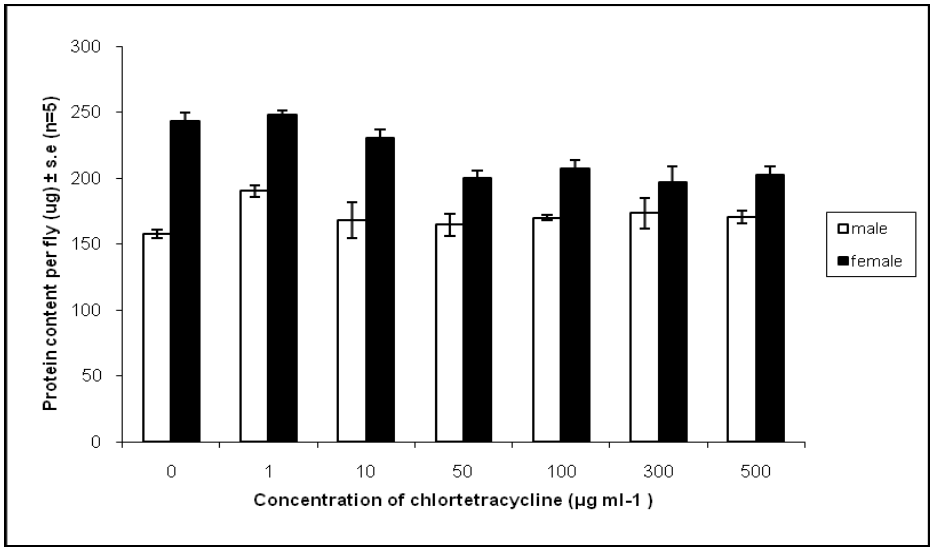


Figure 3.3: The protein content of flies treated with 0-500 µg ml^{-1} chlortetracycline, number of replicates per treatment = 5 (Methods Chapter; Section: 2.5.3, page 46).

3.3.3 Effects of chlortetracycline on Oregon-RS lipid content

Lipid content was the second nutrient to be quantified in chlortetracycline treated flies. As the dry weight of the flies was measured to calculate the lipid content of the flies, the lipid content was normalised to the dry weight of the fly.

An ANOVA was conducted to determine whether there was a significant impact of chlortetracycline treatment on the lipid content of flies. Chlortetracycline had a significant impact on the lipid content of the flies ($F_{6, 54}$ = 15.086, p<0.001). This response to chlortetracycline treatment was observed in both males and females ($F_{6, 54}$ = 1.979, p>0.05). However, there was a significant difference in lipid content between sexes, generally greater in males than in females ($F_{1, 54}$ = 27.222, p<0.001). In females the lipid content (per mg of dry weight) increases from 0.302 mg at 0 µg ml^{-1} to 0.371 mg at 100 µg ml^{-1} (Figure 3.4). In males the lipid content increases from 0.294 mg at 0 µg ml^{-1} to 0.436 mg at 100 µg ml^{-1} (Figure: 3.4).

LSD (Least significant difference) post-hoc statistical test showed that the lipid content of the female flies treated with no chlortetracycline was significantly different from the flies treated with 100 µg ml^{-1}. The lipid content of females peak at 100 µg ml^{-1} of chlortetracycline. This result suggests that it could be an artefact, as the same trend was not observed at the concentrations of 300-500 µg ml^{-1}. In males, post-hoc tests suggest that 0 µg ml^{-1} was significantly different from 1, 50-500 µg ml^{-1} of chlortetracycline.

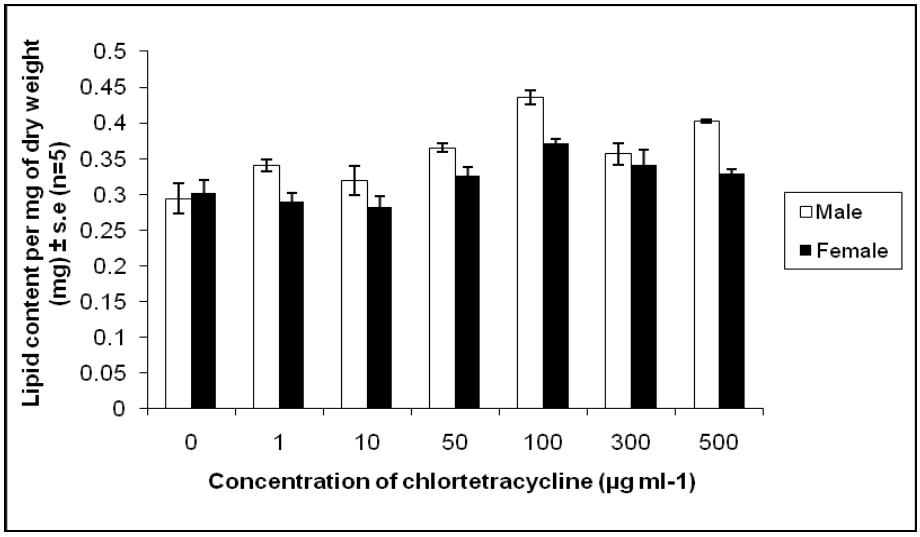


Figure: 3.4: The lipid content of female and male flies treated with 0-500 µg ml^{-1} of chlortetracycline, number of replicates per treatment = 5 (Methods Chapter; Section: 2.5.6, page 48).

Analysis of dry weight of the flies showed that the weight of the chlortetracycline treated flies was significantly affected by chlortetracycline treatment ($F_{6, 54}$ = 9.5, p<0.001). There was a significant difference between male and female flies ($F_{1, 54}$ = 224.738, p<0.001). The response to chlortetracycline differed in male and female flies ($F_{6, 54}$ = 2.661, p<0.05). LSD post-hoc statistical test showed that the weight was significantly different at 100 µg ml^{-1} of chlortetracycline in both male and female flies (Figure: 3.5).

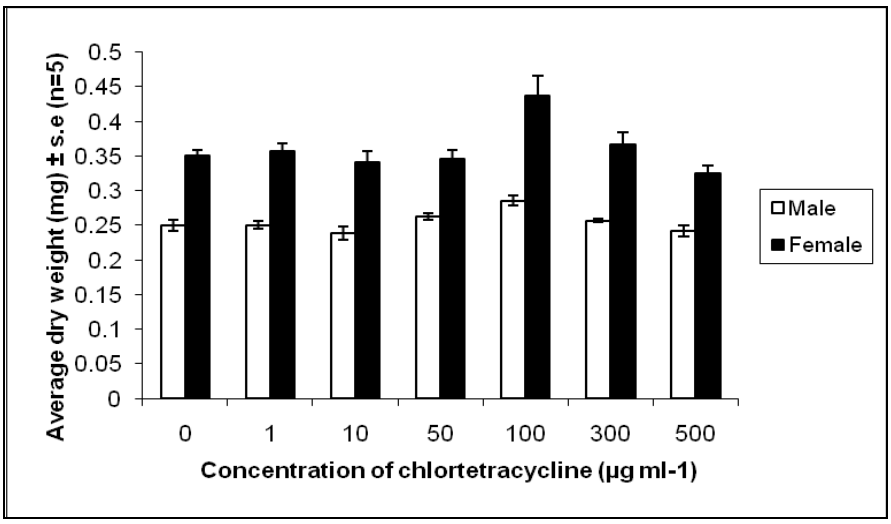


Figure: 3.5: The average dry weight of male and female flies treated with 0- 500 µg ml^{-1} of chlortetracycline, number of replicates per treatment = 5 (Methods Chapter; Section: 2.5.6, page 48).

3.4 Canton-S survival, development, lifespan and fecundity

Experiments with chlortetracycline were repeated with Canton-S, to demonstrate that the observations made in Oregon-RS can also be observed in another wild-type laboratory strain. Furthermore, another method was used to deplete bacteria in the flies via egg dechorionation. A 2X2 factorial experiment was conducted with control flies and flies derived from dechorionated eggs treated with 0, 50 and 300 μg ml^{-1} of chlortetracycline.

3.4.1 Survival to pupae and adulthood

The average survival of the strain Canton-S to pupae and adulthood varied from 39-76%. The survival to pupae and adulthood was not significantly affected by chlortetracycline or by egg dechorionation (Table: 3.4) ($p>0.05$), yet it does appear that chlortetracycline and dechorionation does improve the survival of Canton-S but not when flies derived from dechorionated eggs are treated with chlortetracycline. In control flies the survival was 39-47%, this increased to 57-76% with flies derived from dechorionated eggs without chlortetracycline and control flies treated with chlortetracycline. In flies derived from dechorionation and treated with chlortetracycline the survival was similar to the control flies, which could suggest that the dechorionation and chlortetracycline treatment had a deleterious effect on survival, however chlortetracycline treatment and dechorionation alone improved survival.

Table: 3.4: Survival to pupae and adulthood of control flies and flies derived from dechorionation treated with 0-300 μg ml^{-1} of chlortetracycline and Scheirer-Ray-Hare statistical analysis, number of replicates = 15 (Methods Chapter; Section: 2.3.2.2, page 40).

Concentration of chlortetracycline (μg ml^{-1})	Mean survival (%) n=15			
	Pupae		Adult	
	Control	Dechorionation	Control	Dechorionation
0	47 ± 7	62 ± 4	39 ± 7	57 ± 5
50	71 ± 4	38 ± 6	66 ± 4	35 ± 6
300	76 ± 4	47 ± 5	71 ± 4	47 ± 5
Scheirer-Ray-Hare Test Chlortetracycline Dechorionation Chlortetracycline* Dechorionation	$F_{6, 84} = 0.728$, $p>0.05$ $F_{1, 84} = 0.738$, $p>0.05$ $F_{1, 84} = 2.025$, $p>0.05$		$F_{6, 84} = 0.366$, $p>0.05$ $F_{1, 84} = 1.541$, $p>0.05$ $F_{1, 84} = 2.530$, $p>0.05$	

3.4.2 Development time to pupae and adulthood

The development time to pupation of the strain Canton-S varied significantly with chlortetracycline concentration ($p<0.05$) (Table: 3.5). At 0 µg ml^{-1} chlortetracycline, larvae started to develop into pupae by day 7. Pupae formation was delayed by one day at 50 and 300 µg ml^{-1} of chlortetracycline. Egg dechorionation also caused a significant effect on development time with an extension of one day ($p<0.01$, Table: 3.5). This experiment was repeated and an extension of development was again observed with treated *Drosophila*. Furthermore, the result supports the data obtained with Oregon-RS. It does suggest that the extension in development is due to the removal of bacteria and not a toxicity issue.

Table: 3.5: Median development time (Days) of control flies and flies derived from dechorionation treated with 0-300 µg ml^{-1} of chlortetracycline and Scheirer-Ray-Hare statistical analysis, number of replicates = 15 (Methods Chapter; Section: 2.3.2.2, page 40).

Concentration of chlortetracycline (µg ml^{-1})	Median development time (Days) n=15			
	Pupae		Adult	
	Control	Dechorionation	Control	Dechorionation
0	7	8	11	12
50	8	8	12	12
300	8	8	12	12
Scheirer-Ray-Hare Test Chlortetracycline Dechorionation Chlortetracycline* Dechorionation	$F_{6, 501} = 9.54$, $p<0.05$ $F_{1, 501} = 64.65$, $p<0.001$ $F_{2, 501} = 4.64$, $p>0.05$		$F_{6, 469} = 9.54$, $p<0.05$ $F_{1, 469} = 64.65$, $p<0.05$ $F_{2,469} = 4.64$, $p>0.05$	

3.4.3 Life-span of male Canton-S

To gain an overall understanding about how chlortetracycline treatment and egg dechorionation affect Canton-S, lifespan and fecundity experiments were conducted.

The life-span of male flies varied significantly with chlortetracycline concentration ($p<0.001$), but no significant difference was observed between control and with egg dechorionation ($p>0.05$) and there was no significant difference in response of dechorionated and control flies to chlortetracycline treatment ($p>0.05$) (Table: 3.6). LSD post-hoc tests showed that control male flies on no chlortetracycline had a significantly lower life-span (41 days) compared with 60-64 days of control flies treated with chlortetracycline (Table: 3.6). Flies derived from dechorionated eggs and no treatment with chlortetracycline had an average life-span of 52 days; however, chlortetracycline treatment appears to extend life-span further to 59 and 64 days at 50 and 300 µg ml^{-1}, respectively.

Table: 3.6: Average (mean) male life-span of control flies and flies derived from dechorionated eggs treated with 0-300 µg ml^{-1} of chlortetracycline, number of replicates = 10 (Methods Chapter; Section: 2.3.2.2, page 40).

	Average adult life-span (Days) n=10	
Concentration of chlortetracycline (µg ml^{-1})	Control	Dechorionation
0	41 ± 5.273 (8)	52 ± 3.271 (10)
50	64 ± 2.805 (10)	59 ± 3.437 (10)
300	60 ± 5.842 (10)	64 ± 2.389 (9)
ANOVA Dechorionation Chlortetracycline Chlortetracycline* Dechorionation	 $F_{1,51} = 1.055$, $p>0.05$ $F_{2,51} = 9.419$, $p<0.001$ $F_{2,51} = 1.894$, $p>0.05$	

3.4.4 Fecundity of Canton-S

Female flies were used to assess the fecundity of chlortetracycline treated flies and flies derived from dechorionated eggs. A Scheirer-Ray-Hare test was conducted to determine whether there was a significant effect on the fecundity of control flies and flies derived from dechorionated eggs treated with chlortetracycline. Chlortetracycline treatment was shown to have a significant effect ($p \leq 0.05$, Table: 3.7) on egg laying, with the median egg laying capacity being 113 eggs for the control flies and 49.5 and 72 eggs for 50 and 300 µg ml^{-1}, respectively. Dechorionation had no significant effect on fecundity ($p > 0.05$, Table: 3.7). Flies derived from dechorionated eggs without chlortetracycline treatment were shown to have no significant difference compared with controls (112 and 117, respectively) (Table: 3.7). The response to chlortetracycline of control flies and flies derived from dechorionated eggs was not significantly different ($p > 0.05$, Table: 3.7). Flies derived from dechorionated eggs treated with 50 and 300 µg ml^{-1} chlortetracycline showed an egg laying capacity of 68 and 104 eggs, respectively.

This result suggests that bacteria depletion does not have an effect on the fecundity of *Drosophila*, but the presence of chlortetracycline reduces fecundity. This may be due to 2 reasons; 1) the toxicity of chlortetracycline causes a reduction in the reproduction capacity 2) the females are deterred from ovipositing on the diet containing the antibiotic.

Table: 3.7. Median number of eggs laid over 7 days of control flies and flies derived from dechorionated eggs treated with 0-300 µg ml^{-1} of chlortetracycline and Scheirer-Ray-Hare statistical analysis, number of replicates = 10 (Methods Chapter; Section: 2.3.2.2, page 41).

	Median number of eggs laid over 7 days (n=10)	
Concentration of chlortetracycline (µg ml^{-1})	Control	Dechorionated
0	113	117
50	49.5	68
300	72	104
Scheirer-Ray-Hare Test		
Chlortetracycline	$F_{2,53} = 3.69$, $p<0.05$	
Dechorionation	$F_{1,53} = 0.36$, $p>0.05$	
Chlortetracycline* Dechorionation	$F_{2,53} = 0.35$, $p>0.05$	

3.4.5 Egg Hatching Experiment

Bacteria depleted flies where the eggs were dechorionated have been observed to have an extended development time to pupae and adulthood when compared with control flies. Johnston and Crickmore (2010) reported that axenic (bacteria-free) *Manduca sexta* showed a delayed development; they suggested that the treatment rather than the removal of bacteria had caused the extension. From this suggestion, egg hatching was monitored to determine whether the dechorionation caused the eggs to hatch later than controls. Bacteria depletion did not appear to cause a delay in egg hatching (MWU: Z_1 = -1.463, p>0.05), with the median egg hatching time 19 hours after treatment (Table: 3.8).

Table: 3.8. Median egg hatching time (Hours) of control and dechorionated eggs, number of replicates = 10 (Methods Chapter; Section: 2.3.2.2, page 41).

Treatment	Median egg hatching time (Hours)
Control	19
Dechorionated	19

Egg hatching was also measured to determine whether a delayed egg hatching is responsible for a delay in development time to pupae and adulthood when treated with chlortetracycline. Eggs were transferred to the control diets and diets with 50 and 300 µg ml^{-1} of chlortetracycline. Number of eggs hatched was counted at the beginning and end of each day. Chlortetracycline treatment appears not to have an effect on the egg hatching (H_2 = 0.820, p>0.05), with the median time to hatch of 19 hours after transfer to chlortetracycline or non-chlortetracycline diet (Table: 3.9). A Kruskal-Wallis test was conducted on the data which was shown to have a distribution which was significantly different from normal (p<0.001).

Table: 3.9. Median egg hatching time (Hours) of eggs transferred to diet supplemented with chlortetracycline, number of replicates = 10 (Methods Chapter; Section: 2.3.2.2, page 41).

Concentration of chlortetracycline (µg ml^{-1})	Median egg hatching time (Hours)
0	19
50	19
300	19

Both of these experiments suggest that the delay development time occurs at the larval stage.

3.5 Effects of Chlortetracycline on Canton-S size and nutrition

3.5.1 Effect of chlortetracycline on Canton-S wing area

The size of the flies was inferred from the wing area of the flies and used for nutritional analysis. The wing area of male flies was not significantly affected by either chlortetracycline treatment or by egg dechorionation ($p>0.05$, Table: 3.10). The average wing area of female flies was significantly reduced with chlortetracycline treatment and the treatment with chlortetracycline and egg dechorionation with the exception of dechorionation with treatment of 50 µg ml^{-1} of chlortetracycline diet (Table: 3.10).

Table: 3.10. The wing area of male and female control flies and flies derived from dechorionated eggs (Dechorionation) treated with chlortetracycline (0-300 µg ml^{-1}) and ANOVA statistical analysis, number of replicates = 5 (Methods Chapter; Section: 2.5.1, page 44).

Chlortetracycline concentration (µg ml^{-1})	Wing area (mm^3) Mean ± s.e. (n=5, except *n=3)			
	Males		Females	
	Control	Dechorionation	Control	Dechorionation
0	1.36 ± 0.04	1.36 ± 0.01	1.84 ± 0.02	1.80 ± 0.01
50	1.38 ± 0.01	1.41 ± 0.02	1.75 ± 0.01	1.84 ± 0.02
300	1.39 ±0.03	1.39 ± 0.004*	1.77 ± 0.01	1.78 ± 0.02*
ANOVA Dechorionation Chlortetracycline Chlortetracycline*Dechorionation	 $F_{1,22}$= 0.218, p>0.05 $F_{2,22}$ = 0.988, p>0.05 $F_{2,22}$ = 0.244, p>0.05		 $F_{2,22}$ = 3.750, p>0.05 $F_{2,22}$ = 4.107, p<0.05 $F_{2,22}$ = 10.637, 0.001<p<0.01	

3.5.2 Effect of chlortetracycline on Canton-S triglyceride content

The triglyceride content of the *Drosophila melanogaster* was quantified in order to determine whether bacterial depletion through chlortetracycline treatment and dechorionation affected the lipid nutrition of the insect. Wing area was used as a covariate in this analysis to take into account the size differences between samples. The triglyceride content of male flies was not significantly affected by egg dechorionation, however treatment with chlortetracycline did. In males the triglyceride content decreases from 13.9 µg at 0 µg ml^{-1} to 12.2 and 11.5 µg at 50 and 300 µg ml^{-1} of chlortetracycline, respectively (Table: 3.11). Flies derived from dechorionated eggs and reared on 50 µg ml^{-1} of chlortetracycline actually had a greater triglyceride content (17.2 µg) compared with control flies (13.9 µg), this result was supported by the significant interaction between chlortetracycline and egg dechorionation ($p<0.001$). In females the same pattern was observed, there was no significant effect of egg dechorionation but there was a significant reduction in triglyceride content in the presence of chlortetracycline ($p<0.05$). In females, the triglyceride decreases from 23.5 µg at 0 µg ml^{-1} to 17.3 and 21.2 µg at 50 and 300 µg ml^{-1} respectively (Table: 3.11). The same pattern was observed in flies derived from dechorionated eggs treated with chlortetracycline ($p>0.05$).

Table: 3.11 Triglyceride content of control flies and flies derived from dechorionated eggs treated with 0-300 µg ml^{-1} of chlortetracycline and ANCOVA statistical analysis, number of replicates = 5 (Methods Chapter; Section: 2.5.5, page 47).

Chlortetracycline concentration (µg ml^{-1})	Triglyceride content (µg) per fly Mean ± s.e. (n=5, except *n=3)			
	Males		Females	
	Control	Dechorionation	Control	Dechorionation
0	13.9 ± 0.5	12.0 ± 0.5	23.5 ± 0.6	21.1 ± 1.0
50	12.2 ± 1.1	17.2 ± 0.5	17.3 ± 1.2	17.7 ± 2.5
300	11.5 ± 0.5	10.4 ± 1.3*	21.2 ± 1.2	15.8 ± 0.7*
ANCOVA Wing area covariate Dechorionation Chlortetracycline Chlortetracycline*Dechorionation	$F_{1,21}$= 2.498, p>0.05 $F_{1,21}$ = 0.349, p>0.05 $F_{2,21}$ = 20.799, p<0.001 $F_{2,21}$ = 12.830, p<0.001		$F_{1,22}$ = 0.206, p>0.05 $F_{1,22}$ = 0.521, p>0.05 $F_{2,22}$ = 6.294, 0.05>p>0.01 $F_{2,22}$ = 1.603, p>0.05	

3.5.3 Effect of chlortetracycline on Canton-S protein content

In male flies there was a significant effect of egg dechorionation on the protein content of the flies but chlortetracycline treatment alone had no significant effect on the protein content ($p>0.05$) (Table: 3.12). The protein content was significantly reduced in flies with egg dechorionation and treatment with chlortetracycline (decreases from 102.8 µg in 0 µg ml^{-1} control to 78.2 µg) ($p\leq0.05$). In female flies dechorionation has no significant effect on the protein content, nevertheless chlortetracycline treatment did; protein content decreases from 165.6 µg at 0 µg ml^{-1} to 126.6 and 141.5 µg at 50 and 300 µg ml^{-1}, respectively (Table: 3.12). The same pattern was also observed with flies derived from dechorionated eggs and exposed to chlortetracycline (138.7 and 121.2 µg at 50 and 300 µg ml^{-1}, respectively) which is supported by the non significant result for the interaction between egg dechorionation and chlortetracycline. This result observed in the female flies suggests that the chlortetracycline could be altering protein metabolism/synthesis and not as a result of depleting the bacteria as there was no significant difference with egg dechorionation alone. However, this was not observed in male flies. The only significant reduction was observed in flies with egg dechorionation and treated with chlortetracycline, suggesting that there is an interaction between chlortetracycline and egg dechorionation.

Table: 3.12: The protein content of control flies and flies derived from dechorionated eggs treated with 0-300 µg ml^{-1} of chlortetracycline and ANCOVA statistical analysis, number of replicates = 5 (Methods Chapter; Section: 2.5.3, page 46).

Chlortetracycline concentration (µg ml^{-1})	Protein content (µg) per fly Mean ± s.e. (n=5, except *n=3)			
	Males		Females	
	Control	Dechorionation	Control	Dechorionation
0	102.8 ± 7.3	106.9 ± 4.0	165.6 ± 8.3	152.8 ± 4.6
50	108.7 ± 8.6	87.7 ± 5.2	126.6 ± 10.3	138.7 ± 2.5
300	104.3 ± 6.8	78.2 ± 6.5*	141.5 ± 8.1	121.2 ± 4.8*
ANCOVA				
Wing area covariate	$F_{1,21} = 2.230$, $p>0.05$		$F_{1,22} = 0.147$, $p>0.05$	
Dechorionation	$F_{1,21} = 7.763$, $p<0.05$		$F_{1,22} = 0.582$, $p>0.05$	
Chlortetracycline	$F_{2,21} = 2.644$, $p>0.05$		$F_{2,22} = 5.939$, $0.01>p>0.001$	
Chlortetracycline*Dechorionation	$F_{2,21} = 3.394$, $p\leq0.05$		$F_{2,22} = 1.583$, $p>0.05$	

3.5.4 Effect of chlortetracycline on Canton-S glucose content

The carbohydrate content of the flies were quantified, the carbohydrate sources that were analysed were glucose, trehalose and glycogen. On average, the glucose content of the flies ranged from 4-12 µg (Table: 3.13). The glucose content of male and female flies was approximately 60% greater in flies reared with egg dechorionation excluding the flies also reared with 300 µg ml^{-1}. Chlortetracycline also promoted the quantity of free glucose; still there was only approximately a 40% increase. This result suggests that in the presence of bacteria, free glucose levels are depressed, chlortetracycline does not eliminate all of the bacteria and therefore the effect is less pronounced. The flies with dechorionation and reared on 300 µg ml^{-1} of chlortetracycline did have a lower quantity of glucose, but this could be the result of a build-up effect of a high concentration of chlortetracycline on a fly with an already depleted gut microbiota.

Table: 3.13. The glucose content of control flies and flies derived from dechorionated eggs treated with 0-300 µg ml^{-1} of chlortetracycline and ANCOVA statistical analysis, number of replicates = 5 (Methods Chapter; Section: 2.5.4, page 46).

Chlortetracycline concentration (µg ml^{-1})	Glucose content (µg) per fly Mean ± s.e. (n=5, except *n=3 # n=4)			
	Males		Females	
	Control	Dechorionation	Control	Dechorionation
0	4.9 ± 0.4	8.3 ± 0.6	7.9 ± 0.5	12.3 ± 0.8#
50	5.1 ± 0.3	8.6 ± 0.6	8.1 ± 0.4	13.5 ± 1.3
300	6.7 ± 0.3	5.0 ± 0.5 *	10.7 ± 1.1	4.9 ± 0.3*
ANCOVA Wing area covariate Dechorionation Chlortetracycline Chlortetracycline*Dechorionation	 $F_{1,21}$ = 0.249, p>0.05 $F_{1,21}$ = 18.441, p<0.001 $F_{2,21}$ = 1.943, p>0.05 $F_{2,21}$ = 15.727, p<0.001		 $F_{1,20}$ = 5.830, p<0.05 $F_{1,20}$ = 1.279, p>0.05 $F_{2,20}$ = 5.363, p<0.05 $F_{2,20}$ = 36.873, p<0.001	

3.5.5 Effect of chlortetracycline on Canton-S trehalose content

On average the trehalose content of the flies ranged from 4-14 µg (Table: 3.14). In male flies chlortetracycline treatment appeared to increase the trehalose content of the flies by approximately 30 percent. Again, this supports the theory suggested for the glucose data that the bacteria utilise the sugar and therefore deplete levels within the fly. However, the same pattern was not observed with egg dechorionation, a reduction was actually observed at 0 and 50 µg ml^{-1} of chlortetracycline. In female flies the same pattern was observed as with male flies. An increase in free trehalose for the chlortetracycline treated flies was demonstrated, this increase was substantial with up to a 3 fold change. In female flies with egg dechorionation, an increase was also observed and at a similar quantity as the treatment with 300 µg ml^{-1} (a concentration of antibiotic which depletes most of the bacteria).

Table: 3.14. The trehalose content of control flies and flies derived from dechorionated eggs treated with 0-300 µg ml^{-1} of chlortetracycline and ANCOVA statistical analysis, number of replicates = 5 (Methods Chapter; Section: 2.5.4, page 47).

Chlortetracycline concentration (µg ml^{-1})	Trehalose content (µg) per fly Mean ± s.e. (n=5, except *n=3 # n=4)			
	Males		Females	
	Control	Dechorionation	Control	Dechorionation
0	5.5 ± 0.4	4.1 ± 0.7	4.3 ± 0.4	12.1 ± 2.0#
50	6.3 ± 0.6	2.0 ± 1.4	7.8 ± 1.3	11.3 ± 1.2
300	7.2 ± 1.0	6.1 ± 0.7*	11.8 ± 0.8	14.1 ± 1.3*
ANCOVA				
Wing area covariate	$F_{1,21}$= 0.401, p>0.05		$F_{1,20}$ = 1.324, p>0.05	
Dechorionation	$F_{1,21}$ = 9.035, p<0.01		$F_{1,20}$ = 22.962, p<0.001	
Chlortetracycline	$F_{2,21}$ = 3.766, p<0.05		$F_{2,20}$ = 4.332, p<0.05	
Chlortetracycline*Dechorionation	$F_{2,21}$ = 2.096, p>0.05		$F_{2,20}$ = 1.692, p>0.05	

3.5.6 Effect of chlortetracycline on Canton-S glycogen content

The final carbohydrate that was quantified was glycogen. On average the glycogen content of the flies was 4-22 µg (Table: 3.15). In male flies there was no distinct trend, there was no significant difference between flies with and without egg dechorionation. However, there was a significant difference in glycogen levels of chlortetracycline treated flies at 300 µg ml^{-1} where a 30% increase was observed. The flies without egg dechorionation had a greater quantity of glycogen levels compared to the control flies, but this was not significant ($p>0.05$). At 50 µg ml^{-1} of chlortetracycline with dechorionation, the glycogen levels were approximately 50% greater. This result was supported by the statistical significance of the interaction between egg dechorionation and chlortetracycline. In female flies, a more distinct trend was observed where female flies on high concentrations of chlortetracycline (300 µg ml^{-1}) and with egg dechorionation had a significantly greater quantity of glycogen. A 20-300% increase was observed in females treated with chlortetracycline and reared without egg dechorionation. Female flies reared without egg dechorionation and in the presence of chlortetracycline had a much greater quantity of glycogen compared with control with chlortetracycline; therefore a significant interaction between egg dechorionation and chlortetracycline was observed.

Table: 3.15. The glycogen content of control flies and flies derived from dechorionated eggs treated with 0-300 µg ml^{-1} of chlortetracycline and ANOVA statistical analysis, number of replicates = 5 (Methods Chapter; Section: 2.5.4, page 47).

Chlortetracycline concentration (µg ml^{-1})	Glycogen content (µg) per fly Mean ± s.e. (n=5, except *n=3 # n=4)			
	Males		Females	
	Control	Dechorionation	Control	Dechorionation
0	5.5 ± 0.7	7.0 ± 0.3	7.0 ± 0.3	8.6 ± 1.1*
50	4.1 ± 0.3	8.6 ± 1.0 #	6.7 ± 1.1	10.1 ± 0.5#
300	7.3 ± 1.0	6.9 ± 0.4*	10.2 ± 1.1	22.2 ± 3.9*
ANOVA Wing area covariate Dechorionation Chlortetracycline Chlortetracycline*Dechorionation	$F_{1,20} = 0.205$, $p>0.05$ $F_{1,20} = 2.603$, $p>0.05$ $F_{2,20} = 8.274$, $p<0.01$ $F_{2,20} = 4.113$, $p<0.05$		$F_{1,18} = 0.230$, $p>0.05$ $F_{1,18} = 14.501$, $p<0.01$ $F_{2,18} = 10.074$, $p<0.01$ $F_{2,18} = 5.256$, $p<0.05$	

3.6 Respirometry analysis with chlortetracycline treatment and egg dechorionation reared on the York diet

Respirometry analysis was conducted with male and female Canton-S reared on the York diet, the 2-factor experiment involved; chlorteracycline and dechorionation. The respirometry analysis involved the quantification of oxygen consumption, carbon dioxide production and from these values the repiratory quotient (RQ) value (CO_2/O_2) was calculated.

In male flies the mean oxygen consumption varied from 0.054-0.089 µl per minute with the control flies consuming the greatest oxygen volume per minute (Figure: 3.6a). Carbon dioxide production varied from 0.08-0.10 µl per minute, again with control flies producing the greatest volume (Figure: 3.6a). The respiratory quotient varied from 1.16-1.77, with the greatest RQ value with flies derived from dechorionated eggs treated with 300 µg ml^{-1} chlortetracycline (Figure: 3.7a).

In female flies the oxygen consumption was greater than with male flies which would be expcted as female flies were greater in size. As with male flies the greatest oxygen consumption was observed with control flies, the oxygen consumption across all treatments varied from 0.11-0.18 µl per minute (Figure: 3.6b). Carbon dioxide production varied from 0.10-0.16 µl per minute, again with control flies producing the greatest volume (Figure: 3.6b). The respiratory quotient for females, varied from 0.90-1.21 with the greatest RQ value with flies derived from dechorionated eggs treated with 50 µg ml^{-1} chlortetracycline (Figure: 3.7b).

Analysis of covariance was conducted with oxygen consumption, carbon dioxide production and RQ values with the wing area as the covariate.

Statistical analysis of the oxygen consumption for male and female flies showed that wing area did not differ significantly between the treatments ($F_{1, 65} = 0.118$, $p>0.05$). Chlortetracycline ($F_{6, 65} = 2.219$, $p>0.05$) did not have a significant effect on the oxygen consumption but dechorionation did ($F_{1, 65} = 3.894$, $p=0.05$). Sex did have a significant effect on the oxygen consumption values ($F_{1, 65} = 4.240$, $p<0.05$) with females consuming more oxygen than male flies. The interactions of dechorionation*chlortetracycline treatment also had a significant effect on the oxygen consumption ($F_{2, 65} = 3.724$, $p<0.05$)

with dechorionation and treatment with 50 µg ml^{-1} chlortetracycline of male flies having a greater consumption compared with control flies treated with 50 µg ml^{-1} chlortetracycline. The interactions of sex*chlortetracycline, sex*dechorionation, sex*dechorionation*chlortetracycline did not have a significant value (Figure: 3.6).

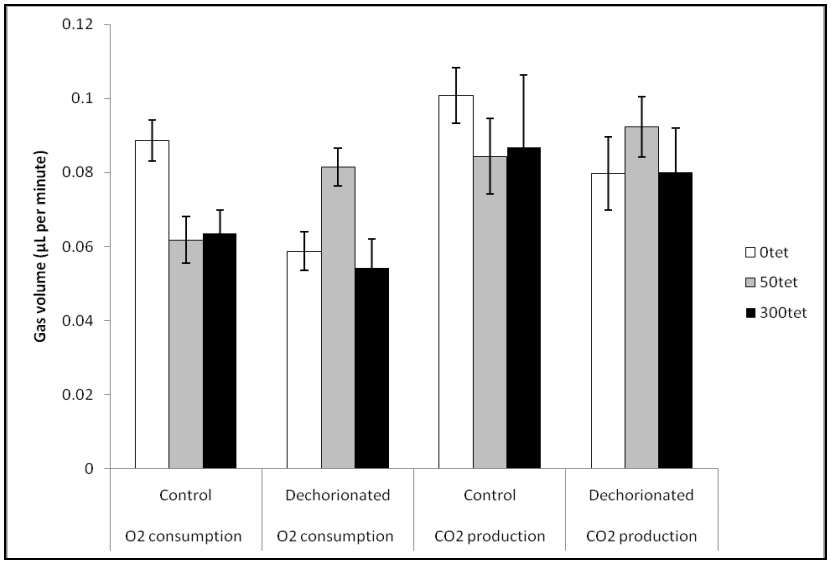
The graphs in Figure: 3.6 suggests that oxygen consumption was significantly decreased with chlortetracycline treatment in comparison to control flies, therefore a separate analysis was conducted with the values gained for chlortetracycline treated and control flies only. An analysis of covariance demonstrate that wing area was not significantly different ($F_{1, 40}$ = 0.442, p>0.05). Chlortetracycline treatment significantly impacted oxygen consumption ($F_{2, 40}$ = 9.157, p<0.01). The same observation was made in male and female flies ($F_{2, 40}$ = 1.054, p>0.05), yet there was a significant difference in oxygen consumption between the 2 sexes ($F_{1, 40}$ = 6.370, p<0.05).

Analysis of carbon dioxide production of male and female flies, showed that wing area did not differ significantly between the treatments ($F_{1, 66}$ = 2.219, p>0.05). Chlortetracycline ($F_{6, 66}$ = 1.040, p>0.05) and dechorionation ($F_{1, 66}$ = 0.816, p>0.05) did not have a significant effect on the carbon dioxide production. Sex also did not have a significant effect on the carbon dioxide production values ($F_{1, 66}$ = 0.097, p>0.05). As with oxygen consumption the interactions of dechorionation*chlortetracycline treatment did also have a significant effect on the carbon dioxide production ($F_{2, 66}$ = 4.244, p<0.05) with dechorionation and treatment with 50 µg ml^{-1} chlortetracycline of female flies having a greater consumption than control flies treated with 50 µg ml^{-1} chlortetracycline. The interactions of sex*chlortetracycline, sex*dechorionation, sex*dechorionation*chlortetracycline did not have a significant value (Figure: 3.6).

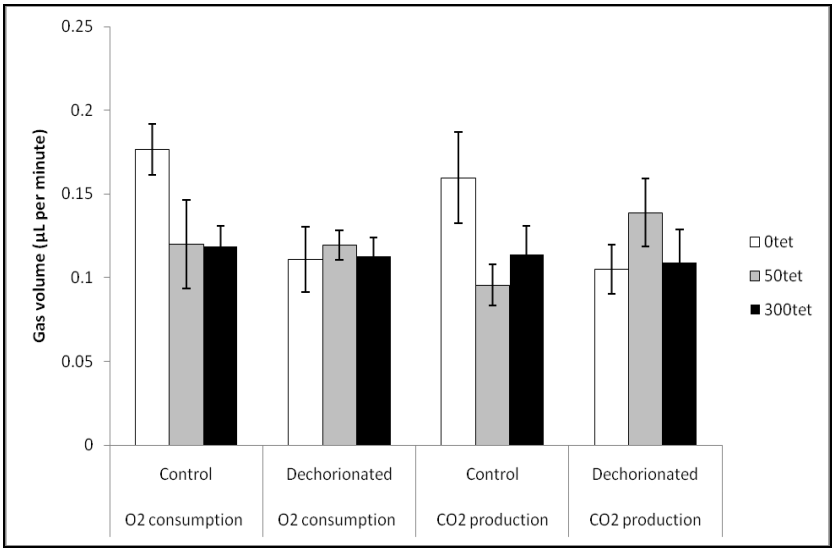
Again the graphs in Figure: 3.6 suggests that carbon dioxide production was significantly decreased with chlortetracycline treatment in comparison to control flies. Once again a separate analysis was conducted with the values gained for chlortetracycline treated and control flies only. ANCOVA statistical analysis demonstrated that wing area was not significantly different ($F_{1, 41}$ = 1.404, p>0.05). Chlortetracycline treatment significantly impacted carbon dioxide production ($F_{2, 41}$ = 5.125, p<0.05). The same observation was observed in male and female flies ($F_{2, 41}$ = 1.269, p>0.05) and there was no significant difference between the 2 sexes ($F_{1, 41}$ = 0.021, p>0.05).

For the RQ data for male and female flies, wing area was not significantly different between the different treatments ($F_{1, 64} = 1.483$, $p>0.05$). Chlortetracycline ($F_{6, 64} = 1.208$, $p>0.05$) and dechorionation ($F_{1, 64} = 2.239$, $p>0.05$) both did not have a significant effect on the RQ values. However, sex did have a significant effect on the RQ values ($F_{1, 64} = 4.587$, $p<0.05$) with females having a lower RQ value than male flies. The interactions of dechorionation*sex, dechorionation*chlortetracycline, sex*chlortetracycline and sex*dechorionation*chlortetracycline all had significant values exceeding the critical value of 0.05 (Figure: 3.7).

a



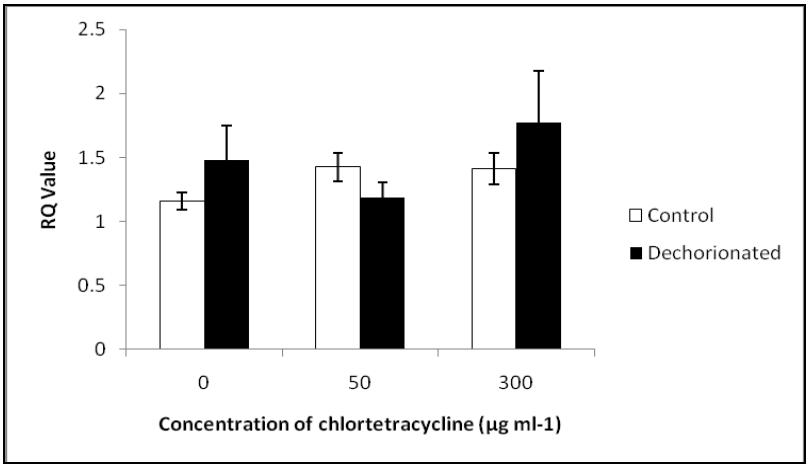
b



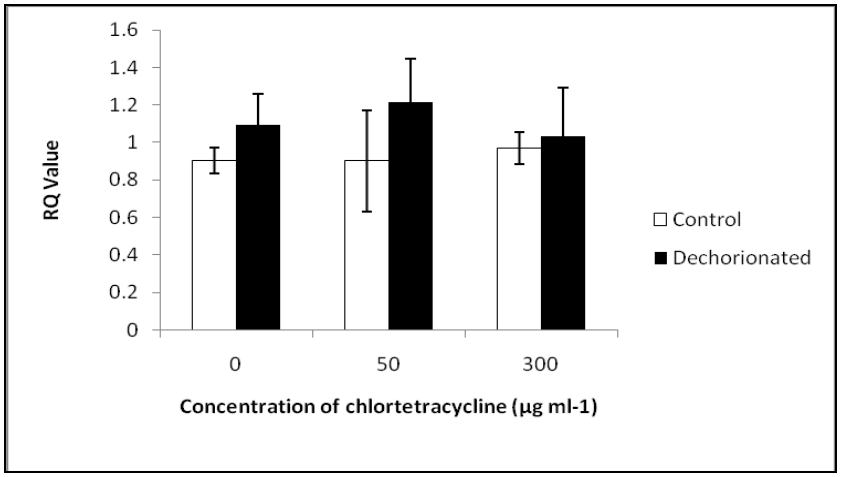
Variable	O_2 Consumption	CO_2 Production
Wing Area	$F_{1, 65} = 0.118$, $p>0.05$	$F_{1, 66} = 2.219$, $p>0.05$
Chlortetracycline	$F_{2, 65} = 2.219$, $p>0.05$	$F_{2, 66} = 1.040$, $p>0.05$
Dechorionation	$F_{1, 65} = 3.894$, $p=0.05$	$F_{1, 66} = 0.816$, $p>0.05$
Sex	$F_{1, 65} = 4.240$, $p<0.05$	$F_{1, 66} = 0.097$, $p>0.05$
Dechorionation*Chlortetracycline	$F_{2, 65} = 3.724$, $p<0.05$	$F_{2, 66} = 4.244$, $p<0.05$
Sex*Chlortetracycline	$F_{2, 65} = 0.347$, $p>0.05$	$F_{2, 66} = 0.139$, $p>0.05$
Sex*Dechorionation	$F_{1, 65} = 0.490$, $p>0.05$	$F_{1, 66} = 0.032$, $p>0.05$
Sex*Dechorionation*Chlortetracycline	$F_{2, 65} = 0.286$, $p>0.05$	$F_{2, 66} = 1.364$, $p>0.05$

Figure: 3.6. O_2 consumption and CO_2 production of male (a) and female (b) flies treated with chlortetracycline and dechorionation with ANCOVA analysis. Number of replicates = Control male flies: 10 0tet, 8 50tet, 11 300tet; Male flies derived from dechorionated eggs: 6 0tet, 7 50tet, 3 300tet; Control female flies: 5 0tet, 3 50tet, 9 300tet; Female flies derived from dechorionated eggs: 5 0tet, 4 50tet, 3 300tet. Methods Chapter; Section: 2.6, page 48.

a



b



Wing Area	$F_{1,64}$ = 1.483, p> 0.05
Dechorionation	$F_{1,64}$ = 2.239 , p>0.05
Chlortetracycline	$F_{2,64}$ = 1.208 , p>0.05
Sex	$F_{1,64}$ = 4.587, p< 0.05
Dechorionation*Sex	$F_{1,64}$ = 0.012 , p>0.05
Dechorionation*Chlortetracycline	$F_{2,64}$ = 1.281 , p>0.05
Sex* Chlortetracycline	$F_{2,64}$ = 0.529 , p>0.05
Dechorionation*Sex*Chlortetracycline	$F_{2,64}$ = 1.259 , p>0.05

Figure: 3.7. RQ values of male (a) and female (b) flies treated with chlortetracycline and dechorionation with ANOVA analysis and ANOVA statistical analysis. Number of replicates = Control male flies: 10 0tet, 8 50tet, 11 300tet; Male flies derived from dechorionated eggs: 6 0tet, 7 50tet, 3 300tet; Control female flies: 5 0tet, 3 50tet, 9 300tet; Female flies derived from dechorionated eggs: 5 0tet, 4 50tet, 3 300tet. Methods Chapter; Section: 2.6, page 48.

3.7 The impacts of chlortetracycline and egg dechorionation on Canton-S reared on a high and low nutrient diet

The survival to pupae with flies reared on the high nutrient diet supplemented with 0-500 µg ml^{-1} of chlortetracycline ranged from 84-72%, with the survival to pupae not significantly affected by the treatment with chlortetracycline (Kruskal-Wallis: $H_6 = 5.854$, $p>0.05$) (Figure: 3.8). However, the survival to adulthood with flies reared on the high nutrient diet supplemented with 0-500 µg ml^{-1} ranged from 7-57%. The lowest survival was at the high concentrations of chlortetracycline of 300-500 µg ml^{-1} with a survival of 7-8%. This result suggests that the mortality occurred at the pupal stage as the survival to pupae was 85-86% at 300-500 µg ml^{-1} compared to 7-8% survival to adulthood. Therefore, 78-79% mortality had occurred at the pupal stage. This difference with chlortetracycline treatment on the survival to adulthood was shown to be significantly different (Kruskal-Wallis: $H_6 = 38.204$, $p<0.001$).

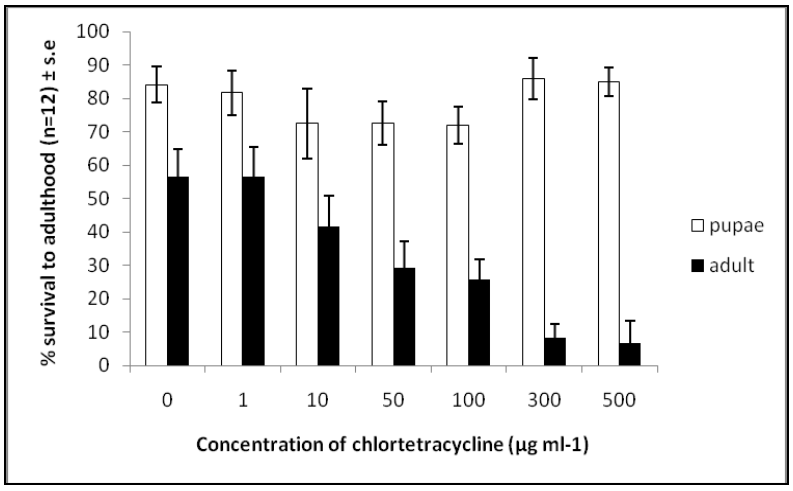


Figure: 3.8. Percent survival from egg of Canton-S reared on the high nutrient diet supplemented with 0-500 µg ml^{-1} of chlortetracycline, number of replicates = 12 with 10 eggs per replicate (Methods Chapter; Section: 2.3.2.1, page 40).

A different response was observed with flies reared on the low nutrient diet compared with those on the high nutrient diet. The survival of the flies to pupae on a diet supplemented with 0-500 µg ml^{-1} of chlortetracycline varied from 83% to 55%, with a significantly lower survival at 100 and 300 µg ml^{-1} of chlortetracycline of 55% and 56%, respectively compared with 75% with control flies (Kruskal-Wallis: $H_6 = 16.138$, $p<0.05$) (Figure: 3.9). The survival to adulthood ranged from 51-81%, a greater survival compared

with the high nutrient diet but still with a significantly lower survival at 100-500 µg ml^{-1} chlortetracycline of 48-52% compared with control flies with a 71% survival (Kruskal-Wallis: $H_6 = 23.043$, $p<0.01$).

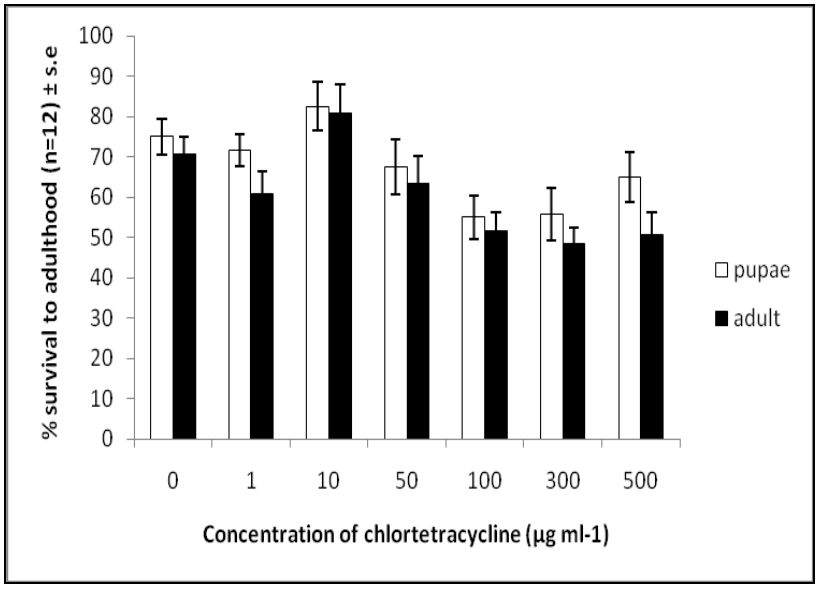


Figure: 3.9. Percent survival from egg of Canton-S reared on the low nutrient diet supplemented with 0-500 µg ml^{-1} of chlortetracycline, number of replicates = 12 with 10 eggs per replicate (Methods Chapter; Section: 2.3.2.1, page 40).

Alongside the experiment with chlortetracycline treatment, bacteria depletion was also conducted via egg dechorionation. The survival to pupae and adulthood of these flies was compared with control flies on the high and low nutrient diets. The survival to pupae of control flies reared on the high nutrient diet was higher than flies derived from dechorionated eggs of 84% and 69% respectively, yet this difference was not significant (Mann Whitney U: $Z_1 = -1.267$, $p>0.05$). The survival to adulthood was lower for both treatments, with a 57% and 39% survival for control and dechorionation respectively. Again, there was no significant difference between the two treatments (Mann Whitney U: $Z_1 = -1.334$, $p>0.05$) was observed (Figure: 3.10).

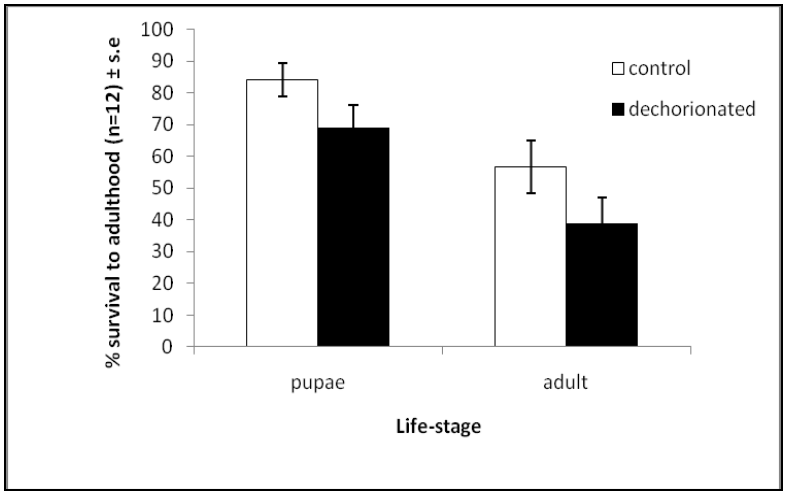


Figure: 3.10. The percent survival of control and flies derived from egg dechorionation on the high nutrient diet, number of replicates = 12 with 10 eggs per replicate (Methods Chapter; Section: 2.3.2.1, page 40).

Flies reared on the low nutrient diet had a lower survival to pupae compared with the high nutrient diet but did have a higher survival to adulthood. The survival to pupae of control flies and with dechorionation was 75% and 64% respectively, this difference was found not to be significantly different (Mann Whitney U: Z_1 = -1.445, p>0.05) (Figure: 3.11). The survival of the flies to adulthood was 71% for control flies and 66% with dechorionation. Again, statistical analysis showed that there was no significant difference between the survival of control flies and with egg dechorionation (Mann Whitney U: Z_1 = -0.874, p>0.05) (Figure: 3.11).

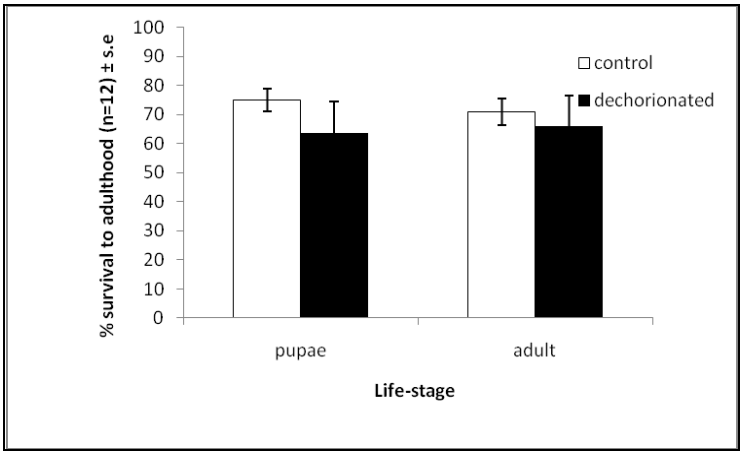


Figure: 3.11. The percent survival of control and flies derived from egg dechorionation on the low nutrient diet, number of replicates = 12 with 10 eggs per replicate (Methods Chapter; Section: 2.3.2.1, page 40).

3.8 Impact of chlortetracycline on *Drosophila* microbiota

3.8.1 Impact of chlortetracycline on *Drosophila* microbiota (Oregon-RS)

Antibiotic treatment was shown to have an effect on the development of *Drosophila.* To determine whether this was in fact due to the removal of bacteria in the flies, larvae were sampled from each treatment and plated onto nutrient agar plates.

This experiment showed a clear difference in bacterial content in the larvae treated with 50-500 µg ml^{-1} chlortetracycline. Even though there were still bacteria present on these plates they were present at a lower number (Table: 3.16). Larvae treated at lower concentration of chlortetracycline had a greater number of bacteria present across the entire agar plate with the number of colony forming units ml^{-1} of homogenate ranging from 26 to 1632 (X^2_6 =6393.36, p<0.05)(Table: 3.16).

Table: 3.16. The Colony Forming Units (CFUs) of the culturable bacteria found in 3rd instar larvae reared on 0-500 µg ml^{-1} chlortetracycline with Chi^2 analysis (Methods Chapter; Section: 2.4.1, page 43).

Concentration of chlortetracycline (µg ml^{-1})	Number of colony forming units per 3rd instar larva (1-2 replicates per treatment).
0	490
1	26; 424
10	1632
50	0; 8
100	1; 4
300	3; 4
500	0; 11

X^2_6 =6393.36, p<0.05

To determine the culturable species of bacteria present in Oregon-RS, 16S rRNA gene sequence analysis was conducted on 10 sampled colonies. BLAST analysis showed that there were several sequences matching the sequence of the 16S rRNA gene of four main bacterial species. These species were *Lactobacillus plantarum, Lactobacillus pentosus, Acetobacter pasteurianus* and *Acetobacter pomorum* (Table: 3.17).

Table: 3.17. The bacterial identification of colonies sampled from Oregon-RS (with number of sequences and the % sequence identity) (Methods Chapter; Section: 2.8.2, page 49).

Species Name	NCBI Accession Number
Lactobacillus plantarum strain HDRS1 16S ribosomal RNA gene, partial sequence (99%) **(5 forward sequences)**	DQ141558.2
Lactobacillus plantarum 16S rRNA gene, clone 6C4 (98-99%) **(5 reverse sequences)**	AM157432.1
Lactobacillus plantarum strain ZDY128 16S ribosomal RNA gene, partial sequence (99%)/ *Lactobacillus pentosus* gene for 16S rRNA, partial sequence, strain: NRIC 1837 (99%) **(1 sequence)**	EU559599.1/AB362758.1
Acetobacter pasteurianus gene for 16S ribosomal RNA, complete sequence, strain: SKU1108 (96-99%) **(4 forward/reverse sequences)**	AB499842.1
Acetobacter pomorum strain EW816 16S ribosomal RNA gene, partial sequence (96-99%) **(3 forward/reverse sequences)**	EU096229.1

3.8.2 Identification of *Wolbachia* in Oregon-R and Canton-S

Tetracycline is often used to remove *Wolbachia* in laboratory-reared *Drosophila* (Fry and Rand, 2002). To identify whether this bacterium was present in the 2 strains of *Drosophila* (Oregon-RS and Canton S), a diagnostic PCR was conducted.

The results suggest Oregon-RS and Canton-S stains do not have *Wolbachia*, this is shown by the absence of a band at ~600 base pairs (Figure: 3.12). The Isogenic (ISO) strain was known to be infected with *Wolbachia* and was used as a positive control.

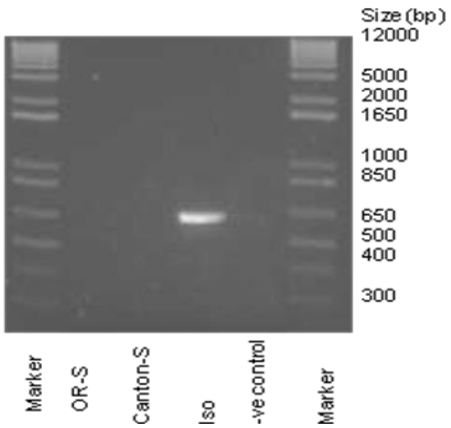


Figure: 3.12. *Wolbachia* detection using PCR, water was used as a negative control and ISO (Isogenic) line as the positive control (band at ~600 bp). Size detection using 1kb plus DNA ladder (Invitrogen). 100ng of DNA added (Methods Chapter; Section: 2.8.3, page 51).

3.8.3 Impact of chlortetracycline on *Drosophila* (Canton S) microbiota

Female adult (7 day old) *Drosophila* were sampled, these flies were homogenised and plated onto nutrient agar. This experiment showed a clear difference in bacterial content in the female *Drosophila* treated with 50 and 300 µg ml^{-1} of chlortetracycline (Table: 3.18). A one-way ANOVA showed that the depletion of bacteria was significant in the presence of chlortetracycline ($F_{2, 29}$ = 53.005, $p<0.001$). Bacteria were also present on plates with 50 µg ml^{-1} of chlortetracycline suggesting that a number of bacteria may be chlortetracycline resistant. No culturable bacteria were found in the flies derived from dechorionated eggs however, 16S rRNA gene analysis suggests that unculturable bacteria were still present in the bacteria depleted flies (presence of a band at 1.5 kb) (Figure: 3.13). Furthermore, secondary bands were present which have not been seen before in previous PCR reactions, this suggests that the higher prevelance of secondary bands with samples of dechorionation/chlortetracycline treatment may have a low number of DNA copies in comparison with control flies. An alteration in the annealing temperature should improve the PCR reaction.

Table: 3.18. The culturable content of control females and females derived from egg dechorionation treated with and without chlortetracycline (0-300 µg ml^{-1}) on nutrient agar plates supplemented with and without of 50 µg ml^{-1} of chlortetracycline, number of replicates = 10 (Methods Chapter; Section: 2.4.1, page 43).

	Log_{10} (CFUs) per fly on non-chlortetracycline plates ± s.e (n=10)		Log_{10} (CFUs) per fly on plates supplemented with 50 µg ml^{-1} of chlortetracycline ± s.e (n=10)	
Concentration of chlortetracycline (µg ml^{-1})	Control	Dechorionated	Control	Dechorionated
0	4.326 ± 0.308	0	2.395 ± 0.426	0
50	2.195 ± 0.304	0	0.749 ± 0.339	0
300	0.349 ± 0.193	0	0	0

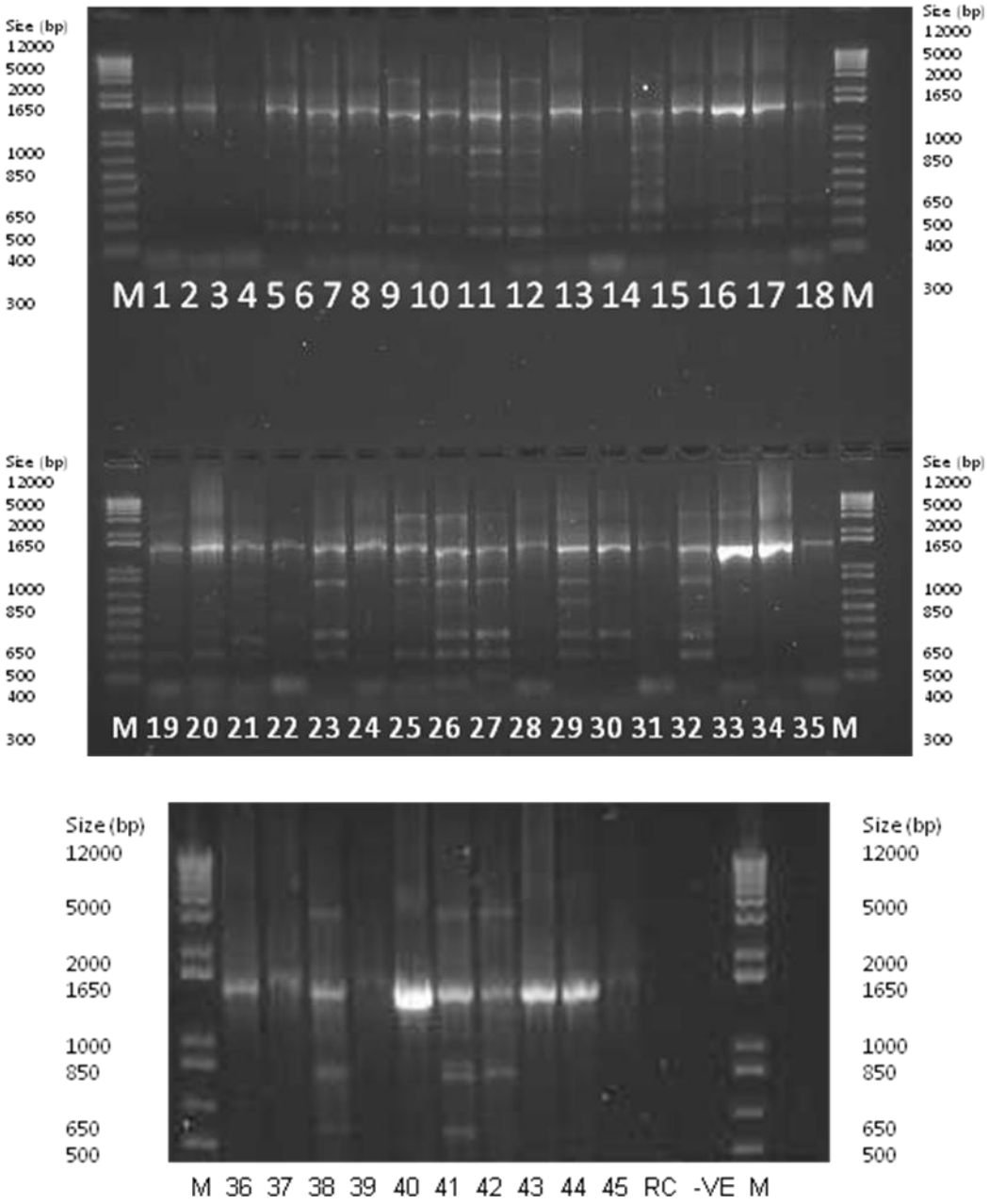


Figure: 3.13: Bacterial 16s rRNA gene analysis using PCR. Separation conditions 1.5% agarose gel, 1 X TAE using a separation voltage of 100 and a PCR product of 1.5 kb. Size detection using 1kb plus DNA ladder (Invitrogen). M = ladder; 1-5, 19-23, 36-37 = egg dechorionation; 6-10, 24-28 = egg dechorionation with 50 µg ml^{-1} of chlortetracycline; 11-15, 29-33, 38-39 = egg dechorionation with 300 µg ml^{-1} of chlortetracycline; 16, 33, 40, 43 = positive controls (untreated); 17, 34, 41, 44 = 50 µg ml^{-1} of chlortetracycline; 18, 35, 42, 45 = 300 µg ml^{-1} of chlortetracycline; RC = reagent control; -ve = negative control (Methods Chapter; Section: 2.8, page 49).

3.8.4 Impact of chlortetracycline on *Drosophila* (Canton S) microbiota diversity

The culturable bacterial colonies reared from flies treated with 0 and 50 μg ml^{-1} of chlortetracycline were identified using 16S rRNA gene analysis (Appendix: Table: 7.1, 7.2). The culturable bacteria in flies reared with no chlortetracycline showed to have a population predominantly *Acetobacter pasteurianus,* with *Acetobacter cerevisiae* and *Acetobacter pomorum* also being identified. Interestingly, the bacterial colonies identified in flies reared on food supplemented with 50 μg ml^{-1} of chlortetracycline had a more diverse population with *Acetobacter pasteurianus, Acetobacter malorum, Acetobacter pomorum, Lactobacillus plantarum, Lactobacillus brevis* and *Micrococcus luteus*. To determine whether the bacterial colonies were chlortetracycline resistant, colonies were reared on nutrient agar plates supplemented with 50 μg ml^{-1} of chlortetracycline (Appendix: Table: 7.3). The bacterial colonies identified in flies reared with no chlortetracycline included *Acetobacter pasteurianus, Acetobacter malorum, Acetobacter pomorum, Lactobacillus plantarum* and *Lactobacillus pentosis.* Similar bacterial species were also identified in flies reared with 50 μg ml^{-1} chlortetracycline with the only bacteria which wasn't identified being *Lactobacillus pentosis*.

The food used to rear the flies was also sampled to determine whether the bacterial diversity within the food was similar to the bacterial community within the fly (Appendix: Table: 7.1, 7.2). The bacterial colonies identified in food with no chlortetracycline included *Acetobacter pasteurianus, Acetobacter cerevisiae, Acetobacter pomorum* and *Lactobacillus plantarum*. Again, similar species were identified on the diet supplemented with 50 μg ml^{-1} of chlortetracycline but *Acetobacter cerevisiae* was not identified.

The 454 analysis showed that *Acetobacter cerevisiae* and *Acetobacter pasteurianus/Acetobacter pomorum* were the dominant symbionts in both control and chlortetracycline treated flies (Appendix: Table 7.4, 7.5; Table: 3.19, Table: 3.20). Further species were identified in control flies; yet these have not been previously identified in *Drosophila* and appear to be contaminants. An example being *Buchnera aphidicola* which is a symbiont of the pea aphid.

Table: 3.19. 454 pyrosequencing analysis of the bacterial species in control flies. The species identified with sequences with greater than 100 hits (Methods Chapter; Section: 2.9, page 51).

Accession number	Bacterial name	Percent Identity	Number of hits
CP001161	*Buchnera aphidicola* str. 5A (*Acyrthosiphon pisum*), complete genome	100	10909
HM080051.1	Uncultured Actinomycetales bacterium clone E153F02 16S ribosomal RNA gene, partial sequence	100	2717
NR_025512.1	*Acetobacter cerevisiae* strain LMG 1625 16S ribosomal RNA, partial sequence >gi|23892796|emb|AJ419843.1| *Acetobacter cerevisiae* 16S rRNA gene, strain LMG 1625	100	2020
GQ477828.1	Uncultured bacterium clone MS-123 16S ribosomal RNA gene, partial sequence	99.6	2020
HM027569.1	*Bacillus subtilis* strain zj2008 16S ribosomal RNA gene, partial sequence	100	1715
AM087199.1	*Asticcacaulis benevestitus* partial 16S rRNA gene, type strain Z-0023T	99.6	312
AB461807.1	*Acinetobacter* sp. M522 gene for 16S rRNA, partial sequence, strain: M522	100	212
EU096229.1 AB308058.1	*Acetobacter pomorum* strain EW816 16S ribosomal RNA gene, partial sequence *Acetobacter pasteurianus* gene for 16S ribosomal RNA, complete sequence	100 100	186
AJ318114.1	Uncultured gamma proteobacterium 16S rRNA gene, clone BIci4	99.6	171

Table: 3.20. 454 pyrosequencing analysis of bacterial species in chlortetracycline treated flies. The species identified with sequences with greater than 100 hits, number of replicates = 12 with 10 eggs per replicate (Methods Chapter; Section: 2.9, page 51).

Accession number	Bacterial name	Percent Identity	Number of hits
NR_025512.1	*Acetobacter cerevisiae* strain LMG 1625 16S ribosomal RNA, partial sequence >gi|23892796|emb|AJ419843.1| *Acetobacter cerevisiae* 16S rRNA gene, strain LMG 1625	100	29544
EU096229.1 AB308058.1	*Acetobacter pomorum* strain EW816 16S ribosomal RNA gene, partial sequence *Acetobacter pasteurianus* gene for 16S ribosomal RNA, complete sequence	100 100	314 314

3.9 Discussion

The results in this chapter do show the importance in commensal bacteria in *Drosophila melanogaster* but further highlights the implications of using antibiotics as a method of removing bacteria. The different methods of bacterial depletion through chlortetracycline (an antibiotic) treatment and dechorionation (surface sterilisation of the egg) have enabled a comparison of the results gained through the 2 different treatments and to distinguish between the effect of toxicity and bacterial depletion.

The major impact of chlortetracycline treatment and dechorionation was the extension of larval development time in both strains of *Drosophila* which was determined by examining the hatch rates of the treated eggs. As the same result was observed by egg dechorionation and antibiotic treatment and bacterial numbers were significantly reduced with chlortetracycline, the extension does appear to be the result of bacterial depletion rather than deleterious effects through the toxicity of chlortetracycline. One explanation is that bacterial depletion results in flies taking longer to reach the critical weight to allow for pupation which may involve changes in the insulin/insulin-like growth factor signalling which has previously been suggested to control the time to pupation and to reach the critical weight for pupation (Edgar, 2006; Beadle *et* al., 1938; Bakker, 1959; Robertson, 1963; Moed *et al*., 1999). Therefore, microbial symbionts may play a critical role in the regulation of this pathway. Changes in ecdysone levels have also been demonstrated to play a significant role in the regulation of the insulin/insulin like growth factor signalling; therefore the microbial symbionts could also regulate the levels of these hormones (Colombani *et al*., 2005). The third reason for the extension could be due to the changes in behaviour of the larvae on the treated diets. As described in Section 3.2.3, larvae on treated diet did not appear to penetrate the diet and the diet had a more solid consistency compared with control diets. Therefore, the larvae may have found it more difficult to feed and as a consequence taken longer to grow, however, the experiment using different concentrations of agar does appear to contradict this theory.

Chlortetracycline did reduce the fecundity of female flies which could be due to two possibilities; 1) the female flies were not as fit when reared on diet with chlortetracycline and had a reduced reproductive capacity and 2) the flies were deterred from laying eggs on a diet containing chlortetracycline. Both reasons are plausible, in Canton-S the

triglyceride content was reduced with chlortetracycline treatment; lipid content of females has been shown to play an important role in reproductive maturation of female animals (Chehab *et al*., 1997; Kennedy and Mitra, 1963) therefore there could be a relationship between these results. Secondly, female flies may detect chemical in the food and did not lay on the food as the fitness of the offspring may be compromised.

The male life-span results were unexpected and contradicted the results gained by Brummel *et al* (2004) and Mair *et al* (2005) but supported the results gained by Ren *et al* (2007). Control flies had a shorter life-span compared with flies with egg dechorionation and chlortetracycline treatment; yet egg dechorionation alone did not significantly affect the life-span.

This extension of lifespan through chlortetracycline treatment could be due to the removal of "pathogenic" bacteria but not all the bacteria allowing the fly to live longer. Further extension in longevity with chlortetracycline treatment and dechorionation could be the result of a combination factors including the removal of bacteria and the chemical itself. This result was interesting as Mair *et al* (2005) did not find a significant effect of tetracycline treatment on life-span. However, this difference could be accounted for as a different strain of *Drosophila* and diet was used. As Cooper *et al* (2004) suggested, the shortening of life-span of control flies on a rich diet could be due to the proliferation of bacteria within the gut, in antibiotic treated flies the tetracycline would control this proliferation resulting in an enhancement in life-span.

Nutrition analysis demonstrated that female flies had a greater response to chlortetracycline treatment and dechorionation due to reasons such as differences in metabolism of male and female animals and the requirement for females to lay eggs and produce offspring. In both sexes an alteration in carbohydrate levels with chlortetracycline treatment and dechorionation suggest that the bacteria consume some of the glucose within the gut of the host. Bacterial depletion will therefore increase the levels of available carbohydrate being absorbed through the gut and resulting in a greater pool of carbohydrate as an energy source.

Oregon-RS and Canton-S female flies had a reduced protein content with chlortetracycline treatment but not with dechorionation (Canton-S only), suggesting that

chlortetracycline (a protein synthesis inhibitor in prokaryotes) could be targeting eukaryotic ribosomes, inhibiting protein synthesis and leading to a reduction in levels of protein. The reduction in triglyceride levels of both sexes with chlortetracycline treatment suggests that the toxicity of the antibiotic was responsible for the change and not bacteria depletion. Two reasons for this could be; 1) chlortetracycline may be targeting lipid metabolism and 2) the flies consumed less food on the diet supplemented with chlortetracycline meaning lower calories were consumed leading to a reduced pool of triglycerides.

Respirometry data showed a significant reduction in respiration rates of flies with bacteria depletion. This result has also been observed in mice and rats (Wostman *et al*., 1982 and Levenson *et al*., 1969). This change could be due to multiple reasons: the removal of bacteria could result in differences in the utilisation of nutrients resulting in a decrease in oxygen consumption (Wostman *et al*., 1982), changes in the morphology of the gut due to the absence of bacteria which have been shown to play a role in gut development (Wostmann *et al*., 1982, Shirkey *et al.,*2006) and in mice and rats it has been suggested that bacteria influence hormones such as nor-epinphrine (octopamine in insects) which may be linked to an increase in oxygen consumption (Levenson *et al*., 1969).

The experiments conducted with the high and low nutrient diets with chlortetracycline and dechorionation treatment have further demonstrated that experimental results do depend on the diet used. What is particularly interesting is that survival is significantly reduced when the flies were treated with high concentrations of chlortetracycline (100-500 µg ml^{-1}) on both diets, with the high nutrient diet showing the greatest decrease in survival. The results with the high nutrient diet does suggest that the chlortetracycline treatment was toxic affecting the survival to adulthood and causing pupal mortality which was greater than observed with the low nutrient diet. This difference between the 2 diets suggests that there may be differences in feeding rates. If this is the case, the flies reared on a high nutrient diet may have consumed more food leading to a greater exposure to and consumption of chlortetracycline.

The experiments with flies derived from dechorionation did not show a significant decrease in survival, therefore, the changes in survival with the chlortetracycline treated flies does again suggest a link with toxicity and not with the removal of bacteria.

The microbial diversity gained through the sequences from culturable bacteria suggested that chlortetracycline treatment may deplete the population of *Acetobacter* allowing the population of *Lactobacillus* to increase. 454 pyrosequencing showed that *Acetobacter* was the dominant species in control flies; this bacterium has previously been found in *Drosophila melanogaster* by Corby-Harris *et al* (2007) and Ren *et al* (2007).

3.10 Conclusion

Experiments with *Drosophila melanogaster* have highlighted the impact of chlortetracycline and egg dechorionation on the insect and have demonstrated that bacterial depletion had a significant impact on the carbohydrate levels in flies, respiration rates and the development time to pupae/adulthood. This does suggest that the microbes play an important role in nutrient acquisition and metabolism of nutrients to allow for growth and development. Lastly, this study has highlighted the deleterious impact of using antibiotics to deplete bacteria which should be considered when studying the role of gut microbes in animal hosts.

Chapter 4: The impact of bacterial depletion on *Drosophila melanogaster* gene expression

4.1 Introduction

The bacterial symbionts in *Drosophila melanogaster* have previously been suggested to be found on the surface of the egg shell (Bakula, 1969). Larvae gain the symbionts through the ingestion of the food where the eggs are laid and the consumption of the chorion of the egg (Bakula, 1969 and observation by myself).

Experiments have demonstrated that these symbionts are important for the performance of *Drosophila melanogaster*, such as life-span enhancement (Brummel *et al*., 2004) and shortening of development time (as demonstrated in Chapter3, Bakula, 1969).

Drosophila melanogaster has become a model for investigating the innate immune response (Hoffman, 2003). *Drosophila* lacks an adaptive immune response and depends on the innate response which includes the use of physical barriers, antimicrobial peptides, hemocytes and reactive oxidative species for protection against pathogens (Hoffman, 2003; Lemaitre and Hoffman, 2007). Two main pathways are involved in the humoral response to microorganisms, the Toll pathway which responds to fungi and Gram-positive bacteria (Hoffman, 2003; Rutschmann *et al.,* 2002) and IMD pathway which mainly responds to Gram-negative bacteria (Hoffman, 2003; Ferrandon *et al*., 2007) (Figure: 4.1).

This activation of the TOLL pathway involves the proteolytic cleavage of Spaetzle (Morisato and Anderson, 1994; Schneider *et al*., 1994; Valanne *et al*., 2011) which binds as a dimer to the Toll ectodomain (Hoffman, 2003; Arnot *et al*., 2010). The intracytoplasmic TIR domain of Toll interacts with three partners, MyD88, Tube and Pelle (Horng and Medzhitov, 2001; Tauszig-Delamasure *et al*., 2002; Sun *et al*., 2002; Xiao *et al*., 1999; Moncrieffe *et al*., 2008; Valanne *et* al., 2011). The Toll pathway then activates the Dorsal-related immunity factor (DIF)/Dorsal which dissociates from the ankyrin-repeat inhibitory protein Cactus through signal-dependent phosphorylation and degradation of Cactus (Wu and Anderson, 1998; Hoffman, 2003; Valanne *et* al., 2011). The activated Dorsal/DIF then directs the expression of antimicrobial peptides in the nucleus (Valanne *et* al., 2011; Hoffman, 2003).

The IMD pathway is activated by Gram-negative bacteria which is detected by PGRP-LC and involves a signalling cascade of IMD, FADD, DREDD and TAK1 (TGFβ-activated kinase) (Gottar *et al*., 2002; Hoffman, 2003). TAK1 activates the IKK-γ and IKK-β complex which phosphorylates and cleaves Relish (Lu *et al*., 2001; Silverman *et al*., 2003; Vidal *et al*., 2003; Ferrandon *et al*., 2007). DREDD (Death-related ced-3/Nedd-2 like protein) and FADD (Fas-associated death domain) can also associate with Relish and cleave Relish (Leulier *et al*., 2002; Naitza *et al.,* 2002; Hoffman, 2003; Leulier *et al*., 2000; Ferrandon *et al*., 2007). The activated Relish can then promote the expression of the antimicrobial peptides (Hoffman, 2003; Ferrandon *et al*., 2007). Furthermore, TAK1 can activate the expression of cytoskeletal proteins in the nucleus (Boutros *et* al., 2002; Hoffman, 2003; Ferrandon *et al*., 2007).

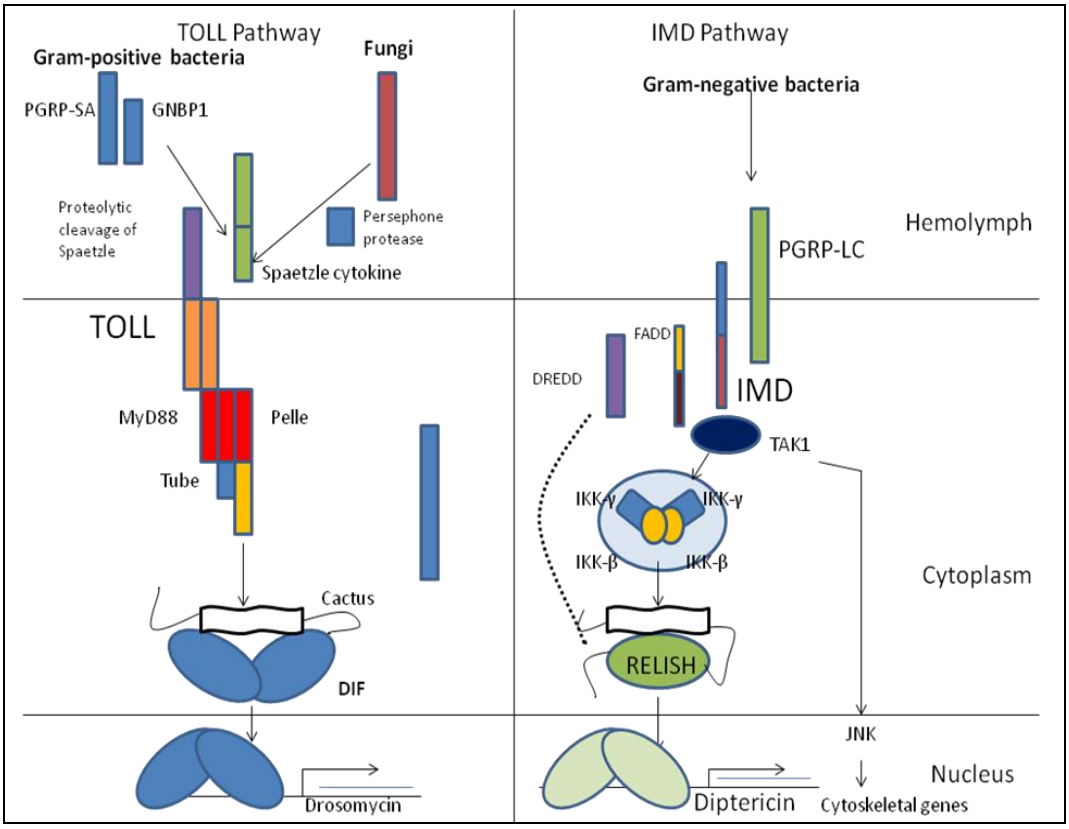


Figure: 4.1. The Toll and IMD pathways, which are activated by Gram-positive, Gram-negative bacteria and fungi. These pathways are induced by peptidoglycan recognition proteins (Hoffman, 2003), the activation of these pathways results in the expression of antimicrobial peptides including Drosomycin (Toll pathway); Diptericin, Cecropins, Attacins and Drosocin (IMD pathway) (Hoffman, 2003). Figure adapted from Hoffman (2003).

A limited number of studies investigating the changes of gene expression of bacteria depleted flies have been performed; studies have mainly concentrated on the response of *Drosophila* immune system to pathogens. Ren *et al* (2007) determined the changes in AMP (antimicrobial peptide) expression in axenic flies. In this paper, qRT-PCR and northern blots were used to assay the gene expression. The expression of AMP genes were found to be reduced in axenic flies, these AMPs included Diptericin, Defensin, Cecropin and Attacin (Ren *et al*., 2007). The possible reason for this response was the reduced bacterial load in axenic flies which reduced the stimulation of immune response.

A genome-wide study has not been conducted to explore the alteration in the transcript levels in bacteria-depleted flies when derived from dechorionated eggs and how this response differs between different diets. Therefore, in this chapter I describe the genome-wide response of flies derived from egg dechorionation and how the response differs on a high and low nutrient diet using microarray analysis with GeneSpring®.

4.2 Microarray study on bacteria-depleted *Drosophila*

4.2.1 The impact of egg dechorionation and diet on the gene expression of *Drosophila*

A genome wide analysis using Agilent microarray chip was performed with control flies and flies derived from dechorionated eggs on a high and low nutrient diet. The analysis involved the comparisons between; dechorionation and control flies on a high nutrient diet; dechorionation and control flies on a low nutrient diet; control flies on the 2 diets and dechorionation on the 2 diets. The analysis has shown that 88-89% of the genes assessed using the microarray had an expression value 2 times greater than the negative controls, indicating that these genes were expressed (Table: 4.1). When the comparisons between the treatments were made, less than one percent of the genes assessed in the microarray were significantly changed in abundance (Table: 4.2). The results have indicated that fewer transcripts (42) were significantly changed in abundance with dechorionation on a low nutrient diet in comparison with dechorionation on the high nutrient diet (136 transcripts). These results demonstrate a difference in response to the 2 diets, which is supported by the significant differences in the abundance of transcripts during the comparison of control flies (89 transcripts) and with dechorionation (212 transcripts) on the 2 diets.

Table: 4.1. The number of sequences expressed in the different treatments.

Treatment	Average number of sequences expressed (n=3) (Expression = 2X negative controls) ± s.e (% sequences expressed)
Egg dechorionation on the rich diet	39017 ± 51 (89%)
Egg dechorionation on the poor diet	38794 ± 327 (89%)
Control on the rich diet	38716 ± 216 (89%)
Control on the poor diet	38512 ± 195 (88%)

Table: 4.2. The number of transcripts with a significant change in abundance of 2 fold or more ($p<0.05$).

Comparison	Sequences significantly expressed of 2 fold or more with $p<0.05$ (change of >3 fold)
High nutrient dechorionation versus high nutrient control	136 (36) (up=47 and down=89)
Low nutrient dechorionation versus low nutrient control	42(15) (up=13 and down=29)
Low nutrient control versus high nutrient control	89(19) (up=45 and down=44)
Low nutrient dechorionation versus high nutrient dechorionation	212 (37) (up=140 and down=72)

An analysis was conducted on the number of transcripts which had been identified to be significantly altered in abundance and which were shared by the treatments, dechorionation on the high nutrient diet (High), dechorionation on the low nutrient diet (Low), control flies on the 2 diets (Controls) and dechorionation samples on the 2 diets (Dechorion). The Venn diagram (Figure: 4.2) has highlighted that with dechorionation on the high diet, a high proportion of transcripts significantly altered in abundance were only found within this treatment; 71 down-regulated and 44 up-regulated. Eleven of the total transcripts (1 up-regulated and 10 down-regulated) were shared with dechorionation on the low nutrient diet, 7 (5 down-regulated and 2 up-regulated) with the control flies on the 2 diets and 1 down-regulated with dechorionation on the 2 diets.

Low nutrient diet with dechorionation had a lower number of transcripts (22; 13 down-regulated and 9 up-regulated) which were significantly altered and only found with this treatment. In comparison, dechorionation on a high nutrient diet (104), control flies on the 2 diets (62) and dechorionation on the 2 diets (182) all had a greater number of transcripts that were only found within each treatment. No transcripts were shared between the dechorionation on the low nutrient diet and the comparison of control (control) flies on the 2 diets and only 8 (5 down-regulated and 3 up-regulated) with dechorionation on the 2 diets. The comparisons between the 2 diets with control flies and flies derived from egg dechorionation only had 19 (7 down-regulated and 12 up-regulated) which were shared between the 2 treatments. No transcripts were shared across the treatments.

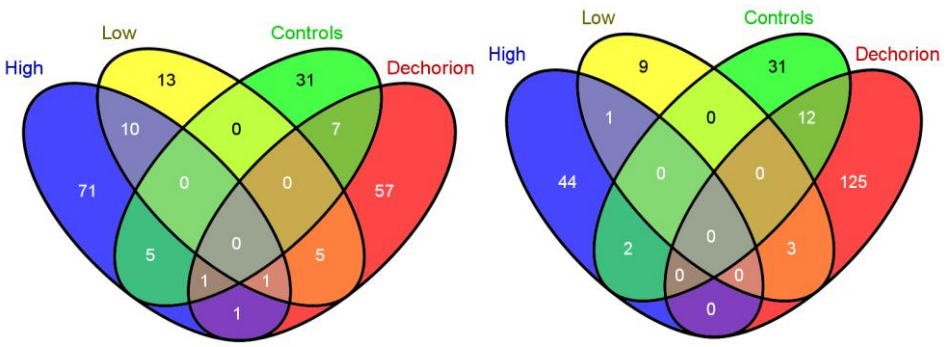


Figure: 4.2. a) Venn diagram showing the comparison of transcripts with a significant decrease in abundance in the treatments: dechorionation versus control on the high nutrient diet (High), dechorionation versus control on the low nutrient diet (Low), control flies on the 2 diets (Controls) and dechorionation samples on the 2 diets (Dechorion) b) Transcripts with a significant increase in abundance. Overlapping numbers show transcripts shared by the treatments and the numbers which are not overlapping are found only in that particular treatment. The Venn diagram was created using an online source produced by Oliveros (2007).

4.2.2 Analysis of the function of the transcripts with a significant change in abundance

Across all comparisons, 40-54% of the sequences with a significant change in abundance had no assigned Gene Ontology term/number. This was due to little experimental evidence to identify the function of the gene or that the gene was a short sequence such as an expression sequence tag or a tentative consensus sequence associated with a gene with no Gene Ontology number. However, 46-60% of the sequences were identified to have a Gene Ontology term with a function which had been experimentally demonstrated or that Blast2Go had suggested that the sequence has similar sequence identity to genes with known functions. Dechorionation on the high nutrient diet (Figure: 4.3a) did show that the majority of the transcripts assigned a function were metabolic (23%) with transcripts associated with the immune system being the second most prominent (12%). A lower number of metabolic transcripts were altered in abundance on the low nutrient diet; with a total of 12%, the same percent as the immune transcripts. The greatest number of transcripts was associated with binding and transport (22%) (Figure: 4.3b). Both the low and high nutrient diets with dechorionation, had the identical proportion of transcripts associated with immunity. For the comparison of control flies reared on the

high and low nutrient diets, no immune transcripts were identified to have a significant change in abundance. More than a quarter of the sequences were associated with metabolism (28%), and 17% with transport and binding (Figure: 4.3c). This was also observed with the dechorionation with the 2 different diets, where the majority of the sequences were metabolic (25%) and binding/transport transcripts (15%). Immune transcripts were also identified to have a significant change in abundance (6%), which was not observed when the control flies were compared (Figure: 4.3d).

In comparison with the major functions of metabolism, immunity and binding/transport, a smaller percentage of the transcripts had miscellaneous and DNA/RNA replication/transcription functions which were identified across all comparisons (0-8%) (Figure: 4.3).

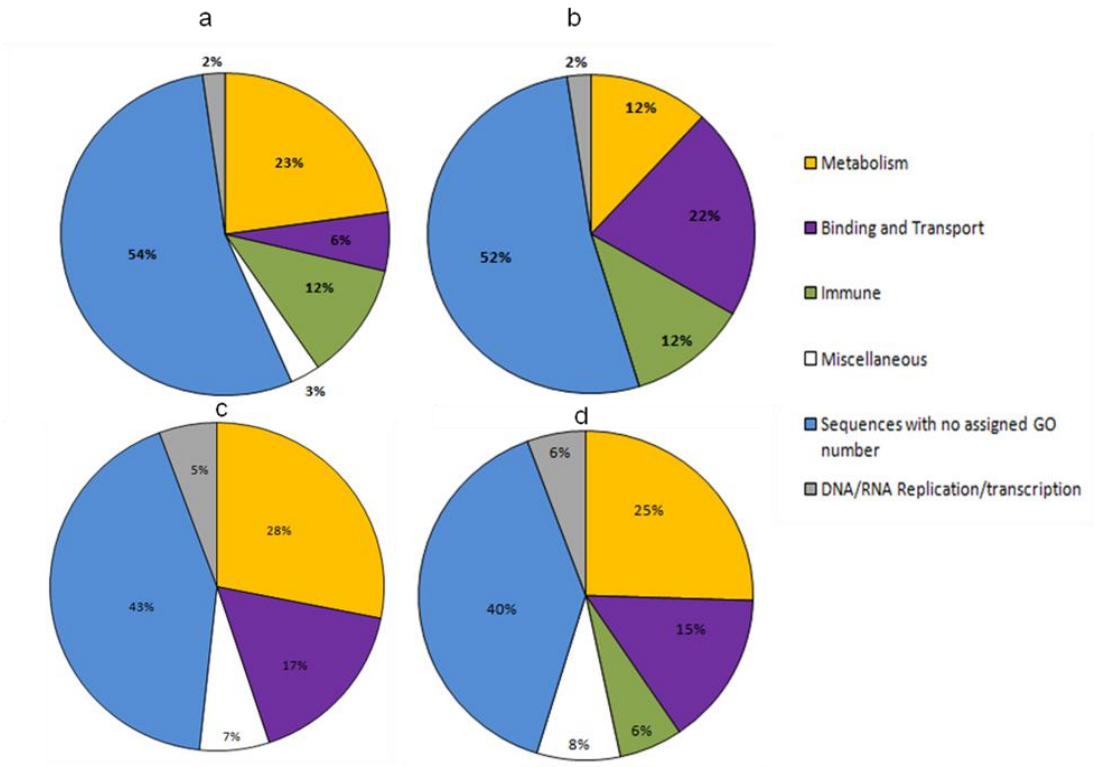


Figure: 4.3. The proportion of the transcripts with a significant change in abundance of 2 fold or more with a Gene Ontology of metabolism (GO:0008152), transport (GO:0006810) and binding (GO:0005488), immunity (GO:0006955), DNA/RNA replication (GO:0006260)/transcription (GO:0009299), miscellaneous and unknown (a = dechorionation versus comventional on the high nutrient diet, b = dechorionation versus control on the low nutrient diet, c = control flies on the high and low nutrient diet, d = dechorionation on the high and low nutrient diet.

4.2.3 Microarray analysis of transcripts associated with *Drosophila melanogaster* immunity

This analysis has demonstrated that dechorionation significantly changes transcripts associated with the immune response (Table: 4.3; Appendix, Table: 7.6, 7.13, and 7.25). The sequences that were significantly changed in abundance on the low nutrient diet were also significantly changed on the high nutrient diet. However, the level of change and the number of transcripts altered in abundance associated with the immune system differed between the 2 diets. On the low nutrient diet, only 5 immune related sequences were identified to have a significant alteration in expression, on the high nutrient diet 16 sequences were identified (Table: 4.3; Appendix, Table: 7.13, 7.6). Furthermore, the transcript with the greatest change in abundance differed with diet. On the low nutrient diet, the greatest change was observed with Diptericin (33.96 fold change) and on the high nutrient, Cecropin C (51.84 fold change). The changes in both of these diets shown in Table: 4.3 suggest that the major response to bacterial depletion is the decrease in the expression of antimicrobial peptides (Diptericin, Attacin C, Cecropin C, Attacin A, Defensin), antifungal genes (Toll pathway) and peptidoglycan recognition proteins. These antimicrobial peptides are associated with the IMD pathway and target gram-negative bacteria (Hoffman, 2003). The remaining sequences were associated with phagocytosis and defence against bacteria.

The comparison between the control flies on the different diets did not show a significant difference in the abundance of immune associated sequences. However, dechorionation on the low and high nutrient diets did (Table: 4.3; Appendix, Table: 7.25). The absolute fold change in abundance (5.4 to 2.0) of the immune transcripts was not as high as the comparison of dechorionation versus control flies (51.8-2.1). Thirteen sequences were identified to have a significant change in abundance of 2 fold or more, with the greatest change observed with the down regulation of a lysozyme precursor (5.38 fold change), and the antimicrobial peptides Diptericin and Defensin (4.96 and 3.86 fold change, respectively). A further 8 transcripts were significantly increased, these included functions of phagocytosis and melanization.

Table: 4.3 Transcripts associated with immunity and significantly changed in abundance of 3 fold or more (NSC=no significant change, SC=significant change, - =down regulated).

				High nutrient diet	Low nutrient diet	Low V high nutrient diet	Low V high nutrient diet
				Dechori-onation V Control	Dechor-ionation V Control	Control flies	Dechori-onation
Sequence No.	Sequence Name	Gene Ontology (GO) Numbers	GO Description	Fold change	Fold change	Fold change	Fold change
CG1373	Cecropin c	GO:0050832 GO:0042742 GO:0005576 GO:0005615 GO:0019731 GO:0050829 GO:0050830	Defense response to fungus Defense response to bacterium Extracellular region Extracellular space Antibacterial humoral response Defense response to Gram-negative bacterium Defense response to Gram-positive bacterium	-51.84	NSC	NSC	NSC
CG8175	Metchnikowin	GO:0019731 GO:0019732 GO:0006952 GO:0050832 GO:0050829 GO:0050830 GO:0005576	Antibacterial humoral response Antifungal humoral response Defense response Defense response to fungus Defense response to Gram-negative bacterium Defense response to Gram-positive bacterium Extracellular region	-36.11	-15.02	NSC	NSC
CG10146	Attacin A	GO:0005615 GO:0019731 GO:0050829	Extracellular space Antibacterial humoral response Defense response to Gram-negative bacterium	-15.85	NSC	NSC	NSC
CG10794	Diptericin b	GO:0019731 GO:0005576	Antibacterial humoral response Extracellular region	-14.94	-13.24	NSC	NSC
BT023384	Defensin	GO:0005615 GO:0019731 GO:0050830 GO:0042742 GO:0006965	Extracellular space Antibacterial humoral response Defense response to Gram-positive bacterium Defense response to bacterium Positive regulation of biosynthetic process of antibacterial peptides active against Gram-positive bacteria	-10.64	NSC	NSC	-3.86-2.428
CG12763	Diptericin	GO:0019731 GO:0050829 GO:0042742 GO:0045087 GO:0005576	Antibacterial humoral response Defense response to Gram-negative bacterium Defense response to bacterium Innate immune response Extracellular region	-10.24	-33.96	NSC	-4.96
CG4740	Attacin C	GO:0019731 GO:0006952 GO:0005615 GO:0042742 GO:0005576	Antibacterial humoral response Defense response Extracellular space Defense response to bacterium Extracellular region	-8.29	-5.67	NSC	NSC
CG15678	Poor imd response upon knock-in	GO:0009609 GO:0005515 GO:0005102 GO:0050777 GO:0045824 GO:0061060	Response to symbiotic bacterium Protein binding Receptor binding Negative regulation of immune response Negative regulation of innate immune response Negative regulation of peptidoglycan recognition protein signaling pathway	-4.43	NSC	NSC	NSC
CG16876	Nimrod c4	GO:0043277 GO:0006911	Apoptotic cell clearance Phagocytosis engulfment	-3.75	NSC	NSC	NSC

		GO:0005886	Plasma membrane				
CG9681	Peptidoglycan recognition protein sb1	GO:0006952 GO:0005576 GO:0008745 GO:0005887 GO:0009253 GO:0042834 GO:0005875 GO:0006955	Defense response Extracellular region N-acetylmuramoyl-L-alanine amidase activity Integral to plasma membrane Peptidoglycan catabolic process Peptidoglycan binding Microtubule associated complex Immune response	-3.34	SC down <-3 fold	NSC	NSC
CG31783	Neither inactivation nor afterpotential d	GO:0006952 GO:0007602 GO:0007603 GO:0007604 GO:0016063 GO:0007155 GO:0005887 GO:0046867 GO:0005044 GO:0016020	Defense response Phototransduction Phototransduction, visible light Phototransduction, UV Rhodopsin biosynthetic process Cell adhesion Integral to plasma membrane carotenoid transport Scavenger receptor activity Membrane	-3.04	NSC	NSC	SC up <3 fold
CG9120	Lysozyme precursor	GO:0005576 GO:0004568 GO:0006952 GO:0016998 GO:0019730 GO:0003796	Extracellular region Chitinase activity Defense response Cell wall macromolecule catabolic process Antimicrobial humoral response Lysozyme activity	NSC	NSC	NSC	-5.38
CG7002	Hemolectin	GO:0042803 GO:0042381 GO:0007599 GO:0035006 GO:0042060 GO:0005576 GO:0008061 GO:0005529 GO:0007155 GO:0006030	Protein homodimerization activity Hemolymph coagulation Hemostasis Melanization defense response Wound healing Extracellular region Chitin binding Sugar binding Cell adhesion Chitin metabolic process	NSC	NSC	NSC	3.08, 2.43, 2.34

4.2.4 Microarray analysis of transcripts associated with *Drosophila melanogaster* metabolism

In total, 5 sequences associated with metabolism were significantly altered in abundance with dechorionation on the low nutrient diet and only one sequence (Obstructor-G) having a 3 or greater fold change (Table: 4.4), this sequence was up-regulated. The sequences with a significant change were associated with a range of functions including proteolysis, cholesterol transport, oxidation reduction and glutathione metabolism (Table: 4.4; Appendix, Table: 7.15) which were all down-regulated except for cholesterol transport.

With dechorionation on the high nutrient diet, 31 metabolic transcripts were significantly changed in abundance at 2 fold or more, 8 of which had a fold change greater than 3 (Table: 4.4; Appendix, Table: 7.8). The metabolic transcripts were predominantly down-regulated, 26 out of 31 sequences. The sequences with a significant change in abundance were associated with proteolysis, carbohydrate metabolism, glutathione biosynthesis/metabolism, glutathione peroxidase activity, lipid/fatty acid metabolism, chitin metabolism and oxidation/reduction. All of which were down-regulated with dechorionation. The gene with the greatest change in abundance was glutamate-cysteine ligase catalytic subunit, where a 4.5 fold decrease was observed. Glutamate-cysteine ligase catalytic subunit was also down-regulated with dechorionation on the low nutrient diet.

For the comparison of the control flies reared on the 2 diets, 25 sequences with functions associated with metabolism were observed to have a significant change in abundance of 2 fold or more (Appendix, Table: 7.20; Table: 4.4). Out of the 25 sequences, 7 had a fold change in abundance of 3 or more. The majority of the transcripts that were significantly changed in abundance had functions associated with having nutrient reservoir activity, chitin metabolism and serine proteolysis. The transcripts associated with nutrient reservoir and storage activity had the greatest change in abundance (29.11-5.44 fold change), and were down-regulated in flies reared on the low nutrient diet. Chitin metabolism was also down-regulated by 7.6-2 fold and serine endopeptidases were down-regulated by 2 fold. Further transcripts were identified to be up-regulated in flies on a low nutrient diet. The gene, phosphoenolpyruvate carboxykinase was increased by

3.3 fold in flies on a low nutrient diet, suggesting the use of gluconeogenesis during energy metabolism.

Flies derived from egg dechorionation on the high and low nutrient diet had 54 metabolic transcripts with significant changes in abundance (Appendix, Table: 7.27; Table: 4.4). Nine transcripts were shown to have a 3-fold or more change in abundance. Similar to the comparison of the control flies on the 2 diets, the major changes occurred with transcripts associated with chitin metabolism (CG7017) which was down-regulated in both of the comparisons. Furthermore, the nutrient reservoir transcripts (Fat body protein 2, Fat body protein 1, Larval serum protein 1 alpha and Larval serum protein 1 beta) were identified to be down-regulated by 7-3.2 fold. Several more transcripts have been identified to have a significant change in abundance; these transcripts included functions of serine proteolysis, glutathione transferase activity and carboxylesterase activity which were all down-regulated in flies reared on a low nutrient diet. The majority of metabolic transcripts were up-regulated which included; glucuronosyltransferase activity, hexokinase, associations with the tricarboxylic acid cycle, glycerol-3-phosphate dehydrogenase activity, glutamine biosynthesis, glycolysis, citrate and transmembrane transporter actvity and pyruvate dehydrogenase activity. The up-regulation of these transcripts suggests an increase in glycolysis (leading to an increase in the TCA cycle).

Table: 4.4. Metabolic transcripts down or up-regulated by greater than threefold (NSC = no significant change, SC = significant change, - = down regulated).

				High nutrient diet	Low nutrient diet	Low V high nutrient diet	Low V high nutrient diet
				Dechori-onation V Control	Dechor-ionation V Control	Control flies	Dechori-onation
Sequence No.	Sequence Name	Gene Ontology (GO) Numbers	GO Description	Fold change	Fold change	Fold change	Fold change
CG2259	Glutamate-cysteine ligase catalytic subunit	GO:0004357 GO:0005515 GO:0006750 GO:0006749 GO:0006974 GO:0017109 GO:0005634 GO:0048471	Glutamate-cysteine ligase activity Protein binding Glutathione biosynthetic process Glutathione metabolic process Response to DNA damage stimulus Glutamate-cysteine ligase complex Nucleus Perinuclear region of cytoplasm,	-4.47	NSC	NSC	NSC
CA804468	Protein farnesyltransferase alpha subunit	GO:0008318 GO:0018346 GO:0005965	Protein prenyltransferase activity Protein amino acid prenylation Protein farnesyltransferase complex	4.072	NSC	NSC	NSC
CG4500	-	GO:0001676, GO:0007498, GO:0004467	Long-chain fatty acid metabolic process Mesoderm development Long-chain-fatty-acid-CoA ligase activity	-3.99	NSC	NSC	NSC
CG14205	-	GO:0016747	Transferase activity, transferring acyl groups other than amino-acyl groups	-3.61	NSC	NSC	NSC
CG7017	-	GO:0005576 GO:0008061 GO:0006030 GO:0016490	Extracellular region Chitin binding Chitin metabolic process structural constituent of peritrophic membrane	-3.60	NSC	-7.60	-5.15
CG33926	Transposase	GO:0006139 GO:0003677 GO:0034960	Nucleobase, nucleoside Nucleotide and nucleic acid metabolic process DNA binding	-3.38	NSC	NSC	NSC
CG12224	-	GO:0055114 GO:0016491 GO:0008076	Oxidation reduction Oxidoreductase activity Voltage-gated potassium channel complex	-3.30	NSC	NSC	NSC
CG12092-RA	Niemann-pick c1	GO:0007417 GO:0007391 GO:0030299 GO:0008158 GO:0016021 GO:0005886 GO:0007422	Central nervous system development Dorsal closure Intestinal cholesterol absorption Hedgehog receptor activity Integral to membrane Plasma membrane peripheral nervous system development	-3.15	NSC	NSC	NSC
CG9781	obsructor-G	GO:0005576 GO:0008061 GO:0006030 GO:0016490	Extracellular region Chitin binding Chitin metabolic process Structural constituent of peritrophic membrane	NSC	3.32	NSC	NSC
CG17285	Fat body protein isoform a	GO:0005344 GO:0008565 GO:0005811 GO:0015032	Oxygen transporter activity Protein transporter activity Lipid particle Storage protein import into fat body	NSC	NSC	-29.1	NSC
CG3763	Fat body protein 2	GO:0055114 GO:0004022	Oxidation reduction Alcohol dehydrogenase (NAD)	NSC	NSC	-23.5	-4.79 to -6.99

		GO:0045735 GO:0005488 GO:0005811	activity Nutrient reservoir activity Binding Lipid particle				
CG4178	Larval serum protein 1 beta	GO:0005344 GO:0005616 GO:0045735 GO:0005811 GO:0006810	Oxygen transporter activity Larval serum protein complex Nutrient reservoir activity Lipid particle Transport	NSC	NSC	-5.44	-3.62
CG10140	Isoform a	GO:0005576 GO:0008061 GO:0006030	Extracellular region Chitin binding Chitin metabolic process	NSC	NSC	-3.88	NSC
CG17725	Phosphoenolpyruvate carboxykinase	GO:0006094 GO:0005525 GO:0016301 GO:0004613 GO:0005739	Gluconeogenesis GTP binding Kinase activity Phosphoenolpyruvate carboxykinase (GTP) activity Mitochondrion	NSC	NSC	3.31	NSC
CG33467	CG33467	GO:0004672 GO:0006468 GO:0005524	Protein kinase activity Protein amino acid phosphorylation ATP binding	NSC	NSC	-3.27	NSC
CG11012	UDP-glycosyltransferase 37a1	GO:0016758 GO:0008152 GO:0015020	Transferase activity, transferring hexosyl groups Metabolic process Glucuronosyltransferase activity	NSC	NSC	NSC	3.55
CG4757	-	GO:0016787 GO:0004091	Hydrolase activity Carboxylesterase activity	NSC	NSC	NSC	-3.38
CG9244	Aconitase-isoform b	GO:0005811 GO:0006099 GO:0051539 GO:0003994 GO:0005739 GO:0006099	Lipid particle Tricarboxylic acid cycle 4 iron, 4 sulfur cluster binding Aconitate hydratase activity Mitochondrion Tricarboxylic acid cycle	NSC	NSC	NSC	3.06
CG17285	Fat body protein isoform a	GO:0005344 GO:0008565 GO:0005811 GO:0015032	Oxygen transporter activity Protein transporter activity Lipid particle Storage protein import into fat body	NSC	NSC	NSC	-7.91
CG2559	Larval serum protein 1 alpha	GO:0005344 GO:0005616 GO:0045735 GO:0005811 GO:0006810 GO:0005576	Oxygen transporter activity Larval serum protein complex Nutrient reservoir activity Lipid particle Transport extracellular region	NSC	NSC	NSC	-3.18
TC213959	NADPH--cytochrome P450 reductase	GO:0009384 GO:0001640 GO:0005515 GO:0004396 GO:0001642 GO:0008237 GO:0008270 GO:0004089 GO:0005516 GO:0030165 GO:0004652 GO:0008761 GO:0042803 GO:0004617 GO:0042169 GO:0016595 GO:003042 GO:000561 GO:0005737 GO:0043025 GO:0042734 GO:0032279 GO:0043679 GO:0048786 GO:0005791 GO:0005829 GO:0005624 GO:0045202	N-acylmannosamine kinase activity Adenylate cyclase inhibiting metabotropic glutamate receptor activity Protein binding Hexokinase activity Group III metabotropic glutamate receptor activity Metallopeptidase activity Zinc ion binding Carbonate dehydratase activity Calmodulin binding PDZ domain binding Polynucleotide adenylyltransferase activity UDP-N-acetylglucosamine 2-epimerase activity Protein homodimerization activity Phosphoglycerate dehydrogenase activity SH2 domain binding Glutamate binding- Dendrite- Extracellular space- Cytoplasm- Cell soma- Asymmetric synapse- Presynaptic active zone	NSC	NSC	NSC	3.16

		GO:0030424 GO:0005634 GO:0043195 GO:0043198 GO:0043234 GO:0005794 GO:0006096 GO:0046380 GO:0007155 GO:0006508 GO:0007196 GO:0007611 GO:0014050 GO:0006054	Rough endoplasmic reticulum Cytosol Membrane fraction Synapse Axon- Nucleus Terminal button Dendritic shaft Protein complex Golgi apparatus Glycolysis N-acetylneuraminate biosynthetic process- Cell adhesion Proteolysis Metabotropic glutamate receptor, adenylate cyclase inhibiting pathway Learning and/or memory Negative regulation of glutamate secretion N-acetylneuraminate metabolic process				

4.2.5 Microarray analysis of transcripts associated with transport and binding in *Drosophila melanogaster*

With dechorionation on the low nutrient diet, 9 had a significant change in abundance with 2 having a change of 3 fold or greater (Appendix, Table: 7.14; Table: 4.5). The significant changes were observed with transcripts associated with odorant binding, RNA/Nucleic acid binding and ion binding. With dechorionation on the high nutrient diet, 8 transcripts also had a significant change in expression of 2 fold and above, these included: protein and metal ion binding (Appendix, Table: 7.7; Table: 4.5). Only 1 of the 8 transcripts had a change in abundance which was 3 fold or greater, Metallothionein A was down-regulated by 3.1 fold.

As with dechorionation on the low nutrient diet, the comparison between the control flies on the high and low nutrient diet demonstrated a difference in abundance of transcripts associated with odorant binding and ion transport and binding (Appendix, Table: 7.19; Table: 4.5). Fifteen transcripts were established to have a significant change in abundance, with only one transcript having a fold change 3 fold or greater. The transcript with the greatest fold change that was significantly up-regulated by 4.2 fold was associated with zinc ion binding. The analysis of the flies derived from egg dechorionation on the 2 diets demonstrated a greater response, with 32 transcripts with a significant change in abundance (Appendix, Table: 7.26; Table: 4.5). The majority of transcripts were down-regulated by 3-2 fold and involved in pheromone/odorant binding.

Table: 4.5. Transport and binding transcripts down or up-regulated by greater than threefold (NSC = no significant change, SC=significant change, - = down regulated).

				High nutrient diet	Low nutrient diet	Low V high nutrient diet	Low V high nutrient diet
				Dechori-onation V Control	Dechor-ionation V Control	Control flies	Dechori-onation
Sequence Name	Sequence Name	Gene Ontology (GO) Numbers	GO Description	Fold change	Fold change	Fold change	Fold change
CG9470	Metallothionein A	GO:0046872	Metal ion binding	-3.10	NSC	NSC	NSC
NM_001 015210	zinc c3hc4 type (ring finger) domain protein	GO:0003676 GO:0008270 GO:0005515	Nucleic acid binding Zinc ion binding protein binding	NSC	-3.26	4.20	NSC
CG11123	mgc69156 protein	GO:0003723	RNA binding	NSC	3.09	NSC	NSC
CG11123	Mgc69156 protein	GO:0003723	RNA binding	NSC	NSC	NSC	4.08
Obp59a	Odorant-binding protein 59a	GO:0006810 GO:0007606 GO:0005549	Transport Sensory perception of chemical stimulus Odorant binding	NSC	NSC	NSC	-3.27
CG4950	Carboxypeptidase n subunit 2	GO:0005515	Protein binding	SC down <3 fold	NSC	NSC	3.24
CG4139	Karl	GO:0005488	Binding	SC down <3 fold	NSC	NSC	3.11
CG6642	Antennal protein 10	GO:0005549 GO:0005550 GO:0007606	Odorant binding Pheromone binding Sensory perception of chemical stimulus	NSC	NSC	NSC	-2.92 to -3.19
CG32975	Nicotinic Acetylcholine Receptor α 34E	GO:0042166 GO:0004889 GO:0016021 GO:0005892 GO:0004889 GO:0004889 GO:0006811 GO:0045211	Acetylcholine binding Nicotinic acetylcholine-activated cation-selective channel activity Integral to membrane Nicotinic acetylcholine-gated receptor-channel complex Nicotinic acetylcholine-activated cation-selective channel activity Nicotinic acetylcholine-activated cation-selective channel activity Ion transport Postsynaptic membrane	NSC	NSC	NSC	3.13

4.2.6 Microarray analysis of transcripts with miscellaneous and DNA/RNA replication or transcription functions in *Drosophila melanogaster*

Eight sequences with miscellaneous functions had a significant change in abundance on the high nutrient diet with none being identified on the low nutrient diet (Appendix; Table: 7.10, Table: 4.6). Out of the 8 sequences, only 1 had a fold change greater than 3. Two of the transcripts were associated with puparial adhesion and were down regulated by 2-7 fold, the remaining transcripts had associations with structural properties, signalling and hemopoiesis.

Six transcripts had a significant change in abundance with control flies reared on the different diets (Appendix, Table: 7.22; Table: 4.6). Four transcripts had a change in abundance of 3-fold or more. Two transcripts associated with the structural constituent of chitin/cuticle had a 9.1-4.6 fold change in abundance, and were down-regulated in flies on a low nutrient diet. Regulators of growth were up-regulated by 3 fold in flies reared on the low nutrient, suggesting differences in the growth rates of the flies reared on the different diets.

Seventeen transcripts were identified to have a significant change in abundance when comparing the flies derived from egg dechorionation on the high and low nutrient diet (Appendix, Table: 7.29; Table: 4.6). The up-regulated transcripts were associated with growth regulation, signalling, cell proliferation and hormone activity. The change in expression was less than 3 fold for all the transcripts except TC215525, which was down-regulated and has a function associated with the structure of chitin and, CG14669 and CG33519 with signalling activities which were up-regulated by 3.4-5 fold.

On the low nutrient diet with dechorionation, only 1 transcript (CG5303) was identified to have a major function involved in DNA/RNA replication or transcription this was upregulated by 2 fold (Appendix, Table: 7.16). Four transcripts were identified to have a significant change in abundance with dechorionation on the high nutrient diet and only one transcript (Cubitus interruptus) with a fold change greater than 3 (Appendix, Table: 7.9; Table: 4.7). With comparison of control flies on the high and low nutrient diet, 5 transcripts had a significant change in abundance; none of these transcripts had a fold change greater than 3 (Appendix, Table: 7.21; Table: 4.7). The comparison of the flies derived from dechorionation on the low and high nutrient diets had a greater number of

transcripts with a significant change in abundance, with 12 transcripts and only 2 with a fold change greater than 3 (CG10110-RA and TC219369) (Appendix, Table: 7.28; Table: 4.7).

Table: 4.6. Miscellaneous transcripts down or up-regulated by greater than threefold (NSC = no significant change, SC = significant change, - = down regulated).

				High nutrient diet	Low nutrient diet	Low V high nutrient diet	Low V high nutrient diet
				Dechori-onation V Control	Dechor-ionation V Control	Control flies	Dechor-ionation
Sequence Number	Sequence Name	Gene Ontology (GO) Numbers	GO Description	Fold change	Fold change	Fold change	Fold change
CG18087	Salivary gland secretion 7	GO:0007594 GO:0005576 GO:0005198	Puparial adhesion Extracellular region Structural molecule activity	-7.42	NSC	NSC	NSC
CG8502	Cuticular protein isoform a	GO:0042302	Structural constituent of cuticle Structural constituent of chitin-based larval cuticle	NSC	NSC	-9.11	NSC
CG7539	Ecdysone-dependent gene 91	GO:0008011	Structural constituent of pupal chitin-based cuticle	NSC	NSC	-4.60	NSC
CG11628/ BT030162	Steppke	GO:0040018 GO:0005086 GO:0032012 GO:0005622	Positive regulation of multicellular organism growth ARF guanyl-nucleotide exchange factor activity Regulation of ARF protein signal transduction Intracellular	NSC	NSC	3.16	SC up <3 fold
CO181664	-	GO:0040018	Positive regulation of multicellular organism growth	NSC	NSC	3.06	NSC
TC215525	Odorant receptor 47a	GO:0005214 GO:0008010	Structural constituent of chitin-based cuticle Structural constituent of chitin-based larval cuticle	NSC	NSC	NSC	-4.66
CG14669	CG14669	GO:0003924 GO:0005525 GO:0007264 GO:0016020	GTPase activity GTP binding Small GTPase mediated signal transduction Membrane	NSC	NSC	NSC	3.40
CG33519	Unc-89	GO:0005524 GO:0004674 GO:0005089 GO:0006468 GO:0035023 GO:0005622 GO:0007527 GO:0045214	ATP binding Protein serine/threonine kinase activity Rho guanyl-nucleotide exchange factor activity Protein amino acid phosphorylation Regulation of Rho protein signal transduction Intracellular Adult somatic muscle development Sarcomere organization	NSC	NSC	NSC	5.022

Table: 4.7. Transcripts associated with DNA/RNA replication/transcription down or up-regulated by greater than threefold (NSC = no significant change, SC = significant change, - = down regulated).

				High nutrient diet	Low nutrient diet	Low V high nutrient diet	Low V high nutrient diet
				Dechori-onation V Control	Dechor-ionation V Control	Control flies	Dechori-onation
Sequence No.	Sequence Name	Gene Ontology (GO) Numbers	GO Description	Fold change	Fold change	Fold change	Fold change
TC219369	Reverse transcriptase	GO:0003723 GO:0003964 GO:0006278	RNA binding RNA-directed DNA polymerase activity RNA-dependent DNA replication	NSC	NSC	NSC	-4.06
CG10110-RA	Cleavage and polyadenylation specificity factor cpsf	GO:0006378 GO:0005847 GO:0003730 GO:0005515 GO:0006379	mRNA polyadenylation mRNA cleavage and polyadenylation Specificity factor complex mRNA 3'-UTR binding, Protein binding mRNA cleavage	NSC	NSC	NSC	3.44
CG2125	Cubitus interruptus (ci)	GO:0010843 GO:0005515 GO:0016563 GO:0035017 GO:0048813 GO:0008544 GO:0048592 GO:0035224 GO:0060914 GO:0035217 GO:0048666 GO:0048666 GO:0048477 GO:0030858 GO:0045750 GO:0045944 GO:0007346 GO:0007367 GO:0007224 GO:0035277 GO:0048100 GO:0005737 GO:0035301 GO:0016020 GO:0005634 GO:0043234 GO:0003704 GO:0003700 GO:0016564 GO:0008270 GO:0007350 GO:0000122 GO:0030707 GO:0045449 GO:0007224 GO:0005634	Promoter binding Protein binding Transcription activator activity Cuticle pattern formation Dendrite morphogenesis Epidermis development Eye morphogenesis Genital disc anterior/posterior pattern formation Heart formation Neuron development, Labial disc development Oogenesis Positive regulation of epithelial cell differentiation Positive regulation of S phase of mitotic cell cycle Positive regulation of transcription from RNA polymerase II promoter Regulation of mitotic cell cycle Segment polarity determination Smoothened signaling pathway Spiracle morphogenesis, open tracheal system Wing disc anterior/posterior pattern formation Cytoplasm Hedgehog signaling complex Membrane Nucleus Protein complex Transcription factor activity Transcription repressor activity Zinc ion binding Blastoderm segmentation Negative regulation of transcription from RNA polymerase II promoter Ovarian follicle cell development Regulation of transcription Smoothened signaling pathway Nucleus	-3.81	NSC	NSC	NSC

4.2.7 Microarray analysis of transcripts with no assigned Gene Ontology number

Overall, 69 sequences were identified to have a significant change in abundance with dechorionation on the high nutrient diet (Appendix, Table: 7.11, 7.12; Table: 4.8). Fourteen of these transcripts, expression sequence tags and tentative annotative sequences with no assigned Gene Ontology number had a change in expression of 3 fold or greater (Appendix, Table: 7.11, 7.12; Table: 4.8). The transcript with the greatest change in expression was CG32185 with a very greatest fold change of 118.8. On the low nutrient diet with dechorionation, 22 sequences with no assigned gene ontology number had a significant change in expression of 2 fold and greater, with 8 having a change in abundance of 3 fold or more (Appendix, Table: 7.17, 7.18; Table: 4.8). As with the high nutrient diet, the greatest change was also observed with CG32185 which was down-regulated by 35 fold. TC218200 and EC265593 were also down-regulated on both diets with dechorionation.

With the comparison of control flies reared on the high and low nutrient diet, 38 sequences with no assigned gene ontology number had a change in expression of 2 fold and greater (Appendix, Table: 7.23, 7.24; Table: 4.8). Seven out of the 38 transcripts had a change in abundance of 3 fold or higher, the greatest change was observed with TC198490. TC198490 was down-regulated by 17-fold in flies reared on the low nutrient diet, and was also down-regulated when the comparison was made between flies derived from egg dechorionation on the 2 diets. A much greater number of sequences were identified with dechorionation on the 2 diets; 84 sequences had a significant change in abundance, 13 with a fold change of 3 or more (Appendix, Table: 7.30, 7.31; Table: 4.8).

Table: 4.8. Sequences with no assigned Gene Ontology number and a fold change of three-fold or greater (NSC = no significant change, SC = significant change, - = down regulated).

		High nutrient diet	Low nutrient diet	Low V high nutrient diet	Low V high nutrient diet
		Dechorionation V Control	Dechorionation V Control	Control flies	Dechorionation
Sequence Number	Sequence Description	Fold change	Fold change	Fold change	Fold change
CG32185	-	-118.89	-35.34	NSC	NSC
EC265593	Expression sequence tag	-40.33	-16.36	NSC	NSC
TC218200	Tentative consensus sequence	-9.01	-11.09	NSC	NSC
CG34143	Ionotropic receptor 10a	7.00	NSC	NSC	NSC
CG18273-RA	-	-4.80	NSC	NSC	NSC
CG33553-RF	-	-4.61	NSC	NSC	NSC
TC212147	Tentative consensus sequence	-4.04	NSC	-3.76	NSC
CG18273	-	3.81	NSC	NSC	NSC
CG16775	-	-3.81	NSC	NSC	NSC
CG31711-RA	-	-3.59	NSC	NSC	NSC
TC213322	Tentative consensus sequence	-3.50	NSC	NSC	NSC
CG41233	-	-3.41	NSC	NSC	NSC
TC217958	Tentative consensus sequence	3.13	NSC	NSC	NSC
CG12998	-	-3.01	NSC	NSC	NSC
TC215502	Tentative consensus sequence	NSC	-5.67	NSC	NSC
TC210124	Tentative consensus sequence	NSC	-5.22	NSC	NSC
TC218367	Tentative consensus sequence	NSC	-4.65	NSC	4.57
TC213314	Tentative consensus sequence	NSC	-4.21	NSC	NSC
CG13445	-	NSC	3.32	SC up <3 Fold	NSC
TC198490	Tentative consensus sequence	NSC	NSC	-17.05	-4.50
CG11370	-	NSC	NSC	-4.50	NSC
TC216174	Tentative consensus sequence	NSC	NSC	3.86	NSC
CG13962	-	NSC	NSC	-4.31	NSC
TC212413	Tentative consensus sequence	NSC	NSC	-3.73	NSC
CG40203	-	NSC	NSC	3.59	NSC
TC217285	Tentative consensus sequence	NSC	NSC	NSC	10.07
TC201533	Tentative consensus sequence	NSC	NSC	NSC	-4.44

TC214613	Tentative consensus sequence	NSC	NSC	NSC	-4.31
TC219844	Tentative consensus sequence	NSC	NSC	NSC	3.85
CG4996	-	NSC	NSC	NSC	3.81
CG34206	-	NSC	NSC	NSC	-3.79
TC212659	Tentative consensus sequence	NSC	NSC	NSC	-3.45
CG17761-RA	-	NSC	NSC	NSC	3.42
AW944513	Expression sequence tag	NSC	NSC	NSC	-3.35
TC216377	Tentative consensus sequence	NSC	NSC	NSC	-3.35
TC201327	Tentative consensus sequence	NSC	NSC	NSC	-3.13

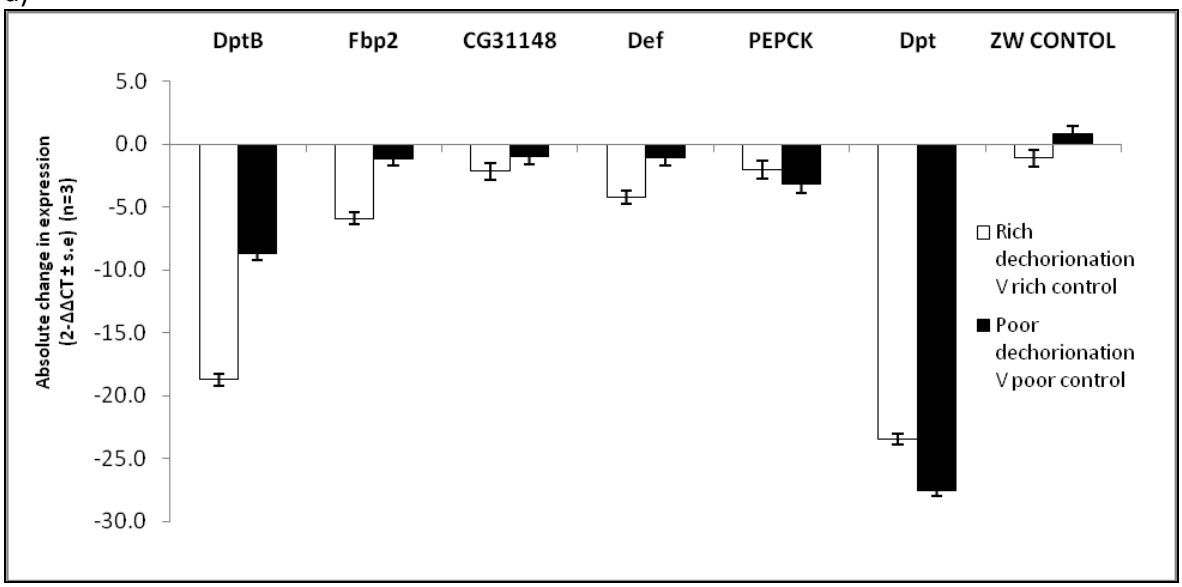
4.3 Quantitative RT-PCR of bacteria-depleted flies on a high and low nutrient diet

Quantitative RT-PCR (qRT-PCR) was conducted to verify the results gained from whole-genome expression profile using microarray analysis. A small group of transcripts were chosen to determine whether the significant difference in abundance of these transcripts was also observed with qRT-PCR.

The qRT-PCR data did support some of the microarray results of flies reared on a high nutrient diet with dechorionation (Figure: 4.4). As with the microarray, qRT-PCR showed that the gene expression of the antimicrobial protein (AMP) Diptericin and Defensin were reduced by 23 and 4 fold, respectively with dechorionation. Furthermore, Diptericin B with an immune-related function was shown to decrease by 18 fold in expression. The gene CG31148, with a role in carbohydrate and lipid metabolism was demonstrated to have a decrease by 2 fold in expression with dechorionation. These were identified to change in expression with dechorionation by both the microarray and qRT-PCR, other transcripts were shown to have a change in abundance with qRT-PCR and not with the microarray. These were Phosphoenol-pyruvate carboxykinase (PEPCK) and Fat body protein 2 with a 2 and 5-fold change, respectively.

Flies reared on the low nutrient diet showed a change in the immune transcripts, Diptericin and Diptericin B with a 23 and 9-fold change in abundance which supports the results gained from the microarray (Figure: 4.4). As with the high nutrient diet, Phosphoenol-pyruvate carboxykinase (PEPCK) was shown to have a 3-fold change in transcript levels unlike the microarray. Low levels of change in expression of Defensin, Fat body protein 2 and CG31148 were observed, but not with a change of greater than 3-fold. An additional control gene, Zwischenferment (involved in glucose-6-phosohate dehydrogenase activity) was quantified with all treatments as the expression of this gene was not significantly altered by 2 fold or more in any of the comparisons during the microarray analysis. The expression of this gene was not significantly altered by 2 fold or more with the high and low nutrient diet when analysed using qRT-PCR (Figure: 4.4).

a)



b)

Gene Name	Gene Number	Up/Down regulation	Microarray Absolute change in expression (n=3)	
			High nutrient diet	Low nutrient diet
			Dechorionation V Control	Dechorionation V Control
Diptericin B (DptB)	CG10794	Down	14.94	7.39-13.24
Fat body protein 2 (FBP2)	CG3763	/	NSC	NSC
CG31148	CG31148	Down	2.62	NSC
Defensin (Def)	BT023384	Down	10.64	NSC
Phosphoenol- pyruvate carboxykinase (PEPCK)	CG17725	Down	NSC	NSC
Diptericin (Dpt)	CG12763	Down	10.24	33.96
Zwischenferment (ZW) Negative Control	CG12529	/	NSC	NSC

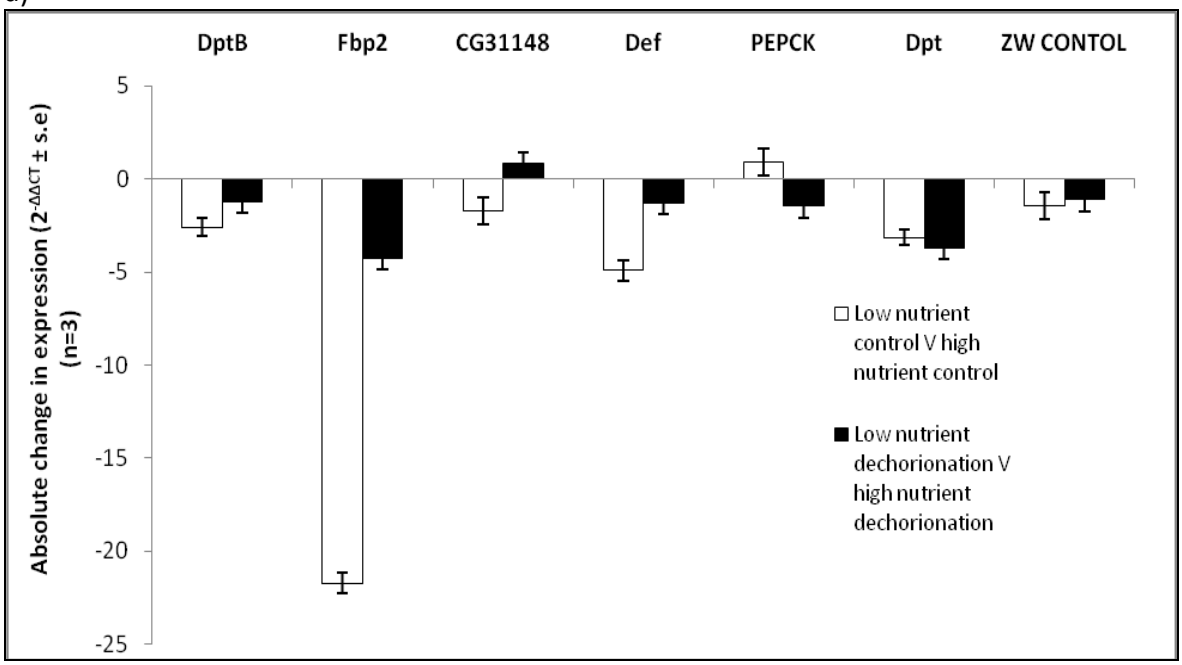
Figure: 4.4. a) qRT-PCR results of DptB = Diptericin B; Fbp2 = Fat body protein 2; CG31148; Def = Defensin; PEPCK = Phosphoenol-pyruvate carboxykinase; Dpt = Diptericin; ZW = Zwischenferment. These were selected for the comparison with the microarray for the treatments of dechorionation on a high and low nutrient diet b) Microarray results for the genes selected for qRT-PCR (NSC = no significant change).

Not only was the impact of dechorionation on the transcript abundance in *Drosophila* analysed but also the impact of diet. Two comparisons were made with the first comparing control flies on the high and low nutrient diets and the second, with the flies derived from dechorionated eggs on the 2 diets.

The qRT-PCR data with control flies reared on the high and low nutrient diet did show some support with the microarray results, however some differences were also observed (Figure: 4.5). As with the microarray, qRT-PCR showed that the gene expression of Fat body protein 2 did decrease in the low nutrient diet, with a 22 fold change. However, Phosphoenol-pyruvate carboxykinase (PEPCK) which had a 3 fold change, was not shown to have a significant change with qRT-PCR. Furthermore, qRT-PCR did show that there was a change in expression of Defensin, Diptericin B and Diptericin with a 5, 3 and 3-fold change, which was not shown by the microarray. Both CG31148 and the control gene, Zw did not show a fold change of 2 or more.

The results for dechorionation with the high and low nutrient diet, again showed a change in transcript abundance of the immune gene, Diptericin by 4 fold and the metabolic gene Fat body protein 2 with a 4 fold change (Figure: 4.5). However, the gene Defensin which was identified by the microarray with a significant change in abundance did not show a change of greater than 2 fold. Diptericin B, CG31148, PEPCK and the control gene Zw were all shown not to have a change in expression of 2 fold or greater by qRT-PCR and the microarray (Figure: 4.5).

a)



b)

Gene Name	Gene Number	Up/Down regulation	Microarray Absolute change in expression (n=3)	
			Control flies	Dechorionation
Diptericin B (DptB)	CG10794	Down	NSC	NSC
Fat body protein 2 (FBP2)	CG3763	Down	23.54	4.79
CG31148	CG31148	/	NSC	NSC
Defensin (Def)	BT023384	Down	NSC	2.43
Phosphoenol- pyruvate carboxykinase (PEPCK)	CG17725	Up	3.31	NSC
Diptericin (Dpt)	CG12763	Down	NSC	4.96
Zwischenferment (ZW) Negative Control	CG12529	/	NSC	NSC

Figure: 4.5. a) qRT-PCR results of DptB = Diptericin B; Fbp2 = Fat Body Protein 2; CG31148; Def = Defensin; PEPCK = Phosphoenol- pyruvate carboxykinase; Dpt = Diptericin; ZW = Zwischenferment. These were selected for the comparison with the microarray for the treatment, controls on a high and low nutrient diet and dechorionation on a high and low nutrient diet b) Microarray results for the genes selected for qRT-PCR (NSC = no significant change).

4.4 Discussion

Unlike many genome-wide studies, the results described in this chapter with the treatment egg dechorionation, had a small number of genes with a significant change in expression. The major observation was the change in abundance of immune transcripts which were decreased in flies derived from dechorionated eggs on both the high and low nutrient diet. This change suggests that the depletion of bacteria at the embryo stage has resulted in a reduction in immune regulation. The presence of bacteria would activate the immune system and suggests that the bacteria contribute to the regulation of the IMD pathway and the Toll pathway. Furthermore, this result suggests that these pathways may be required to control the microbial symbionts found within the gut (Reynolds and Rolff, 2008). This response is supported by Ren *et al* (2007) where a decrease in expression of antimicrobial peptides was observed in axenic flies.

When dechorionation and control comparisons were made with the low nutrient diet, a smaller number of transcripts were identified compared with the high nutrient diet. This suggests that the response to bacterial depletion was not as strong with the low nutrient diet as the response on the high nutrient diet. This demonstrates that diet has an important role in the response of *Drosophila* to treatments and also shows that the difference in the response of *Drosophila* demonstrated by different research labs could be due to the use of different diets.

The greatest change in adundance was observed in transcripts which were associated with immunity and not metabolism, suggesting that the dominant response to bacterial depletion was immune related and not metabolic. The metabolic transcripts where the expression was significantly altered were part of different metabolic pathways and therefore, proved more difficult to discuss compared with the immune transcripts. A decrease in the abundance of transcripts associated with carbohydrate metabolism, lipid metabolism, aminoacyl transferase activity and serine proteases have been shown to be down-regulated with dechorionation on the high nutrient diet. This reduction in metabolism does suggest that the bacteria do play a role in the metabolism in *Drosophila*. These changes may occur as bacteria have been shown to aid nutrient degradation (Hooper, 2009; Savage, 1986) and the reduction in bacteria could result in changes in the quantity of nutrients available, leading to changes in metabolism.

The comparison between the control flies on a high and low nutrient diet showed that the transcripts which had a significant change in expression were mainly metabolic transcripts. One example was the significant reduction in Fat body protein 1 which has a function of transporting proteins into the fat body. The low nutrient diet contained 20 g L^{-1} of yeast but the high nutrient diet contained a much greater content of 80 g L^{-1} of yeast. Therefore, the reduced availability of protein in the low nutrient diet could have resulted in a reduced store of protein and a reduction in the transport of proteins into the fat body. Fat body protein 2 and Larval serum protein 1 beta (both act as a nutrient reservoir) also had a reduced abundance which would be expected on a low nutrient diet as less nutrients would be available to be stored. Furthermore, a gene associated with the process of gluconeogenesis was increased in abundance in flies reared on the low nutrient diet. The limited supply of carbohydrates in a diet such as the low nutrient diet could lead to the gluconeogenesis (a metabolic pathway providing glucose using a non-carbohydrate, such as pyruvate) and is commonly increased in animals which are starved or on a low carbohydrate diet (Berg *et al*., 2002, p425-463 and Westman *et al*., 2007). However, the change in abundance of phosphoenolpyruvate carboxykinase (involved in gluconeogenesis) was not observed with qRT-PCR.

Unlike the control flies, the comparison of dechorionation with a high and low nutrient diet demonstrated a significant difference in the expression of the immune genes including; Defensin and Diptericin. All of which have a reduced transcript abundance with flies reared on a low nutrient diet. This suggests that the immune system has responded greater on a high nutrient diet highlighting the role of diet in the response of *Drosophila*. A quarter of all the transcripts with a significant change in abundance were metabolic transcripts. Genes that were identified with the control flies on the 2 diets were also identified with the flies derived from dechorionation. These included the nutrient reservoir genes (Fat body protein 2 and Larval serum protein 1 beta) which were also identified to be down-regulated by 7-3.2 fold. The transcript levels of several genes associated with the tricarboxylic acid cycle were significantly up-regulated suggesting an increase in glycolysis in flies without symbionts on the low nutrient diet in comparison with the high nutrient diet. This observation was different to the comparison of the control flies on the 2 diets. The transcript level of Phosphoenolpyruvate carboxykinase was significantly changed in control flies using the microarray analysis but was not

significantly altered in abundance using qRT-PCR. Therefore, the changes in glycolysis (all 3 fold or less) with dechorionation on the 2 diets would need to be confirmed using qRT-PCR.

QRT-PCR has been proven to be an important tool to verify the results gained from the microarray. Out of 28 reactions, 21 correlated well with the microarray, however 7 did not. The transcripts with large changes in abundance of greater than 4 fold change, did correlate well with the results gained through qRT-PCR. This would suggest that transcripts with a fold change close to 2 may produce results that did not correspond as well with the results gained through qRT-PCR. As shown by Morey *et al* (2006), transcripts exhibiting at least a 1.4 fold change and a p-value of 0.0001 or less show a strong correlation with microarray data however those with a lower significance should be approached with more caution when verifying microarray results using qRT-PCR. The microarray analysis provides an overall overview of the changes in transcript levels however, to gain more accurate results qRT-PCR would be a preferred method of determining changes in expression.

4.5 Conclusion

The microarray has demonstrated that the major result of removing bacterial symbionts from *Drosophila melanogaster* is the depletion in the immune system, particularly transcripts associated with the Toll/IMD pathway. This result suggests that the Toll/IMD pathway is involved in controlling the symbionts found within the insect gut. Lastly, the microarray with bacterial depletion on a high and low nutrient diet has demonstrated differences in response to different diets.

Chapter 5: Minimal chlortetracycline concentrations with the RIDL® mosquito, LA513 and the impact of chlortetracycline on wild-type *Aedes aegypti*

5.1 Introduction

5.1.1 RIDL® Mosquitoes

As described in Section 1.5, RIDL® has been used with *Aedes aegypti*. LA513A is an example of a RIDL® mosquito (*Aedes aegypti*) that has a late-acting dominant lethal genetic system which causes the death of both male and female mosquitoes at L4-pupal stage (Phuc *et al*., 2007, Section: 1.5). This tetracycline-repressible genetic system requires the insects to be reared with chlortetracycline, which suppresses the expression of the lethal gene and allows rearing of the insects. Oxitec Ltd currently rear LA513A in water supplemented with 30 μg ml^{-1} of chlortetracycline to suppress the expression of tTav.

In this chapter the minimum concentration of chlortetracycline required to suppress the late-acting dominant lethal gene being expressed in LA513A was determined. This was conducted by rearing the mosquitoes with a range of chlortetracycline concentrations (0-30 μg ml^{-1}). The fitness was determined by measuring survival and development time to pupae/adulthood and the life-span of the adults.

This experiment will allow reductions in the cost of chlortetracycline and waste during mass-rearing, which will limit the quantities of chlortetracycline being released into the environment. Furthermore, a reduction in the concentration used during rearing of the mosquitoes could potentially reduce the impact on beneficial bacteria found within the mosquito and promote performance and fitness.

5.1.2 Wild-type *Aedes aegypti* and

To complement the experiments with LA513, studies were made to determine the impact of using chlortetracycline on the insect host and its microbiota. As described in Chapter 1, little is known about the diversity of the gut microbiota of *Aedes aegypti*. Only one research group has published data regarding the diversity of the microbes found within the gut of *Aedes aegypti*. What we do know from the published data is that *Bacillus,*

Asaia, Enterobacter, Klebsiella and Serratia are the dominant genera found in the gut of *Aedes aegypti* (Gusmão *et al*., 2007; Gusmão *et al*., 2010).

The role of these individual gut symbionts to the insect host is still unknown however we do know what happens to the performance of the insect when all the bacteria in *Aedes aegypti* are eliminated. Lang *et al* (1972) identified the effects of eliminating bacteria on the performance of *Aedes aegypti*. It was suggested that the removal of bacteria did not affect of the development time, survival and protein content. Nevertheless, bacterial depletion did impact the lipid content, water content and extended the life-span of the insect. This indicates that the performance of the mosquito was not compromised as much as shown in *Drosophila melanogaster* (Chapter 3) but the nutritional content of the insect was affected. Furthermore, it was shown that bacteria-free mosquitoes had an extended life-span which is similar to *Drosophila melanogaster* where an extension was observed with antibiotic treated.

There have not been any studies that have shown the impacts of chlortetracycline treatment on *Aedes aegypti* and the associated microbes. Therefore, I determined the impacts of chlortetracycline on wild-type Asian *Aedes aegypti*, a strain which was reared at Oxitec Ltd and originally isolated from Malaysia in 1974 and wild-type Mexican *Aedes aegypti* reared at Cornell University since 2006. The reason for the use of both strains was to determine whether there was a difference between a strain that may have had prior contact with chlortetracycline in the laboratory and one at Cornell University which had not. Both strains were treated with a range of chlortetracycline concentrations (0-100 µg ml^{-1}). Survival and development to pupae and adulthood, lifespan and the nutrition of the mosquitoes were measured. 454 pyrosequencing and 16S rRNA gene analysis was conducted to gain more insight into the diversity of bacteria within larvae and adult *Aedes aegypti* and to identify the changes in the diversity when the insects were treated with chlortetracycline.

5.2 LA513 (Bisex-Lethal) performance with varying concentrations of chlortetracycline

5.2.1 Survivorship to pupae and adulthood of LA513

Survival to pupae and adult stages were measured using 6 replicates of 150 mosquito larvae. The survival to pupae and adulthood varied significantly with chlortetracycline concentration: with very high mortality at 0 and 0.01 µg ml^{-1}; and >80% survival at greater concentrations (Pupae - KW: H_6 = 34.512, p<0.001; Adult - KW: H_6 = 37.840, p<0.001) (Figure 5.1). Pupae mortality was observed at 0.1 µg ml^{-1} with 29% of total pupae failing to emerge as adults. At 0.5 µg ml^{-1} and above, the percentage that died was smaller, ranging from 11-20%. Survival to adulthood was lower than survival to pupae, mosquitoes reared on 0.1 µg ml^{-1} showed the biggest decrease with only 58% surviving to adulthood. Mosquitoes reared on 0.5 µg ml^{-1} and above showed a much greater survival of 75-91%.

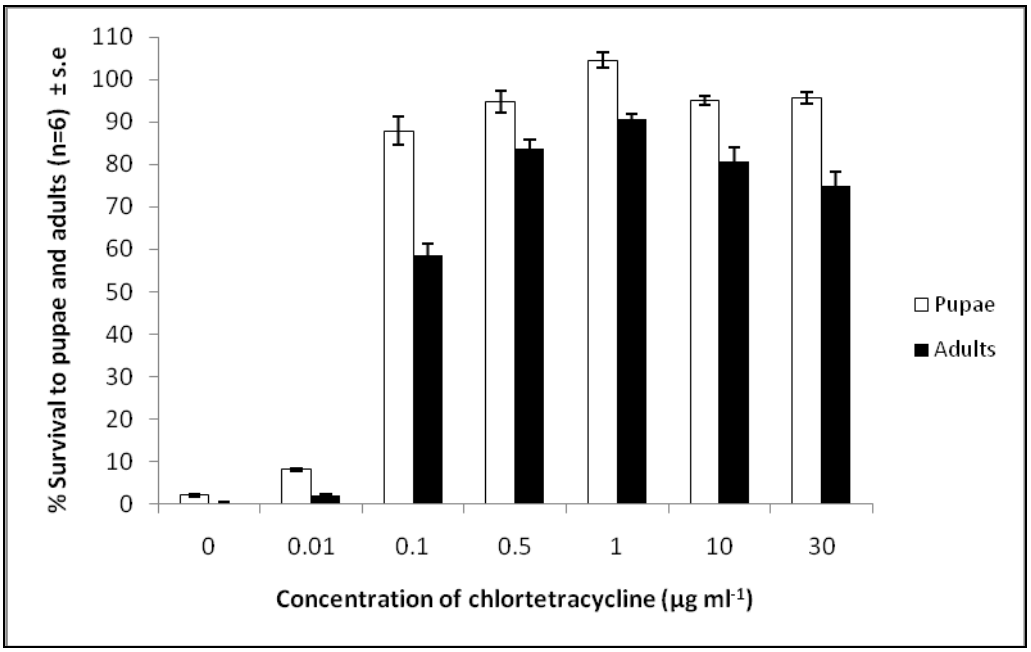


Figure: 5.1. Percent survival to pupae of mosquitoes treated with 0-30 µg ml^{-1} of chlortetracycline, number of replicates = 6 with 150 larvae per replicate (Methods Chapter; Section: 2.3.3, page 41-42).

5.2.2 Development time of LA513

Development time to pupae and adulthood was measured for three reasons; 1) experiments with *Drosophila* showed extension of development time when treated with chlortetracycline (Chapter 3), 2) development time could be affected by the expression of the lethal gene and 3) the development time is important when mass rearing. Any change in development time due to changes in chlortetracycline concentration need to be identified so the rearing schedule can be adjusted accordingly.

The development time to pupae was determined using 6 replicates of 150 larvae and analysed using Kruskal-Wallis analysis. This analysis demonstrated that development time to pupae was significantly different in males (KW: H_4 = 103.073, $p<0.001$) and females (KW: H_4 = 149.947, $p<0.001$) when treated with different concentrations of chlortetracycline. At 0.1 and 1 µg ml^{-1} the median development was the same for males (10 days) and females (11 days). However, at 10 and 30 µg ml^{-1} the median development time was reduced to 9 days in males but in females the development time remained the same from 0.1 to 30 µg ml^{-1} (Figure: 5.2).

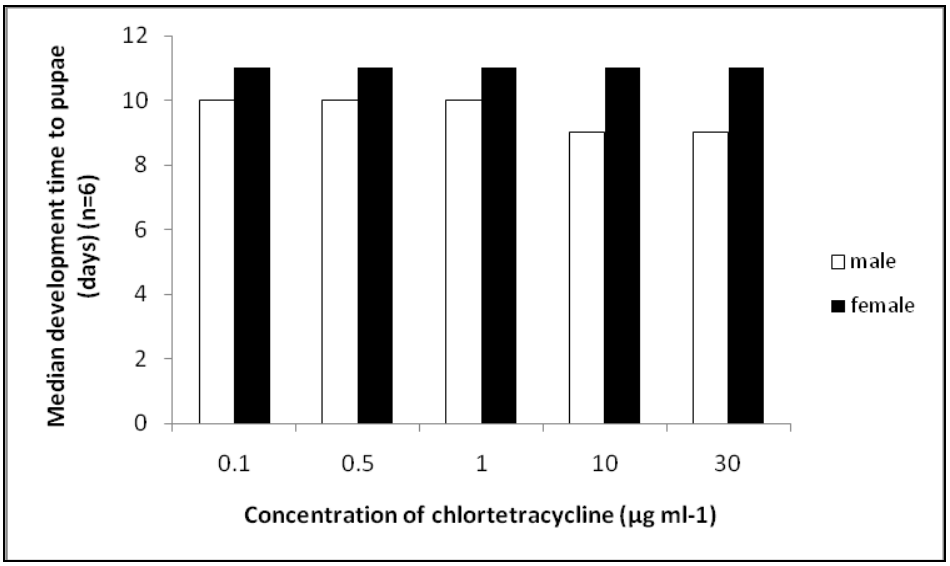


Figure: 5.2. Median development time to pupae of LA513 reared with 0.1-30 µg ml^{-1} of chlortetracycline, number of replicates = 6 with 150 larvae per replicate (Methods Chapter; Section: 2.3.3, page 41-42).

The development time to adulthood was also significantly different in males (KW: H_4 = 121.411, p<0.001 and females (KW: H_4 = 130.528, P<0.001) when treated with different concentrations of chlortetracycline. At 0.1 µg ml^{-1} both male and female have the same median development time of 12 days, however above this concentration the development time reduced to 11 days in males and increased to 13 days in females (Figure: 5.3).

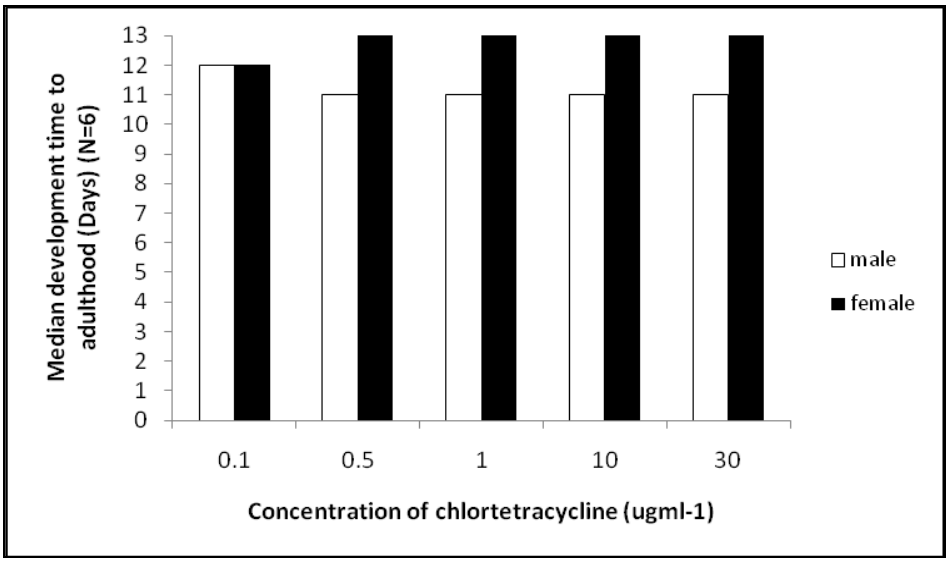
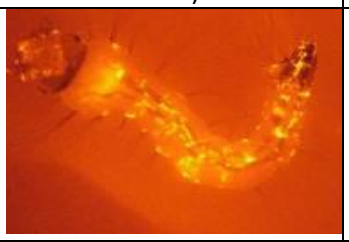
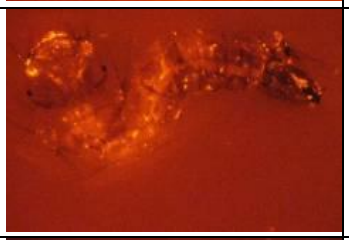
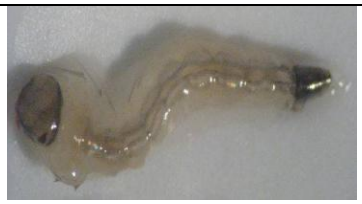
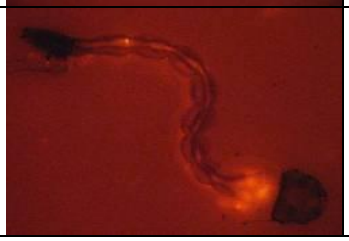


Figure: 5.3. Median development time to adulthood with 0.1-30 µg ml^{-1} of chlortetracycline, number of replicates = 6 with 150 larvae per replicate (Methods Chapter; Section: 2.3.3, page 41-42).

5.2.3 Observations of DsRed fluorescence in LA513A treated with 0-10 µg ml^{-1} of chlortetracycline

It has been suggested that the level of DsRed expression is affected by the chlortetracycline concentration and that DsRed expression may be enhanced by the positive feedback loop of the RIDL® system, linking the fluorescence expression with the RIDL® expression. Therefore, fluorescence images were taken to examine the expression of DsRed in LA513A. DsRed expression was continually observed to have a more pronounced fluorescence in mosquitoes reared on 0 and 0.01 µg ml^{-1} of chlortetracycline (Table: 5.1).

Table: 5.1. Fluorescence of LA513 reared on different concentrations of chlortetracycline.

Chlortetracycline concentration (µg ml^{-1})	Larvae viewed using the filters for red fluorescence (excitation 510-550, emission 590LP)	Larvae viewed under normal light
0		
0.01		
0.1		
10		

5.2.4 Life-span of LA513

Life-span is an important measurement with both male and female LA513. Male life-span needs to be long enough to ensure that the transgenic mosquitoes mate with the female mosquitoes in the wild. In addition female mosquitoes need to have a lifespan long enough in the mass rearing facility to mate with male mosquitoes and produce sufficient eggs for production needs. Adult lifespan data varied significantly with chlortetracycline concentration in females (KW: H_4 = 80.778, $p<0.001$) and in males (KW: H_4 = 86.372, $p<0.001$), being shorter on 0.1 µg ml^{-1} chlortetracycline compared with treatments 0.5 µg ml^{-1} and above. The difference was more pronounced in males (median lifespan 15 days on 0.1 µg ml^{-1} and 29 days on 0.5 µg ml^{-1} chlortetracycline) than in females (31 days and 39 days, respectively) (Figure: 5.4, Figure: 5.5).

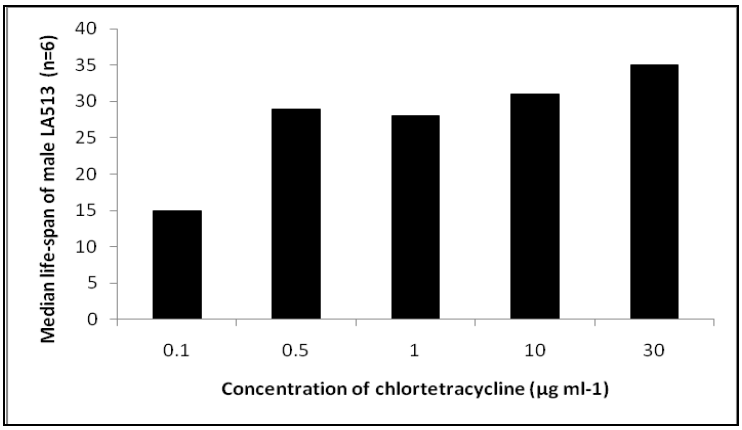


Figure: 5.4. Life-span of male LA513 treated with 0.1-30 µg ml^{-1} of chlortetracycline, number of replicates = 6 with 20 adult mosquitoes per replicate (Methods Chapter; Section: 2.3.3, page 41-42).

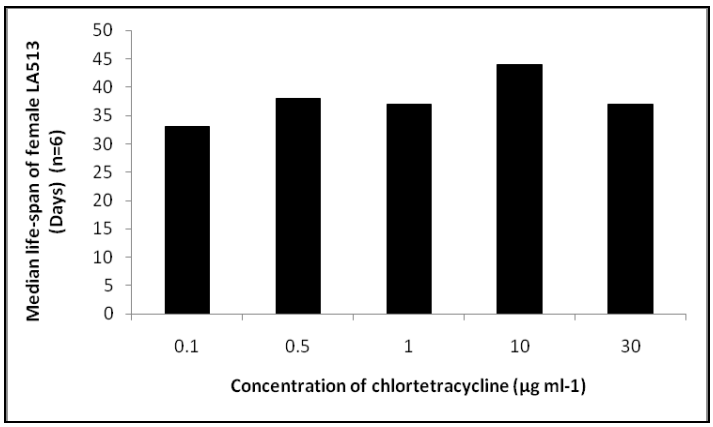


Figure: 5.5. Life-span of female LA513 treated with 0.1-30 µg ml^{-1} of chlortetracycline, number of replicates = 6 with 20 adults per replicate (Methods Chapter; Section: 2.3.3, page 41-42).

5.3 The impacts of chlortetracycline treatment on WT *Aedes aegypti*

The second section of this chapter will determine the impact of chlortetracycline on two wild-type strains of *Aedes aegypti*; Asian *Aedes aegypti* which was used to create LA513 at Oxitec Ltd and secondly, Mexican *Aedes aegypti.*

5.3.1 Survival of Asian *Aedes aegypti* treated with 0-100 µg ml^{-1} of chlortetracycline

The survival of Asian wild-type (WT) was the first performance parameter to be measured using 7 replicates of 300 larvae. The percent survival to pupae and adult mosquitoes varied from 69-98% (Figure: 5.6). Control mosquitoes had a survival to pupae and adulthood of 90% and 78% respectively. Angular transformation of the percentage data was conducted and an ANOVA was used to identify the significance of the results; for pupae there was no significant effect on survival ($F_{6, 34}$ = 1.186, p>0.05) between any of the chlortetracycline concentrations, this was also observed with adult data ($F_{6, 41}$ = 1.359, p>0.05).

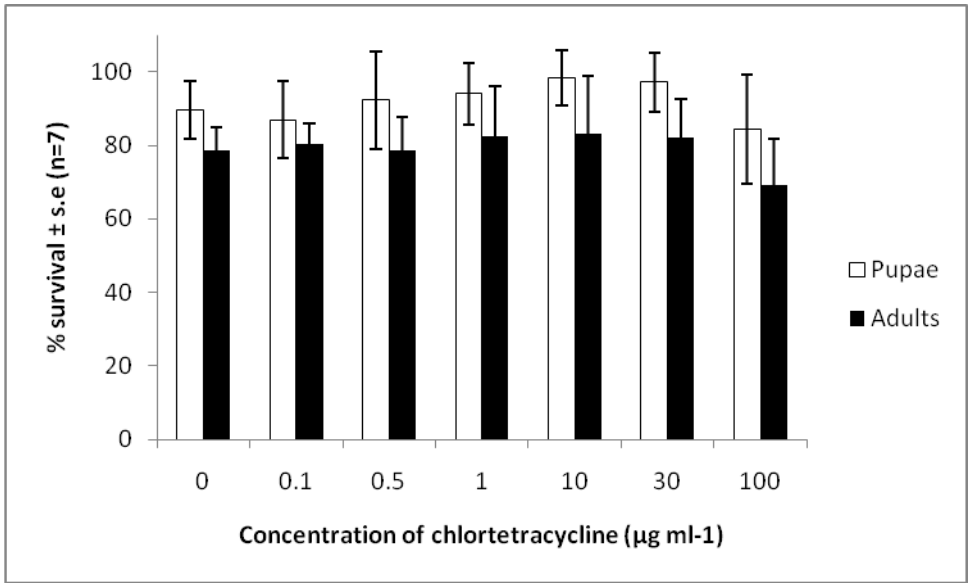


Figure: 5.6. Percent survival to pupae and adulthood of Asian *Aedes aegypti* treated with 0-100 µg ml^{-1} of chlortetracycline, number of replicates = 7 with 300 larvae per replicate (Methods Chapter; Section: 2.3.3, page 41-42).

5.3.2 Development time of Asian *Aedes aegypti* treated with chlortetracycline

The development time to adulthood was assessed in male and female mosquitoes. In male mosquitoes, control mosquito emergence peaked at 11 days. Chlortetracycline treatments at the concentrations; 0.5, 10, 30 and 100 µg ml^{-1} also had a peak emergence of 11 days. The treatments of 0.1 and 1 µg ml^{-1} had a pupation peak a day later at 12 days (Figure: 5.7). Statistical analysis showed that the development time was significantly different with chlortetracycline treatment for males (Kruskal-Wallis: H_6 = 40.451, $p<0.001$).

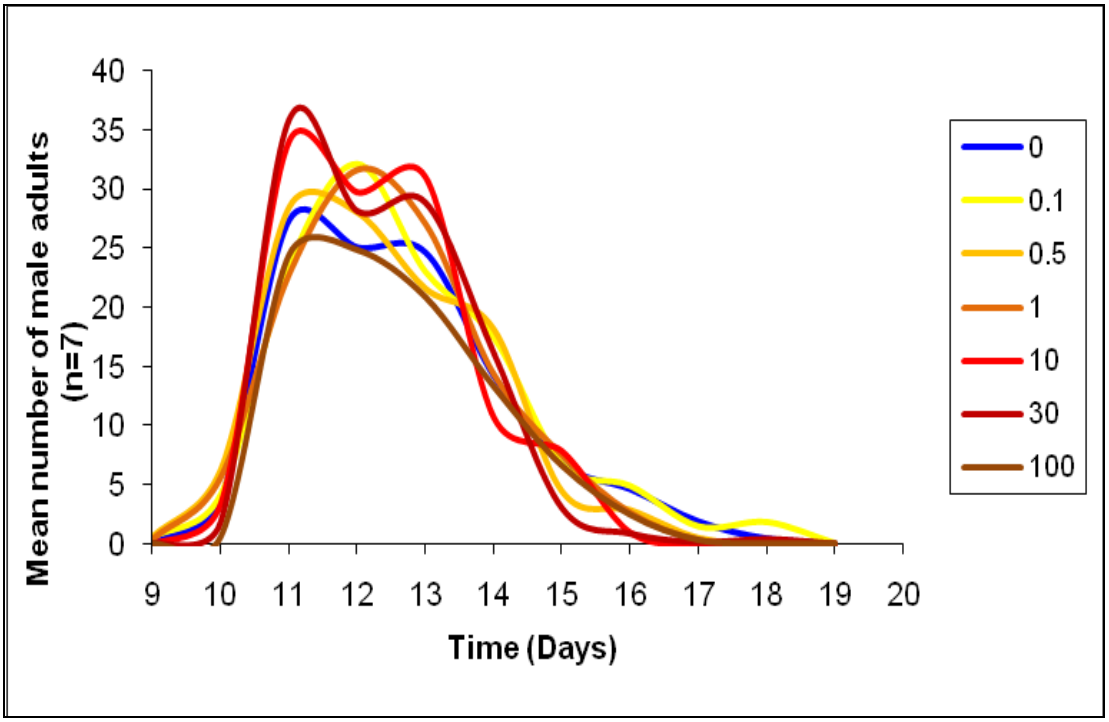


Figure: 5.7. Development curve to adulthood of male mosquitoes treated with 0-100 µg ml^{-1} of chlortetracycline, number of replicates = 7 with 300 larvae per replicate (Methods Chapter; Section: 2.3.3, page 41-42).

For female mosquitoes pupation curves indicated a peak emergence of 14 days across all treatments, however statistical analysis suggested that chlortetracycline did significantly impact development time to adulthood (Kruskal-Wallis: H_6 = 21.588, $p<0.01$) (Figure: 5.8).

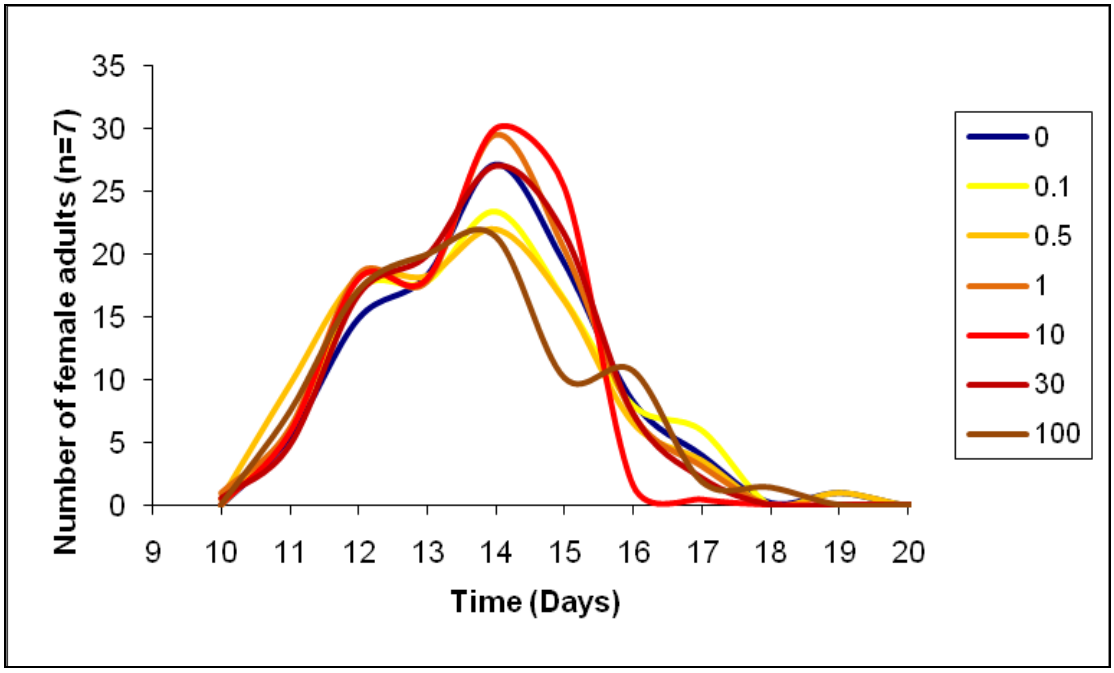


Figure: 5.8. Development curve to adulthood of female mosquitoes treated with 0-100 µg ml^{-1} of chlortetracycline, number of replicates = 7 with 300 larvae per replicate (Methods Chapter; Section: 2.3.3, page 41-42).

5.3.3 Life-span of *Aedes aegypti*

A Cox's regression statistical analysis was conducted with life-span data as the experiment was performed for a limited time and before all the mosquitoes died (50-55 days). The life-span of female mosquitoes was significantly affected by the treatment of chlortetracycline, compared with the control treatment of 0 µg ml^{-1} ($p<0.001$). Control mosquitoes showed a reduction in life-span (median lifespan of 30 days) compared with other treatments (33-38.5 median lifespan) which suggests that chlortetracycline treatment could promote life-span (Figure: 5.9. and Table: 5.2.). The life-span of male mosquitoes was not significantly altered with chlortetracycline treatment (median lifespan of 23-29 days) ($p>0.05$) (Figure: 5.10 and Table: 5.3).

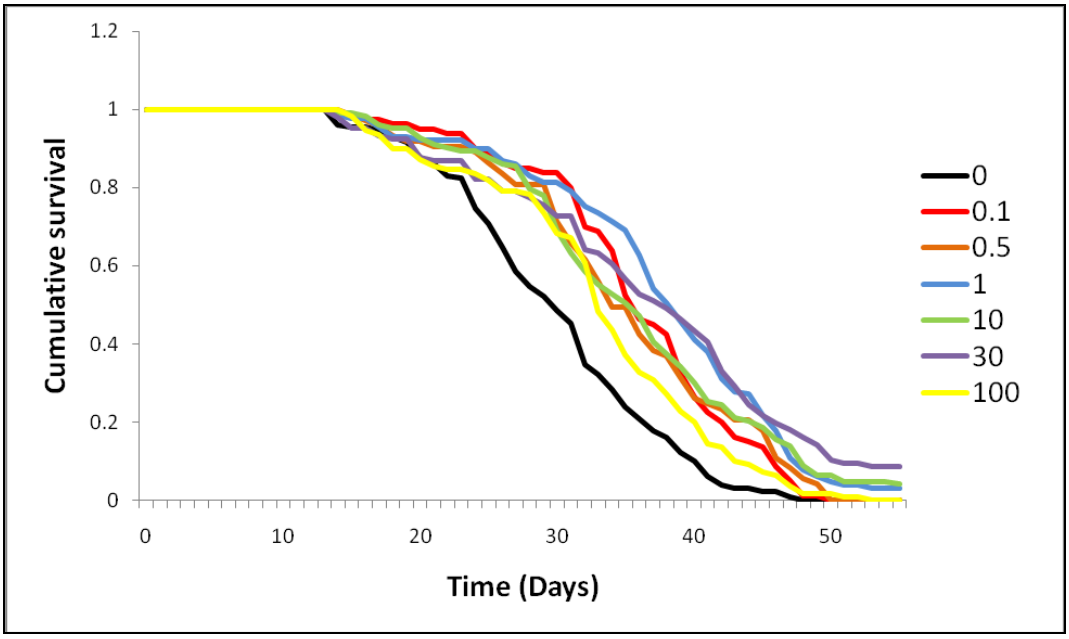


Figure: 5.9. Cumulative survival of female Asian *Aedes aegypti* treated with 0-100 µg ml^{-1} chlortetracycline, number of replicates = 5 with 30 adults per replicate (Methods Chapter; Section: 2.3.3, page 41-42).

Table: 5.2. Results of Cox's regression analysis of the life-span of female Asian *Aedes aegypti* treated with 0-100 µg ml^{-1} chlortetracycline. For each treatment the estimated regression coefficient (B), experimental Exp(B), standard error (S.E), degrees of freedom (d.f) and Wald-Statistic are given.

Covariate	B	Exp(B)	S.E	Wald Statistic	d.f	Significance
Replicate	-0.130	0.878	0.033	16.090	1	<0.001
Chlortetracycline treatment				93.529	6	<0.001
0.1	0.530	1.698	0.132	16.068	1	<0.001
0.5	-0.323	0.724	0.150	4.672	1	<0.05
1	-0.263	0.769	0.152	2.996	1	NS
10	-0.485	0.616	0.132	13.579	1	<0.001
30	-0.296	0.744	0.134	4.888	1	<0.05
100	-0.654	0.520	0.141	21.466	1	<0.001

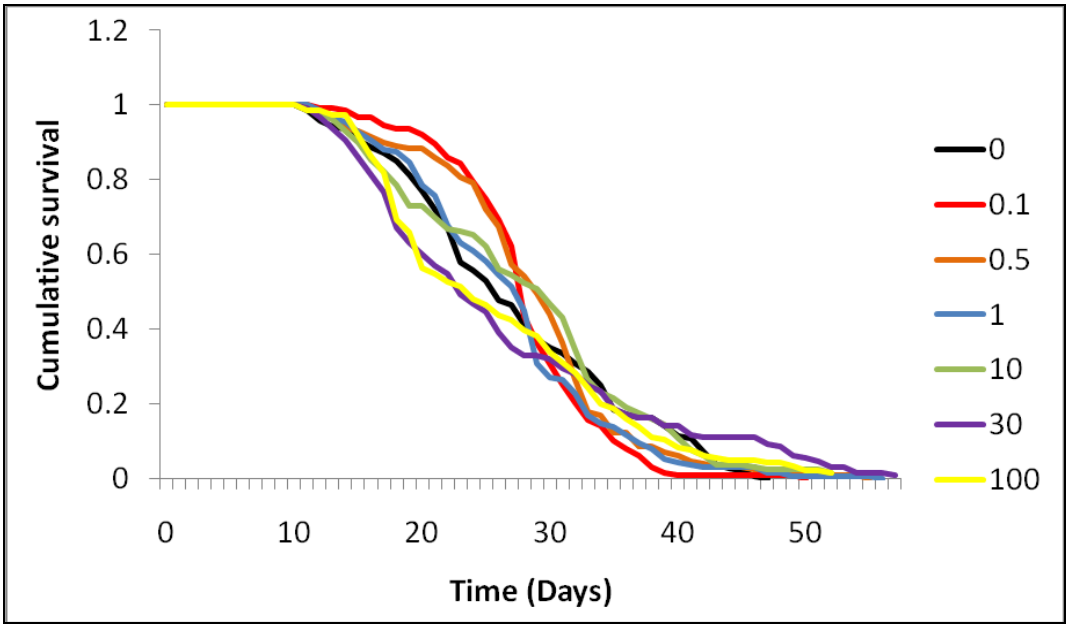


Figure: 5.10. Cumulative survival of male Asian *Aedes aegypti* treated with 0-100 µg ml^{-1} chlortetracycline, number of replicates = 5 with 30 adults per replicate (Methods Chapter; Section: 2.3.3, page 41-42).

Table: 5.3. Results of Cox's regression analysis of the life-span of male Asian *Aedes aegypti* treated with 0-100 µ µg ml^{-1} of chlortetracycline. For each treatment the estimated regression coefficient (B), experimental Exp(B), standard error (S.E), degrees of freedom (d.f) and Wald-Statistic are given.

Covariate	B	Exp(B)	S.E	Wald Statistic	d.f	Significance
Replicate	0.054	1.055	0.023	5.431	1	<0.05
Chlortetracycline treatment				4.483	6	>0.05
0.1	-0.111	0.895	0.120	0.862	1	>0.05
0.5	0.038	1.039	0.124	0.093	1	>0.05
1	-0.078	0.925	0.123	0.408	1	>0.05
10	0.052	1.054	0.121	0.189	1	>0.05
30	-0.145	0.865	0.122	1.411	1	>0.05
100	-0.066	0.936	0.124	0.285	1	>0.05

5.3.4 Total lipid content of Asian *Aedes aegypti* treated with chlortetracycline

The lipid content was consistently greater in males compared with female mosquitoes and the values ranged from 0.308 and 0.485 mg per mg of dry weight. Chlortetracycline treatment did not have a significant effect on the lipid content of male and female mosquitoes (Table: 5.4). A 2-way ANOVA showed that chlortetracycline does not have a significant effect on the lipid content within the mosquitoes ($F_{6, 130} = 1.588$, $p>0.05$) at the concentrations of chlortetracycline tested in this study. This response to chlortetracycline treatment was observed in both males and females ($F_{6, 130} = 0.907$, $p>0.05$). As described above, the lipid content between male and female mosquitoes was significantly different ($F_{1, 130} = 147.245$, $p<0.001$).

Table: 5.4. Total lipid content of Asian *Aedes aegypti* treated with 0-100 µg ml^{-1} chlortetracycline, number of replicates = 5 (Methods Chapter; Section: 2.5.6, page 48).

Concentration of chlortetracycline (µg ml^{-1})	Mean lipid content per mosquito (mg) per mg of dry weight ± s.e (n=10)	
	Male	Female
0	0.453 ±0.017	0.319 ±0.015
0.1	0.412 ±0.023	0.327 ±0.011
0.5	0.433 ±0.013	0.338 ±0.020
1	0.485 ±0.027	0.352 ±0.012
10	0.431 ±0.012	0.335 ±0.017
30	0.435 ±0.029	0.335 ±0.015
100	0.449 ±0.010	0.308 ±0.006

The dry weight of the mosquitoes ranged from 0.295 to 0.645 mg, with female mosquitoes having the greater weight. This statistical test (ANOVA) showed a significant effect with chlortetracycline treatment ($F_{6, 130} = 6.696$, $p<0.001$) and sex (males smaller than females, $F_{1, 130} = 569.426$, $p<0.001$) and also a significant interaction between sex and chlortetracycline treatment ($F_{1, 130} = 2.356$, $0.05>p>0.01$). LSD (Least significant difference) post hoc test revealed that male mosquitoes had a significantly greater dry weight when treated with 100 µg ml^{-1} chlortetracycline than all other treatments. In female mosquitoes the dry weight was significantly increased at 10 µg ml^{-1} (0.621 µg) and 100 µg ml^{-1} (0.645 µg) (Table: 5.5).

Table: 5.5. Dry weight of Asian *Aedes aegypti* treated with 0-100 µg ml^{-1} of chlortetracycline, number of replicates = 5 (Methods Chapter; Section: 2.5.6, page 48).

Concentration of chlortetracycline (µg ml^{-1})	Average dry weight per mosquito (mg) ± s.e (n=10)	
	Male	Female
0	0.302 ± 0.003	0.526 ± 0.022
0.1	0.299 ± 0.004	0.519 ± 0.026
0.5	0.299 ± 0.004	0.569 ± 0.028
1	0.295 ± 0.009	0.561 ± 0.026
10	0.310 ± 0.010	0.621 ± 0.026
30	0.310 ± 0.016	0.562 ± 0.030
100	0.359 ± 0.010	0.645 ± 0.028

5.3.5 Wing length of Asian *Aedes aegypti* treated with 0-100 µg ml^{-1} of chlortetracycline

Wing length was used as another measure of size (Chapter 2) to compare different chlortetracycline treatments. Wing length of male and female mosquitoes ranged from 1.422 to 2.177 mm, with females having longer wings compared with males (Table: 5.6). A 2-way ANOVA showed that the wing length did vary significantly with chlortetracycline treatment ($F_{6, 331}$= 38.038, $p<0.001$), there was a significant difference between male and female mosquitoes (shorter in males than females, $F_{1, 331}$ = 1.033, $p<0.001$) with no significant interaction between chlortetracycline and sex ($F_{6, 331}$ = 1.784, $p>0.05$). LSD post hoc analysis revealed that males reared with 0 and 100 µg ml^{-1} of chlortetracycline had significantly shorter wings (1.42 mm) than males reared with intermediate chlortetracycline concentrations. A difference between treatments was obtained for females with a significantly smaller wing length at 0 and 100 µg ml^{-1} of chlortetracycline (Table: 5.6).

Table: 5.6. Wing length of Asian *Aedes aegypti* treated with 0-100 µg ml^{-1} of chlortetracycline, number of replicates = 5 (Methods Chapter; Section: 2.5.1, page 44).

Concentration of chlortetracycline (µg ml^{-1})	Average wing length (mm) ± s.e (n=6)	
	Male	Female
0	1.425 ± 0.018	1.843 ± 0.017
0.1	1.561 ± 0.070	2.083 ± 0.023
0.5	1.685 ± 0.018	2.177± 0.023
1	1.615 ± 0.083	1.916 ± 0.085
10	1.663 ± 0.021	2.050 ± 0.080
30	1.597 ± 0.023	2.107 ± 0.021
100	1.422 ± 0.021	1.874 ± 0.035

5.4 Chlortetracycline effects on the performance of Mexican *Aedes aegypti*

5.4.1 Survival of Mexican *Aedes aegypti* treated with 0-100 µg ml^{-1} of chlortetracycline

The survival to pupae and adulthood was measured for the different chlortetracycline treatments, the percent survival to adults ranged from 51-79% (Figure: 5.11). The percentage data was angular transformed and an ANOVA was conducted. The statistical analysis showed that the mosquitoes were significantly affected by chlortetracycline treatment at pupae ($F_{6, 35}$ = 4.246, p<0.01) and adulthood ($F_{6, 35}$ = 7.372, p<0.001). Pupal survival was significanty reduced at 0.1 and 100 µg ml^{-1} of chlortetracycline where survival reduced from 75% with control mosquitoes to 67% at 0.1 and 100 µg ml^{-1}. Adult survival showed a greater response to chlortetracycline than pupae, survival was 73% with control mosquitoes, however the survival was significantly reduced to 51% at 100 µg ml^{-1} of chlortetracycline.

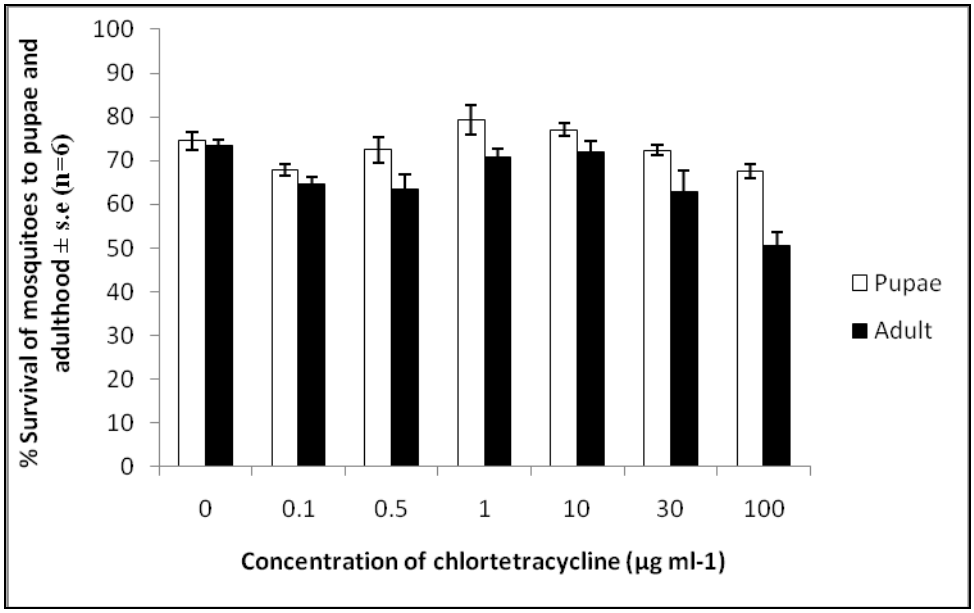


Figure: 5.11. Percent survival to pupae and adulthood of Mexican *Aedes aegypti* treated with 0-100 µg ml^{-1} of chlortetracycline, number of replicates = 6 with 150 larvae per replicate (Methods Chapter; Section: 2.3.3, page 41-42).

5.4.2 Development time to adulthood of Mexican *Aedes aegypti* treated with chlortetracycline

To assess the impact of chlortetracycline development rates were determined for male and female mosquitoes.

For male mosquitoes, development curves demonstrate that adult emergence peaked at 11 days for control mosquitoes and at 30-100 µg ml^{-1} of chlortetracycline. Peak emergence of 0.1-10 µg ml^{-1} of chlortetracycline was reduced to day 10 (Figure: 5.12). Statistical analysis showed that the development time was significantly different with chlortetracycline treatment for males (Kruskal-Wallis: $H_6 = 36.459$, $p<0.001$).

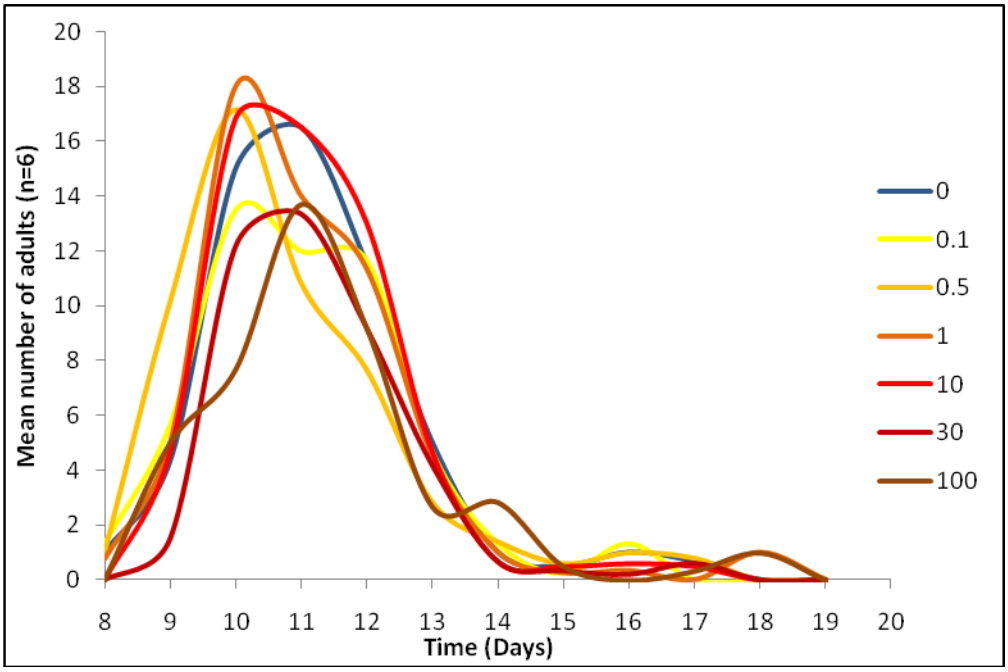


Figure: 5.12. Development curves to adulthood of male mosquitoes treated with 0-100 µg ml^{-1} chlortetracycline, number of replicates = 6 with 150 larvae per replicate (Methods Chapter; Section: 2.3.3, page 41-42).

For female mosquitoes, development curves demonstrated an extension in the time to emergence in control mosquitoes, with a 12 and 13 day time period for adult emergence (Figure: 5.13). A 12 day period was also observed for 10 and 100 µg ml^{-1} of chlortetracycline and a reduction to 11 days at 0.1, 0.5, 1, 30 µg ml^{-1} of chlortetracycline (Figure: 5.13). Kruskal-Wallis analysis indicated that the development time was significantly different between treatments in female mosquitoes (Kruskal-Wallis: $H_6 = 38.372$, $p<0.01$).

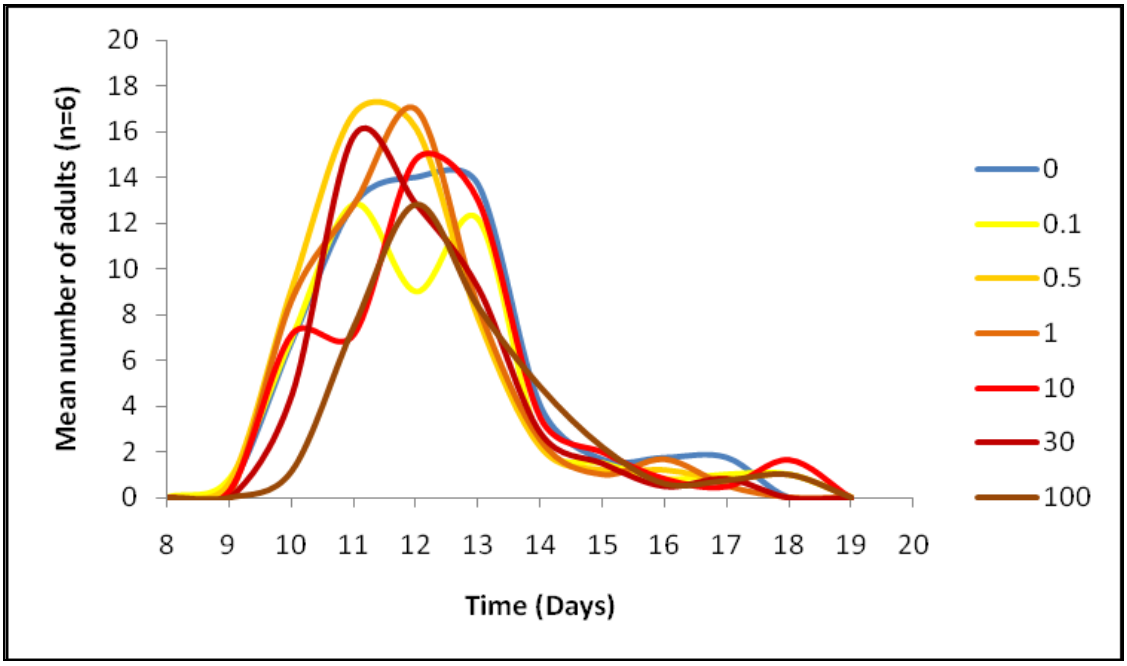


Figure: 5.13. Development curves to adulthood of female mosquitoes treated with 0-100 µg ml^{-1} chlortetracycline, number of replicates = 6 with 150 larvae per replicate (Methods Chapter; Section: 2.3.3, page 41-42).

5.4.3 Life-span of Mexican *Aedes aegypti* treated with chlortetracycline

In females, the median life-span varied from 35.5 to 51.5 days (Figure: 5.14). Statistical analysis showed that the life-span of female mosquitoes varied significantly with chlortetracycline treatment (KW: H_6 = 89.545, p<0.001). With control mosquitoes, the median life-span was 51.5 days, however at 100 µg ml^{-1} the lifespan was significantly reduced to 40 days, with the lowest life-span being at 30 µg ml^{-1} (35.5 days).

The life-span of male mosquitoes was also measured, the median life-span was similar to female mosquitoes of 35-58 days (Figure: 5.15). A Kolmogorov-Smirnov (KS) test was conducted to assess the normality of the data for the male life-span data before the statistical analysis was conducted. The distribution was not significantly different from the normal distribution (p>0.05), therefore a one-way ANOVA was conducted to determine whether there was a significant effect on the life-span of male mosquitoes.

The life-span of male mosquitoes varied significantly with chlortetracycline treatment ($F_{6, 653}$ =11.382, p<0.001). With control mosquitoes, the median life-span was 55.5 days, however at 100 µg ml^{-1}, the lifespan was significantly reduced to 35 days, with the lowest life-span being at 30 and 100 µg ml^{-1}. LSD post-hoc statistical test supported this result

and showed that at 0.5 to 100 µg ml^{-1} of chlortetracycline had a significantly different life-span than the control treatment.

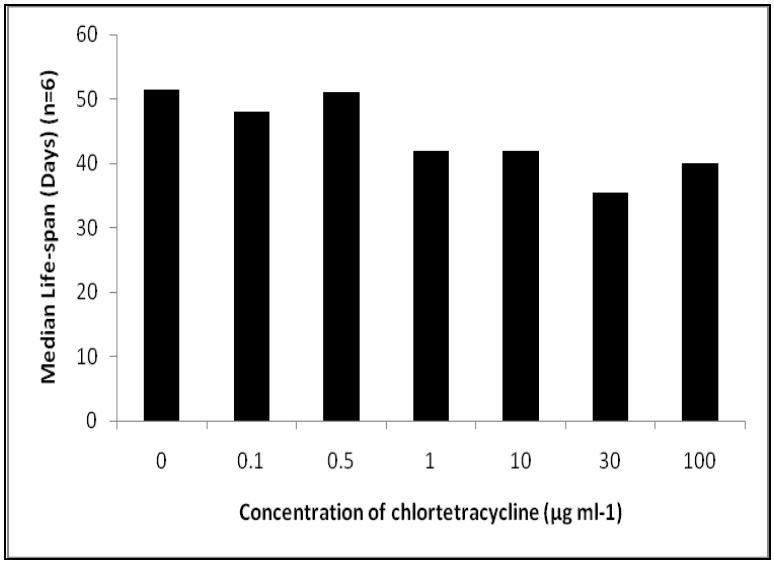


Figure: 5.14. Median life-span of female Mexican *Aedes aegypti* treated with 0-100 µg ml^{-1} of chlortetracycline, number of replicates = 6 with 20 adults per replicate (Methods Chapter; Section: 2.3.3, page 41-42).

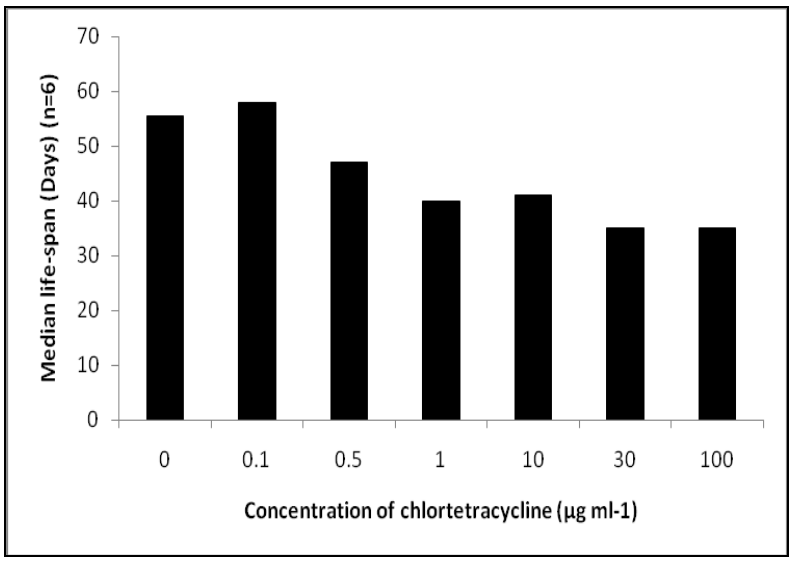


Figure: 5.15. Median life-span of male Mexican *Aedes aegypti* treated with 0-100 µg ml^{-1} of chlortetracycline, number of replicates = 6 with 20 adults per replicate (Methods Chapter; Section: 2.3.3, page 41-42).

5.5 Nutritional Status of Mexican *Aedes aegypti*

The carbohydrate, lipid and protein content of the insect was analysed to determine how chlortetracycline treatment affected the nutrition of the insect. Further measurements were conducted, including dry weight and wing length to determine the effects of chlortetracycline on the size of the insect.

5.5.1 Wing length of Mexican *Aedes aegypti* treated with 0-100 µg ml^{-1} of chlortetracycline.

The size was inferred from the wing length of the mosquitoes and used for nutritional analysis. The wing length of the mosquitoes varied from 2-2.8 mm, with females having a longer wing length (Table: 5.7). An ANOVA demonstrated that chlortetracycline had no significant effect on the wing length of either males or females ($F_{6, 56}$ = 1.070, p>0.05). However, there was a significant difference between the sexes ($F_{1, 56}$ = 198.910, p<0.001). The response of male and female mosquitoes to chlortetracycline was not significantly different ($F_{6, 56}$ = 0.636, p>0.05).

Table: 5.7. Wing length of Mexican *Aedes aegypti* treated with 0-100 µg ml^{-1} of chlortetracycline, number of replicates = 5 (Methods Chapter; Section: 2.5.1, page 44).

Concentration of chlortetracycline (µg ml^{-1})	Mean wing length (mm) ± s.e (n=5)	
	Male	Female
0	2.094 ± 0.038	2.703 ± 0.026
0.1	2.104 ± 0.014	2.734 ±0.029
0.5	2.112 ± 0.043	2.836 ± 0.055
1	2.115 ± 0.041	2.807 ± 0.037
10	2.132 ± 0.017	2.810 ± 0.039
30	2.139 ± 0.049	2.705 ± 0.068
100	2.151 ± 0.040	2.823 ± 0.039

5.5.2 Glucose content of Mexican *Aedes aegypti* treated with 0-100 µg ml^{-1} of chlortetracycline.

The glucose content varied between 1.25 and 3.1 µg, with a greater glucose content observed in female mosquitoes (Table: 5.8). To determine whether there was a significant effect of antibiotic treatment on the glucose content of mosquitoes, a 2-way ANCOVA was conducted with wing length used a size covariate. The statistical analysis

showed that wing length varied significantly ($F_{1, 55}$ = 7.324, p<0.05). Chlortetracycline appears to have a significant effect on the glucose content within the mosquitoes ($F_{6, 55}$ = 4.294, p<0.01), with a greater response in females. Female glucose content reduced as the chlortetracycline concentration increased. There was also a significant difference between sexes ($F_{1, 55}$ = 4.232, p<0.05), a greater content observed in females compared with males. The response to chlortetracycline was shown to be different in male and female mosquitoes ($F_{6, 55}$ = 5.344, p<0.001). The glucose content of male mosquitoes did not significantly alter with treatment of chlortetracycline ($F_{6, 28}$ = 1.933, p>0.05) (Table: 5.8). However, in females, the glucose content was significantly decreased at 30 µg ml^{-1} of chlortetracycline where the mean glucose content decreased from 2.53 µg in control mosquitoes to 1.61 µg ($F_{6, 28}$ = 9.416, p>0.001) (Table: 5.8).

Table: 5.8. Glucose content of mosquitoes treated with chlortetracycline (0-100 µg ml^{-1}), number of replicates = 5 (Methods Chapter; Section: 2.5.4, page 46).

Concentration of chlortetracycline (µg ml^{-1})	Mean glucose content µg per fly ± s.e (n=5)	
	Male	Female
0	1.42 ± 0.13	2.53 ± 0.22
0.1	1.51 ± 0.09	3.09 ±0.24
0.5	1.66± 0.13	3.10 ± 0.36
1	1.75 ± 0.10	2.10 ± 0.21
10	1.25 ± 0.06	2.44 ± 0.16
30	1.63 ± 0.08	1.61 ± 0.05
100	1.74 ± 0.07	2.46 ± 0.12

5.5.3 Glycogen content of Mexican *Aedes aegypti* treated with 0-100 µg ml^{-1} of chlortetracycline.

Glycogen was quantified in both male and female mosquitoes, the glycogen content of the mosquitoes ranged on average between 2.70 and 5.73 µg (Table: 5.9). Male mosquitoes showed a greater content of glycogen compared with female mosquitoes. A 2-way ANCOVA (wing length as the covariate) showed that wing length was statistically significantly different ($F_{1, 54}$ = 4.202, p≤0.05). Chlortetracycline concentration appears to have no significant effect on the glycogen content within the flies ($F_{6, 54}$ = 1.752, p>0.05) but a significant difference was observed between sexes ($F_{1, 54}$ = 26.630, p<0.001). Furthermore, there was a no significant interaction between chlortetracycline treatment

and sex ($F_{6, 54}$ = 2.133 p>0.05), showing that the response was the same in both sexes. The content of glycogen of male mosquitoes ranged from 4.26 µg in control mosquitoes to 5.73 µg at 100 µg ml^{-1} of chlortetracycline. Little change was observed in female mosquitoes, in control mosquitoes the glycogen content was 3.42 µg and at 100 µg ml^{-1} of chlortetracycline the quantity was 3.53 µg.

Table: 5.9. Glycogen content of mosquitoes treated with chlortetracycline (0-100 µg ml^{-1}), number of replicates = 5 (Methods Chapter; Section: 2.5.4, page 46).

Concentration of chlortetracycline (µg ml^{-1})	Mean glycogen content µg per fly ± s.e (n=5,*4)	
	Male	Female
0	4.26 ±0.30	3.42 ± 0.21
0.1	5.51 ± 0.18	4.59 ± 0.53
0.5	5.72 ± 0.53	4.63 ± 0.47
1	5.38 ± 0.75	3.12 ± 0.30*
10	5.35 ± 0.59	4.00 ± 0.52
30	5.60 ± 0.65	2.70 ± 0.32
100	5.73 ± 0.26	3.53 ± 0.36

5.5.4 Trehalose content of Mexican *Aedes aegypti* treated with 0-100 µg ml^{-1} of chlortetracycline.

The trehalose content varied from 1.81 to 5.66 µg, with similar trehalose content in male and female mosquitoes (Table: 5.10). An ANCOVA was conducted to determine whether there was a significant impact of antibiotic treatment on the trehalose content of the mosquitoes. Wing length was used as the covariate and was shown to be significantly different ($F_{1, 54}$ = 5.257, p<0.05). Chlortetracycline appears to have a significant effect on the trehalose content within the mosquitoes ($F_{6, 54}$ = 9.091, p<0.001). There was no significant difference between the 2 sexes ($F_{1, 54}$ = 0.033, p>0.05). Furthermore, the same response to chlortetracycline was observed in both sexes ($F_{6, 54}$ = 2.074, p>0.05). The trehalose content of male mosquitoes ranged from 4.65 µg in control mosquitoes to 3.96 µg at 100 µg ml^{-1} of chlortetracycline, the lowest trehalose content was observed at 0.1 µg ml^{-1} of chlortetracycline with 1.81 µg. In control female mosquitoes, the trehalose content was 5.70 µg, however at 0.1-30 µg ml^{-1} of chlortetracycline the trehalose quantity was reduced to 3.75-4.14 µg. At 100 µg ml^{-1} of chlortetracycline the trehalose content was 5.66 µg, not significantly different to control mosquitoes.

Table: 5.10. Trehalose content of mosquitoes treated with chlortetracycline (0-100 µg ml^{-1}), number of replicates = 5 (Methods Chapter; Section: 2.5.4, page 46).

Concentration of chlortetracycline (µg ml^{-1})	Trehalose content µg per fly ± s.e (n=5,*4)	
	Male	Female
0	4.65 ± 0.17	5.70 ± 0.38
0.1	1.81 ± 0.15	3.75 ± 0.23*
0.5	2.60 ± 0.17	4.01 ± 0.52
1	3.30 ± 0.29	4.10 ± 0.28
10	3.55 ± 0.76	4.04 ± 0.72
30	4.60 ± 0.23	4.14 ± 0.54
100	3.96 ± 0.24	5.66 ± 0.35

5.5.5 Total lipid content of Mexican *Aedes aegypti* treated with 0-100 µg ml^{-1} of chlortetracycline.

The total lipid content of male and female mosquitoes varied from 0.243 to 0.378 mg, with the greatest lipid content being in male mosquitoes (Table: 5.11). Using a 2-way ANOVA, chlortetracycline treatment appears to have a significant effect on the lipid content within the mosquitoes ($F_{6, 56}$ = 3.129, $p<0.05$) with a significant difference between sexes ($F_{1, 56}$ = 220.023, $p<0.001$). However, there was a significant difference in the response to chlortetracycline by the 2 sexes ($F_{6, 56}$ = 3.910, $p<0.01$). In female mosquitoes, the lipid content was significantly different at 1 and 30 µg ml^{-1} of chlortetracycline, where the lipid content increased from 0.246 mg in control mosquitoes to 0.278-0.300 mg. In males, the lipid content was shown to significantly increase from 0.328 mg in control mosquitoes to 0.363-0.378 mg at 0.5, 10 and 30 µg ml^{-1} of chlortetracycline.

Table: 5.11. Total lipid content of mosquitoes treated with 0-100 µg ml^{-1} of chlortetracycline, number of replicates = 5 (Methods Chapter; Section: 2.5.6, page 48).

Concentration of chlortetracycline (µg ml^{-1})	Mean lipid content per mg of dry weight (mg) per fly ± s.e (n=5)	
	Male	Female
0	0.328 ± 0.015	0.246 ± 0.003
0.1	0.326 ± 0.013	0.249 ± 0.003
0.5	0.378 ± 0.014	0.243 ± 0.008
1	0.340 ± 0.015	0.300 ± 0.006
10	0.363 ± 0.013	0.252 ± 0.017
30	0.367 ± 0.011	0.278 ± 0.008
100	0.362 ± 0.009	0.264 ± 0.017

5.5.6 Dry weight of Mexican *Aedes aegypti* treated with 0-100 µg ml^{-1} of chlortetracycline

The dry weight of the mosquitoes varied from 0.298 mg to 0.572 mg, with females having the greater weight (Table: 5.12). A 2-way ANOVA showed that chlortetracycline had a significant effect on the dry weight of the mosquitoes ($F_{6, 56}$ = 4.133, p<0.01). There was also a significant difference between male and female mosquitoes as described above ($F_{1, 56}$ = 321.540, p<0.001). However, there was not a significant interaction between chlortetracycline treatment and sex ($F_{6, 56}$ = 1.970, p>0.05), suggesting that the response to chlortetracycline was the same in both sexes. In female mosquitoes, the weight range increased to 0.540-0.572 mg compared with control mosquitoes with a weight of 0.456 mg. In male mosquitoes, the dry weight also increased to 0.351 mg at 100 µg ml^{-1} of chlortetracycline compared with control mosquitoes with a dry weight of 0.304 mg.

Table: 5.12. Dry weight of mosquitoes treated with 0-100 µg ml^{-1} of chlortetracycline, number of replicates = 5 (Methods Chapter; Section: 2.5.6, page 48).

Concentration of chlortetracycline (µg ml^{-1})	Mean dry weight (mg) per fly ± s.e (n=5)	
	Male	Female
0	0.304 ± 0.009	0.456 ± 0.015
0.1	0.298 ± 0.008	0.493 ± 0.007
0.5	0.374 ± 0.020	0.487 ± 0.033
1	0.308 ± 0.028	0.544 ± 0.012
10	0.349 ± 0.017	0.506 ± 0.036
30	0.337 ± 0.008	0.540 ± 0.028
100	0.351 ± 0.010	0.572 ± 0.026

5.5.7 Protein content of Mexican *Aedes aegypti* treated with 0-100 µg ml^{-1} of chlortetracycline

The protein content was quantified in male and female mosquitoes which varied from 79-177 µg with females having a larger quantity than males (Table: 5.13). Wing length was used as a size covariate. The observed difference in protein content between male and female was supported by the ANCOVA statistical analysis ($F_{1, 54}$ = 24.196, p<0.001). The wing length was significantly different ($F_{1, 54}$ = 11.768, p<0.05). The statistical analysis demonstrated that there was a significant effect of chlortetracycline treatment on protein content ($F_{6, 54}$ = 2.792, p<0.05), however different responses were observed with the 2 sexes ($F_{6, 54}$ = 7.034, p<0.001). In female mosquitoes, there does not appear to be a

trend with protein content and increasing concentration of chlortetracycline. However, in males a decrease in protein content was observed in treated mosquitoes (1-100 µg ml^{-1}) compared with control; the protein content reduced from 113.95 µg to 94.63-78.98 µg.

Table: 5.13: Protein content of mosquitoes treated with 0-100 µg ml^{-1} of chlortetracycline, number of replicates = 5 (Methods Chapter; Section: 2.5.3, page 46).

Concentration of chlortetracycline (µg ml^{-1})	Mean protein content µg per fly ± s.e (n=5,*4)	
	Male	Female
0	113.95 ± 5.53	124.28 ± 6.95
0.1	86.55 ± 2.90	140.23 ± 5.09
0.5	86.84 ± 6.35	157.35 ± 9.06
1	78.98 ± 4.25	147.22 ± 10.52
10	91.78 ± 5.31	177.34 ± 7.18*
30	91.95 ± 5.30	126.15 ± 4.71
100	94.63 ± 5.52	154.74 ± 6.25

5.6 Impacts of chlortetracycline on the culturable bacterial numbers and diversity in WT *Aedes aegypti*

The impact of chlortetracycline on the colony forming units and the diversity of the bacteria cultured from the larvae and water treated with chlortetracycline was assessed. 454 pyrosequencing was used to gain an insight into the diversity of culturable and non-culturable bacteria of larvae, adult male and adult female mosquitoes. The diversity of bacteria within control adult males was also compared with chlortetracycline treated males at 30 µg ml^{-1} of chlortetracycline. The diversity of the bacteria in chlortetracycline treated adult males was chosen rather than female mosquitoes as RIDL® male mosquitoes are released into the wild to compete with male mosquitoes in the wild.

5.6.1 Impacts of chlortetracycline on the culturable bacterial numbers in Asian WT *Aedes aegypti* and the rearing water for Asian WT *Aedes aegypti*

To investigate the impact of chlortetracycline treatment on the bacterial numbers in Asian WT, larvae reared with 0-100 µg ml^{-1} of the antibiotic and the rearing water were plated onto agar plates (+/- 50 µg ml^{-1} of chlortetracycline) with 5 dilution factors. The number of colony forming units (CFUs) varied from 10^3 to 10^8 per larva (Table: 5.14). The number of CFUs found in larvae and water at different concentrations of chlortetracycline was reduced at 30 and 100 µg ml^{-1} (100,000 times lower than other treatments). Colonies grew on plates supplemented with 50 µg ml^{-1} of chlortetracycline indicating that the

larvae and water bore chlortetracycline-resistant bacteria. In addition, with larval and water samples the number of colonies was reduced by 100-1000 fold on chlortetracycline plates relative to chlortetracycline-free plates. In water and larvae, the number of CFUs on antibiotic plates was similar to antibiotic-free plates at high chlortetracycline concentrations, 30-100 μg ml^{-1} (Table: 5.14).

Table: 5.14. Log_{10} CFUs of Asian wild-type mosquito larvae and water treated with 0-100 μg ml^{-1} of chlortetracycline, number of replicates = 1 (Methods Chapter; Section: 2.4.2, page 43).

Chlortetracycline concentration (μg ml^{-1})	Log_{10} CFUs per larvae (n=1)		Log_{10} CFUs per ml of water (n=1)	
	Minus chlortetracycline plates	Plus chlortetracycline plates	Minus chlortetracycline plates	Plus chlortetracycline plates
0	8.346	4.797	8.598	6.659
0.1	7.226	4.178	8.742	6.483
0.5	6.705	5.251	8.809	5.699
1	7.267	6.426	8.812	5.813
10	6.962	3.828	8.812	6.301
30	3.951	3.085	6.124	5.494
100	3.186	3.693	6.556	6.107

The culturable bacteria of Asian *Aedes aegypti* larvae and rearing water were sampled from the agar plates (+/- 50 μg ml^{-1} of chlortetracycline), up to 5 colonies with different morphology were identified from each treatment.

In control larvae the bacteria identified included: *Microbacteria* (Actinobacteria), *Chryseobacterium meningosepticum* (Bacteroidetes), *Serratia marcescens/Pseudomonas fluorescens* (Proteobacteria) and *Leucobacter* (Actinobacteria) (Appendix: Table: 7.32, 7.33). The Genera/species that were continually identified in the larvae across all treatments and in the water samples were; *Serratia marcescens (Proteobacteria), Leucobacter* (Actinobacteria)*, Chryseobacterium meningosepticum* (Bacteroidetes) and *Microbacteria.* Genera/species that were not commonly found in other treatments and were only found in water samples were; *Bacillus cereus* (Firmicutes) in control water and *Delftia* (*Proteobacteria*) found at 100 μg ml^{-1} of chlortetracycline rearing water (Appendix: Table: 7.32, 7.33).

These results suggest that the bacterial diversity in larvae treated with different concentrations of chlortetracycline does not appear to significantly change. Nevertheless, the relative total number of bacteria was significantly lowered with 30 and 100 ug ml^{-1} treatments of chlortetracycline. Water and larvae showed similar species diversity. The results have demonstrated that *Microbacteria* (Actinobacteria), *Leucobacter* (Actinobacteria)*, Serratia marcescens* (Proteobacteria) and *Chryseobacterium meningosepticum* (Bacteroidetes) are the dominant culturable species in Asian *Aedes aegypti* larvae and in the rearing water.

5.6.2 Chlortetracycline effects on the culturable bacterial numbers in Mexican WT *Aedes aegypti* and the rearing water for Mexican WT *Aedes aegypti*

During the performance studies with Mexican wild-type mosquitoes, the number of colony forming units (CFUs) was quantified in the mosquito larvae. The number of colony forming units (CFUs) varied from 10^3 to 10^8 per larva (Table: 5.15), showing a similar number of CFUs measured with Asian WT (Table: 5.14).

Table: 5.15. Log_{10} CFUs of Mexican wild-type mosquito larvae and water treated with 0-100 µg ml^{-1} of chlortetracycline during the performance studies, number of replicates = 1 (Methods Chapter; Section: 2.4.2, page 43).

Chlortetracycline concentration (µgml^{-1})	Log_{10} CFUs per larvae (n=1)		Log_{10} CFUs per ml of water (n=1)	
	Minus chlortetracycline plates	Plus chlortetracycline plates	Minus chlortetracycline plates	Plus chlortetracycline plates
0	8.028	2.263	8.602	4.283
0.1	8.150	2.301	7.556	5.134
0.5	6.368	3.222	6.00	4.477
1	6.233	2.689	6.862	5.079
10	4.590	3.699	6.903	5.079
30	4.146	3.768	5.903	8.795
100	4.865	4.768	5.326	5.903

A smaller and separate experiment investigating the impact of chlortetracycline on the bacterial content in Mexican wild-type larvae was also conducted with replicates to confirm the above results.

A Kolmogorov-Smirnov (KS) test was performed for the Log_{10} CFU data of larvae and water samples before the statistical analysis was conducted. The data was significantly

different from the normal distribution ($p<0.05$), therefore the data was rank transformed before a Three-Way ANOVA was conducted. It was shown that there was a significant effect of chlortetracycline ($F_{6, 28} = 8.148$, $p<0.001$) on the culturable bacterial content of mosquitoes and the water, with a significant difference between the presence and absence of chlortetracycline in the agar plates ($F_{1, 28} = 184.254$, $p<0.001$) (Table: 5.16). A greater number of CFUs was observed with water samples at higher concentrations of chlortetracycline on antibiotic plates, with a 100 fold greater CFUs (Table: 5.16). Statistical analysis showed that there was a difference between the water and larvae samples ($F_{1, 28} = 48.395$, $p<0.001$). The response of the water and larvae samples to chlortetracycline treatment was significantly different ($F_{6, 28} = 3.337$, $p<0.05$), the number of CFUs measured in larvae was reduced by 1000 fold, however, in water it was only reduced by 8 fold (Table: 5.16). On chlortetracycline supplemented agar plates, the CFUs observed were significantly reduced to 10^6 in control samples ($F_{1, 28} = 5.994$, $p<0.05$) (Table: 5.16). Furthermore, the difference between chlortetracycline supplemented agar plates and non-supplemented plates was less pronounced with treatments at higher concentrations of chlortetracycline (30-100 µg ml^{-1}) with less than a 2 fold difference ($F_{6, 28} = 22.676$, $p<0.001$). Statistical analysis also showed that the three-way interaction (Treatment*Sample Type*+/-Chlortetracycline on agar plates) was not significantly different ($H_{6, 28} = 49.244$, $p>0.05$).

Table: 5.16. Log_{10} CFUs of Mexican wild-type mosquito larvae and rearing water treated with 0-100 µg ml^{-1} of chlortetracycline, number of replicates = 2 (Methods Chapter; Section: 2.4.2, page 43).

Chlortetracycline concentration (µg ml^{-1})	Mean Log_{10} CFUs per larvae (n=2) ± s.e		Mean Log_{10} CFUs per ml of water (n=2) ± s.e	
	Minus chlortetracycline plates	Plus chlortetracycline plates	Minus chlortetracycline plates	Plus chlortetracycline plates
0	8.199 ± 0.24	1.673 ± 0.15	8.846 ± 0.02	3.628 ± 1.15
0.1	8.308 ± 0.13	3.184 ± 1.49	8.947 ± 0.07	5.396 ± 0.07
0.5	7.539 ± 0.21	4.068 ± 0.60	8.375 ± 0.21	4.758 ± 0.06
1	7.495 ± 0.001	4.885 ± 0.11	8.360 ± 0.34	4.690 ± 0.54
10	7.689 ± 0.27	7.990 ± 0.06	8.322 ± 0.28	7.789 ± 0.06
30	5.409 ± 0.56	5.588 ± 1.11	7.288 ± 0.39	6.681 ± 0.10
100	4.539 ± 0.97	4.518 ± 0.78	7.906 ± 0.01	7.981 ± 0.06

The culturable bacteria were also sampled from the agar plates in which the numbers of CFUs were counted, up to 6 colonies were identified from each treatment, including both samples of water and larvae and on plates supplemented with chlortetracycline. In control larvae the bacteria identified included: *Microbacterium laevaniformans* (Actinobacteria)*, Elizabethkingia meningoseptica* (Bacteroidetes) and *Serratia marcescens* (Proteobacteria) (Appendix: Table: 7.34, 7.35). The following Genera/species were continually identified in all treatments, *Elizabethkingia meningoseptica* (Bacteroidetes), *Serratia marcescens* (Proteobacteria) and *Microbacteria.* However, new Genera/species including *Leucobacter* (Actinobacteria) (0.1 μg ml^{-1} of chlortetracycline), *Arthrobacter woluwensis* (Actinobacteria) (0.5 μg ml^{-1} of chlortetracycline) and *Klebsiella pneumoniae* (Proteobacteria) (10 and 100 μg ml^{-1} of chlortetracycline) were also identified in other treatments (Appendix: Table: 7.34, 7.35). In the water samples, the Genera/species that were identified were very similar to those identified in the larvae with the only difference being the identification of *Ochrobactrum* (Proteobacteria) and *Delftia* (Proteobacteria) found at 0.5 μg ml^{-1} of chlortetracycline and *Chryseobacterium meningosepticum* (Bacteroidetes) at 1 μg ml^{-1} of chlortetracycline (Appendix: Table: 7.34, 7.35).

5.6.3 454 pyrosequencing analysis of Mexican WT *Aedes aegypti*

The 454 pyrosequencing analysis of control Mexican WT larvae showed that the diversity of microbes found in the larvae were predominantly Actinobacteria with a low number of Proteobacteria (Appendix: Table: 7.36). The member of the phylum, Actinobacteria which made up 95% of the population was *Microbacterium laevaniformans* strain NML, this bacterium was also identified during culturable techniques (Appendix: Table: 7.34, 7.35). However, in adult male mosquitoes, the diversity was altered; the member of the phylum Bacteroidetes made up 93% of the sequence hits showing that the diversity of bacteria shifted from Actinobacteria (which was also identified using culturable techniques) to Bacteroidetes from larvae to adulthood (Appendix: Table: 7.37). In adult females, a similar observation was observed with a majority of 80% of the reads belonging to the phylum Bacteroidetes with 15% belonging to Proteobacteria (Appendix: Table: 7.39). When the adult male mosquitoes were treated with chlortetracycline, the dominant bacterium present was a member of the phylum Proteobacteria with 76% of the total reads and Bacteroidetes having 17% (Appendix: Table: 7.38).

5.7 Discussion

Preliminary experiments with LA513A treated with 0-100 µg ml^{-1} suggested that LA513 were able to survive on 0.1 µg ml^{-1} and above. The experiment was therefore repeated with 0.01-30 µg ml^{-1} to determine the minimal concentration. Survival to pupae and adulthood suggest that 0.5 µg ml^{-1} and above of chlortetracycline was required to suppress the expression of tTAV. However, at 0.1 µg ml^{-1} survival to pupae was high, 29% of the pupae failed to emerge as adults, suggesting that the tTAV was still expressed at a high enough level to cause lethality. The fluorescence images of the expression of DsRed in the larvae showed a reduction in DsRed fluorescence in the mosquito larvae at 0.1 µg ml^{-1} and above. This may suggest that the level of DsRed expression is affected by the chlortetracycline concentration and that the DsRed expression may be enhanced by the positive feedback loop of the RIDL® system, which is being expressed in the absence of chlortetracycline, linking the fluorescence expression with the RIDL® expression. The link with fluorescence and the enhanced DsRed expression with the RIDL® system is also supported by the survival data which suggest that at concentrations below 0.1 µg ml^{-1} of chlortetracycline, poor survival is observed suggesting that the lethal gene is being expressed.

As RIDL® mosquitoes will be released into the wild; life-span was considered an important performance assay to ensure that the RIDL® males will live long enough to mate with wild females. Life-span results showed that 10 and 30 µg ml^{-1} of chlortetracycline gave the longest life-span. Interestingly, in males 0.5-1 µg ml^{-1} of chlortetracycline doubled the life-span compared with 0.1 µg ml^{-1}. This result supports the survival data measured during this study, where it was demonstrated that at 0.1 µg ml^{-1} of chlortetracycline the level of survival was not as high as at 0.5 µg ml^{-1} and above of chlortetracycline. The life-span data also suggest that males are more sensitive to chlortetracycline concentration with a greater difference between 0.1 and 0.5 µg ml^{-1} and above compared with females.

The conclusions made with the life-span data is also supported by the development data which showed that the development time of male mosquitoes to pupae and adulthood decreased to 9 days with 10-30 µg ml^{-1} of chlortetracycline and 11 days with 0.5-30 µg ml^{-1} of chlortetracycline, respectively. In females there was no difference in development time to pupae however, at 0.5 µg ml^{-1} and above an increase in development time to

adulthood was observed. This response was not observed in Asian wild type mosquitoes at this concentration, therefore there could be an interaction between the transgene and chlortetracycline.

As with *Drosophila melanogaster*, experiments with wild-type *Aedes aegypti* did indicate a significant impact on the time to peak emergence with chlortetracycline treatment. Experiments with Mexican *Aedes aegypti* indicated that control and high concentrations of chlortetracycline significantly extended peak emergence and low to medium concentrations of 0.1-10 μg ml^{-1} reduced the time to peak emergence in Mexican *Aedes aegypti.* This result suggests that chlortetracycline could promote development rates by removing deleterious bacteria. Extension of peak emergence at high concentrations could be the result of the toxic effect of the antibiotic. Unlike Mexican *Aedes aegypti,* Asian *Aedes aegypti* peak emergence did not show a trend with chlortetracycline treatment.

The survival data to adulthood differed for Mexican and Asian mosquitoes; there was no significant effect of the survival with Asian WT but with Mexican WT a decrease in survival was observed at 100 μg ml^{-1} of chlortetracycline. This result suggests that Mexican WT was more sensitive to chlortetracycline treatment unlike Asian WT which had previous exposure to chlortetracycline (through exposure from contaminated trays and water) and therefore was more tolerant to the antibiotic.

Asian WT female mosquitoes had an extended life-span when treated with chlortetracycline, which suggests that the antibiotic could eliminate bacteria that are deletrious on the performance of the insect, this result is also supported by the findings of Lang *et al* (1972), which showed that the axenic mosquitoes had a greater life-span. Nevertheless, Mexican WT mosquitoes had a life-span that was significantly reduced in both sexes when treated with chlortetracycline (0.5-100 μg ml^{-1}). This suggests that the shortening of life-span could be due to the depletion of bacteria as the reduction was observed at concentrations of chlortetracycline where the bacterial CFUs were reduced from 10^{8} to 10^{3-7} in larvae. Furthermore, the reduction was observed at low concentrations of chlortetracycline as well as high concentrations, which could eliminate toxicity as the main reason for the reduction. Another reason for the reduction in life-span may not necessarily be a due to a decrease in bacterial numbers but it could be due

to the change in diversity of the bacteria within the mosquito. Mexican WT mosquitoes were recently isolated from Mexico (2006), therefore the greater sensitivity may be due to the fact that this strain has been reared in laboratory conditions for less time in comparison to Asian WT.

Interestingly, wing length was significantly altered with Asian not Mexican mosquitoes. In Asian mosquitoes, wing length was decreased at 100 µg ml^{-1} which would suggest that the high concentration of chlortetracycline had a negative impact on the insect. Furthermore, the wing length of control flies was significantly reduced which suggests that high bacterial numbers (10^8 CFUs in larvae) could have had a negative impact on the mosquito and the presence of a small amount of chlortetracycline could actually remove deleterious bacteria and promote mosquito performance.

Chlortetracycline was established to have a significant impact on the dry weight of both Asian and Mexican mosquitoes. In both of these strains, it was demonstrated that the mosquito dry weight was significantly increased when treated with chlortetracycline (Tables: 5.5, 5.12) in both male and female adults upon emergence. This result was unexpected as the quantity of protein (in Mexican WT only) decreased in males with chlortetracycline treatment and lipid (Asian WT only) did not significantly alter in both male and female mosquitoes. The reason for the decrease in protein content of male Mexican mosquitoes could be due to one or a combination of factors such as the removal of bacteria, a change in the diversity of the bacteria and toxicity of chlortetracycline.

The lipid content of the mosquitoes were not significantly changed in Asian WT, and only significantly increased at 1 and 30 µg ml^{-1} of chlortetracycline with female Mexican WT and 0.5, 10 and 30 µg ml^{-1} of chlortetracycline with male Mexican WT. The publication by Lang *et al* (1972) suggested that the percent lipid content of pupae was significantly reduced in axenic mosquitoes, however this change was not observed in the experiments described in this chapter using adult mosquitoes. Chlortetracycline treatment in this study did not eliminate all the bacteria found in the mosquitoes and pupae were not sampled, therefore if the mosquitoes were produced axenically and different life-stages were sampled then a difference may have been identified.

Carbohydrate quantification results for chlortetracycline treated mosquito gave more variable results. With glycogen, there was no significant effect of chlortetracycline treatment on the quantities within the insects of either sex. The reason for this may be that chlortetracycline does not eliminate all the bacteria within the mosquitoes and experiments with axenic mosquitoes may show a stronger effect. In general, the trehalose concentration was shown to be reduced in male and female mosquitoes when treated with chlortetracycline compared with control mosquitoes. However, in female mosquitoes the decrease occurred with all concentrations of chlortetracycline except at 100 µg ml^{-1} of chlortetracycline where the trehalose quantity was similar to control mosquitoes. This increase of trehalose at this high chlortetracycline concentrations suggest that chlortetracycline itself may be causing the change as it does not follow the trend with the other concentrations of chlortetracycline. The reduction in trehalose could also be explained by a change in diversity of the bacteria found within the mosquitoes which may not affect the glycogen levels but impact the concentration of trehalose circulating in the hemolymph.

The experiments investigating the impact of the chlortetracycline treatment on the microbiota demonstrated that culturable bacteria (CFUs) were still found in Asian and Mexican *Aedes aegypti* even at high concentrations of chlortetracycline treatments. Furthermore, colonies grew on plates supplemented with 50 µg ml^{-1} of chlortetracycline suggesting that the larvae and water bore chlortetracycline-resistant bacteria and could explain why the impact of chlortetracycline on the mosquitoes was not as great when compared with *Drosophila melanogaster* treated with chlortetracycline.

The culturable bacterial diversity in larvae in control and chlortetracycline treatment did not appear to alter, 454 analysis would provide data to confirm this result. Species/Genera such as *Microbacteria, Elizabethkingia meningoseptica*, *Leucobacter* and *Serratia marcescens* were continually identified in all samples. The rearing water also contained similar species which does suggest that the mosquitoes gain the bacteria from the water. One possibility is that the bacteria may originate from within the egg or on the egg surface and the introduction to water allows growth and replication of the bacteria, providing a food source. Fish food was another possibility, which was fed to the larvae and may have provided the symbionts. One argument against this hypothesis is that

similar bacteria have been found by other research groups with mosquitoes obtained from the wild and with other species of mosquitoes (Demaio *et al.,* 1996; Gusmão *et al*., 2010) suggesting that the fish food may not be solely responsible for the species found within the mosquitoes.

The larval 454 data showed similarities with the data obtained with culturable data, which suggests that many of the bacteria found within the mosquitoes are culturable. Interestingly, the 454 data analysis with control versus chlortetracycline treated adult male mosquitoes demonstrated a change in bacterial diversity. In control mosquitoes the major bacterium was *Elizabethkingia meningoseptica* and with chlortetracycline treatment *Raoultella sp.* was dominant and *Elizabethkingia meningoseptica* being the second most common species. This change in diversity may be due to the reduction in chlortetracycline sensitive species which allows for the replication and growth of those which were resistant.

The 454 and culturable bacterial analysis has shown that the majority of the microbes that inhabit the mosquitoes are common soil and water bacteria. *Klebsiella*, *Serratia*, *Enterobacter* and *Arthrobacter* are such bacteria (Madigan *et al*., 2003, p379). This would be expected as mosquitoes develop in rain water fed small pools of water.

The presence of bacteria at 100 µg ml^{-1} of chlortetracycline does suggest that the microbes are antibiotic resistant. This was perhaps not surprising with the strain reared at Oxitec Ltd, where both transgenic mosquitoes requiring chlortetracycline and wild-type mosquitoes were reared in the same room and in the same rearing trays, allowing wild-type mosquitoes to be exposed to residual chlortetracycline. In the Mexican WT mosquitoes resistant bacteria were also found, which was unexpected. It has became apparent that this was not uncommon and even in areas where waste containing antibiotics was rare, such as the Arctic where low levels of Ampicillin resistance was observed in bacteria of Polar bear faeces (Glad *et al*., 2010). Antibiotic resistance exists naturally but the excessive use of chlortetracycline in agriculture in countries such as the USA and Europe to promote health and growth of cattle has meant that the selection pressure has increased leading to bacterial resistant strains to tetracycline to rise (Frappaolo and Guest, 1986; Dibner and Richards, 2005; Allen *et al*., 2010).

5.8 Conclusion

Aedes aegypti are mainly colonised by bacteria commonly found in soil and water such as *Klebsiella*, *Serratia* and *Arthrobacter* (Madigan *et al*., 2003, p379). The bacteria found in these mosquitoes show chlortetracycline resistance. However, there does appear to be a distinct change in the diversity of the bacteria found in Mexican WT treated with chlortetracycline compared with control mosquitoes where the the dominant bacterium switched from *Elizabethkingia meningoseptica* to *Raoultella sp* with chlortetracycline treatment.

Chlortetracycline affected the survival, life-span and the nutrition of wild-type *Aedes aegypti*. However, the chlortetracycline treatment did not entirely eliminate the microbiota and there were still negative impacts on performance. This could be due to a reduction in the bacterial population, a change in diversity and/or due to the direct effects of chlortetracycline on the insects.

The performance experiments with the transgenic line, LA513 demonstrate that these mosquitoes can be reared on chlortetracycline at concentrations lower than currently used (30 μg ml^{-1}). The results suggest that concentrations as low as 0.5 μg ml^{-1} can be used to rear LA513 nevertheless, the recommendation for rearing would be a concentration of 10 μg ml^{-1}. This concentration maintains an efficacy margin of more than an order of magnitude for error and accounts for chlortetracycline degradation (chlortetracycline is light sensitive) and the biodegradation of chlortetracycline by the insects.

Chapter 6: Discussion

The research described in this thesis has demonstrated the deleterious impacts of chlortetracycline treatment on two dipteran insects; *Drosophila melanogaster* and *Aedes aegypti*. Egg dechorionation was also used during this project, an alternative and preferable method of removing bacteria from *Drosophila* as it removes bacteria and does not damage the egg. This method of eliminating bacteria in *Drosophila* improved the understanding of the impact of chlortetracycline and the result of eliminating bacteria. An alternative method of bacterial depletion in mosquitoes would also create a greater understanding of the role of the microbes found within this insect.

6.1 The study of insect-microbe interactions in *Drosophila melanogaster* and *Aedes aegypti*

The study of microbes within insects has become increasingly popular for several reasons; to increase the understanding of the insect-microbe interaction as a tool for the control of insect populations, and to increase the understanding of the role of gut microbes in humans. The results gained from the experiments using *Drosophila melanogaster* and *Aedes aegypti* during this project can be used for different applications.

Firstly, *Drosophila* can be used as a model for determining the role of gut microbes in humans. The experiments with *Drosophila* have improved our understanding of the role of bacteria in the nutrition and metabolism of the fly. *Drosophila* is a suitable model to use as it has a very low diversity of bacteria in the gut (with the flies on the York diet consisting of mainly *Acetobacter* species), can be easily manipulated, the environment and diet can be tightly controlled, the whole genome has been sequenced (Adams *et al*., 2000) and mutant flies can be produced with relative ease compared with other insects.

In mice, mutants have been created to understand the relationship between the human gut and *Bacteroides* (Salyers and Pajeau 1989) and the role of bacteria in inflammatory diseases (Boivin *et al*., 1997). Therefore, the creation of *Drosophila* mutants will: 1) identify the relationship between Acetobacter/*Lactobacillus* and the *Drosophila* host and their role in metabolism and growth and 2) identify the role of microbes in immunity and disease. Mutant *Drosophila* may be useful in identifying the reason for the extension in development time when bacteria are depleted in the gut. This could be the result of

changes in the insulin/insulin-like growth factor signalling which has previously been shown to control the time to pupation and reach the critical weight for pupation (Edgar, 2006; Bakker, 1959; Beadle *et* al., 1938; Moed *et al*., 1999; Robertson, 1963). These changes can be confirmed using qRT-PCR and microarray data with first instar, second instar, third instar, prepupa and pupa. Mutants could also be used to confirm the role of genes through inactivation and comparing the development of wild-type flies. Furthermore, the use of mutants could improve the understanding of the relationship between the host and gut bacteria and their role in the *Drosophila* immune system where a reduction in antimicrobial peptide gene expression has been demonstrated in this thesis and by Ren *et al* (2007) in bacteria depleted flies.

Another major advantage of using Drosophila to examine the role of gut microbes is the differences in response to diet. The difference was emphasised in this thesis, by Ren *et al* (2007) and Brummel *et al* (2004). As aging and metabolic studies using *Drosophila* have shown, diet impacts the fecundity and life-span of the fly (Chapter 1, Piper *et al*., 2005). The ratio of protein and carbohydrates have been demonstrated to be responsible for the changes in life-span (1:16 protein:carbohydrate ratio) and fecundity (1:2 protein:carbohydrate ratio) and not dietary restriction (Lee *et al*., 2008 and Ja *et al*., 2009). Feeding behaviour was analysed by Ja *et al* (2009) to eliminate the difference in feeding rates for the reason for changes during dietary restriction. One advantage with *Drosophila*, is that the development of the CAFE (Capillary feeder) assay by Ja *et al* (2007) has enabled the quantification and the monitor of feeding behaviour of the flies. This technique could be used with antibiotic treated and dechorionated flies to determine whether the nutrition of the fly and extension in development time is of the result of bacterial depletion or changes in feeding behaviour. The quantification of food intake will allow for an approximation of the concentration of chlortetracycline that the flies are consuming and not just exposed to. Furthermore, the difference in the response to diet by *Drosophila* will enable the study of microbe and diet interactions.

With mosquitoes (mainly with *Anopheles gambiae*), research with bacterial symbionts has concentrated on a method of controlling the insect populations rather than using the insect as a model for human health. The increasing interest in using bacteria to control insect pests is due to the limitations of other methods for reducing the mosquito

populations or prevalence of vector related diseases. An example is to genetically engineer resident microbes to express anti-plasmodium factors (Riehle *et al*., 2007) and to identify resident microbes which reduce the susceptibility to plasmodium infection (Dong *et al*., 2009).

The relative ease (not as easy as *Drosophila*) to genetically transform *Aedes aegypti* could allow for potential methods to 1) gain increasing knowledge regarding the relationship between the mosquito host and the bacterial species found within the host and 2) manipulate the insect host to disrupt the beneficial relationship between the host and bacteria, allowing for a method of controlling the population of the insect.

The identification of bacteria found within *Aedes aegypti* could allow for experiments involving the rearing of axenic mosquitoes. Infection studies with the identified bacterial species including *Serratia* have the potential to inform us about the role of bacteria in mosquitoes which would allow for manipulation of certain species that may play a key role in sugar and blood metabolism. Targeting these bacteria could limit the metabolism of key nutrients, leading to the malaise of the insect host and eventual death, an effective method of controlling insect vectors of human diseases.

6.2 The use of antibiotics and bacterial depletion

The experiments using chlortetracycline to deplete bacterial communities within *Drosophila melanogaster* and *Aedes aegypti* have demonstrated that the two insects and their symbionts respond differently to the antibiotic treatment. *Drosophila melanogaster* have been confirmed to not entirely depend on their gut symbionts, this was also observed by Brummel *et al* (2004), Ren *et al* (2007) and Bakula (1969). This response is very different to aphids, where the elimination of its symbionts significantly reduces the size and fecundity of the insect (Houk and Griffiths, 1980; Mittler, 1971; Sasaki *et al*., 1991). In fact, *Drosophila* appears to have an improved survival and no reduction in fecundity and size.

Unlike *Drosophila,* chlortetracycline treatment of *Aedes aegypti* did not eliminate all the bacteria within the insect (still containing several thousand colony forming units at 100 μg ml^{-1}) suggesting that the use of chlortetracycline may not be a useful method to eliminate all the bacterial population within *Aedes aegypti*. A higher concentration of

chlortetracycline may have reduced the numbers further, however, a combination of antibiotics or rearing axenically (Lang *et* al., 1972) are other methods that could be used. As deleterious impacts of using an antibiotic have been identified during this project, it may be preferable to rear the mosquitoes axenically rather than using a combination of antibiotics.

From this thesis it is demonstrated that chlortetracycline and dechorionation have produced different results, with the performance traits including fecundity, life-span and metabolism. A significant impact of dechorionation with immune gene expression was observed in this thesis; however, the impact of chlortetracycline on the gene expression was not conducted. This experiment would prove an important tool in explaining the differences between the 2 methods of dechorionation and chlortetracycline treatment and gain more information regarding the impact of chlortetracycline and an insight into the result of using certain methods of bacterial depletion.

The elimination of entire populations of bacteria within the insect host is a crude method of developing our understanding of the role of microbes within the host. There are several considerations to be made when planning to eliminate bacteria as described previously, total elimination of bacteria can lead to a general malaise of the insect. Furthermore, the complete removal of bacteria provides information regarding the impact of eliminating the entire community but does not provide information regarding the role of each species of bacteria. Introduction studies with axenic flies and exposure to bacterial symbionts such as *Acetobacter* will develop the understanding of the function of individual species within the gut. Moreover, these experiments could be developed further by introducing a mix of bacterial symbionts to determine how certain bacterial communities impact the insect host. Infection studies with symbionts and pathogens could also provide information regarding the role of these symbionts in the protection of the host against pathogens such as *Serratia* and *Enterococcus* (Flyg *et al*., 1980; Basset *et al*., 2000).

6.3 The treatment of insects with chlortetracycline and its implications for Oxitec Ltd

This thesis has emphasized the implications of using antibiotics to deplete bacteria. As results have shown, chlortetracycline has a greater deleterious impact on *Drosophila* when compared with dechorionation. This has highlighted the implications of using chlortetracycline to conduct experiments where bacterial depletion is required or for the use of tetracycline on/off genetic expression systems. Whereas a lower number of deleterious impacts of chlortetracycline were observed on the performance of *Aedes aegypti* in comparison with *Drosophila*, other insects may respond in a similar manner to *Drosophila*. Therefore, this implication should be acknowledged by companies who regularly use antibiotics such as Oxitec Ltd.

For Oxitec Ltd, it is crucial to ensure that when comparisons between the performance of wild-type and transgenic insects are made, that a chlortetracycline treated wild-type and transgenic be included in the analysis to differentiate between the deleterious impacts of the insertion of a transgene and the treatment with chlortetracycline.

The treatment of small insects with chlortetracycline by Oxitec Ltd, does need to be at a concentration which is high enough to suppress the transgene but low enough to limit the deleterious impact on the insect and bacterial symbionts. As with the experiments conducted in this thesis, it was shown that 10 μg ml^{-1} of chlortetracycline was the advised quantity required for LA513 to suppress the bi-sex lethal gene. This concentration was lower than 30-100 μg ml^{-1} where deleterious impacts were observed on wild-type mosquitoes. One important fitness trait which was addressed was the life-span of the insect host when treated with chlortetracycline; this experiment suggested that high concentrations (30-100 μg ml^{-1}) significantly reduced the life-span of the mosquitoes (Mexican only).

Life-span is one of the most important performance measurements for Oxitec Ltd as they will require the mosquito to live long enough to mate with wild female mosquitoes. If the life-span is significantly reduced then the release of transgenic mosquitoes to control mosquito populations will prove to be unsuccessful. Another important performance trait is fecundity, experiments with *Drosophila melanogaster* did suggest that

chlortetracycline did significantly impact the fecundity of female flies. Time limitations prevented this experiment to be conducted with mosquitoes, however, it may prove a crucial experiment for the assessment of transgenic mosquitoes.

On the other hand, the deleterious impact of the antibiotic on the mosquito may have been proved to not significantly alter the fitness of the mosquito to the point where it is unable to perform as well as mosquitoes in the wild. The recent successful field studies conducted by Oxitec Ltd with LA513 in Grand Cayman did show that the transgenic population successfully mated with mosquitoes in the wild and significantly reduced the population of the dengue carrying *Aedes aegypti* (http://www.oxitec.com/wp-content/uploads/2010/11/Oxitec-MRCU-press-release.pdf, www.newscientist.com).

Despite the fact that chlortetracycline-resistant bacteria in mosquitoes have limited the deleterious impact of the antibiotic on the mosquito, with *Drosophila* the deleterious impact of antibiotic treatment was an example where chlortetracycline could substantially impact the insect host. Oxitec Ltd is also developing RIDL® in other insects including the Mediterranean fruit fly, Mexican fruit fly and the olive fly. The olive fly (*Bactrocera oleae*) has been demonstrated to have a major symbiont, *Acetobacter* (Kounatidis *et al*., 2009). This symbiont was also the major symbiont of the *Drosophila* used in the studies described in this thesis, therefore could the treatment of chlortetracycline remove the major symbiont of the olive fly and impact the performance of the insect? If so, it could prove more difficult for a RIDL® insect to be produced and compete successfully with insects in the wild. In addition, *Drosophila melanogaster* could be used as model for investigating the impact of removing the major symbionts of *Lactobacillus* and *Acetobacter* from the host. This research could improve the understanding of the role of these symbionts which could provide methods of pest control with insects where their major symbionts are *Lactobacillus* and *Acetobacter*. If reminiscent of *Drosophila*, the olive fly could undergo changes in nutrition, metabolism and development and these symbionts could be used as a method to control these agricultural pests.

As described previously and during this thesis, a difference in response to chlortetracycline was observed with mosquitoes and in *Drosophila*. This result should be considered by groups including Oxitec Ltd when treating other insects with

chlortetracycline, it would be advised to determine the impact of this antibiotic on the performance on the insect before continuing with experiments and techniques such as RIDL®. However, bacterial depletion in the 2 insects tested during this project were not affected as much as insects with a tight interaction such as aphids and tsetse flies, the use of antibiotics should be decided with caution as an insect may be suitable for RIDL® but the fitness may be compromised through the use of chlortetracycline.

If the fitness of the insect was significantly compromised due to bacterial depletion through chlortetracycline treatment, the identification and culture of the beneficial symbionts could allow for the re-introduction of these symbionts into the insect host. Niyazi *et al* (2004) re-introduced two symbionts (*Enterobacter agglomerans* and *Klebsiella pneumoniae*) into Mediterranean fruit flies through feeding. This introduction improved their survival and mating numbers, highlighting the importance of the gut microbes in the fitness of the host (Niyazi *et al*., 2004). This re-introduction with insects used by Oxitec Ltd would be difficult as it would not be possible to provide the bacteria in the food which is also supplemented with an antibiotic. However, when rearing transgenic mosquitoes such as the strain LA513, chlortetracycline treatment is only required at the larval to pupal stage and not at the adult stage. Therefore, bacteria could be re-introduced into the adult male populations before they are released into the wild to mate with females.

6.4 The use of chlortetracycline, antibiotic resistance, the microbial community and its implications for Oxitec Ltd

Species of bacteria were still present within *Aedes aegypti* treated with 0-100 µg ml^{-1} of chlortetracycline, suggesting the presence of chlortetracycline resistant strains within the mosquitoes. To ensure these bacteria were chlortetracycline resistant the bacteria were also cultured overnight in nutrient broth +/- chlortetracycline. Further methods to detect chlortetracycline resistance by identifying the presence of different groups of tetracycline resistance genes including Tet(A), Tet(M) and Tet(O) using conventional polymerase chain reaction or multiplex PCR (Ng *et al*., 2001) would improve the understanding of the prevalence of tetracycline resistance.

Chlortetracycline resistance was observed in mosquitoes reared at Oxitec Ltd and with a population with no prior contact with chlortetracycline in the laboratory. This suggests

that wild populations of mosquitoes may already contain antibiotic resistant strains. The exposure to this antibiotic in the laboratory could promote the establishment of a gut microbiota dominated by bacterial populations that are already resistant to the antibiotic and therefore, treatment could change the overall diversity within the insect gut which was observed in adult male mosquitoes in Chapter 5.

Not only should chlortetracycline be considered as an antibiotic, but also as a signalling molecule. Antibiotics play a role in the communication between bacteria within complex bacterial communities (Yim *et al*., 2007). The experiments with *Aedes aegypti* did not show a change in the number of bacteria at treatments of low concentrations of chlortetracycline. However, the community/diversity may still have altered through the introduction of this signalling molecule resulting in changes in the communication between bacteria within the insect gut (Yim *et al*., 2007). The introduction of these antibiotics could alter the maintenance of the community (Yim *et al*., 2007). Celli and Trieu-Cuot (1998) demonstrated that exposure to tetracycline enhanced horizontal gene transfer. Therefore, Oxitec Ltd should consider the environmental impacts when using large quantities of chlortetracycline, especially when using chlortetracycline water when rearing mosquito larvae. Such volumes of chlortetracycline rearing water could be substantial when mass rearing mosquitoes. As demonstrated through the over-use of antibiotics in agriculture (Kümmerer *et al*., 2004) the release of the chlortetracycline into the water systems could increase chlortetracycline resistant bacteria within the environment, and the persistence of microbes with chlortetracycline resistance. Moreover, high usage of the antibiotic could result in increasing levels of gene transfer between bacterial communities and an alteration in the communication between bacterial communities found within the environment.

To limit the release of chlortetracycline into the environment, Oxitec Ltd should consider treating the water with UV light and high temperatures, 2 conditions which chlortetracycline is sensitive. In addition, limit the exposure of wild-type mosquitoes at Oxitec Ltd. These insects should be kept separately from the transgenic mosquitoes and the rearing equipment should be thoroughly decontaminated to prevent unnecessary exposure to chlortetracycline and to chlortetracycline-resistant bacteria found within the mosquitoes.

Not only should the impact on the host and environment be considered but also the employees who work with the insects. The antibiotic-resistant bacteria could be exposed to employees and if an infection was to occur, then treatment with antibiotics could prove to be more difficult. Furthermore, the bacteria carrying chlortetracycline resistance genes within the mosquitoes could transfer chlortetracycline resistant genes to pathogens. The development of chlortetracycline resistance could reduce the ability to treat infections within animals and humans if exposed to these mosquitoes in the laboratory and if/when they are released into the wild. If Oxitec Ltd were to release mosquitoes carrying bacteria with the tetracycline resistance gene, these bacteria could be introduced into the environment and create problems with treating infections.

Bacteria identified in *Aedes aegypti* have also been implicated in human diseases. One of the bacteria identified in *Aedes aegypti* was *Chryseobacterium meningosepticum/Elizabethkingia meningiseptica*. This continual identification of this bacterium by myself and by others (Dong *et al*., 2009) suggests that it is not a pathogen of mosquitoes, however, this bacterium has been documented to cause meningitis and sepsis in infants and immunocompromised individuals in Taiwan (Chui *et al*., 2000). In addition, *Serratia marcescens* found in *Aedes aegypti* has been identified as an opportunistic pathogen infecting and causing death of individuals during an epidemic and also carrying antibiotic resistance (Schaberg *et al*., 1976). The presence of pathogens and the suggestion that these bacteria are antibiotic resistant highlights the importance of ensuring that exposed individuals are prevented from being infected with these bacteria.

6.5 Overall conclusion

In conclusion, the work in this thesis has revealed the importance of host-symbiont interactions of *Drosophila melanogaster* and *Aedes aegypti*. The deleterious effects of chlortetracycline treatment emphasise the implications of using antibiotics to control genetic systems for insect pest management and as a method to determine the impact of bacterial depletion. Egg dechorionation with *Drosophila melanogaster* has provided an alternative tool to evaluate bacterial depletion and to determine the impact of chlortetracycline treatment.

7.0 Appendix

7.1. Bacterial identification of culturable bacteria in *Drosophila* treated with and without chlortetracycline and the rearing food

Table: 7.1. Bacterial identities from 16S rRNA gene analysis in control *Drosophila melanogaster* and the food used to rear the insects.

Larvae	Food
EU096229.1 - *Acetobacter pomorum* strain EW816 16S ribosomal RNA gene, partial sequence (97%)	EU096229.1 - *Acetobacter pomorum* strain EW816 16S ribosomal RNA gene, partial sequence (97%)
GQ359863.1 - *Acetobacter* sp. 6-C-2 16S ribosomal RNA gene, partial sequence (97%)	GQ359863.1 - *Acetobacter* sp. 6-C-2 16S ribosomal RNA gene, partial sequence (98%)
EU096229.1 - *Acetobacter pomorum* strain EW816 16S ribosomal RNA gene, partial sequence (98%)	EU096229.1 - *Acetobacter pomorum* strain EW816 16S ribosomal RNA gene, partial sequence (94%)
FN429068.1 - *Acetobacter pasteurianus* partial 16S rRNA gene, strain SX461 (98%)	FJ227313.1 - *Acetobacter pasteurianus* strain bh12 16S ribosomal RNA gene, partial sequence (98%)
GQ359863.1 - *Acetobacter* sp. 6-C-2 16S ribosomal RNA gene, partial sequence (98%)	FN429074.1 - *Acetobacter pasteurianus* partial 16S rRNA gene, strain ZJ362 (99%)
FN429068.1 - *Acetobacter* pasteurianus partial 16S rRNA gene, strain SX461 (98%)	EU807752.1 - *Lactobacillus plantarum* strain ML5-1 16S ribosomal RNA gene, partial sequence (98%)
FN429068.1 - *Acetobacter pasteurianus* partial 16S rRNA gene, strain SX461 (97%)	DQ981282.1 - Uncultured bacterium clone thom_k16 16S ribosomal RNA gene, partial sequence (99%)
FN429068.1 - *Acetobacter pasteurianus* partial 16S rRNA gene, strain SX461 (99%)	NR_025512.1 - *Acetobacter cerevisiae* strain LMG 1625 16S ribosomal RNA, partial sequence (98%)
EU096229.1 - *Acetobacter pomorum* strain EW816 16S ribosomal RNA gene, partial sequence (98%)	HM562995.1 - *Acetobacter cerevisiae* strain T0-PCP23 16S ribosomal RNA gene, partial sequence (98%)
FN429068.1 - *Acetobacter pasteurianus* partial 16S rRNA gene, strain SX461 (99%)	FN429068.1 - *Acetobacter pasteurianus* partial 16S rRNA gene, strain SX461 (98%)
GQ359863.1 - *Acetobacter* sp. 6-C-2 16S ribosomal RNA gene, partial sequence (98%)	FN429068.1 - *Acetobacter pasteurianus* partial 16S rRNA gene, strain SX461 (98%)
EU096229.1 - *Acetobacter pomorum* strain EW816 16S ribosomal RNA gene, partial sequence (99%)	FJ227313.1 - *Acetobacter pasteurianus* strain bh12 16S ribosomal RNA gene, partial sequence (98%)
EU096229.1 - *Acetobacter pomorum* strain EW816 16S ribosomal RNA gene, partial sequence (98%)	EU096229.1 - *Acetobacter pomorum* strain EW816 16S ribosomal RNA gene, partial sequence (97%)
FN429068.1 - *Acetobacter pasteurianus* partial 16S rRNA gene, strain SX461 (98%)	GQ359863.1 - *Acetobacter* sp. 6-C-2 16S ribosomal RNA gene, partial sequence (99%)
EU096229.1 - *Acetobacter pomorum* strain EW816 16S ribosomal RNA gene, partial sequence (99%)	
NR_025512.1 - *Acetobacter cerevisiae* strain LMG 1625 16S ribosomal RNA, partial sequence (97%)	
FN429068.1 - *Acetobacter pasteurianus* partial 16S rRNA gene, strain SX461 (98%)	
DQ981281.1 - Uncultured bacterium clone thom_c06 16S ribosomal RNA gene, partial sequence (98%)	

FJ227313.1 - *Acetobacter pasteurianus* strain bh12 16S ribosomal RNA gene, partial sequence (97%)	
NR_025512.1 - *Acetobacter cerevisiae* strain LMG 1625 16S ribosomal RNA, partial sequence (99%)	
EU096229.1 - *Acetobacter pomorum* strain EW816 16S ribosomal RNA gene, partial sequence (98%)	
GQ359863.1 - *Acetobacter* sp. 6-C-2 16S ribosomal RNA gene, partial sequence (98%)	
GQ359863.1 - *Acetobacter* sp. 6-C-2 16S ribosomal RNA gene, partial sequence (99%)	
GQ359863.1 - *Acetobacter* sp. 6-C-2 16S ribosomal RNA gene, partial sequence (99%)	
FN429074.1 - *Acetobacter pasteurianus* partial 16S rRNA gene, strain ZJ362 (97%)	
EU096229.1 - *Acetobacter pomorum* strain EW816 16S ribosomal RNA gene, partial sequence (97%)	
FJ227313.1 - *Acetobacter pasteurianus* strain bh12 16S ribosomal RNA gene, partial sequence (99%)	
GQ359863.1 - *Acetobacter* sp. 6-C-2 16S ribosomal RNA gene, partial sequence (96%)	
GQ359863.1- *Acetobacter* sp. 6-C-2 16S ribosomal RNA gene, partial sequence (99%)	
GQ359863.1- *Acetobacter* sp. 6-C-2 16S ribosomal RNA gene, partial sequence (99%)	
EU096229.1- *Acetobacter pomorum* strain EW816 16S ribosomal RNA gene, partial sequence (99%)	
GQ359863.1- *Acetobacter* sp. 6-C-2 16S ribosomal RNA gene, partial sequence (98%)	
GQ359863.1- *Acetobacter* sp. 6-C-2 16S ribosomal RNA gene, partial sequence (99%)	

Table: 7.2. Bacterial identities from 16S rRNA gene analysis in *Drosophila melanogaster* and the food used to rear the insects at 50 µg ml^{-1}.

Larvae	Food
EU096229.1 - *Acetobacter pomorum* strain EW816 16S ribosomal RNA gene, partial sequence (98%)	GQ359860.1 - *Lactobacillus* sp. 0-C-2 16S ribosomal RNA gene, partial sequence (96%)
EU096229.1 - *Acetobacter pomorum* strain EW816 16S ribosomal RNA gene, partial sequence (99%)	EU096229.1 - *Acetobacter pomorum* strain EW816 16S ribosomal RNA gene, partial sequence (99%)
EU096229.1 - *Acetobacter pomorum* strain EW816 16S ribosomal RNA gene, partial sequence (98%)	GQ359863.1 - *Acetobacter* sp. 6-C-2 16S ribosomal RNA gene, partial sequence (97%)
GQ359863.1 - *Acetobacter* sp. 6-C-2 16S ribosomal RNA gene, partial sequence (97%)	HM462422.1 - *Lactobacillus plantarum* strain ChR-I-str20 16S ribosomal RNA gene, partial sequence (99%)
GQ359863.1 - *Acetobacter* sp. 6-C-2 16S ribosomal RNA gene, partial sequence (96%)	FN429068.1 - *Acetobacter pasteurianus* partial 16S rRNA gene, strain SX461 (98%)
GQ359863.1 - *Acetobacter* sp. 6-C-2 16S ribosomal RNA gene, partial sequence (98%)	FJ227313.1 - *Acetobacter pasteurianus* strain bh12 16S ribosomal RNA gene, partial sequence (98%)
GQ359863.1 - *Acetobacter* sp. 6-C-2 16S ribosomal RNA gene, partial sequence (97%)	GQ359860.1 - *Lactobacillus* sp. 0-C-2 16S ribosomal RNA gene, partial sequence (99%)
GQ359863.1 - *Acetobacter* sp. 6-C-2 16S ribosomal RNA gene, partial sequence (98%)	FN429068.1 - *Acetobacter pasteurianus* partial 16S rRNA gene, strain SX461 (96%)
FN429068.1 - *Acetobacter pasteurianus* partial 16S rRNA gene, strain SX461 (99%)	EU096229.1 - *Acetobacter pomorum* strain EW816 16S ribosomal RNA gene, partial sequence (99%)
EU096229.1 - *Acetobacter pomorum* strain EW816 16S ribosomal RNA gene, partial sequence (97%)	GQ359863.1 - *Acetobacter* sp. 6-C-2 16S ribosomal RNA gene, partial sequence (98%)
FJ227313.1 - *Acetobacter pasteurianus* strain bh12 16S ribosomal RNA gene, partial sequence (98%)	EU096229.1 - *Acetobacter pomorum* strain EW816 16S ribosomal RNA gene, partial sequence (98%)
FN429068.1 - *Acetobacter pasteurianus* partial 16S rRNA gene, strain SX461 (99%)	FJ227313.1 - *Acetobacter pasteurianus* strain bh12 16S ribosomal RNA gene, partial sequence (98%)
GU253891.1 - *Lactobacillus pentosus* strain N3 16S ribosomal RNA gene, partial sequence (98%)	GQ359860.1 - *Lactobacillus* sp. 0-C-2 16S ribosomal RNA gene, partial sequence (98%)
HM449702.1 - *Micrococcus luteus* strain PCSB6 16S ribosomal RNA gene, partial sequence (97%)	GQ359863.1 - *Acetobacter* sp. 6-C-2 16S ribosomal RNA gene, partial sequence (98%)
GQ359863.1 - *Acetobacter* sp. 6-C-2 16S ribosomal RNA gene, partial sequence (98%)	FJ227313.1 - *Acetobacter pasteurianus* strain bh12 16S ribosomal RNA gene, partial sequence (97%)
GQ359863.1 - *Acetobacter* sp. 6-C-2 16S ribosomal RNA gene, partial sequence (98%)	
EU096229.1 - *Acetobacter pomorum* strain EW816 16S ribosomal RNA gene, partial sequence (98%)	
GU369767.1 - *Lactobacillus brevis* strain JS-7-2 16S ribosomal RNA gene, partial sequence (99%)	
EU096229.1 - *Acetobacter pomorum* strain EW816 16S ribosomal RNA gene, partial sequence (98%)	
EU096229.1 - *Acetobacter pomorum* strain EW816 16S ribosomal RNA gene, partial sequence (98%)	
GQ359863.1 - *Acetobacter* sp. 6-C-2 16S ribosomal RNA gene, partial sequence (99%)	
HM218620.1 - *Acetobacter malorum* strain NM156-4 16S ribosomal RNA gene, partial sequence (97%)	
FJ227317.1 - *Lactobacillus brevis* strain b4 16S ribosomal RNA gene, partial sequence (98%)	
GQ359863.1 - *Acetobacter* sp. 6-C-2 16S ribosomal RNA gene, partial sequence (98%)	
GQ359863.1 - *Acetobacter* sp. 6-C-2 16S ribosomal RNA gene, partial sequence (98%)	
AB494721.1 - *Lactobacillus plantarum* gene for 16S ribosomal RNA, partial sequence, strain: KL23 (99%)	
GQ359863.1 - *Acetobacter* sp. 6-C-2 16S ribosomal RNA gene, partial sequence (99%)	
GU253891.1 - *Lactobacillus pentosus* strain N3 16S ribosomal RNA gene, partial sequence (98%)	
GQ359863.1 - *Acetobacter* sp. 6-C-2 16S ribosomal RNA gene, partial sequence (98%)	
EU096229.1 - *Acetobacter pomorum* strain EW816 16S ribosomal RNA gene, partial sequence (97%)	

Table: 7.3. Bacterial identities from 16S rRNA gene analysis in *Drosophila melanogaster* on nutrient agar plates supplemented with 50 µg ml^{-1}.

Control flies	*Drosophila* treated with 50 µg ml^{-1} of chlortetracycline
FJ227313.1 - *Acetobacter pasteurianus* strain bh12 16S ribosomal RNA gene, partial sequence (98%)	HM218620.1 - *Acetobacter malorum* strain NM156-4 16S ribosomal RNA gene, partial sequence (98%)
GQ359863.1 - *Acetobacter* sp. 6-C-2 16S ribosomal RNA gene, partial sequence (98%)	AB510752.1 - *Lactobacillus plantarum* gene for 16S ribosomal RNA, partial sequence, strain: I041715 (97%)
EU096229.1 - *Acetobacter pomorum* strain EW816 16S ribosomal RNA gene, partial sequence (98%)	HM218620.1 - *Acetobacter malorum* strain NM156-4 16S ribosomal RNA gene, partial sequence (98%)
EU096229.1 - *Acetobacter pomorum* strain EW816 16S ribosomal RNA gene, partial sequence (99%)	GQ359863.1 - *Acetobacter* sp. 6-C-2 16S ribosomal RNA gene, partial sequence (98%)
GQ359863.1 - *Acetobacter* sp. 6-C-2 16S ribosomal RNA gene, partial sequence (98%)	GQ359860.1 - *Lactobacillus* sp. 0-C-2 16S ribosomal RNA gene, partial sequence (98%)
FN429068.1 - *Acetobacter pasteurianus* partial 16S rRNA gene, strain SX461 (97%)	FJ462692.1 - *Lactobacillus* sp. strain E-1 16S ribosomal RNA gene, partial sequence (99%)
FN429074.1 - *Acetobacter pasteurianus* partial 16S rRNA gene, strain ZJ362 (96%)	EU096229.1 - *Acetobacter pomorum* strain EW816 16S ribosomal RNA gene, partial sequence (99%)
GQ359863.1 - *Acetobacter* sp. 6-C-2 16S ribosomal RNA gene, partial sequence (98%)	FJ227313.1 - *Acetobacter pasteurianus* strain bh12 16S ribosomal RNA gene, partial sequence (96%)
FJ751793.1 - *Lactobacillus plantarum* strain DSPV 354T 16S ribosomal RNA gene, partial sequence (99%)	EU789400.1 - *Lactobacillus plantarum* strain M01210 16S ribosomal RNA gene, partial sequence (97%)
HM218620.1 - *Acetobacter malorum* strain NM156-4 16S ribosomal RNA gene, partial sequence (99%)	HQ286594.1 - *Lactobacillus plantarum* strain H2 16S ribosomal RNA gene, partial sequence (98%)
HM562999.1 - *Lactobacillus plantarum* strain T30-PCM02 16S ribosomal RNA gene, partial sequence (99%)	FJ751793.1 - *Lactobacillus plantarum* strain DSPV 354T 16S ribosomal RNA gene, partial sequence (98%)
AB368905.1 - *Lactobacillus plantarum* gene for 16S rRNA, partial sequence, strain: T3-10 (99%)	GQ359863.1 - *Acetobacter* sp. 6-C-2 16S ribosomal RNA gene, partial sequence (99%)
FJ227313.1 - *Acetobacter pasteurianus* strain bh12 16S ribosomal RNA gene, partial sequence (97%)	GQ359863.1 - *Acetobacter* sp. 6-C-2 16S ribosomal RNA gene, partial sequence (98%)
FN429068.1 - *Acetobacter pasteurianus* partial 16S rRNA gene, strain SX461 (99%)	EU096229.1 - *Acetobacter pomorum* strain EW816 16S ribosomal RNA gene, partial sequence (99%)
FJ227313.1 - *Acetobacter pasteurianus* strain bh12 16S ribosomal RNA gene, partial sequence (98%)	EU096229.1 - *Acetobacter pomorum* strain EW816 16S ribosomal RNA gene, partial sequence (99%)
GQ359863.1 - *Acetobacter* sp. 6-C-2 16S ribosomal RNA gene, partial sequence (99%)	GQ359863.1 - *Acetobacter* sp. 6-C-2 16S ribosomal RNA gene, partial sequence (98%)
EU096229.1 - *Acetobacter pomorum* strain EW816 16S ribosomal RNA gene, partial sequence (98%)	GQ359863.1 - *Acetobacter* sp. 6-C-2 16S ribosomal RNA gene, partial sequence (98%)
GU253891.1 - *Lactobacillus pentosus* strain N3 16S ribosomal RNA gene, partial sequence (98%)	GQ359863.1 - *Acetobacter* sp. 6-C-2 16S ribosomal RNA gene, partial sequence (98%)
GQ359863.1 - *Acetobacter* sp. 6-C-2 16S ribosomal RNA gene, partial sequence (98%)	EU096229.1 - *Acetobacter pomorum* strain EW816 16S ribosomal RNA gene, partial sequence (97%)
GQ359863.1 - *Acetobacter* sp. 6-C-2 16S ribosomal RNA gene, partial sequence (98%)	FJ227313.1 - *Acetobacter pasteurianus* strain bh12 16S ribosomal RNA gene, partial sequence (98%)
GQ359863.1 - *Acetobacter* sp. 6-C-2 16S ribosomal RNA gene, partial sequence (98%)	EU096229.1 - *Acetobacter pomorum* strain EW816 16S ribosomal RNA gene, partial sequence (98%)
GQ359863.1 - *Acetobacter* sp. 6-C-2 16S ribosomal RNA gene, partial sequence (99%)	EU096229.1 - *Acetobacter pomorum* strain EW816 16S ribosomal RNA gene, partial sequence (98%)
	FN429068.1 - *Acetobacter pasteurianus* partial 16S rRNA gene, strain SX461 (96%)
	EU096229.1 - *Acetobacter pomorum* strain EW816 16S ribosomal RNA gene, partial sequence (98%)
	FJ227313.1 - *Acetobacter pasteurianus* strain bh12 16S ribosomal RNA gene, partial sequence (98%)

7.2. Bacterial identification using 454 pyrosequencing of *Drosophila* guts treated with and without chlortetracycline

Table: 7.4. 454 Sequencing results of bacterial identities for control *Drosophila* guts

Number of reads	% Identity	Accession Number	Identity	Lineage
10909	100	CP001161	*Buchnera aphidicola* str. 5A (*Acyrthosiphon pisum*), complete genome	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae*; Buchnera.*
2717	100	HM080051	Uncultured Actinomycetales bacterium clone E153F02 16S ribosomal RNA gene, partial sequence	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales;
2020	100	NR_025512	*Acetobacter cerevisiae* strain LMG 1625 16S ribosomal RNA, partial sequence >gi|23892796|emb|AJ419843.1| Acetobacter cerevisiae 16S rRNA gene, strain LMG 1625	Bacteria; Proteobacteria; Alphaproteobacteria; Rhodospirillales; Acetobacteraceae*; Acetobacter*.
2020	99.6	GQ477828	Uncultured bacterium clone MS-123 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
1715	100	HM027569	*Bacillus subtilis* strain zj2008 16S ribosomal RNA gene, partial sequence	Bacteria; Firmicutes; Bacillales; *Bacillaceae*; *Bacillus*.
312	99.6	AM087199	*Asticcacaulis benevestitus* partial 16S rRNA gene, type strain Z-0023T	Bacteria; Proteobacteria; Alphaproteobacteria;Caulobacterales; Caulobacteraceae; *Asticcacaulis*.
212	100	AB461807	*Acinetobacter* sp. M522 gene for 16S rRNA, partial sequence, strain: M522	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Moraxellaceae*; Acinetobacte*r.
186	100	EU096229	*Acetobacter pomorum* strain EW816 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Alphaproteobacteria; Rhodospirillales; Acetobacteraceae; *Acetobacter*.
	100	AB308058	*Acetobacter pasteurianus* gene for 16S ribosomal RNA, complete sequence	Bacteria; Proteobacteria; Alphaproteobacteria; Rhodospirillales; Acetobacteraceae; *Acetobacter.*
171	99.6	AJ318114	Uncultured gamma proteobacterium 16S rRNA gene, clone BIci4	Bacteria; Proteobacteria; Gammaproteobacteria; environmental
82	100	AB050446	*Spiroplasma* sp. YR-2 gene for 16S rRNA, partial sequence	Bacteria; Tenericutes; Mollicutes; Entomoplasmatales; Spiroplasmataceae; *Spiroplasma*.
64	100	HM344691	Uncultured bacterium clone ncd1060g01c1 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples
63	100	EF433462	*Devosia* sp. IPL18 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Alphaproteobacteria;Rhizobiales; Hyphomicrobiaceae; *Devosia*
57	98.9	AY673373	Streptomycetaceae bacterium Ellin7207 16S ribosomal RNA gene, partial sequence	Bacteria; Actinobacteria; Actinobacteridae;Actinomycetales; Streptomycineae; Streptomycetaceae
55	96.0	AJ247194	*Asticcacaulis excentricus* partial 16S rRNA gene for 16S ribosomal RNA, strain DSM 4724(T)	Bacteria; Proteobacteria; Alphaproteobacteria; Caulobacterales;Caulobacteraceae; *Asticcacaulis*.
38	100	HM334791	Uncultured bacterium clone ncd991e01c1 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
34	100	HM337834	Uncultured bacterium clone ncd1107h11c1 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
25	100	HM344642	Uncultured bacterium clone ncd1060b08c1 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.

23	99.6	GQ206310	*Wolbachia* endosymbiont of *Sogatella furcifera* clone A3H1M1 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Alphaproteobacteria; Rickettsiales;Rickettsiaceae; Wolbachieae; *Wolbachia*
21	98.9	GQ988635	Uncultured bacterium clone GI_AR_U_G0 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
20	100	AF078368	Grassland soil clone sl2_508 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
14	100	HM335477	Uncultured bacterium clone ncd1004g08c1 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
14	99.6	HM333436	Uncultured bacterium clone ncd1098f07c1 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
14	100	GU635382	Uncultured bacterium clone RW0038 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
13	98.9	FJ665195	Uncultured bacterium clone BCSAS2P1C1 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
13	100	FJ436049	*Burkholderia vietnamiensis* strain SIr-665 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Burkholderiaceae; *Burkholderia*; *Burkholderia cepacia* complex
12	99.6	HM322590	Uncultured bacterium clone ncd400a04c1 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
12	98.9	HM286801	Uncultured bacterium clone ncd634h05c1 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
11	99.3	HM278405	Uncultured bacterium clone ncd554d11c1 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
10	98.5	AF253413	*Acidocella* sp. LGS-3 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Alphaproteobacteria; Rhodospirillales; Acetobacteraceae; *Acidocella*
8	94.97	DQ413077	Uncultured bacterium clone 18 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
8	100	GU643701	Uncultured bacterium clone RW8357 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
8	99.6	HM257287	Uncultured bacterium clone ncd103d11c1 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
8	99.6	EU372971	*Kocuria* sp. E7 16S ribosomal RNA gene, partial sequence	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Micrococcaceae; *Kocuria*
8	99.2	EU776263	Uncultured bacterium clone IR_aaa03d07 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
8	98.8	FJ897521	*Pedobacter* sp. N1d-b1 16S ribosomal RNA gene, partial sequence	Bacteria; Bacteroidetes; Sphingobacteria; Sphingobacteriales;Sphingobacteriaceae; *Pedobacter*
8	100	GU644300	Uncultured bacterium clone RW8956 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
7	99.6	AB271048	*Microbacterium ginsengisoli* gene for 16S rRNA, partial sequence	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Microbacteriaceae; *Microbacterium*
7	100	EU630302	Uncultured *Actinomyces* sp. clone NST3Q1b12 16S ribosomal RNA gene, partial sequence	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Actinomycineae; Actinomycetaceae; *Actinomyces*; environmental samples
7	91.2	AF507713	Uncultured soil bacterium clone S166 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
7	88.9	FJ916286	Uncultured delta proteobacterium clone DE1C1 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Deltaproteobacteria; environmental
7	100	HM314555	Uncultured bacterium clone ncd425b11c1 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.

7	99.3	EF219646	Uncultured beta proteobacterium clone AI-1M_A05 16S gene, partial sequence	Bacteria; Proteobacteria; Betaproteobacteria; environmental
7	97.8	AM400943	Flavobacteriaceae bacterium JJ-2987 partial 16S rRNA gene, isolate JJ-2987	Bacteria; Bacteroidetes; Flavobacteria; Flavobacteriales;Flavobacteriaceae
7	100	L33977	*Sphaerotilus natans* 16S ribosomal RNA (16S rRNA)	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; *Sphaerotilus*
7	100	FJ797394	*Comamonas* sp. G4 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Betaproteobacteria;Burkholderiales; Comamonadaceae; *Comamonas*
6	98.5	AM988902	*Chryseobacterium* sp. AKB-2008-VA6 partial 16S rRNA gene, strain AKB-2008-VA6	Bacteria; Bacteroidetes; Flavobacteria; Flavobacteriales;Flavobacteriaceae; *Chryseobacterium*
6	100	GQ246690	*Janibacter* sp. M2T2B13 16S ribosomal RNA gene, partial sequence	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Intrasporangiaceae; *Janibacter*
6	97.8	AM396913	*Carnobacterium* sp. NJ-46 16S rRNA gene, strain NJ-46	Bacteria; Firmicutes; Lactobacillales; Carnobacteriaceae; *Carnobacterium*
6	98.0	AM936584	Uncultured candidate division TM7 bacterium partial 16S rRNA gene, clone EMP2	Bacteria; candidate division TM7; environmental samples
6	100	AB538964	*Methylomonas* sp. Fw12E-Y gene for 16S rRNA, partial sequence	Bacteria; Proteobacteria; Gammaproteobacteria;Methylococcale; Methylococcaceae; *Methylomonas*
6	100	HM338449	Uncultured bacterium clone ncd1119e03c1 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
5	97.8	AY332104	*Microbacterium* sp. GWS-BW-H145 16S ribosomal RNA gene, partial sequence	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Microbacteriaceae; *Microbacterium*
5	100	EU440980	*Sphingopyxis* sp. 2PR58-1 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales; Sphingomonadaceae; *Sphingopyxis*
5	92.7	HM308241	Uncultured bacterium clone ncd893f05c1 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
5	99.6	GQ891704	*Caulobacter leidyia* strain W1 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales; Sphingomonadaceae
5	100	FM176596	Uncultured Acidimicrobiales bacterium partial 16S rRNA gene, clone CL5.H403	Bacteria; Actinobacteria; Acidimicrobidae; Acidimicrobiales;
5	99.2	GQ391570	Uncultured organism clone G07-1-PTM2 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
5	100	HM343804	Uncultured bacterium clone ncd1051a04c1 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
5	90.7	FJ475509	Uncultured delta proteobacterium clone AhedenP24 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Deltaproteobacteria; environmental
5	100	HM079530	Uncultured *Lactobacillaceae* bacterium clone E105G12 16S ribosomal RNA gene, partial sequence	Bacteria; Firmicutes; Lactobacillales; *Lactobacillaceae*
5	93.0	GQ339139	Uncultured bacterium clone IS-32 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
5	78.1	EU436157	*Anaplasma phagocytophilum* genotype APV 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Alphaproteobacteria; Rickettsiales; Anaplasmataceae; *Anaplasma*; phagocytophilum group
5	97.8	HM057788	Uncultured bacterium clone A8W_114 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
5	99.6	EF061026	Uncultured Flavobacteria bacterium clone LiUU-22-10 16S ribosomal RNA gene, partial sequence	Bacteria; Bacteroidetes; Flavobacteria; environmental samples

4	98.9	EU876624	Uncultured *Flavisolibacter* sp. clone KL2-18 16S ribosomal RNA gene, partial sequence	Bacteria; Bacteroidetes;Sphingobacteria; Sphingobacteriales;Chitinophagaceae; *Flavisolibacter*; environmental samples
4	99.6	HM340514	Uncultured bacterium clone ncd1057b08c1 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
4	100	EU300429	Uncultured *Propionibacteriaceae* bacterium clone GASP-KC3W1_F10 16S ribosomal RNA gene, partial sequence	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Propionibacteriaceae; environmental samples.
4	90.4	AF047568	Candidate division OP11 clone LGd1 16S ribosomal RNA gene, partial sequence	Unknown classification
4	98.5	FJ002227	*Navicula* sp. C21 16S ribosomal RNA gene, partial sequence; chloroplast	Eukaryota; stramenopiles; Bacillariophyta;Bacillariophyceae;Bacillariophycidae; Naviculales; Naviculaceae; *Navicula*
4	99.6	GU225981	Uncultured bacterium clone 192 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
4	100	HM339571	Uncultured bacterium clone ncd1008e06c1 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
4	99.6	AB265906	Uncultured Chloroflexi bacterium gene for 16S rRNA, partial sequence, clone: UH-12	Bacteria; Chloroflexi; environmental samples
4	100	GU408433	*Leptotrichia* sp. oral taxon 215 clone HU062 16S ribosomal RNA gene, partial sequence	Bacteria; Fusobacteria; Fusobacteriales; Fusobacteriaceae; *Leptotrichia*
4	93.4	DQ829135	Uncultured proteobacterium clone DOK_NOFERT_clone140 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; environmental samples
4	98.5	GU473087	Uncultured *Janthinobacterium* sp. clone BfP10 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Oxalobacteraceae; *Janthinobacterium*; environmental samples
4	97.8	FJ200295	*Streptomyces* sp. CLS28 16S ribosomal RNA gene, partial sequence	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Streptomycineae; Streptomycetaceae; *Streptomyces*
4	82.5	GQ391003	Uncultured organism clone D01-5-410 16S ribosomal RNA gene, partial sequence	Unknown classification
4	82.3	AM040129	Uncultured delta proteobacterium partial 16S rRNA gene, clone Sylt 33	Bacteria; Proteobacteria; Deltaproteobacteria; environmental
4	94.9	FJ598048	*Lutibacter* sp. S7-2 16S ribosomal RNA gene, partial sequence	Bacteria; Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; *Lutibacter*.
4	96.0	AY922021	Uncultured Bacteroidetes bacterium clone AKYG467 16S ribosomal RNA gene, partial sequence	Bacteria; Bacteroidetes; environmental samples
4	96.3	FJ542898	Uncultured Microbacteriaceae bacterium clone A09-05G 16S ribosomal RNA gene, partial sequence	Bacteria; Actinobacteria; Actinobacteridae;Actinomycetales; Micrococcineae; Microbacteriaceae; environmental samples
4	99.6	GU415459	*Streptococcus anginosus* clone WW062 16S ribosomal RNA gene, partial sequence	Bacteria; Firmicutes; Lactobacillales; Streptococcaceae;*Streptococcus*; *Streptococcus anginosus* group
4	99.6	FM176343	Uncultured candidate division SR1 bacterium partial 16S rRNA gene, clone BF.A2	Bacteria; candidate division SR1; environmental samples
4	99.6	NR_029345	*Staphylococcus condimenti* strain F-2 16S ribosomal RNA, complete sequence >gi|2673873|emb|Y15750.1| Staphylococcus condimenti 16S rRNA gene, strain F-2 T, DSM 11674 T	Bacteria; Firmicutes; Bacillales; *Staphylococcus*
4	98.5	FN668139	Uncultured Flavobacterium sp. partial 16S rRNA gene, clone ZS-2-61	Bacteria; Bacteroidetes; Flavobacteria; Flavobacteriales;Flavobacteriaceae; Flavobacterium; environmental samples
4	99.6	GQ994674	Uncultured organism clone supp_mic9 16S ribosomal RNA gene, partial sequence	Unclassified sequences; environmental samples

4	98.1	GQ402641	Uncultured bacterium clone PW134 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
4	98.1	HM308483	Uncultured bacterium clone ncd897d02c1 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
4	94.0	DQ676299	Uncultured Chlorobi bacterium clone MVP-23 16S ribosomal RNA gene, partial sequence	Bacteria; Chlorobi; environmental samples
4	84.6	AM943204	Uncultured bacterium partial 16S rRNA gene, isolate KA-001.0.36	Bacteria; environmental samples
4	95.6	GU643527	Uncultured bacterium clone RW8183 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
4	98.9	EU982453	Uncultured bacterium clone DYB14 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
4	98.2	GU929374	Uncultured *Cellvibrio* sp. clone 45d_B7 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; *Cellvibrio*; environmental samples
4	99.6	AY655732	*Cellulomonas parahominis* strain W7387 16S ribosomal RNA gene, partial sequence	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Cellulomonadaceae; *Cellulomonas*
4	99.3	FJ859687	*Ochrobactrum pseudogrignonense* strain BIHB 340 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Alphaproteobacteria;Rhizobiales; Brucellaceae; *Ochrobactrum*
3	99.3	FM872717	Uncultured bacterium partial 16S rRNA gene, clone FB01A04	Bacteria; environmental samples
3	97.0	HM307004	Uncultured bacterium clone ncd874f01c1 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
3	99.3	DQ337515	*Microbacterium* sp. BBDP82 16S ribosomal RNA gene, partial sequence	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Microbacteriaceae; *Microbacterium*
3	94.85294 1176	FJ482172	Uncultured candidate division OD1 bacterium clone Pav-OD9 16S ribosomal RNA gene, partial sequence	Bacteria; candidate division OD1; environmental samples
3	97.8	AM990702	Rhodobacteraceae bacterium MOLA 435 partial 16S rRNA gene, culture collection MOLA:435	Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae
3	87.8	FM176343	Uncultured candidate division SR1 bacterium partial 16S rRNA gene, clone BF.A2	Bacteria; candidate division SR1; environmental samples
3	91.5	EU914095	Uncultured bacterium clone D6ENV_87G11 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
3	96.3	DQ827966	Uncultured Bacteroidetes bacterium clone DOK_BIODYN_clone272 16S ribosomal RNA gene, partial sequence	Bacteria; Bacteroidetes; environmental samples
3	99.6	GU429487	Beta proteobacterium oral taxon B96 clone ST047 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Betaproteobacteria
3	99.3	HM270636	Uncultured bacterium clone ncd268g09c1 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
3	100	FM176037	Uncultured *Rhodoblastus* sp. partial 16S rRNA gene, clone CL4.E185	Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Bradyrhizobiaceae; *Rhodoblastus*; environmental samples
3	100	AY882019	*Streptomyces yanglinensis* strain 317 16S ribosomal RNA gene, partial sequence	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Streptomycineae; Streptomycetaceae; *Streptomyces*
3	96.7	HM125151	*Burkholderia* sp. CPA4 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Burkholderiaceae; *Burkholderia*
3	100	EF668276	Uncultured Geobacteraceae bacterium clone M22_1608 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Deltaproteobacteria; Desulfuromonadales; Geobacteraceae; environmental samples
3	80.3	GQ423809	Uncultured bacterium clone R1B24H 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.

3	100	GU181268	*Variovorax* sp. SGM1-15 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae; *Variovorax*
3	99.6	EF220978	Uncultured Bacteroidetes bacterium clone D04_SGPO01 16S gene, partial sequence	Bacteria; Bacteroidetes; environmental samples
3	100	FJ875714	Uncultured beta proteobacterium clone D-08-ClB03 small subunit ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Betaproteobacteria; environmental
3	99.3	D84617	*Variovorax* sp. S23408 gene for 16S ribosomal RNA, partial sequence	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae; *Variovorax*
3	87.7	CU922275	Uncultured Acidobacteria bacterium 16S rRNA gene from clone QEDR1BF06	Bacteria; environmental samples.
3	87.6	GQ263674	Uncultured bacterium clone FW3_65C 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
3	100	GQ159514	Uncultured bacterium clone 16slp92-01e03.q1k 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
3	91.6	FJ694279	Uncultured bacterium clone KL201F02 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
3	100	HM328779	Uncultured bacterium clone ncd499f12c1 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
3	100	EF121241	*Microcystis aeruginosa* strain SPC 777 16S ribosomal RNA gene, partial sequence	Bacteria; Cyanobacteria; Chroococcales; *Microcystis*
3	93.4	FJ542953	Uncultured gamma proteobacterium clone B02-03F 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Gammaproteobacteria; environmental
3	100	GU956686	Uncultured Firmicutes bacterium clone LI3-309 16S ribosomal RNA gene, partial sequence	Bacteria; Firmicutes; environmental samples
3	95.1	AM940560	Uncultured alpha proteobacterium partial 16S rRNA gene, clone A6-42	Bacteria; Proteobacteria; Alphaproteobacteria; environmental
3	94.9	EF639389	*Hymenobacter* sp. BSw20462 16S ribosomal RNA gene, partial sequence	Bacteria; Bacteroidetes; Cytophagia; Cytophagales; Cytophagaceae; *Hymenobacter*
3	95.6	FM175743	Uncultured *Rickettsia* sp. partial 16S rRNA gene, clone CL2.C528	Bacteria; Proteobacteria; Alphaproteobacteria; Rickettsiales; Rickettsiaceae; Rickettsieae; *Rickettsia*; environmental samples
3	92.7	AY673182	Actinobacteridae bacterium Ellin7016 16S ribosomal RNA gene, partial sequence	Bacteria; Actinobacteria; Actinobacteridae
3	99.6	FN554975	*Chryseobacterium* sp.R4-1A partial 16S rRNA gene, type strain R4-1AT	Bacteria; Bacteroidetes; Flavobacteria; Flavobacteriales;*Flavobacteriaceae*; *Chryseobacterium*
3	100	AB362615	*Lactobacillus brevis* gene for 16S rRNA, partial sequence, strain: NRIC 0134	Bacteria; Firmicutes; Lactobacillales; Lactobacillaceae; *Lactobacillus*
2	92.3	AY193185	Uncultured candidate division OD1 bacterium clone DA23 16S ribosomal RNA gene, partial sequence	Bacteria; candidate division OD1; environmental samples
2	99.6	GQ404080	Uncultured bacterium clone BD289 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
2	92.6	DQ190785	Uncultured proteobacterium clone JAB NFA1 88 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; environmental samples
2	100	CU926027	Uncultured Unclassified bacterium 16S rRNA gene from clone QEDN7DE10	Bacteria; environmental samples.
2	93.4	AY395155	Uncultured actinobacterium clone E07ST 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
2	100	CP000721	*Clostridium beijerinckii* NCIMB 8052, complete genome	Bacteria; Firmicutes; Clostridia; Clostridiales; Clostridiaceae; *Clostridium*
2	93.0	FM176368	Uncultured Cystobacteraceae bacterium partial 16S rRNA gene, clone CL5.H118	Bacteria; Proteobacteria; Deltaproteobacteria; Myxococcales; Cystobacterineae; Cystobacteraceae; environmental samples
2	97.1	GU636726	Uncultured bacterium clone RW1382 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.

2	99.3	FM176093	Uncultured *Pseudorhodobacter* sp. partial 16S rRNA gene, clone CL4.E259	Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; *Pseudorhodobacter*; environmental samples
2	97.3	DQ129127	Uncultured soil bacterium clone CWT SM03_G11 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
2	98.2	GQ397014	Uncultured bacterium clone AK1DE2_05G 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
2	100	GU643321	Uncultured bacterium clone RW7977 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
2	100	FN563432	*Mesorhizobium* sp. LSE1 partial 16S rRNA gene, strain LSE1	Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Phyllobacteriaceae; *Mesorhizobium*
2	100	GU640437	Uncultured bacterium clone RW5093 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
2	93.0	CU923796	Uncultured Actinobacteria bacterium 16S rRNA gene from clone QEDP3BH09	Bacteria; environmental samples.
2	100	HM015669	*Spiroplasma citri* isolate UPM 16S ribosomal RNA gene, partial sequence; 16S-23S ribosomal RNA intergenic spacer, complete sequence; and 23S ribosomal RNA gene, partial sequence	Bacteria; Tenericutes; Mollicutes; Entomoplasmatales; Spiroplasmataceae; *Spiroplasma*
2	97.0	GU643071	Uncultured bacterium clone RW7727 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
2	99.6	FM177077	Uncultured *Rhodoferax* sp. partial 16S rRNA gene, clone CL6-7.L499	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae; *Rhodoferax*; environmental samples
2	96.7	CP000675	*Legionella pneumophila* str. Corby, complete genome	Bacteria; Proteobacteria; Gammaproteobacteria; Legionellales; Legionellaceae; *Legionella*
2	99.6	GU385867	*Paenibacillus* sp. QT21 16S ribosomal RNA gene, partial sequence	Bacteria; Firmicutes; Bacillales; Paenibacillaceae; *Paenibacillus*
2	94.5	FM176448	Uncultured *Aquiflexum* sp. partial 16S rRNA gene, clone CL5.H221	Bacteria; Bacteroidetes; Cytophagia; Cytophagales;Cyclobacteriaceae; *Aquiflexum*; environmental samples
2	96.3	AB365060	*Nocardioides oleivorans* gene for 16S rRNA, partial sequence	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Nocardioidaceae; *Nocardioides*
2	96.7	CP001828	*Legionella pneumophila* 2300/99 Alcoy, complete genome	Bacteria; Proteobacteria; Gammaproteobacteria; Legionellales; Legionellaceae; *Legionella*
2	98.15498155	FM176577	Uncultured Polyangiaceae bacterium partial 16S rRNA gene, clone CL5.H380	Bacteria; Proteobacteria; Deltaproteobacteria; Myxococcales; Sorangiineae; Polyangiaceae; environmental samples
2	97.4	AJ244650	*Brevundimonas*-like sp. LMG 11050 16S rRNA gene	Bacteria; Proteobacteria; Alphaproteobacteria; Caulobacterales; Caulobacteraceae
2	100	EF612342	*Methylobacterium* sp. K6-11 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Methylobacteriaceae; *Methylobacterium*
2	99.3	EU558285	*Paenibacillus* sp. B3a 16S ribosomal RNA gene, partial sequence	Bacteria; Firmicutes; Bacillales; Paenibacillaceae; *Paenibacillus*
2	90.8	FM176113	Uncultured *Desulforegula* sp. partial 16S rRNA gene, clone CL4.E284	Bacteria; Proteobacteria; Deltaproteobacteria; Desulfobacterales; Desulfobacteraceae; *Desulforegula*; environmental samples
2	97.8	GU643314	Uncultured bacterium clone RW7970 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
2	99.6	HM345221	Uncultured bacterium clone ncd1152d06c1 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
2	92.9	FJ620939	Uncultured soil bacterium clone FACE.R1.EC.C09 small subunit ribosomal RNA gene, partial sequence	Bacteria; environmental samples.

2	97.8	AM988899	*Chryseobacterium* sp. AKB-2008-HE92 partial 16S rRNA gene, strain AKB-2008-HE92	Bacteria; Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; *Chryseobacterium*
2	90.4	FJ694279	Uncultured bacterium clone KL201F02 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
2	100	AY436793	*Methylophilus* sp. ECd4 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Betaproteobacteria; Methylophilales; Methylophilaceae; *Methylophilus*
2	94.4	FM206085	Uncultured bacterium partial 16S rRNA gene, clone GW_7	Bacteria; environmental samples.
2	99.6	GQ284336	*Arthrobacter nicotinovorans* strain THWCSN3 16S ribosomal RNA gene, partial sequence	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Micrococcaceae; *Arthrobacter*
2	100	GU992398	*Lactococcus lactis* subsp. lactis strain RIBB1 16S ribosomal RNA gene, partial sequence	Bacteria; Firmicutes; Lactobacillales; Streptococcaceae; *Lactococcus*
1	99.6	GU902766	Uncultured bacterium clone PP254-b02 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
1	94.8	GQ354973	Uncultured Spirochaetales bacterium clone 4-217 16S ribosomal RNA gene, partial sequence	Bacteria; Spirochaetes; Spirochaetales; environmental samples
1	100	HM333401	Uncultured bacterium clone ncd1098d02c1 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
1	80.2	EF663250	Uncultured proteobacterium clone GASP-MA2W2_F07 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; environmental samples
1	91.2	DQ294012	Uncultured epsilon proteobacterium clone BRIC27 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Epsilonproteobacteria; environmental samples
1	99.6	FJ827889	Uncultured actinobacterium clone ME012E8 16S ribosomal RNA gene, partial sequence	Bacteria; Actinobacteria; environmental samples
1	98.9	AB190066	*Comamonas* sp. N-31-25-4 gene for 16S rRNA, partial sequence	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae; *Comamonas*
1	92.7	GQ339250	Uncultured bacterium clone IS-195 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; environmental samples
1	90.5	AF069496	*Trojanella thessalonices* 16s ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Alphaproteobacteria; Rickettsiales; *Candidatus Odyssella*
1	100	GQ157139	Uncultured bacterium clone 16slp101-3h05.p1k 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
1	99.3	FJ827881	Uncultured actinobacterium clone ME011D4 16S ribosomal RNA gene, partial sequence	Bacteria; Actinobacteria; environmental samples
1	97.8	GQ302554	Uncultured Gemmatimonadetes bacterium clone sw-xj18 16S ribosomal RNA gene, partial sequence	Bacteria; Gemmatimonadetes; environmental samples.
1	85.8	EU245242	Uncultured organism clone MAT-CR-H4-F07 16S ribosomal RNA gene, partial sequence	Unclassified; environmental samples.
1	98.1	DQ453128	*Comamonas odontotermitis* strain Dant 3-8 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae; *Comamonas*
1	92.6	FM176882	Uncultured *Waddlia* sp. partial 16S rRNA gene, clone CL6-7.L258	Bacteria; Chlamydiae; Chlamydiales; Waddliaceae; *Waddlia*; environmental samples
1	87.5	AY988769	Uncultured soil bacterium clone L1A.3D03 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
1	92.7	GQ339243	Uncultured bacterium clone IS-186 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples
1	97.8	FJ894731	Uncultured bacterium clone nbt40f08 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples
1	95.9	FJ764216	Uncultured beta proteobacterium clone EW1-085 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Betaproteobacteria; environmental samples

1	93.3	GU305806	Uncultured bacterium clone YHY25 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
1	90.5	HM269021	Uncultured bacterium clone ncd241g11c1 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples
1	99.3	AY958085	*Staurastrum punctulatum* chloroplast, complete genome	Eukaryota; Viridiplantae; Streptophyta; Zygnemophyceae; Desmidiales; Desmidiaceae; *Staurastrum*
1	91.7	HM335512	Uncultured bacterium clone ncd1001d03c1 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
1	91.5	AY532578	Uncultured bacterium clone 1013-28-CG38 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
1	84.7	FJ712836	Uncultured *Rhizobium* sp. clone Cvi12 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Rhizobiaceae;*Rhizobium/Agrobacterium* group; *Rhizobium* environmental samples
1	88.6	EU723941	*Aeromonas* sp. AE100 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Gammaproteobacteria;Aeromonadales; Aeromonadaceae; *Aeromonas*
1	90.1	EU676408	Uncultured bacterium clone 44P1 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
1	93.9	HM341023	Uncultured bacterium clone ncd1005g12c1 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
1	97.0	FJ517700	Uncultured Bdellovibrionales bacterium clone 26-2_8 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Deltaproteobacteria; Bdellovibrionales; environmental samples
1	100	EU117887	Uncultured actinobacterium clone RC1B2 16S ribosomal RNA gene, partial sequence	Bacteria; Actinobacteria; environmental samples
1	94.5	FJ475456	Uncultured Acetobacteraceae bacterium clone AhedenP18 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Alphaproteobacteria; Rhodospirillales; Acetobacteraceae; environmental samples
1	97.4	HM269134	Uncultured bacterium clone ncd243e10c1 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
1	85.6	EF018867	Uncultured bacterium clone Amb_16S_1350 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
1	94.9	EU979051	Uncultured delta proteobacterium clone g42 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Deltaproteobacteria; environmental
1	89.7	DQ532275	Uncultured bacterium clone JSC9-H2 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
1	88.6	EU723933	*Aeromonas* sp. AE99 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Gammaproteobacteria;Aeromonadales; Aeromonadaceae; *Aeromonas*
1	99.3	EU131002	*Sphingomonas* sp. BAC318 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Alphaproteobacteria;Sphingomonadales; Sphingomonadaceae; *Sphingomonas*
1	97.7	FJ719670	*Eutreptia viridis* strain SAG1226-1c 16S ribosomal RNA gene, partial sequence; chloroplast	Eukaryota; Euglenozoa; Euglenida; Eutreptiales; *Eutreptia*
1	94.1	GQ859797	Uncultured bacterium clone AA105 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
1	98.3	EF072459	Uncultured delta proteobacterium clone GASP-WA1W3_B08 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Deltaproteobacteria; environmental
1	95.6	HM317816	Uncultured bacterium clone ncd328a02c1 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
1	99.6	AB100608	*Swingsiella fulva* gene for 16S rRNA, partial sequence	Bacteria; Proteobacteria; Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae; *Rhodanobacter*
1	94.6	DQ676307	Uncultured candidate division OD1 bacterium clone MVP-35 16S ribosomal RNA gene, partial sequence	Bacteria; candidate division OD1; environmental samples.

1	88.3	FM176464	Uncultured Polyangiaceae bacterium partial 16S rRNA gene, clone CL5.H245	Bacteria; Proteobacteria; Deltaproteobacteria; Myxococcales; Sorangiineae; Polyangiaceae; environmental samples
1	95.2	EU914095	Uncultured bacterium clone D6ENV_87G11 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples
1	100	NR_025513	*Acetobacter malorum* strain LMG 1746 16S ribosomal RNA, partial sequence	Bacteria; Proteobacteria; Alphaproteobacteria; Rhodospirillales; Acetobacteraceae; *Acetobacter*
1	93.0	AJ867896	uncultured betaproteobacterium partial 16S rRNA gene, clone A3	Bacteria; Proteobacteria; Betaproteobacteria; environmental
1	96.3	DQ828676	Uncultured proteobacterium clone DOK_CONFYM_clone423 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; environmental samples
1	99.3	FM173120	*Corynebacterium lubricantis* partial 16S rRNA gene, strain KSS-4Se	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Corynebacterineae; Corynebacteriaceae; *Corynebacterium*
1	99.3	AB264798	*Chitinophaga ginsengisegetis* gene for 16S rRNA, partial sequence	Bacteria; Bacteroidetes; Sphingobacteria; Sphingobacteriales; Chitinophagaceae; *Chitinophaga*
1	94.8	DQ501318	Uncultured Bacteroidetes bacterium clone FSW11-13 16S ribosomal RNA gene, partial sequence	Bacteria; Bacteroidetes; environmental samples
1	95.2	AY673182	Actinobacteridae bacterium Ellin7016 16S ribosomal RNA gene, partial sequence	Bacteria; Actinobacteria; Actinobacteridae.
1	96.7	AY268295	Uncultured bacterium clone A2 16S small subunit ribosomal RNA gene, partial sequence	Bacteria; environmental samples
1	98.4	AY731468	Uncultured Cellulomonadaceae bacterium clone mM3 16S ribosomal RNA gene, partial sequence	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Cellulomonadaceae; environmental samples
1	96.0	AM935633	Uncultured *Sphingomonas* sp. partial 16S rRNA gene, clone AMDH2	Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales; Sphingomonadaceae; *Sphingomonas*; environmental samples
1	98.5	EU423300	*Nocardioides* sp. LnR5-15 16S ribosomal RNA gene, partial sequence	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Nocardioidaceae; *Nocardioides*
1	88.7	FJ673881	Uncultured bacterium clone 130-6J1 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples
1	99.6	GU642496	Uncultured bacterium clone RW7152 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples
1	99.6	FM173386	*Flavobacterium* sp. CL1.3 partial 16S rRNA gene, isolate CL1.3	Bacteria; Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; *Flavobacterium*
1	91.6	FJ936832	Uncultured bacterium clone kab115 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples
1	97.8	DQ501338	Uncultured beta proteobacterium clone ST11-40 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Betaproteobacteria; environmental
1	98.1	GU416464	Streptococcus cristatus clone VF065 16S ribosomal RNA gene, partial sequence	Bacteria; Firmicutes; Lactobacillales; Streptococcaceae; Streptococcus
1	92.2	CU922275	Uncultured Acidobacteria bacterium 16S rRNA gene from clone QEDR1BF06	Bacteria; environmental samples
1	92.3	EU135203	Uncultured bacterium clone FFCH1186 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples
1	96.0	FM175683	Uncultured Micromonosporineae bacterium partial 16S rRNA gene, clone CL2.C451	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Micromonosporineae; environmental samples
1	94.5	EF540429	Uncultured soil bacterium clone MK27b 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples
1	94.1	EF470923	Uncultured bacterium clone 156-21F 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples

1	99.3	AM884298	*Mycobacterium gordonae* partial 16S rRNA gene, strain 126/1/03	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Corynebacterineae; Mycobacteriaceae; *Mycobacterium*.
1	96.703296703	FJ793551	*Alcaligenes* sp. GR24-5 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Alcaligenaceae; *Alcaligenes*
1	94.1	EF020290	Uncultured bacterium clone Elev_16S_1827 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples
1	99.6	GU642018	Uncultured bacterium clone RW6674 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples
1	99.6	AF125877	Dehydroabietic acid-degrading bacterium DhA-73 16S ribosomal RNA gene, complete sequence	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae
1	98.1	AB184555	*Streptomyces mucoflavus* gene for 16S rRNA, partial sequence, strain: NBRC 13973	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Streptomycineae; Streptomycetaceae; *Streptomyces*
1	97.8	DQ521555	Uncultured bacterium clone ANTLV9_C10 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples
1	100	AB252938	Uncultured Nitrospirae bacterium gene for 16S rRNA, partial sequence, clone: 480	Bacteria; Nitrospirae; environmental samples

Table: 7.5. 454 Sequencing results of bacterial identities for *Drosophila* guts treated with 50 µg ml^{-1} of chlortetracycline.

Number of reads	% Identity	Accession Number	Identity	Lineage
29544	100	NR_025512	*Acetobacter cerevisiae* strain LMG 1625 16S ribosomal RNA, partial sequence >gi|23892796|emb|AJ419843.1| Acetobacter cerevisiae 16S rRNA gene, strain LMG 1625	Bacteria; Proteobacteria; Alphaproteobacteria; Rhodospirillales; Acetobacteraceae; *Acetobacter.*
314	100	EU096229	*Acetobacter pomorum* strain EW816 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Alphaproteobacteria; Rhodospirillales; Acetobacteraceae; *Acetobacter*.
54	100	CP001161	*Buchnera aphidicola* str. 5A (Acyrthosiphon pisum), complete genome	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; *Buchnera*.
12	100	HM334791	Uncultured bacterium clone ncd991e01c1 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
2	100	GQ246723	*Lactobacillus* sp. M3T1B5 16S ribosomal RNA gene, partial sequence	Bacteria; Firmicutes; Lactobacillales; Lactobacillaceae;*Lactobacillus*
2	100	HM344642	Uncultured bacterium clone ncd1060b08c1 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
2	96.3	EF668276	Uncultured Geobacteraceae bacterium clone M22_1608 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Deltaproteobacteria; Desulfuromonadales;Geobacteraceae; environmental samples
2	97.4	FJ444721	Uncultured *Sinorhizobium* sp. clone 4h-12 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Rhizobiaceae; *Sinorhizobium*/Ensifer group; *Sinorhizobium*; environmental samples
2	97.4	EU839288	Soil bacterium 05G-03 16S ribosomal RNA gene, partial sequence	Soil bacterium
2	97.0	DQ153941	*Brevibacterium* sp. SK8B10 16S ribosomal RNA gene, partial sequence	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales;Micrococcineae; Brevibacteriaceae; *Brevibacterium*
2	100	FJ887890	*Bacillus malacitensis* strain TP12 16S ribosomal RNA gene, partial sequence	Bacteria; Firmicutes; Bacillales; Bacillaceae; *Bacillus*
2	98.1	FJ654577	Uncultured alpha proteobacterium clone 012_E03_06-017477 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Alphaproteobacteria; environmental
2	98.9	GU129070	Porphyromonadaceae bacterium 62bF 16S ribosomal RNA gene, partial sequence	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales;Porphyromonadaceae; unclassified Porphyromonadaceae
2	99.6	HM328284	Uncultured bacterium clone ncd491c09c1 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
1	100	HM027569	*Bacillus subtilis* strain zj2008 16S ribosomal RNA gene, partial sequence	Bacteria; Firmicutes; Bacillales; Bacillaceae; *Bacillus*.
1	100	GQ246660	*Brevundimonas* sp. M1T2B6 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Alphaproteobacteria; Caulobacterales;Caulobacteraceae; *Brevundimonas*
1	91.2	AF507713	Uncultured soil bacterium clone S166 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.

1	100	FJ875714	Uncultured beta proteobacterium clone D-08-ClB03 small subunit ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Betaproteobacteria; environmental
1	98.2	AB252934	Uncultured alpha proteobacterium gene for 16S rRNA, partial sequence, clone: 225	Bacteria; Proteobacteria; Alphaproteobacteria; environmental
1	99.3	GU208440	Uncultured prokaryote clone Fr3-5 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
1	99.6	GU902766	Uncultured bacterium clone PP254-b02 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
1	96.7	NR_025512	*Acetobacter cerevisiae* strain LMG 1625 16S ribosomal RNA, partial sequence	Bacteria; Proteobacteria; Alphaproteobacteria; Rhodospirillales; Acetobacteraceae; Acetobacter.
1	97.0	GU916225	Uncultured bacterium clone F5K2Q4C04IIOUV 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
1	98.9	EU982453	Uncultured bacterium clone DYB14 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
1	100	GU124493	*Arthrobacter* sp. endosymbiont of Nilaparvata lugens clone A300 16S ribosomal RNA gene, partial sequence	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales;Micrococcineae; Micrococcaceae; *Arthrobacter*
1	90	AY988665	Uncultured soil bacterium clone L1A.1H04 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
1	81.4	FM176408	Uncultured candidate division OD1 bacterium partial 16S rRNA gene, clone BF.A2	Bacteria; candidate division OD1; environmental samples
1	97.8	GU643314	Uncultured bacterium clone RW7970 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
1	100	EU004565	*Paenibacillus* sp. HM06-03 16S ribosomal RNA gene, partial sequence	Bacteria; Firmicutes; Bacillales; Paenibacillaceae; *Paenibacillus*
1	95.2	GU472572	Uncultured *Rhodocyclaceae* bacterium clone Rh60A4 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Betaproteobacteria; Rhodocyclales;Rhodocyclaceae; environmental samples
1	86.7	EF020290	Uncultured bacterium clone Elev_16S_1827 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
1	100	HM333643	Uncultured bacterium clone ncd1107g12c1 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
1	99.6	HM336914	Uncultured bacterium clone ncd1087f06c1 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
1	95.2	GQ023532	Uncultured bacterium clone nbu319g02c1 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
1	99.6	HM146606	Uncultured bacterium clone SD102-3_d06 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
1	99.6	HM332517	Uncultured bacterium clone ncd991c11c1 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
1	93.0	HM099641	Lachnospiraceae bacterium oral taxon F15 strain UY038 16S ribosomal RNA gene, partial sequence	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae
1	99.3	FN646601	*Brachybacterium* sp. SS-2009-PON14 partial 16S rRNA gene, strain PON14	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales;Micrococcineae; Dermabacteraceae; *Brachybacterium*

1	89.7	EU803767	Uncultured bacterium clone 5C231389 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
1	99.3	FJ482194	Uncultured candidate division OP11 bacterium clone Pav-OP27 16S ribosomal RNA gene, partial sequence	Bacteria; candidate division OP11; environmental samples
1	99.6	EU775345	Uncultured bacterium clone gir_aah93g05 16S ribosomal RNA gene, partial sequence	environmental
1	98.1	AF236006	Beta proteobacterium A0618 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Betaproteobacteria
1	99.3	AY876630	Uncultured Gemmatimonadetes bacterium clone Nsp8b 16S ribosomal RNA gene, partial sequence	Bacteria; Gemmatimonadetes; environmental samples
1	91.6	FJ155589	*Methylobacterium* sp. SW08-7 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales;Methylobacteriaceae; *Methylobacterium*
1	99.6	NR_025512	*Acetobacter cerevisiae* strain LMG 1625 16S ribosomal RNA, partial sequence	Bacteria; Proteobacteria; Alphaproteobacteria; Rhodospirillales; Acetobacteraceae; *Acetobacter.*
1	95.6	AF141504	Uncultured gamma proteobacterium clone CRE-PA17 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Gammaproteobacteria; environmental
1	93.1	AB079644	Green non-sulfur bacterium AK-6 gene for 16S ribosomal RNA, partial sequence	Bacteria; Chloroflexi.
1	93.1	AB079639	*Kouleothrix aurantiaca* gene for 16S rRNA, partial sequence, strain:MYSI-A	Bacteria; Chloroflexi; *Kouleothrix*
1	100	HM329712	Uncultured bacterium clone ncd980d04c1 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.

7.3 Microarray transcripts with 2 fold or more change in abundance ($p<0.05$)

Table: 7.6. Dechorionation on the high nutrient diet: Immune transcripts

Gene Number	Gene Name	Up/Down regulation	Absolute fold change	Gene Ontology (GO) number	GO term
CG1373	Cecropin c	Down	51.84	GO:0050832 GO:0042742 GO:0005576 GO:0005615 GO:0019731 GO:0050829 GO:0050830	Defence response to fungus Defence response to bacterium Extracellular region Extracellular space Antibacterial humoral response Defence response to Gram-negative bacterium Defence response to Gram-positive bacterium
CG8175	Metchnikowin	Down	36.11	GO:0019731 GO:0019732 GO:0006952 GO:0050832 GO:0050829 GO:0050830 GO:0005576	Antibacterial humoral response Antifungal humoral response Defence response Defence response to fungus Defence response to Gram-negative bacterium Defence response to Gram-positive bacterium Extracellular region
CG10146	Attacin A	Down	15.85	GO:0005615 GO:0019731 GO:0050829	Extracellular space Antibacterial humoral response Defence response to Gram-negative bacterium
CG10794	Diptericin b	Down	14.94	GO:0019731 GO:0005576	Antibacterial humoral response Extracellular region
BT023384	Defensin	Down	10.64	GO:0005615 GO:0019731 GO:0050830 GO:0042742 GO:0006965	Extracellular space Antibacterial humoral response Defence response to Gram-positive bacterium Defence response to bacterium Positive regulation of biosynthetic process of antibacterial peptides active against gram-positive bacteria
CG12763	Diptericin	Down	10.24	GO:0019731 GO:0050829 GO:0042742 GO:0005576 GO:0045087 GO:0005576	Antibacterial humoral response Defence response to Gram-negative bacterium Defence response to bacterium Innate immune response Extracellular region
CG4740	Attacin C	Down	8.29	GO:0019731 GO:0006952 GO:0005615 GO:0042742 GO:0005576	Antibacterial humoral response Defence response Extracellular space, Defence response to bacterium Extracellular region
CG15678	Poor imd response upon knock-in	Down	4.43	GO:0009609 GO:0005515 GO:0005102 GO:0050777 GO:0045824 GO:0061060	Response to symbiotic bacterium Protein binding Receptor binding Negative regulation of immune response Negative regulation of innate immune response Negative regulation of peptidoglycan recognition protein signalling pathway
CG16876	Nimrod c4	Down	3.75	GO:0043277 GO:0006911 GO:0005886	Apoptotic cell clearance Phagocytosis engulfment Plasma membrane
CG9681	Peptidoglycan recognition protein sb1	Down	3.34	GO:0006952 GO:0005576 GO:0008745 GO:0005887 GO:0009253 GO:0042834 GO:0005875	Defence response Extracellular region N-acetylmuramoyl-L-alanine amidase activity Integral to plasma membrane Peptidoglycan catabolic process Peptidoglycan binding

				GO:0006955	Microtubule associated complex Immune response
CG31783	Neither inactivation nor afterpotential d	Down	3.04	GO:0006952 GO:0007602 GO:0007603 GO:0007604 GO:0016063 GO:0007155 GO:0005887 GO:0046867 GO:0005044 GO:0006952 GO:0016020	Defence response, Phototransduction, Phototransduction, visible light, Phototransduction, UV Rhodopsin biosynthetic process Cell adhesion Integral to plasma membrane carotenoid transport Scavenger receptor activity Defence response Membrane
CG9080	Listericin	Down	2.97	GO:0050829 GO:0050830 GO:0061057 GO:0061059	Defence response to Gram-negative bacterium Defence response to Gram-positive bacterium Peptidoglycan recognition protein signalling pathway Positive regulation of peptidoglycan recognition protein signalling pathway
CG7496	Peptidoglycan recognition protein sd	Down	2.57	GO:0005887 GO:0045087 GO:0009253 GO:0005515 GO:0008745 GO:0042834 GO:0005576 GO:0050830 GO:0006955 GO:0006952	Integral to plasma membrane Innate immune response Peptidoglycan catabolic process Protein binding N-acetylmuramoyl-L-alanine amidase Peptidoglycan binding activity Extracellular region Defence response to Gram-positive bacterium Immune response Defence response
CG14704	Peptidoglycan recognition protein sb2	Down	2.43	GO:0005515 GO:0050830 GO:0005576 GO:0004040 GO:0008745 GO:0042834 GO:0006952 GO:0016045 GO:0006955 GO:0009253 GO:0000270 GO:0005887	Protein binding, Defence response to Gram-positive bacterium, Extracellular region Amidase activity N-acetylmuramoyl-L-alanine amidase Peptidoglycan binding activity Defence response Detection of bacterium Immune response Peptidoglycan catabolic process Peptidoglycan metabolic process Integral to plasma membrane
SD22390	Cg6124- partial	Down	2.29	GO:0006910 GO:0008367	Phagocytosis Recognition
CG4099	Scavenger receptor class C, type I	Down	2.09	GO:0030247 GO:0005044 GO:0006952 GO:0050829 GO:0006955 GO:0006909 GO:0009617 GO:0005887 GO:0016020	Polysaccharide binding Scavenger receptor activity Defence response Defence response to Gram-negative bacterium Immune response Phagocytosis response to bacterium Integral to plasma membrane membrane

Table: 7.7. Dechorionation on the high nutrient diet: Binding and transport transcripts

Gene Number	Gene Name	Up/Down regulation	Absolute fold change	Gene Ontology (GO) number	GO term
CG9470	Metallothionein A	Down	3.10	GO:0046872	Metal ion binding
BT022784	Mitochondrial dicarboxylate carrier	Up	2.48	GO:0016021 GO:0005488 GO:0006810	Integral to membrane Transport Binding
CG10943	Viral a-type inclusion protein	Down	2.39	GO:0005488	Binding
BT025105	Class vii uncontrol myosin	Up	2.35	GO:0016459 GO:0005524 GO:0003774	Myosin complex ATP binding Motor activity
BT023209	Metallothionein c	Down	2.24	GO:0005507 GO:0006875	Copper ion binding Cellular metal ion homeostasis
CG4950	Carboxypeptidase n subunit 2	Down	2.14	GO:0005515	Protein binding
CG33192	Metallothionein D	Down	2.14	GO:0046872	Metal ion binding
CG4139	Karl (isoform a)	Down	2.05	GO:0005488	Binding

Table: 7.8. Dechorionation on the high nutrient diet: Metabolic transcripts

Gene Number	Gene Name	Up/Down regulation	Absolute fold change	Gene Ontology (GO) number	GO term
CG2259	Glutamate-cysteine ligase catalytic subunit	Down	4.47	GO:0004357 GO:0005515 GO:0006750 GO:0006749 GO:0006974 GO:0017109 GO:0005634 GO:0048471	Glutamate-cysteine ligase activity Protein binding Glutathione biosynthetic process Glutathione metabolic process Response to DNA damage stimulus Glutamate-cysteine ligase complex Nucleus Perinuclear region of cytoplasm,
CA804468	Protein farnesyltransferase alpha subunit	Up	4.07	GO:0008318 GO:0018346 GO:0005965	Protein prenyltransferase activity Protein amino acid prenylation Protein farnesyltransferase complex
CG4500	Bgml_drome ame: full=long-chain-fatty-acid-- ligase bubblegum-like	Down	3.99	GO:0001676 GO:0007498 GO:0004467	Long-chain fatty acid metabolic process Mesoderm development Long-chain-fatty-acid-CoA ligase activity
CG14205	-	Down	3.61	GO:0016747	Transferase activity, transferring acyl groups other than amino-acyl groups
CG7017	-	Down	3.60	GO:0005576 GO:0008061 GO:0006030 GO:0016490	Extracellular region Chitin binding Chitin metabolic process structural constituent of peritrophic membrane
CG33926	Transposase	Down	3.38	GO:0006139 GO:0003677 GO:0034960	Nucleobase, nucleoside Nucleotide and nucleic acid metabolic process DNA binding
CG12224	-	Down	3.30	GO:0055114 GO:0016491 GO:0008076	Oxidation reduction Oxidoreductase activity Voltage-gated potassium channel complex

CG12092-RA	Niemann-pick c1	Down	3.15	GO:0007417 GO:0007391 GO:0030299 GO:0008158 GO:0016021 GO:0005886 GO:0007422	Central nervous system development Dorsal closure Intestinal cholesterol absorption Hedgehog receptor activity Integral to membrane Plasma membrane Peripheral nervous system development
CG11512	Glutathione s transferase d4	Down	2.94	GO:0004602 GO:0006979 GO:0004364	Glutathione peroxidase activity Response to oxidative stress Glutathione transferase activity
CG15533	Acid sphingomyelinase	Down	2.93	GO:0004767 GO:0006685 GO:0006684	Sphingomyelin phosphodiesterase activity Sphingomyelin catabolic process Sphingomyelin metabolic process
CG10814	-	Down	2.72	GO:0008336 GO:0055114	Gamma-butyrobetaine dioxygenase activity Oxidation reduction
l(2)k05819	Lethal isoform b	Up	2.72	GO:0006754 GO:0006812 GO:0016020 GO:0015662	ATP biosynthetic process Cation transport Membrane ATPase activity, coupled to transmembrane movement of ions, phosphorylative mechanism
CG15534	-	Down	2.72	GO:0004767 GO:0006685	Sphingomyelin phosphodiesterase activity Sphingomyelin catabolic process
CG12766	Aldo-keto reductase	Down	2.66	GO:0016491 GO:0055114 GO:0004032	Oxidoreductase activity Oxidation reduction Aldehyde reductase activity
CG31148	-	Down	2.62	GO:0005975 GO:0043169 GO:0006665 GO:0005764 GO:0004348 GO:0007040	Carbohydrate metabolic process Cation binding Sphingolipid metabolic process Lysosome Glucosylceramidase activity Lysosome organization
BT022430	tpa_inf: hdc06756	Down	2.61	GO:0006508 GO:0004252	Proteolysis Serine-type endopeptidase activity
CG30098	tpa_inf: hdc06756	Down	2.44	GO:0006508 GO:0004252	Proteolysis Serine-type endopeptidase activity
TC209893	CG9444-PA	Up	2.43	GO:0005427 GO:0004550	Proton-dependent oligopeptide secondary active transmembrane transporter activity Nucleoside diphosphate kinase activity
EC216908	Synaptic vesicle membrane protein vat-1 homolog-like	Up	2.33	GO:0008270 GO:0055114 GO:0016491	Zinc ion binding Oxidation reduction Oxidoreductase activity
CG11459	-	Down	2.23	GO:0004197 GO:0006508	Cysteine-type endopeptidase activity Proteolysis
CG12242	Glutathione s transferase d5	Down	2.22	GO:0004602 GO:0006979 GO:0004364	Glutathione peroxidase activity Response to oxidative stress Glutathione transferase activity
CG17234	Serine protease	Down	2.20	GO:0006508 GO:0004252	Proteolysis Serine-type endopeptidase activity
CG30287	Isoform a	Down	2.21	GO:0006508 GO:0004252	Proteolysis Serine-type endopeptidase activity
CG14219	-	Down	2.19	GO:0016747	Transferase activity, transferring acyl groups other than amino-acyl groups
CG34357	CG34357	Down	2.19	GO:0005524 GO:0004383	ATP binding Guanylate cyclase activity

				GO:0004672 GO:0006182 GO:0023034 GO:0006468	Protein kinase activity cGMP biosynthetic process Intracellular signalling pathway Protein amino acid phosphorylation
EC216839	Bifunctional purine biosynthesis protein	Up	2.13	GO:0004643 GO:0003937 GO:0006188	Phosphoribosylaminoimidazolec arboxamide formyltransferase activity IMP cyclohydrolase activity IMP biosynthetic process
CG9989	Mitochondrial endonuclease	Down	2.12	GO:0046872 GO:0003676 GO:0016787	Metal ion binding Nucleic acid binding Hydrolase activity
CG8693	Isoform a	Down	2.12	GO:0005975 GO:0043169 GO:0003824 GO:0004558	Carbohydrate metabolic process Cation binding Catalytic activity, alpha-glucosidase activity
CG7715	-	Down	2.09	GO:0008061 GO:0006030 GO:0005576	Chitin binding Chitin metabolic process Extracellular region
CG41624	Spookier	Down	2.03	GO:0004497 GO:0009055 GO:0020037 GO:0006697 GO:0007591 GO:0055114	Monooxygenase activity Electron carrier activity Heme binding Ecdysone biosynthetic process Molting cycle, chitin-based cuticle Oxidation-reduction process
CG2958	Lectin-24Db	Down	2.02	GO:0042806 GO:0005537 GO:0005534	Fucose binding Mannose binding Galactose binding

Table: 7.9. Dechorionation on the high nutrient diet: DNA/RNA replication/transcription transcripts

Gene Number	Gene Name	Up/Down regulation	Absolute fold change	Gene Ontology (GO) number	GO term
CG2125	Cubitus interruptus (ci)	Down	3.81	GO:0010843 GO:0005515 GO:0016563 GO:0035017 GO:0048813 GO:0008544 GO:0048592 GO:0035224 GO:0060914 GO:0035217 GO:0048666 GO:0048666 GO:0048477 GO:0030858 GO:0045750 GO:0045944 GO:0007346 GO:0007367 GO:0007224 GO:0035277 GO:0048100 GO:0005737 GO:0035301 GO:0016020 GO:0005634 GO:0043234 GO:0003704 GO:0003700 GO:0016564 GO:0008270 GO:0007350 GO:0000122 GO:0030707 GO:0045449 GO:0007224 GO:0005634	Promoter binding Protein binding Transcription activator activity Cuticle pattern formation Dendrite morphogenesis Epidermis development Eye morphogenesis Genital disc anterior/posterior pattern formation Heart formation Neuron development Labial disc development Oogenesis Positive regulation of epithelial cell differentiation Positive regulation of S phase of mitotic cell cycle Positive regulation of transcription from RNA polymerase II promoter Regulation of mitotic cell cycle Segment polarity determination Smoothened signalling pathway Spiracle morphogenesis, open tracheal system Wing disc anterior/posterior pattern formation Cytoplasm Hedgehog signalling complex Membrane Nucleus Protein complex Transcription factor activity Transcription repressor activity Zinc ion binding Blastoderm segmentation Negative regulation of transcription from RNA polymerase II promoter Ovarian follicle cell development Regulation of transcription Smoothened signalling pathway Nucleus
EC267473 (CG1705)	Methoprene -tolerant	Up	2.72	GO:0005500 GO:0046982 GO:0042803 GO:0030528 GO:0050793 GO:0006355 GO:0005634 GO:0003700 GO:0004871 GO:0006355 GO:0007165	Juvenile hormone binding Protein heterodimerization activity Protein homodimerization activity Transcription regulator activity Regulation of developmental process Regulation of transcription, DNA-dependent Nucleus Sequence-specific DNA binding transcription factor activity Signal transducer activity Regulation of transcription, DNA-dependent signal transduction
TC219913	CG7564-PA	Up	2.20	GO:0005685 GO:0005634 GO:0000398 GO:0045843	snRNP U1-CFB Nucleus Nuclear mRNA splicing, via spliceosome Negative regulation of striated muscle development
CK662469	LD13130p	Up	2.15	GO:0005730 GO:0000176 GO:0003676 GO:0008408 GO:0005652 GO:0006396	Nucleolus Nuclear exosome (RNase complex) Nucleic acid binding 3'-5' exonuclease activity Nuclear lamina RNA processing

Table: 7.10. Dechorionation on the high nutrient diet: Miscellaneous transcripts

Gene Number	Gene Name	Up/Down regulation	Absolute fold change	Gene Ontology (GO) number	GO term
CG18087	Salivary gland secretion 7	Down	7.42	GO:0007594 GO:0005576 GO:0005198	Puparial adhesion Extracellular region Structural molecule activity
BP553587 (CG17082)	-	Up	2.93	GO:0007165 GO:0005622	Signal transduction Intracellular
CG11720-RA	Salivary gland secretion 3	Down	2.78	GO:0007594 GO:0005576 GO:0005198	Puparial adhesion Extracellular region Structural molecule activity
CG7548	-	Down	2.53	GO:0005214	Structural constituent of chitin-based cuticle
CO316961 (CG34341)	Phosphodiesterase 11	Down	2.52	GO:0004115 GO:0047555 GO:0046058 GO:0046068 GO:0007165	3',5'-cyclic-AMP phosphodiesterase activity 3',5'-cyclic-GMP phosphodiesterase activity cAMP metabolic process cGMP metabolic process Signal transduction
AA978453 (CG14217)	Tao-1	Up	2.28	GO:0004674 GO:0006915 GO:0005886 GO:0005524 GO:0004702 GO:0006468	Protein serine/threonine kinase activity Apoptosis Plasma membrane ATP binding Receptor signaling protein serine/threonine kinase activity Protein phosphorylation
CA807003 (CG9181)	Protein tyrosine phosphatase 61F	Up	2.27	GO:0005515 GO:0004725 GO:0007411 GO:0071456 GO:0007377 GO:0071456 GO:0007377 GO:0000278 GO:0050732 GO:0048477 GO:0006470 GO:0032880 GO:0031647 GO:0005737 GO:0005634 GO:0048471 GO:0006470	Protein binding Protein tyrosine phosphatase activity Axon guidance Cellular response to hypoxia Germ-band extension Cellular response to hypoxia Germ-band extension Mitotic cell cycle Negative regulation of peptidyl-tyrosine phosphorylation Oogenesis Protein dephosphorylation Regulation of protein localization Regulation of protein stability Cytoplasm Nucleus Perinuclear region of cytoplasm Protein dephosphorylation
BT023292	Tetraspanin isoform a	Up	2.14	GO:0016021 GO:0030097	Integral to membrane Hemopoiesis

Table: 7.11. Dechorionation on the high nutrient diet: Tentative consensus sequences and Expression Sequence Tags with no assigned gene ontology.

Sequence Number	Sequence description	Up/Down regulation	Absolute fold change
EC265593 (CG14322)	Expression sequence tag	Down	40.33
TC218200 (Imaginal discs, adulthead, larval-pupal stage)	Tentative consensus sequence	Down	9.01
TC212147 (Head)	Tentative consensus sequence	Down	4.04
TC213322	Tentative consensus sequence	Down	3.50
TC217958 (Mixed stage embryos, adult heads and imaginal discs)	Tentative consensus sequence	Up	3.13
TC212294 (Adult male and female heads)	Tentative consensus sequence	Up	2.91
TC219398 (Mixed stage embryos, adult heads and imaginal discs)	Tentative consensus sequence	Up	2.00-2.85
TC207643 (Larval and early pupal stage)	Tentative consensus sequence	Up	2.69
TC211395 (Head)	Tentative consensus sequence	Down	2.64
CO340976	Expression sequence tag	Up	2.60
TC218608 (Mixed stage embryos, adult heads and imaginal discs)	Tentative consensus sequence	Up	2.52
TC220267	Tentative consensus sequence	Down	2.50
TC217746 (Mixed stage embryos, adult heads and imaginal discs)	Tentative consensus sequence	Up	2.49
TC202144	Tentative consensus sequence	Down	2.47
TC198329 (Mixed stage embryos, adult heads and imaginal discs)	Tentative consensus sequence	Up	2.39
EC251496	Expression sequence tag	Down	2.37
TC217470	Tentative consensus sequence	Up	2.36
TC216814	Tentative consensus sequence	Down	2.30
EC251372	Expression sequence tag	Up	2.30
TC214127 (Mixed stage embryos, adult heads and imaginal discs)	Tentative consensus sequence	Up	2.29
TC218787 (Mixed stage embryos, adult heads and imaginal discs)	Tentative consensus sequence	Up	2.29
TC216582	Tentative consensus sequence	Up	2.28
TC221202	Tentative consensus sequence	Up	2.27
AT28783	Expression sequence tag	Down	2.25
TC215744	Tentative consensus sequence	Down	2.24
TC219887 (Mixed stage embryos, adult heads and imaginal discs)	Tentative consensus sequence	Up	2.20
TC217326 (Embryo)	Tentative consensus sequence	Down	2.20
TC216236 (Mixed stage embryos, adult heads and imaginal discs)	Tentative consensus sequence	Down	2.19
TC204335 (Larval early pupae)	Tentative consensus sequence	Down	2.19
TC201532	Tentative consensus sequence	Up	2.18
TC218281 (Mixed stage embryos, adult heads and imaginal discs)	Tentative consensus sequence	Up	2.13
CK133206	Expression sequence tag	Up	2.10
CA806439	Expression sequence tag	Up	2.09
CA805541 (CG43139)	Expression sequence tag	Up	2.06
CO335149	Expression sequence tag	Down	2.06
LP20693	Expression sequence tag	Down	2.06
TC214720	Tentative consensus sequence	Down	2.05
TC216455 (Mixed stage embryos, adult heads and imaginal discs)	Tentative consensus sequence	Up	2.04

TC220402 (Embryo)	Tentative consensus sequence	Up	2.03
TC215707 (Mixed stage embryos, adult heads and imaginal discs)	Tentative consensus sequence	Up	2.02
TC215821 (Mixed stage embryos, adult heads and imaginal discs)	Tentative consensus sequence	Up	2.02
EL871925	Expression sequence tag	Up	2.01
EC265111	Expression sequence tag	Down	2.00
CO300471	Expression sequence tag	Down	2.00

Table: 7.12. Dechorionation on the high nutrient diet: Sequences with no assigned gene ontology.

Gene/Sequence Number	Up/Down regulation	Absolute fold change
CG32185	Down	118.89
CG34143 (Ionotropic receptor 10a)	Up	7.00
CG18273-RA	Down	4.80
CG18273	Up	3.81
CG33553-RF	Down	4.61
CG16775	Down	3.82
CG31711-RA	Down	3.59
CG41233	Down	3.41
CG12998	Down	3.01
NM_168143 (CG32408)	Down	2.86
NM_167853 (CG9094)	Up	2.86
CG31410 (Niemann-Pick type C-2e)	Down	2.72
CG33460	Down	2.71
CG13641	Down	2.65-2.74
CG18539	Up	2.62
CG14639 (TwdlF)	Down	2.42
CG31698	Up	2.41
CG7953	Down	2.28
CG31554	Up	2.28
CG7968	Down	2.26
CG13640	Down	2.15
NM_144221	Down	2.02
CG40137	Up	2.01
CG9616	Down	2.01

Table: 7.13. Dechorionation on the low nutrient diet: Immune transcripts.

Gene Number	Gene Name	Up/Down regulation	Absolute fold change	Gene Ontology (GO) number	GO term
CG12763	Diptericin	Down	33.96	GO:0019731 GO:0050829 GO:0042742 GO:0005576 GO:0045087 GO:0005576	Antibacterial humoral response Defence response to Gram-negative bacterium Defence response to bacterium Innate immune response Extracellular region
CG8175	Metchnikowin	Down	15.02	GO:0019731 GO:0019732 GO:0006952 GO:0050832 GO:0050829 GO:0050830 GO:0005576	Antibacterial humoral response Antifungal humoral response Defence response Defence response to fungus Defence response to Gram-negative bacterium Defence response to Gram-positive bacterium Extracellular region
CG10794	Diptericin b	Down	13.24	GO:0019731 GO:0005576	Antibacterial humoral response Extracellular region
CG4740	Attacin C	Down	5.67	GO:0019731 GO:0006952 GO:0005615 GO:0042742 GO:0005576	Antibacterial humoral response Defence response Extracellular space Defence response to bacterium Extracellular region
CG9681	Peptidoglycan recognition protein sb1	Down	2.89-2.77	GO:0006952 GO:0005576 GO:0008745 GO:0005887 GO:0009253 GO:0042834 GO:0005875 GO:0006955	Defence response Extracellular region N-acetylmuramoyl-L-alanine amidase activity Integral to plasma membrane Peptidoglycan catabolic process Peptidoglycan binding Microtubule associated complex Immune response

Table: 7.14. Dechorionation on the low nutrient diet: Binding and transport transcripts.

Gene Number	Gene Name	Up/Down regulation	Absolute fold change	Gene Ontology (GO) number	GO term
NM_001015210	Zinc c3hc4 type (ring finger) domain protein	Down	3.26	GO:0003676 GO:0008270 GO:0005515	Nucleic acid binding Zinc ion binding protein binding
CG11123	Mgc69156 protein	Up	3.09	GO:0003723	RNA binding
EC240045	Coatomer subunit alpha	Up	2.90	GO:0005515 GO:0016192 GO:0006886 GO:0030126 GO:0005198	Protein binding Vesicle-mediated transport Intracellular protein transport COPI vesicle coat Structural molecule activity
CG32704	Glutamate receptor 1	Down	2.78	GO:0016020 GO:0004970 GO:0006811 GO:0005234	Membrane Ionotropic glutamate receptor activity Ion transport Extracellular-glutamate-gated ion channel activity
CG12754	Odorant receptor 42b	Down	2.35	GO:0007608 GO:0004984 GO:0005549 GO:0016021 GO:0016020	Sensory perception of smell Olfactory receptor activity Odorant binding Integral to membrane Plasma membrane
CG3250	Os-C	Down	2.09	GO:0005550	Pheromone binding
CG11748	Odorant-binding protein 19a	Down	2.09	GO:0005549 GO:0005576 GO:0042048 GO:0019236 GO:0007606 GO:0006810	Odorant binding Extracellular region Olfactory behaviour Response to pheromone Sensory perception of chemical stimulus Transport
CG8807	Lush	Down	2.09	GO:0035275 GO:0042048 GO:0019236 GO:0005549 GO:0045471 GO:0007606 GO:0007608 GO:0005576 GO:0006810	Dibutyl phthalate binding Olfactory behaviour Response to pheromone Odorant binding Response to ethanol Sensory perception of chemical stimulus Sensory perception of smell Extracellular region Transport
CG6642	Antennal protein 10	Down	2.09-2.20	GO:0005549 GO:0005550 GO:0007606	Odorant binding Pheromone binding Sensory perception of chemical stimulus

Table: 7.15. Dechorionation on the low nutrient diet: Metabolic transcripts.

Gene Number	Gene Name	Up/Down regulation	Absolute fold change	Gene Ontology (GO) number	GO term
CG9781	Obstructor-G	Up	3.32	GO:0005576 GO:0008061 GO:0006030 GO:0016490	Extracellular region Chitin binding Chitin metabolic process Structural constituent of peritrophic membrane
CG2259	Glutamate-cysteine ligase catalytic subunit	Down	2.89	GO:0004357 GO:0005515 GO:0006750 GO:0006749 GO:0006974 GO:0017109 GO:0005634 GO:0048471	Glutamate-cysteine ligase activity Protein binding Glutathione biosynthetic process Glutathione metabolic process Response to DNA damage stimulus Glutamate-cysteine ligase complex Nucleus Perinuclear region of cytoplasm
CO334625	Cholesterol transporter tart1	Up	2.74	GO:0017127	Cholesterol transporter activity
CG12224	CG12224	Down	2.34	GO:0016491 GO:0055114	Oxidoreductase activity Oxidation reduction
CG32473	CG32473	Down	2.30	GO:0004177 GO:0008237 GO:0008270 GO:0006508	Aminopeptidase activity Metallopeptidase activity Zinc ion binding Proteolysis

Table: 7.16. Dechorionation on the low nutrient diet: DNA/RNA replication/transcription transcripts.

Gene Number	Gene Name	Up/Down regulation	Absolute fold change	Gene Ontology (GO) number	GO term
AA440503 (CG5303)	Meiotic from via Salaria 332	Up	2.30	GO:0007143 GO:0007140 GO:0007062 GO:0005694 GO:0000775 GO:0045132 GO:0007132 GO:0007062 GO:0005634	Female meiosis Male meiosis Sister chromatid cohesion Chromosome Chromosome, centromeric region Meiotic chromosome segregation Meiotic metaphase I Sister chromatid cohesion Nucleus

Table: 7.17. Dechorionation on the low nutrient diet: Tentative consensus sequences and Expression sequence tags with no assigned gene ontology.

Sequence Number	Sequence description	Up/Down regulated	Absolute fold change
EC265593	Expression sequence tag	Down	16.36
TC218200 (Larval early pupal)	Tentative consensus sequence	Down	11.09
TC215502	Tentative consensus sequence	Down	4.65
TC210124(BI628134)	Tentative consensus sequence	Down	4.21
TC218367 (Embryo)	Tentative consensus sequence	Up	3.75
TC213314 (Embryo)	Tentative consensus sequence	Down	3.67
TC203290 (Adult testis)	Tentative consensus sequence	Up	2.89
TC218479 (mixed stage embryos, imaginal disks, and adult heads)	Tentative consensus sequence	Down	2.64
TC221383	Tentative consensus sequence	Up	2.45
TC196107 (Embryo)	Tentative consensus sequence	Down	2.32
TC217326 (Embryo)	Tentative consensus sequence	Down	2.28
TC220675	Tentative consensus sequence	Up	2.28
EC235662	Expression sequence tag	Down	2.20
TC217270 (Male and female adult head)	Tentative consensus sequence	Up	2.18
TC212583 (Embryo)	Tentative consensus sequence	Down	2.17
CK133206	Expression sequence tag	Up	2.11
TC221392	Tentative consensus sequence	Down	2.07
TC216434	Tentative consensus sequence	Up	2.00

Table: 7.18. Dechorionation on the low nutrient diet: Sequences with no assigned gene ontology.

Gene/Sequence Number	Up/Down regulated	Absolute fold change
CG32185 (BT023614)	Down	35.34
CG13445	Down	3.11
CG34336 (RT07405p)	Up	2.18
CG15820	Down	2.02

Table: 7.19. Control flies on the high and low nutrient diet: Binding and transport transcripts.

Gene Number	Gene Name	Up/Down regulation	Absolute fold change	Gene Ontology (GO) number	GO term
CG40343 (NM_001015210)	CG40343	Up	4.20	GO:0003676 GO:0008270	Nucleic acid binding Zinc ion binding
CG8585	I_h channel	Up	2.96	GO:0005221 GO:0005244 GO:0005249 GO:0006813 GO:0055085 GO:0016020 GO:0005886	Intracellular cyclic nucleotide activated Cation channel activity Voltage-gated ion channel activity Voltage-gated potassium channel activity Potassium ion transport Transmembrane transport Membrane Plasma membrane
CG7592	Odorant-binding protein 99b	Down	2.94	GO:0005549 GO:0007606 GO:0006810 GO:0035071 GO:0019236 GO:0005576 GO:0042048 GO:0048102	Odorant binding Sensory perception of chemical stimulus Transport Salivary gland cell autophagic cell death Response to pheromone Extracellular region Olfactory behaviour Autophagic cell death
CO265995	Secis-binding protein 2	Down	2.70	GO:0046872	Metal ion binding
CG41087	-	Up	2.42	GO:0006457 GO:0031072 GO:0051082	Protein folding Heat shock protein binding Unfolded protein binding
BP557102 (CG10706)	Small conductance calcium-activated potassium channel	Up	2.33	GO:0015269 GO:0005516 GO:0016286 GO:0006813 GO:0016021	Calcium-activated potassium channel activity Calmodulin binding Small conductance calcium-activated potassium channel activity Potassium ion transport Integral to membrane
EL882428	Isoform b	Down	2.33	GO:0016021 GO:0006814 GO:0005215	Integral to membrane Sodium ion transport Transporter activity
CG32284	CG14957 protein	Down	2.24	GO:0005576 GO:0008061	Extracellular region Chitin binding
CG8177	-	Up	2.11	GO:0015301 GO:0005452 GO:0006820 GO:0016021	Anion:anion antiporter activity Inorganic anion exchanger activity Anion transport Integral to membrane
CG12944	Odorant-binding protein 47a	Up	2.09	GO:0006810 GO:0007606 GO:0005549	Transport Sensory perception of chemical stimulus Odorant binding
CG4465	-	Up	2.09	GO:0008513 GO:0055085	Secondary active organic cation transmembrane

					transporter activity Transmembrane transport
CG10293-RC	Held out wings	Up	2.06	GO:0005634 GO:0005737 GO:0030154 GO:0003729 GO:0007525 GO:0045214 GO:0000381 GO:0007438 GO:0007498 GO:0008078 GO:0008347 GO:0009790 GO:0008366 GO:0007475 GO:0003730	Nucleus Cytoplasm Cell differentiation mRNA binding Somatic muscle development Sarcomere organization Regulation of alternative nuclear mRNA splicing, via spliceosome Oenocyte development Mesoderm development Mesodermal cell migration Glial cell migration Embryonic development Axon ensheathment Apposition of dorsal and ventral imaginal disc-derived wing surfaces mRNA 3'-UTR binding
CG4898	Tropomyosin 1	Up	2.01	GO:0048813 GO:0045451 GO:0010591 GO:0030017 GO:0003779 GO:0048813 GO:0006936 GO:0048477 GO:0007315 GO:0005862	Dendrite morphogenesis Poleplasm oskar mRNA localization Regulation of lamellipodium assembly Sarcomere Actin binding Dendrite morphogenesis Muscle contraction Oogenesis Poleplasm assembly Muscle thin filament tropomyosin
AI517949 (CG2520)	Like-AP180	Up	2.01	GO:0007270 GO:0042331 GO:0006898 GO:0007268 GO:0048488 GO:0005905 GO:0005545 GO:0030276 GO:0048268 GO:0007269 GO:0016183 GO:0048489 GO:0030131 GO:0030118 GO:0008021	Nerve-nerve synaptic transmission Phototaxis Receptor-mediated endocytosis Synaptic transmission Synaptic vesicle endocytosis coated pit 1-phosphatidylinositol binding Clathrin binding Clathrin coat assembly Neurotransmitter secretion Synaptic vesicle coating Synaptic vesicle transport Clathrin adaptor complex Clathrin coat Synaptic vesicle
CG11326	Thrombospondin	Up	2.00	GO:0008201 GO:0033627 GO:0016203 GO:0007517	Heparin binding Cell adhesion mediated by integrin Muscle attachment

				GO:0031012 GO:0005927 GO:0043234 GO:0005509	Muscle organ development Extracellular matrix Muscle tendon junction Protein complex Calcium ion binding

Table: 7.20. Control flies on the high and low nutrient diet: Metabolic transcripts

Gene Number	Gene Name	Up/Down regulation	Absolute fold change	Gene Ontology (GO) number	GO term
CG17285	Fat body protein 1	Down	29.12	GO:0005344 GO:0008565 GO:0005811 GO:0015032	Oxygen transporter activity Protein transporter activity Lipid particle Storage protein import into fat body
CG3763	Fat body protein 2	Down	23.54	GO:0055114 GO:0004022 GO:0045735 GO:0005488 GO:0005811	Oxidation reduction Alcohol dehydrogenase (NAD) activity Nutrient reservoir activity Binding Lipid particle
CG7017	-	Down	7.60	GO:0005576 GO:0008061 GO:0006030 GO:0016490	Extracellular region Chitin binding Chitin metabolic process structural constituent of peritrophic membrane
CG4178	Larval serum protein 1 beta	Down	5.44	GO:0005344 GO:0005616 GO:0045735 GO:0005811 GO:0006810	Oxygen transporter activity Larval serum protein complex Nutrient reservoir activity Lipid particle Transport
CG10140	Isoform a	Down	3.88	GO:0005576 GO:0008061 GO:0006030	Extracellular region Chitin binding Chitin metabolic process
CG17725	Phosphoenolpyruvate carboxykinase	Up	3.31	GO:0006094 GO:0005525 GO:0016301 GO:0004613 GO:0005739	Gluconeogenesis GTP binding Kinase activity Phosphoenolpyruvate carboxykinase (GTP) activity Mitochondrion
CG33467	-	Down	3.27	GO:0004672 GO:0006468 GO:0005524	Protein kinase activity Protein amino acid phosphorylation ATP binding
CG32564	-	Down	2.87	GO:0009055 GO:0020037 GO:0005506 GO:0004497	Electron carrier activity Heme binding Iron ion binding Monooxygenase activity
CG32464	l(3)82Fd	Up	2.83	GO:0016998	Cell wall macromolecule catabolic process
TC209631	CG4346-PA	Down	2.71	GO:0004623 GO:0007615 GO:0008016 GO:0008355	Phospholipase A2 activity Anesthesia-resistant memory Regulation of heart contraction Olfactory learning
CG13744	Serine protease	Down	2.54	GO:0006508 GO:0004252	Proteolysis Serine-type endopeptidase activity
CG41624	Spookier	Down	2.44	GO:0004497 GO:0009055 GO:0020037 GO:0006697 GO:0007591 GO:0055114	Monooxygenase activity Electron carrier activity Heme binding Ecdysone biosynthetic process Molting cycle, chitin-based cuticle Oxidation-reduction process
CG9663	-	Up	2.38	GO:0042626 GO:0005524 GO:0005215 GO:0043190	ATPase activity, coupled to transmembrane movement of substances ATP binding

				GO:0016021	Transporter activity ATP-binding cassette (ABC) transporter complex Integral to membrane
TC207646	-	Down	2.38	GO:0051018 GO:0008104	Protein kinase A binding Protein localization
CG14957	-	Down	2.34	GO:0005576 GO:0008061 GO:0006030	Extracellular region Chitin binding Chitin metabolic process
TC209811	Lectin type C	Up	2.39	GO:0005534	Galactose binding
BT025118	Serine protease	Down	2.20	GO:0005576 GO:0005198 GO:0006508 GO:0004252	Extracellular region Structural molecule activity Proteolysis Serine-type endopeptidase activity
CG30098	tpa_inf: hdc06756	Down	2.28	GO:0006508 GO:0004252	Proteolysis Serine-type endopeptidase activity
BT022430	tpa_inf: hdc06756	Down	2.25	GO:0006508 GO:0004252	Proteolysis Serine-type endopeptidase activity
EL876446	CG11126-pa	Down	2.17	GO:0016787 GO:0009166	Hydrolase activity Nucleotide catabolic process
CG8256	Gpo-1 (glycerol-3-phosphate dehydrogenase)	Up	2.11	GO:0005743 GO:0007629 GO:0006072 GO:0005811 GO:0006127 GO:0005509 GO:0004368 GO:0009331	Mitochondrial inner membrane Flight behaviour Glycerol-3-phosphate metabolic process Lipid particle Glycerophosphate shuttle Calcium ion binding Glycerol-3-phosphate dehydrogenase activity Glycerol-3-phosphate dehydrogenase complex
TC209893	CG9444-PA	Up	2.08	GO:0005427 GO:0004550	Proton-dependent oligopeptide secondary active transmembrane transporter activity Nucleoside diphosphate kinase activity
CG3986	Chitinase 4	Down	2.04	GO:0005576 GO:0004568 GO:0008061 GO:0043169 GO:0006032	Extracellular region Chitinase activity Chitin binding Cation binding Chitin catabolic process
CG11771	Oligopeptidase a	Up	2.04	GO:0004222 GO:0006508	Metalloendopeptidase activity Proteolysis
TC215745	CG5087-PA	Up	2.01	GO:0004842	Ubiquitin-protein ligase activity

Table: 7.21. Control flies on the high and low nutrient diet: DNA/RNA replication/transcription transcripts.

Gene Number	Gene Name	Up/Down regulation	Absolute fold change	Gene Ontology (GO) number	GO term
CG41130	MIP04163p	Up	2.47	GO:0045449 GO:0030528 GO:0005634	Regulation of transcription Transcription regulator activity Nucleus
BG639986	Exosome complex exonuclease rrp45	Up	2.14	GO:0000176 GO:0000175 GO:0000177 GO:0003723 GO:0006396	Nuclear exosome (RNase complex) 3'-5'-exoribonuclease activity Cytoplasmic exosome (RNase complex) RNA binding RNA processing
CG11518	Pygopus	Up	2.14	GO:0005515 GO:0016563 GO:0060232 GO:0048813 GO:0009880 GO:0035214 GO:0048526 GO:0030177 GO:0006351 GO:0007472 GO:0016055 GO:0007223 GO:0005634 GO:0008270	Protein binding Transcription activator activity Delamination Dendrite morphogenesis Embryonic pattern specification Eye-antennal disc development Imaginal disc-derived wing expansion Positive regulation of Wnt receptor signalling pathway Transcription, DNA-dependent Wing disc morphogenesis Wnt receptor signalling pathway Wnt receptor signalling pathway, calcium modulating pathway Nucleus Zinc ion binding
Dis3	Mitotic control protein dis3	Up	2.10	GO:0004540 GO:0003723	Ribonuclease activity RNA binding
CG11491	Broad	Up	2.01	GO:0005634 GO:0005622 GO:0035070 GO:0035071 GO:0006355 GO:0040034 GO:0007552 GO:0035072 GO:0008219 GO:0008270 GO:0003704 GO:0003700 GO:0009608 GO:0035075 GO:0040034 GO:0007458 GO:0048477 GO:0048747 GO:0048808 GO:0035193 GO:0007562 GO:0001752 GO:0006914 GO:0016566 GO:0003677	Nucleus Intracellular Salivary gland histolysis Salivary gland cell autophagic cell death Regulation of transcription, DNA-dependent Regulation of development Metamorphosis Ecdysone-mediated induction of salivary gland cell autophagic cell death Cell death Zinc ion binding Specific RNA polymerase II transcription factor activity Transcription factor activity Response to symbiont Response to ecdysone Regulation of development, heterochronic Heterochronic progression of morphogenetic furrow involved in compound eye morphogenesis Oogenesis Muscle fiber development Male genitalia morphogenesis Larval central nervous system remodelling Eclosion Compound eye photoreceptor fate commitment Autophagy Specific transcriptional repressor activity DNA binding

Table: 7.22. Control flies on the high and low nutrient diet: Miscellaneous transcripts.

Gene Number	Gene Name	Up/Down regulation	Absolute fold change	Gene Ontology (GO) number	GO term
CG8502	Cuticular protein isoform 49Ac	Down	9.11	GO:0042302	Structural constituent of cuticle Structural constituent of chitin-based larval cuticle
CG7539	Ecdysone-dependent gene 91	Down	4.60	GO:0008011	Structural constituent of pupal chitin-based cuticle
CG11628	Steppke	Up	3.16	GO:0040018 GO:0005086 GO:0032012 GO:0005622	Positive regulation of multicellular organism growth ARF guanyl-nucleotide exchange factor activity Regulation of ARF protein signal transduction Intracellular
CO181664	-	Up	3.06	GO:0040018	Positive regulation of multicellular organism growth
TC215268	CG7941-PA	Up	2.61	GO:0005214 GO:0008010	Structural constituent of chitin-based cuticle Structural constituent of chitin-based larval cuticle
CG13586	Ion transport peptide	Up	2.05	GO:0005179 GO:0005184 GO:0007218 GO:0005576	Hormone activity Neuropeptide hormone activity Neuropeptide signalling pathway Extracellular region

Table: 7.23. Control flies on the high and low nutrient diet: Tentative consensus sequences and expression sequence tags with no assigned gene ontology number.

Sequence Number	Sequence description	Up/Down regulation	Absolute fold change
TC198490	Tentative consensus sequence	Down	17.05
TC216174 (Embryo, imaginal disks and head)	Tentative consensus sequence	Up	3.86
TC212147 (Head)	Tentative consensus sequence	Down	3.76
TC212413 (Head)	Tentative consensus sequence	Down	3.73
TC215541 (Embryo, imaginal disks and head)	Tentative consensus sequence	Up	2.86
TC217270 (Adult head)	Tentative consensus sequence	Down	2.79
TC221252 (Embryo, imaginal disks and head)	Tentative consensus sequence	Down	2.73
BI568522	Expression sequence tag	Down	2.66
TC211303 (S2 cell)	Tentative consensus sequence	Up	2.64
TC220055 (Embryo, imaginal disks and head)	Tentative consensus sequence	Up	2.63
CO183923	Expression sequence tag	Down	2.49
CO184327	Expression sequence tag	Down	2.41
TC215981 (Salivary glands)	Tentative consensus sequence	Up	2.38
TC216561 (Embryo, imaginal disks and head)	Tentative consensus sequence	Up	2.36
BP553587	Expression sequence tag	Up	2.36
TC219436	Tentative consensus sequence	Up	2.35
TC217125 (Embryo, imaginal disks and head)	Tentative consensus sequence	Up	2.27
TC209931 (Head)	Tentative consensus sequence	Down	2.20
TC219340 (Head)	Tentative consensus sequence	Down	2.19
TC211084 (Head)	Tentative consensus sequence	Down	2.17

TC217379 (Head)	Tentative consensus sequence	Up	2.15
TC215879 (Head)	Tentative consensus sequence	Down	2.10
TC210443 (Embryo)	Tentative consensus sequence	Up	2.08
TC221262	Tentative consensus sequence	Down	2.07
TC217757	Tentative consensus sequence	Up	2.04
AI517949	Expression sequence tag	Up	2.01
TC218077 (Embryo, imaginal disks and head)	Tentative consensus sequence	Down	2.01

Table: 7.24. Control flies on the high and low nutrient diet: Sequences with no assigned gene ontology number.

Gene/Sequence Number	Up/Down regulation	Absolute fold change
CG11370	Down	4.50
CG13962	Down	4.31
CG40203	Up	3.59
CG13445	Up	2.71
GM04319	Down	2.61
BT028806	Down	2.37
AT28783	Down	2.29
CG14563	Down	2.20
CG32182	Down	2.18
CG15212	Up	2.04
CG30395	Up	2.02
CG41581	Up	2.00

Table: 7.25. Dechorionation on the high and low nutrient diet: Immune transcripts.

Gene Number	Gene Name	Up/Down regulation	Absolute fold change	Gene Ontology (GO) number	GO term
CG9120	Lysozyme precursor	Down	5.38	GO:0005576 GO:0004568 GO:0006952 GO:0016998 GO:0019730 GO:0003796	Extracellular region Chitinase activity Defence response Cell wall macromolecule catabolic process Antimicrobial humoral response Lysozyme activity
CG12763	Diptericin	Down	4.96	GO:0019731 GO:0050829 GO:0042742 GO:0005576 GO:0045087 GO:0005576	Antibacterial humoral response Defence response to Gram-negative bacterium Defence response to bacterium Innate immune response Extracellular region
BT023384	Defensin	Down	3.86-2.43	GO:0005615 GO:0019731 GO:0050830 GO:0042742 GO:0006965	Extracellular space Antibacterial humoral response Defence response to Gram-positive bacterium Defence response to bacterium Positive regulation of biosynthetic process of antibacterial peptides active against Gram-positive bacteria
CG15825	Fondue	Up	2.71	GO:0042381 GO:0007552 GO:0005811	Hemolymph coagulation Metamorphosis Lipid particle
CG31783	Neither	Up	2.65	GO:0006952	Defence response

	inactivation nor afterpotential d			GO:0007602 GO:0007603 GO:0007604 GO:0016063 GO:0007155 GO:0005887 GO:0046867 GO:0005044 GO:0006952 GO:0016020	Phototransduction Phototransduction, visible light Phototransduction, UV Rhodopsin biosynthetic process Cell adhesion Integral to plasma membrane carotenoid transport Scavenger receptor activity Defence response Membrane
CG6124-RA	-	Up	2.61	GO:0051635 GO:0006910	Bacterial cell surface binding Phagocytosis, recognition
SD22390	Cg6124-partial	Up	2.47	GO:0006910 GO:0008367	Phagocytosis Recognition
CG7002	Hemolectin	Up	2.43, 2.34, 3.08	GO:0042803 GO:0042381 GO:0007599 GO:0035006 GO:0042060 GO:0005576 GO:0008061 GO:0005529 GO:0007155 GO:0006030	Protein homodimerization activity Hemolymph coagulation Hemostasis Melanization defence response Wound healing Extracellular region Chitin binding Sugar binding Cell adhesion Chitin metabolic process
CG8942	Nimrod c1	Up	2.28	GO:0017147 GO:0006909 GO:0016055	Wnt-protein binding Phagocytosis Wnt receptor signalling pathway
CG33956	Kayak	Up	2.21	GO:0019730 GO:0007298 GO:0048749 GO:0007391 GO:0001736 GO:0046529 GO:0007254 GO:0007297 GO:0007464 GO:0031660 GO:0006355 GO:0009611 GO:0016330 GO:0051124 GO:0035220 GO:0042060 GO:0005737 GO:0005634 GO:0003677 GO:0005515 GO:0046983 GO:0046982 GO:0003702 GO:0043565 GO:0003700 GO:0003704 GO:0008134	Antimicrobial humoral response Border follicle cell migration Compound eye development Dorsal closure Establishment of planar polarity Imaginal disc fusion, thorax closure JNK cascade Ovarian follicle cell migration R3/R4 cell fate commitment Regulation of cyclin-dependent protein kinase activity involved in G2/M Regulation of transcription, DNA-dependent Response to wounding Second mitotic wave involved in compound eye morphogenesis Synaptic growth at neuromuscular junction Wing disc development Wound healing Cytoplasm Nucleus DNA binding Protein binding Protein dimerization activity Protein heterodimerization activity RNA polymerase II transcription factor activity Sequence-specific DNA binding Sequence-specific DNA binding transcription factor activity Specific RNA polymerase II transcription factor activity Transcription factor binding
CG1106	Gelsolin	Up	2.18-2.75	GO:0006911 GO:0005884 GO:0005829 GO:0005576 GO:0003779	Phagocytosis, engulfment, Actin filament Cytosol Extracellular region Actin binding
BT030437	Neuroligin 3	Up	2.10	GO:0006911 GO:0042043	Phagocytosis, engulfment Neurexin binding
CG4099	Scavenger receptor class C, type I	Up	2.04	GO:0030247 GO:0005044 GO:0006952 GO:0050829 GO:0006955 GO:0006909	Polysaccharide binding Scavenger receptor activity Defence response Defence response to Gram-negative bacterium Immune response

				GO:0009617 GO:0005887 GO:0016020	Phagocytosis response to bacterium Integral to plasma membrane membrane

Table: 7.26. Dechorionation on the high and low nutrient diet: Transport and binding transcripts.

Gene Number	Gene Name	Up/Down regulation	Absolute fold change	Gene Ontology (GO) number	GO term
CG11123	Mgc69156 protein	Up	4.082	GO:0003723	RNA binding
BT024441	Odorant-binding protein 59a	Down	3.27	GO:0006810 GO:0007606 GO:0005549	Transport Sensory perception of chemical stimulus Odorant binding
CG4950	Carboxypeptidase n subunit 2	Up	3.24	GO:0005515	Protein binding
CG32975	Nicotinic Acetylcholine Receptor α 34E	Up	3.13	GO:0042166 GO:0004889 GO:0016021 GO:0005892 GO:0006811 GO:0045211	Acetylcholine binding Nicotinic acetylcholine-activated cation-selective channel activity Integral to membrane Nicotinic acetylcholine-gated receptor-channel complex Ion transport Postsynaptic membrane
CG4139	Karl	Up	3.11	GO:0005488	Binding
CG6642	Antennal protein 10	Down	2.92 to 3.19	GO:0005549 GO:0005550 GO:0007606	Odorant binding Pheromone binding Sensory perception of chemical stimulus
U02542	Odorant-binding protein 83a	Down	2.90	GO:0005615 GO:0005550 GO:0007606 GO:0008145 GO:0006810	Extracellular space Pheromone binding Sensory perception of chemical stimulus Phenylalkylamine binding Transport
EL870510	Antennal protein 5	Down	2.89	GO:0000785 GO:0001745 GO:0006333 GO:0008429 GO:0003682 GO:0005634	Chromatin Compound eye morphogenesis Chromatin assembly or disassembly Phosphatidylethanolamine binding Chromatin binding Nucleus
CG5430	Phosphatidylethanolamine-binding protein (A5)	Down	2.85	GO:0000785 GO:0001745 GO:0006333 GO:0008429 GO:0003682 GO:0005634	Chromatin Compound eye morphogenesis Chromatin assembly or disassembly Phosphatidylethanolamine binding Chromatin binding Nucleus
CG18408	CAP	Up	2.82	GO:0005925 GO:0048190 GO:0016246 GO:0008103 GO:0008360 GO:0008154 GO:0016442 GO:0042052 GO:0005509 GO:0008407 GO:0002168 GO:0008179	Focal adhesion Wing disc dorsal/ventral pattern formation RNA interference Oocyte Microtubule cytoskeleton polarization Regulation of cell shape Cohesin complex ATP binding ATP-dependent RNA helicase activity

				GO:0008278 GO:0005524 GO:0004004 GO:0007062 GO:0005811 GO:0043186 GO:0007286 GO:0003676 GO:0003779 GO:0017166	Sister chromatid cohesion Lipid particle Granule Spermatid development Nucleic acid binding Actin binding Adenylate cyclase binding Instar larval development Bristle morphogenesis Calcium ion binding Rhabdomere development RNA-induced silencing complex Actin polymerization or depolymerization Vinculin binding
CG11748	Odorant-binding protein 19a	Down	2.80	GO:0005576 GO:0042048 GO:0019236 GO:0007606 GO:0005549 GO:0006810	Extracellular region Olfactory behaviour Response to pheromone Sensory perception of chemical stimulus Odorant binding Transport
CA805378	Odorant-binding protein 99b	Down	2.69	GO:0005549 GO:0007606 GO:0006810 GO:0035071 GO:0019236 GO:0005576 GO:0042048	Odorant binding Sensory perception of chemical stimulus Transport Salivary gland cell Autophagic cell death Response to pheromone Extracellular region Olfactory behaviour
CG41087	-	Up	2.61	GO:0006457 GO:0031072 GO:0051082	Protein folding Heat shock protein binding Unfolded protein binding
CG10436	Pheromone-binding protein-related protein 1	Down	2.60-2.57	GO:0005549 GO:0006810 GO:0008145 GO:0005550 GO:0007606 GO:0005576	Odorant binding Transport Phenylalkylamine binding Pheromone binding Sensory perception of chemical stimulus Extracellular region
BT029288	Zinc finger protein	Down	2.51	GO:0003676 GO:0005622 GO:0008270	Nucleic acid binding Zinc ion binding Intracellular
CG3250	Os-C	Down	2.51-2.86	GO:0005550	Pheromone binding
CG11326	Thrombospondin	Up	2.49	GO:0008201 GO:0033627 GO:0016203 GO:0007517 GO:0031012 GO:0005927 GO:0043234 GO:0005509	Heparin binding Cell adhesion mediated by integrin Muscle attachment Muscle organ development Extracellular matrix Muscle tendon junction Protein complex Calcium ion binding
CG15279	Sodium shloride dependent amino acid transporter	Up	2.43	GO:0005887 GO:0005328 GO:0005416 GO:0005416 GO:0006836 GO:0005326	Integral to plasma membrane Neurotransmitter:sodium symporter activity Cation:amino acid symporter activity Neurotransmitter transport Neurotransmitter transporter activity
CG7454	Odorant receptor 85a	Down	2.34	GO:0007186 GO:0016021 GO:0004984 GO:0050896	G-protein coupled receptor protein signalling pathway Integral to membrane

				GO:0005549 GO:0005886 GO:0007608 GO:0016020	Olfactory receptor activity Response to stimulus Odorant binding Plasma membrane Sensory perception of smell Membrane
CG8807	Lush	Down	2.30	GO:0035275 GO:0042048 GO:0019236 GO:0005549 GO:0045471 GO:0007606 GO:0007608 GO:0005576 GO:0006810	Dibutyl phthalate binding Olfactory behaviour Response to pheromone Odorant binding Response to ethanol Sensory perception of chemical stimulus Sensory perception of smell, Extracellular region Transport
CG5670	Na pump α subunit (Atpalpha)	Up	2.26	GO:0008324 GO:0005391 GO:0008344 GO:0006812 GO:0008340 GO:0001700 GO:0007626 GO:0050905 GO:0008360 GO:0035158 GO:0009612 GO:0009266 GO:0009612 GO:0019991 GO:0051124 GO:0007268 GO:0001894 GO:0005634 GO:0005886 GO:0005918 GO:0005886 GO:0005524 GO:0005391 GO:0006754 GO:0006812 GO:0015672 GO:0035152 GO:0005886 GO:0005890	Cation transmembrane transporter activity Sodium:potassium-exchanging ATPase activity Adult locomotory behavior Cation transport Determination of adult lifespan Embryonic development via the syncytial blastoderm Locomotory behavior Neuromuscular process Regulation of cell shape Regulation of tube diameter, open tracheal system Response to mechanical stimulus Septate junction assembly Synaptic growth at neuromuscular junction Synaptic transmission Tissue homeostasis Plasma membrane ATP binding Sodium:potassium-exchanging ATPase activity ATP biosynthetic process Cation transport Monovalent inorganic cation transport Regulation of tube architecture, open tracheal system Plasma membrane Sodium:potassium-exchanging ATPase complex
CG6600-RA	Mfs transporter	Up	2.24	GO:0006810 GO:0016021 GO:0005215	Transport Integral to membrane Transporter activity
CG1176	Pheromone-binding protein isoform a	Down	2.24	GO:0005576 GO:0005550 GO:0007606 GO:0008145 GO:0005549 GO:0006810	Extracellular region Pheromone binding Sensory perception of chemical stimulus Phenylalkylamine binding Odorant binding Transport

AA141263	AA141263	Down	2.19	GO:0005515	Protein binding
CG8497	Rhophilin	Up	2.17	GO:0017049 GO:0007165 GO:0005622	GTP-Rho binding Signal transduction Intracellular
EL881596	EL881596	Down	2.15	GO:0005267 GO:0006813 GO:0016020	Potassium channel activity Potassium ion transport Membrane
CG41520	CG41520	Up	2.14	GO:0005102 GO:0007165	Receptor binding Signal transduction
BT029057	Dpr3	Up	2.09	GO:0007500 GO:0006468 GO:0007523 GO:0007280 GO:0008360 GO:0032234 GO:0048542 GO:0048747 GO:0016021 GO:0007506 GO:0007419 GO:0008347 GO:0007513 GO:0005007 GO:0007525 GO:0008078 GO:0007431 GO:0008543 GO:0007493 GO:0005886 GO:0010002 GO:0005524	Mesodermal cell fate determination Protein amino acid phosphorylation Larval visceral muscle development Pole cell migration Regulation of cell shape Regulation of calcium ion transport via store-operated calcium channel activity Lymph gland development Muscle fiber development Integral to membrane Gonadal mesoderm development Ventral cord development Glial cell migration Pericardial cell differentiation Fibroblast growth factor receptor activity Somatic muscle development Mesodermal cell migration Salivary gland development Fibroblast growth factor receptor signalling pathway Endodermal cell fate determination Plasma membrane Cardioblast differentiation ATP binding
GH07418	Isoform b	Up	2.09	GO:0005743 GO:0022857 GO:0005811 GO:0006839 GO:0005509 GO:0016021	Mitochondrial inner membrane Transmembrane transporter activity Lipid particle Mitochondrial transport Calcium ion binding Integral to membrane
CG12754	Odorant receptor 42b	Down	2.08	GO:0007608 GO:0004984 GO:0005549 GO:0016021 GO:0016020	Sensory perception of smell Olfactory receptor activity Odorant binding Integral to membrane Plasma membrane
CG1915-RA	Sallimus	Up	2.07	GO:0003779 GO:0008307 GO:0040011 GO:0007498 GO:0007076 GO:0016203 GO:0007520 GO:0045214 GO:0007062 GO:0007519 GO:0000794	Actin binding Structural constituent of muscle Locomotion Mesoderm development Mitotic chromosome condensation Muscle attachment Myoblast fusion Sarcomere organization Sister chromatid cohesion

				GO:0005875 GO:0030017 GO:0030018 GO:0004687 GO:0005089 GO:0007517 GO:0035023 GO:0004687	Skeletal muscle tissue development Condensed nuclear chromosome Microtubule associated complex Sarcomere Z disc Rho guanyl-nucleotide exchange factor activity Muscle organ development Regulation of Rho protein signal transduction Myosin light chain kinase activity
CG6641	Pheromone-binding protein 5	Down	2.06	GO:0005576 GO:0005550 GO:0007606 GO:0008145 GO:0006810 GO:0005549	Extracellular region Pheromone binding Sensory perception of chemical stimulus Phenylalkylamine binding Transport Odorant binding

Table: 7.27. Dechorionation on the high and low nutrient diet: Metabolic transcripts.

Gene Number	Gene Name	Up/Down regulation	Absolute fold change	Gene Ontology (GO) number	GO term
CG17285	Fat body protein 1	Down	7.91	GO:0005344 GO:0008565 GO:0005811 GO:0015032	Oxygen transporter activity Protein transporter activity Lipid particle Storage protein import into fat body
CG7017	-	Down	5.15	GO:0005576 GO:0008061 GO:0006030 GO:0016490	Extracellular region Chitin binding Chitin metabolic process structural constituent of peritrophic membrane
CG3763	Fat body protein 2	Down	4.79 to 6.99	GO:0055114 GO:0004022 GO:0045735 GO:0005488 GO:0005811	Oxidation reduction Alcohol dehydrogenase (NAD) activity Nutrient reservoir activity Binding Lipid particle
CG4178	Larval serum protein 1 beta	Down	3.62	GO:0005344 GO:0005616 GO:0045735 GO:0005811 GO:0006810	Oxygen transporter activity Larval serum protein complex Nutrient reservoir activity Lipid particle Transport
CG11012-RA	Idp-glycosyltransferase 37a1	Up	3.55	GO:0016758 GO:0008152 GO:0015020	Transferase activity, transferring hexosyl groups Metabolic process Glucuronosyltransferase activity
CG4757	-	Down	3.38	GO:0016787 GO:0004091	Hydrolase activity Carboxylesterase activity
CG2559	Larval serum protein 1 alpha	Down	3.18	GO:0005344 GO:0005616 GO:0045735 GO:0005811 GO:0006810 GO:0005576	Oxygen transporter activity Larval serum protein complex Nutrient reservoir activity Lipid particle Transport extracellular region
TC213959	NADPH--cytochrome	Up	3.16	GO:0009384 GO:0001640	N-acylmannosamine kinase activity

	P450 reductase			GO:0005515 GO:0004396 GO:0001642 GO:0008237 GO:0008270 GO:0004089 GO:0005516 GO:0030165 GO:0004652 GO:0008761 GO:0042803 GO:0004617 GO:0042169 GO:0016595 GO:003042 GO:000561 GO:0005737 GO:0043025 GO:0042734 GO:0032279 GO:0043679 GO:0048786 GO:0005791 GO:0005829 GO:0005624 GO:0045202 GO:0030424 GO:0005634 GO:0043195 GO:0043198 GO:0043234 GO:0005794 GO:0006096 GO:0046380 GO:0007155 GO:0006508 GO:0007196 GO:0007611 GO:0014050 GO:0006054	Adenylate cyclase inhibiting metabotropic glutamate receptor activity Protein binding Hexokinase activity Group III metabotropic glutamate receptor activity Metallopeptidase activity Zinc ion binding Carbonate dehydratase activity Calmodulin binding PDZ domain binding Polynucleotide adenylyltransferase activity UDP-N-acetylglucosamine 2- epimerase activity Protein homodimerization activity Phosphoglycerate dehydrogenase activity SH2 domain binding Glutamate binding- Dendrite- Extracellular space- Cytoplasm- Cell soma- Asymmetric synapse- Presynaptic active zone Rough endoplasmic reticulum Cytosol Membrane fraction Synapse Axon- Nucleus Terminal button Dendritic shaft Protein complex Golgi apparatus Glycolysis N-acetylneuraminate biosynthetic process- Cell adhesion Proteolysis Metabotropic glutamate receptor, adenylate cyclase inhibiting pathway Learning and/or memory Negative regulation of glutamate secretion N-acetylneuraminate metabolic process
CG9244	Aconitase	Up	3.06	GO:0005811 GO:0006099 GO:0051539 GO:0003994 GO:0005739 GO:0006099	Lipid particle Tricarboxylic acid cycle 4 iron, 4 sulfur cluster binding Aconitate hydratase activity Mitochondrion Tricarboxylic acid cycle
CG10357	Fbn28 protein	Down	2.84	GO:0005576 GO:0003676 GO:0003824 GO:0005622 GO:0008270 GO:0006629 GO:0004806	Extracellular region Nucleic acid binding Catalytic activity Intracellular Zinc ion binding Lipid metabolic process Triglyceride lipase activity
CG5999	-	Up	2.80	GO:0016758 GO:0008152 GO:0015020	Transferase activity Transferring hexosyl groups Metabolic process Glucuronosyltransferase activity

CG33273	Insulin-like peptide 5	Down	2.80	GO:0005576 GO:0005179 GO:0008286 GO:0060180 GO:0005158	Extracellular region Hormone activity Insulin receptor signalling pathway Female mating behavior Insulin receptor binding
BT030185	Isoform c	Up	2.78	GO:0016887 GO:0005524 GO:0043190 GO:0005215	ATPase activity ATP binding ATP-binding cassette (ABC) transporter complex Transporter activity
CG13643	-	Up	2.73	GO:0003777 GO:0005875 GO:0008061 GO:0005524 GO:0007018 GO:0005576 GO:0006030	Microtubule motor activity Microtubule associated complex, Chitin binding ATP binding Microtubule-based movement, Extracellular region Chitin metabolic process
CG11661	Neural conserved at 73EF	Up	2.72	GO:0005875 GO:0004591 GO:0030976 GO:0006096 GO:0006099 GO:0009353	Microtubule associated complex Oxoglutarate dehydrogenase (succinyl-transferring) activity Thiamin pyrophosphate binding Glycolysis, tricarboxylic acid cycle Mitochondrial oxoglutarate dehydrogenase complex
BT025118	Serine protease	Down	2.66	GO:0005576 GO:0005198 GO:0006508 GO:0004252	Extracellular region Structural molecule activity Proteolysis Serine-type endopeptidase activity
CG6865	Anionic trypsin-2	Down	2.61	GO:0006508 GO:0004252	Proteolysis Serine-type endopeptidase activity
CG32304	Obstructor-I	Down	2.57	GO:0005576 GO:0008061 GO:0006030	Extracellular region Chitin binding Chitin metabolic process
CG3725	Calcium ATPase at 60A	Up	2.57 to 2.90	GO:0005515 GO:0007274 GO:0051282 GO:0005783 GO:0005811 GO:0005635 GO:0005524 GO:0005388 GO:0006754 GO:0006816 GO:0005789 GO:0016021 GO:0016529	Protein binding Neuromuscular synaptic transmission Regulation of sequestering of calcium ion Endoplasmic reticulum Lipid particle Nuclear envelope ATP binding Calcium-transporting ATPase activity ATP biosynthetic process Calcium ion transport Endoplasmic reticulum membrane Integral to membrane Sarcoplasmic reticulum
CG8256	Glycerol-3-phosphate dehydrogenase	Up	2.57	GO:0005743 GO:0007629 GO:0006072 GO:0005811 GO:0006127 GO:0005509 GO:0004368 GO:0009331	Mitochondrial inner membrane Flight behaviour Glycerol-3-phosphate metabolic process Lipid particle Glycerophosphate shuttle Calcium ion binding Glycerol-3-phosphate dehydrogenase activity Glycerol-3-phosphate dehydrogenase complex

CG30035	Trehalose transporter 1-1	Up	2.46	GO:0015771 GO:0016020 GO:0005355 GO:0008643 GO:0055085 GO:0016021	Trehalose transport Membrane Glucose transmembrane transporter activity Carbohydrate transport Transmembrane transport Integral to membrane
CG6193	Adenomatous polyposis coli tumor sUppressor homolog 2	Up	2.45	GO:0007411 GO:0035293 GO:0008362 GO:0030720 GO:0016337 GO:0007405 GO:0045892 GO:0008017 GO:0030178 GO:0008258 GO:0040001 GO:0032154 GO:0045179 GO:0005875 GO:0016327 GO:0005912 GO:0007447 GO:0005634 GO:0045862 GO:0021550 GO:0035190 GO:0000910	Axon guidance Chitin-based larval cuticle pattern formation Chitin-based embryonic cuticle biosynthetic process Oocyte localization during germarium-derived egg chamber formation Cell-cell adhesion Neuroblast proliferation Negative regulation of transcription DNA-dependent Microtubule binding Negative regulation of Wnt receptor signalling pathway Head involution Establishment of mitotic spindle localization Cleavage furrow Apical cortex Microtubule associated complex Apicolateral plasma membrane Adherens junction Imaginal disc pattern formation Nucleus Positive regulation of proteolysis Medulla oblongata development Syncytial nuclear migration Cytokinesis
CG7399	Henna	Up	2.42	GO:0006726 GO:0004505 GO:0005811 GO:0005506 GO:0004510 GO:0055114 GO:0016597 GO:0006911 GO:0006559	Eye pigment biosynthetic process Phenylalanine 4-monooxygenase activity Lipid particle Iron ion binding Tryptophan 5-monooxygenase activity Oxidation reduction Amino acid binding Phagocytosis, engulfment, L-phenylalanine catabolic process
CG33159	-	Down	2.37	GO:0006508 GO:0004252	Proteolysis Serine-type endopeptidase activity
CG4067	Pugilist	Up	2.36	GO:0004488 GO:0004477 GO:0005524 GO:0008652 GO:0004329 GO:0009396 GO:0005811 GO:0055114	Methylenetetrahydrofolate dehydrogenase (NADP+) activity Methenyltetrahydrofolate cyclohydrolase activity ATP binding Cellular amino acid biosynthetic process Formate-tetrahydrofolate ligase activity Folic acid and derivative biosynthetic process Lipid particle Oxidation reduction

TC206789	CG6865-PA	Down	2.34	GO:0004295 GO:0006508	Trypsin activity Proteolysis
TC196046	-	Down	2.34	GO:0009384 GO:0001640 GO:0005515 GO:0004396 GO:0001642 GO:0008237 GO:0008270 GO:0004089 GO:0005516 GO:0030165 GO:0004652 GO:0008761 GO:0042803 GO:0004617 GO:0042169 GO:0016595 GO:0030425 GO:0005615 GO:0005737 GO:0043025 GO:0042734 GO:0032279 GO:0043679 GO:0048786 GO:0005791 GO:0005829 GO:0005624 GO:0045202 GO:0030424 GO:0005634 GO:0043195 GO:0043198 GO:0043234 GO:0005794 GO:0006096 GO:0046380 GO:0007155 GO:0006508 GO:0007196 GO:0007611 GO:0014050 GO:0006054	N-acylmannosamine kinase activity Adenylate cyclase inhibiting metabotropic glutamate receptor activity Protein binding- Hexokinase activity Group III metabotropic glutamate receptor activity- Metallopeptidase activity Zinc ion binding- Carbonate dehydratase activity Calmodulin binding PDZ domain binding Polynucleotide adenylyltransferase activity UDP-N-acetylglucosamine 2- epimerase activity Protein homodimerization activity Phosphoglycerate dehydrogenase activity SH2 domain binding Glutamate binding Dendrite Extracellular space Cytoplasm- Cell soma Presynaptic membrane- Asymmetric synapse Nerve terminal Presynaptic active zone Rough endoplasmic reticulum Cytosol- Membrane fraction Synapse Axon Nucleus Terminal button Dendritic shaft Protein complex Golgi apparatus Glycolysis N-acetylneuraminate biosynthetic process Cell adhesion Proteolysis- Metabotropic glutamate receptor, adenylate cyclase inhibiting pathway Learning and/or memory Negative regulation of glutamate secretion N-acetylneuraminate metabolic process
CG5887	Desat1	Up	2.30	GO:0006723 GO:0042811 GO:0005811 GO:0004768 GO:0006633 GO:0006629 GO:0055114	Cuticle hydrocarbon biosynthetic process Pheromone biosynthetic process Lipid particle Stearoyl-CoA 9-desaturase activity Fatty acid biosynthetic process Lipid metabolic process Oxidation reduction
BT022319	Glutathione s transferase	Down	2.29	GO:0004364	Glutathione transferase activity

	e4				
CG11391	IP11920p	Down	2.29	GO:0003824 GO:0008152	Catalytic activity Metabolic process
CO194525	CG7900	Down	2.28	GO:0016884	Carbon-nitrogen ligase activity, with glutamine as amido-N-donor
CG40801	Phosphoribosylaminoimidazole carboxylase	Up	2.27	GO:0004638 GO:0006189 GO:0004639 GO:0009320 GO:0005524	Phosphoribosylaminoimidazole carboxylase activity De novo' IMP biosynthetic process Phosphoribosylaminoimidazolesuccinocarboxamide synthase activity Phosphoribosylaminoimidazole carboxylase complex ATP binding
CG1743	Glutamine synthetase 2 (glutamate-amonia ligase)	Up	2.22	GO:0005737 GO:0004356 GO:0006538 GO:0007416 GO:0045213 GO:0006542	Cytoplasm Glutamate-ammonia ligase activity Glutamate catabolic process Synapse assembly Neurotransmitter receptor metabolic process Glutamine biosynthetic process
CG17525	Glutathione s transferase e4	Down	2.19	GO:0004364	Glutathione transferase activity
CG8424	Juvenile hormone esterase dUplication	Down	2.18 to 2.30	GO:0004091	Carboxylesterase activity
CG9485	isoform d	Up	2.17	GO:0004135 GO:0005978 GO:0043169 GO:0004134	Amylo-alpha-1,6-glucosidase activity Glycogen biosynthetic process Cation binding 4-alpha-glucanotransferase activity
CG7910	-	Up	2.17	GO:0016884 GO:0017064	Carbon-nitrogen ligase activity, with glutamine as amido-N-donor Fatty acid amide hydrolase activity
CG4347	UGP	Up	2.16	GO:0003983 GO:0008152	UTP:glucose-1-phosphate uridylyltransferase activity Metabolic process
CG2958	Lectin-24Db	Up	2.16	GO:0042806 GO:0005537 GO:0005534	Fucose binding Mannose binding Galactose binding
NM_141223	Isoform a	Up	2.15	GO:0006182, GO:0006468, GO:0007242, GO:0004713, GO:0005524, GO:0004383	cGMP biosynthetic process Protein amino acid phosphorylation Protein tyrosine kinase activity ATP binding Guanylate cyclase activity
CG3001	Hexokinase (Hex-A)	Up	2.14	GO:0005524 GO:0006096 GO:0004396	ATP binding Glycolysis Hexokinase activity
CG3972	Cyp4g1(cytochrome p450)	Up	2.12	GO:0020037 GO:0016020 GO:0055114 GO:0004497 GO:0009055 GO:0006629 GO:0005792	Heme binding Membrane Oxidation reduction, Monooxygenase activity Electron carrier activity Lipid metabolic process Microsome
CG8808	Pyruvate dehydrogenase kinase	Up	2.08	GO:0005524 GO:0004740 GO:0000155 GO:0018106	ATP binding Pyruvate dehydrogenase (acetyl-transferring) kinase activity

				GO:0006090 GO:0007165 GO:0005759	Two-component sensor activity Peptidyl-histidine phosphorylation Pyruvate metabolic process Signal transduction Mitochondrial matrix
CG5028	Isocitrate dehydrogenase	Up	2.08	GO:0005875 GO:0004449 GO:0000287 GO:0051287 GO:0006099 GO:0005759 GO:0005739	Microtubule associated complex Isocitrate dehydrogenase (NAD+) activity Magnesium ion binding NAD or NADH binding Tricarboxylic acid cycle Mitochondrial matrix Mitochondrion
TC216824	Retrotransposon-like family member (retr-1)-like	Up	2.06	GO:0003676 GO:0044238 GO:0003824	Nucleic acid binding Primary metabolic process Catalytic activity
CG34357	-	Up	2.05	GO:0005524 GO:0004383 GO:0004672 GO:0006182 GO:0023034 GO:0006468	ATP binding Guanylate cyclase activity Protein kinase activity cGMP biosynthetic process Intracellular signalling pathway Protein amino acid phosphorylation
CG3979	I'm not dead yet	Up	2.05	GO:0015137 GO:0015141 GO:0008340 GO:0010889 GO:0005886 GO:0015142 GO:0006814 GO:0055085	Citrate transmembrane transporter activity Succinate transmembrane transporter activity Determination of adult lifespan Regulation of sequestering of triglyceride Plasma membrane Tricarboxylic acid transmembrane transporter activity Sodium ion transport Transmembrane transport
CG4329	Isoform a	Up	2.04	GO:0006754 GO:0005524 GO:0016020 GO:0015662	ATP biosynthetic process ATP binding Membrane ATPase activity, Coupled to transmembrane movement of ions, phosphorylative mechanism
CG10924	-	Up	2.03	GO:0005525 GO:0004613 GO:0006094 GO:0005739	GTP binding Phosphoenolpyruvate carboxykinase (GTP) activity Gluconeogenesis Mitochondrion
CG6784	Tissue factor pathway inhibitor 2	Down	2.03	GO:0004867	Serine-type endopeptidase inhibitor activity
LD28657	Mlf1-adaptor molecule	Up	2.03	GO:0006468 GO:0012505 GO:0005794 GO:0004672 GO:0005524 GO:0006888 GO:0005829 GO:0042803	Protein amino acid phosphorylation Endomembrane system Golgi apparatus Protein kinase activity ATP binding ER to Golgi vesicle-mediated transport Cytosol Protein homodimerization activity
CG33103	Papilin	Up	2.02 to 2.23	GO:0005201 GO:0030198	Extracellular matrix structural constituent

				GO:0005604 GO:0004222 GO:0004867 GO:0008270 GO:0005578	Extracellular matrix organization Basement membrane Metalloendopeptidase activity Serine-type endopeptidase inhibitor activity Zinc ion binding Proteinaceous extracellular matrix
CG3902	Acyl-dehydrogenase	Up	2.02	GO:0008152 GO:0009055 GO:0003995 GO:0050660	Metabolic process Electron carrier activity Acyl-CoA dehydrogenase activity FAD binding
TC201205	CG2790-PA	Up	2.00	GO:0008411 GO:0007163 GO:0009061	4-hydroxybutyrate CoA-transferase activity Establishment and/or maintenance of cell polarity Anaerobic respiration

Table: 7.28. Dechorionation on the high and low nutrient diet: RNA/DNA replication/transcription transcripts.

Gene Number	Gene Name	Up/Down regulation	Absolute fold change	Gene Ontology (GO) number	GO term
TC219369	Reverse transcriptase	Down	4.06	GO:0003723 GO:0003964 GO:0006278	RNA binding RNA-directed DNA polymerase activity RNA-dependent DNA replication
CG10110-RA	Cleavage and polyadenylation specificity factor cpsf	Up	3.44	GO:0006378 GO:0005847 GO:0003730 GO:0005515 GO:0006379	mRNA polyadenylation ,mRNA cleavage and polyadenylation Specificity factor complex mRNA 3'-UTR binding, Protein binding mRNA cleavage
CG2932	Bteb2	Up	2.83	GO:0003702 GO:0003676 GO:0005634 GO:0008270 GO:0005622	RNA polymerase II transcription factor activity Nucleic acid binding Nucleus Zinc ion binding Intracellular
TC197177	Pol protein	Up	2.60	GO:0004519 GO:0003964 GO:0003677 GO:0006278 GO:0003723 GO:0015074	Endonuclease activity RNA-directed DNA polymerase activity DNA binding RNA-dependent DNA replication RNA binding DNA integration
CG32353	CG32353	Up	2.54	GO:0045449 GO:0030176 GO:0043565 GO:0030528 GO:0003677 GO:0005634	Regulation of transcription, DNA-dependent Integral to endoplasmic reticulum membrane Sequence-specific DNA binding Regulation of transcription Transcription regulator activity

					DNA binding Nucleus
CG40351	CG40351	Up	2.52	GO:0003676 GO:0005634 GO:0000166 GO:0018024 GO:0006338	Nucleic acid binding Nucleus Nucleotide binding Histone-lysine N-methyltransferase activity Chromatin remodeling
CG6269	Unc-4	Down	2.49	GO:0043565 GO:0003700 GO:0045449 GO:0006355 GO:0005634 GO:0003700	Sequence-specific DNA binding Transcription factor activity Regulation of transcription Regulation of transcription, DNA-dependent Nucleus Transcription factor activity
CG13906	Nervous fingers 1	Down	2.47	GO:0007411 GO:0006357 GO:0008270 GO:0048663 GO:0048813 GO:0005634 GO:0003702 GO:0048666 GO:0003676 GO:0005622	Axon guidance Regulation of transcription from RNA polymerase II promoter Zinc ion binding Neuron fate commitment Dendrite morphogenesis Nucleus RNA polymerase II transcription factor activity Neuron development Nucleic acid binding Intracellular
CG11049	Shaven	Up	2.17	GO:0048813 GO:0007517 GO:0048666 GO:0003677 GO:0003702 GO:0042676 GO:0045449 GO:0005634 GO:0003700	Dendrite morphogenesis Muscle organ development Neuron development DNA binding RNA polymerase II transcription factor activity Compound eye cone cell fate commitment Regulation of transcription Nucleus Transcription factor activity
CG10293-RC	Held out wings	Up	2.14	GO:0005634 GO:0005737 GO:0030154 GO:0003729 GO:0007525 GO:0045214 GO:0000381 GO:0007438 GO:0007498 GO:0008078 GO:0008347 GO:0009790 GO:0008366 GO:0007475 GO:0003730	Nucleus Cytoplasm Cell differentiation mRNA binding Somatic muscle development Sarcomere organization Regulation of alternative nuclear mRNA splicing, via spliceosome Oenocyte development Mesoderm development Mesodermal cell migration Glial cell migration Embryonic development Axon ensheathment Apposition of dorsal and ventral imaginal disc-derived wing surfaces mRNA 3'-UTR binding

TC194836	-	Down	2.11	GO:0001584 GO:0042562 GO:0003705 GO:0008188 GO:0005515 GO:0003682 GO:0003700 GO:0008022 GO:0008138 GO:0003677 GO:0004930 GO:0030273 GO:0019182 GO:0016933 GO:0019992 GO:0016500 GO:0008301 GO:0016020 GO:0005737 GO:0005887 GO:0019183 GO:0005667 GO:0016021 GO:0005886 GO:0005575 GO:0005634 GO:0007166 GO:0001756 GO:0007218 GO:0048468 GO:0007193 GO:0045472 GO:0006091 GO:0042475 GO:0045944 GO:0007631 GO:0030534 GO:0051928 GO:0009636 GO:0006357 GO:0007186 GO:0007268 GO:0007204	Rhodopsin-like receptor activity Hormone binding RNA polymerase II transcription factor activity, enhancer binding Neuropeptide receptor activity Protein binding Chromatin binding Transcription factor activity Protein C-terminus binding Protein tyrosine/serine/threonine phosphatase activity DNA binding G-protein coupled receptor activity Melanin-concentrating hormone receptor activity Histamine-gated chloride channel activity Extracellular-glycine- gated ion channel activity Diacylglycerol binding Protein-hormone receptor activity DNA bending activity Cytoplasm Integral to plasma membrane Histamine-gated chloride channel complex- Transcription factor complex Integral to membrane Plasma membrane Cellular component Nucleus Cell surface receptor linked signal transduction- Somitogenesis Neuropeptide signalling pathway Cell development G-protein signalling, adenylate cyclase inhibiting pathway Response to ether Generation of precursor metabolites and energy Odontogenesis of dentine-containing teeth Positive regulation of transcription from RNA polymerase II promoter Feeding behavior Adult behaviour Positive regulation of calcium ion transport Response to toxin Regulation of transcription from RNA polymerase II promoter G-protein coupled receptor protein signalling pathway Synaptic transmission

					Elevation of cytosolic calcium ion concentration
CG6103	Cyclic-AMP response element binding protein B at 17A	Up	2.06	GO:0003677 GO:0003702 GO:0043565 GO:0007616 GO:0072375 GO:0010552 GO:0006355 GO:0030431 GO:0005634 GO:0046983 GO:0003702 GO:0003700 GO:0007623 GO:0007611 GO:0045475 GO:0045187 GO:0007622 GO:0005634	DNA binding RNA polymerase II transcription factor activity Sequence-specific DNA binding Long-term memory Medium-term memory Positive regulation of gene-specific transcription from RNA polymerase II promoter Regulation of transcription, DNA-dependent Sleep Nucleus Protein dimerization activity RNA polymerase II transcription factor activity Transcription factor activity Circadian rhythm Learning or memory Locomotor rhythm Regulation of circadian sleep/wake cycle, sleep Rhythmic behaviour Nucleus

Table: 7.29. Dechorionation on the high and low nutrient diet: Miscellaneous transcripts.

Gene Number	Gene Name	Up/Down regulation	Absolute fold change	Gene Ontology (GO) number	GO term
CG33519	Unc-89	Up	5.02	GO:0005524 GO:0004674 GO:0005089 GO:0006468 GO:0035023 GO:0005622 GO:0007527 GO:0045214	ATP binding Protein serine/threonine kinase activity Rho guanyl-nucleotide exchange factor activity Protein amino acid phosphorylation Regulation of Rho protein signal transduction Intracellular Adult somatic muscle development Sarcomere organization
TC215525	Odorant receptor 47 a	Down	4.66	GO:0005214 GO:0008010	Structural constituent of chitin-based cuticle Structural constituent of chitin-based larval cuticle
CG14669	-	Up	3.40	GO:0003924 GO:0005525 GO:0007264 GO:0016020	GTPase activity GTP binding Small GTPase mediated signal transduction Membrane
AF254371	Stretchin- isoform d	Up	2.74	GO:0005200 GO:0004683 GO:0006468 GO:0004687 GO:0005524 GO:0010447	Structural constituent of cytoskeleton Calmodulin-dependent protein kinase activity Protein amino acid phosphorylation

				GO:0005021 GO:0042597	Myosin light chain kinase activity ATP binding Response to acidity Vascular endothelial growth factor receptor activity Periplasmic space
CG1228-RD	Ptpmeg	Up	2.57	GO:0048102 GO:0048846 GO:0048036 GO:0016319 GO:0035071 GO:0008092 GO:0004725 GO:0006470 GO:0005737 GO:0005856 GO:0019898	Autophagic cell death Axon extension involved in axon guidance Central complex development Mushroom body development Salivary gland cell Cytoskeletal protein binding Protein tyrosine phosphatase activity Protein amino acid dephosphorylation Cytoplasm Cytoskeleton Extrinsic to membrane
TC219478	-	Up	2.48	GO:0005634	Nucleus
BT030162	Steppke	Up	2.45	GO:0040018 GO:0005086 GO:0032012 GO:0005622	Positive regulation of multicellular organism growth ARF guanyl-nucleotide exchange factor activity Regulation of ARF protein signal transduction Intracellular
TC220202	-	Down	2.35	GO:0005634 GO:0005681 GO:0000398 GO:0000381 GO:0009792	Nucleus Spliceosome Nuclear mRNA splicing via spliceosome Regulation of alternative nuclear mRNA splicing, via spliceosome Embryonic development ending in birth or egg hatching
GH15083	Isoform a	Up	2.22	GO:0031410 GO:0008355 GO:0042127 GO:0007611	Cytoplasmic vesicle Olfactory learning Regulation of cell proliferation Learning or memory
LD02307	Isoform b	Up	2.15	GO:0005576 GO:0008083	Extracellular region Growth factor activity
CG31004	CG31004	Up	2.10	GO:0007160	Cell-matrix adhesion
CG33960	CG33960	Up	2.10	GO:0007411 GO:0005886	Axon guidance, Plasma membrane
CG7533	Charybde	Up	2.09	GO:0008219 GO:0008258 GO:0045926 GO:0009968 GO:0005737	Cell death Head involution Negative regulation of growth Negative regulation of signal transduction Cytoplasm
CG8927	Isoform a	Up	2.08	GO:0042302	Structural constituent of cuticle
CG8201-RM	Par-1	Up	2.08	GO:0004672 GO:0050321 GO:0007015 GO:0019730 GO:0009798	Protein kinase activity Tau-protein kinase activity Actin filament organization Antimicrobial humoral response

				GO:0030709 GO:0007298 GO:0001737 GO:0007276 GO:0007294 GO:0000226 GO:0090176 GO:0016325 GO:0051663 GO:0030707 GO:0007318 GO:0008360 GO:0007317 GO:0051124 GO:0016323 GO:0005938 GO:0045169 GO:0045172 GO:0031594 GO:0061174 GO:0005524 GO:0004674 GO:0009948 GO:0007294 GO:0045185 GO:0007314 GO:0009994 GO:0007314 GO:0009994 GO:0016325 GO:0045451 GO:0006468 GO:0030111	Axis specification Border follicle cell delamination Border follicle cell migration Establishment of imaginal disc-derived wing hair orientation Gamete generation Germarium-derived oocyte fate determination Microtubule cytoskeleton organization Microtubule cytoskeleton organization involved in Establishment of planar polarity Oocyte microtubule cytoskeleton organization Oocyte nucleus localization involved in oocyte Dorsal/ventral axis specification Ovarian follicle cell development Pole plasm protein localization Regulation of cell shape Regulation of pole plasm oskar mRNA localization Synaptic growth at neuromuscular junction Basolateral plasma membrane Cell cortex Fusome Germline ring canal Neuromuscular junction Type I terminal button ATP binding Protein serine/threonine kinase activity Anterior/posterior axis specification Germarium-derived oocyte fate determination Maintenance of protein location Oocyte anterior/posterior axis specification Oocyte differentiation Oocyte microtubule cytoskeleton organization Pole plasm oskar mRNA localization Protein amino acid phosphorylation Regulation of Wnt receptor signalling pathway
CG13586	Ion transport peptide	Up	2.06	GO:0005179 GO:0005184 GO:0007218 GO:0005576	Hormone activity Neuropeptide hormone activity Neuropeptide signalling pathway Extracellular region
CG18255	Stretchin-Mlck	Up	2.00 to 2.91	GO:0004683 GO:0004687 GO:0005875 GO:0005524 GO:0004674 GO:0005200	Calmodulin-dependent protein kinase activity Myosin light chain kinase activity Microtubule associated complex

				GO:0006468	ATP binding Protein serine/threonine kinase activity Structural constituent of cytoskeleton Protein amino acid phosphorylation

Table: 7.30. Dechorionation on the high and low nutrient diet: Tentative consensus sequences and expression sequence tags with no assigned gene ontology.

Sequence Number	Sequence description	Up/Down regulation	Absolute fold change
TC217285 (Embryo)	Tentative consensus sequence	Up	10.07
TC218367 (Embryo)	Tentative consensus sequence	Up	4.57
TC198490 (Lsp1α-PA)	Tentative consensus sequence	Down	4.50
TC201533	Tentative consensus sequence	Down	4.44
TC214613	Tentative consensus sequence	Down	4.31
TC219844	Tentative consensus sequence	Up	3.85
TC212659 (Head)	Tentative consensus sequence	Down	3.45
AW944513 (CG6340)	Expression sequence tag	Down	3.35
TC216377 (Head)	Tentative consensus sequence	Down	3.35
TC201327(CG4757-RA)	Tentative consensus sequence	Down	3.13
TC221143 (Embryo)	Tentative consensus sequence	Up	2.91
TC218749 (Embryos, imaginal disks and adults)	Tentative consensus sequence	Up	2.86
TC215215 (3rd instar larvae challenged with gram+/- bacteria in fat body)	Tentative consensus sequence	Down	2.73
TC219201 (Embryos, imaginal disks and adults)	Tentative consensus sequence	Up	2.66
TC203290	Tentative consensus sequence	Up	2.66
TC213534 (Head)	Tentative consensus sequence	Up	2.62
BI568522	Expression sequence tag	Down	2.60
TC215821 (Embryos, imaginal disks and adults)	Tentative consensus sequence	Down	2.54
TC213341 (Head)	Tentative consensus sequence	Up	2.51
TC214171 (Embryos, imaginal disks and adults)	Tentative consensus sequence	Up	2.47
TC216854 (Embryos, imaginal disks and adults)	Tentative consensus sequence	Up	2.44
TC213388 (Head)	Tentative consensus sequence	Up	2.43
TC217757	Tentative consensus sequence	Up	2.42
TC215541 (Embryos, imaginal disks and adults)	Tentative consensus sequence	Up	2.34
CB305266	Expression sequence tag	Down	2.33
TC219559	Tentative consensus sequence	Up	2.32

TC220354	Tentative consensus sequence	Up	2.32
TC211303 (S2 cells)	Tentative consensus sequence	Up	2.29
TC217125 (Embryos, imaginal disks and adults)	Tentative consensus sequence	Up	2.27
TC220379	Tentative consensus sequence	Up	2.27
TC216368	Tentative consensus sequence	Up	2.24
TC210404 (Head)	Tentative consensus sequence	Down	2.22
TC216551 (Head)	Tentative consensus sequence	Up	2.20
CA805541 (CG43139)	Expression sequence tag	Down	2.20
TC213188 (Embryo)	Tentative consensus sequence	Up	2.16
TC213947 (Salivary glands)	Tentative consensus sequence	Down	2.15
TC219494 Embryo and S2 cell culture)	Tentative consensus sequence	Up	2.13
TC218163 (Ovary)	Tentative consensus sequence	Up	2.13
TC218584	Tentative consensus sequence	Up	2.12
TC220906	Tentative consensus sequence	Up	2.10
TC209214 (Head)	Tentative consensus sequence	Up	2.10
CO280900	Expression sequence tag	Up	2.10
TC215995	Tentative consensus sequence	Up	2.06
TC217151 (Embryo and S2 cell culture)	Tentative consensus sequence	Up	2.05
TC210032 (Head)	Tentative consensus sequence	Down	2.05
TC212461 (Head, ovary and embryo)	Tentative consensus sequence	Up	2.05
TC212954 (Head)	Tentative consensus sequence	Up	2.03
TC218875 (Embryos, imaginal disks and adults)	Tentative consensus sequence	Up	2.02
TC213630 (Testis)	Tentative consensus sequence	Down	2.01

Table: 7.31. Dechorionation on the high and low nutrient diet: Sequences with no assigned gene ontology.

Gene/Sequence Number	Up/Down regulation	Absolute fold change
CG4996	Up	3.81
CG34206	Down	3.79
CG17761-RA	Up	3.42
CG40119-RA	Down	2.91
l(2)01289	Up	2.91
CG40119	Up	2.80
CG6544 (Fau)	Up	2.79
CG31008	Up	2.69
NM_001015169 (CG41063)	Up	2.51
CG15597	Down	2.47
NR_003123	Down	2.44
pncr015:3L	Down	2.42
nimB5	Up	2.40
CG34394	Up	2.37
CG7502-RA	Up	2.37
CG17944	Down	2.32
CG41130	Up	2.27
CG41581	Up	2.26
CG40626	Up	2.25
CG11592	Up	2.23
CG17839	Up	2.21
CG34383	Up	2.18
CR42217	Up	2.16
CG32564	Down	2.16
CG40137	Down	2.14
AT10144	Down	2.12
CG3246	Up	2.12
CG14406	Up	2.08
CG14066-RB	Up	2.06
CG31526	Down	2.05
CG30296	Up	2.05
BT024213	Up	2.05
NM_001015218 (CG40159)	Up	2.04
CG41529	Up	2.04
CG6454	Up	2.01

7.4 Bacterial identities of culturable bacteria in Asian and Mexican *Aedes aegypti* larvae and water samples at 0-100 µg ml^{-1}chlortetracycline

Table: 7.32. Bacterial identities found in Asian *Aedes aegypti* larvae and water samples at the different chlortetracycline concentrations (0-100 µg ml^{-1}) (percent identity).

Chlortetracycline concentration (µg ml^{-1})	Water	Larvae
0	HQ113217.1 - *Microbacterium* sp. Z5 16S ribosomal RNA gene, partial sequence (98%)	FJ652595.1 - *Pseudomonas fluorescens* strain PSY-11 16S ribosomal RNA gene, partial sequence AY514432.1 - *Serratia marcescens* strain N1.8 16S ribosomal RNA gene, partial sequence (99%)
	HQ236076.1 - *Bacillus cereus* strain TBD3-2 16S ribosomal RNA gene, partial sequence (99%)	AJ704545.1 - *Chryseobacterium meningosepticum* partial 16S rRNA gene, strain ATCC 51720 (99%)
	GQ504012.1 - *Leucobacter* sp. NAL101 16S ribosomal RNA gene, partial sequence (97%)	GQ504012.1 - *Leucobacter* sp. NAL101 16S ribosomal RNA gene, partial sequence (97%)
	EU346911.1 - *Leucobacter chironomi* strain MM2LB 16S ribosomal RNA gene, partial sequence (98%)	HQ113217.1 - *Microbacterium paraoxydans* strain CL-9.11a 16S ribosomal RNA gene, partial sequence (98%)
	HM573359.1 - *Bacillus* sp. EB353 16S ribosomal RNA gene, partial sequence	GQ504012.1 - *Leucobacter* sp. NAL101 16S ribosomal RNA gene, partial sequence (97%)
0.1	AJ704545.1 - *Chryseobacterium meningosepticum* partial 16S rRNA gene, strain ATCC 51720 (99%)	GQ504012.1 - *Leucobacter* sp. NAL101 16S ribosomal RNA gene, partial sequence (97%)
	HM820111.1 - Uncultured bacterium clone nby372d11c1 16S ribosomal RNA gene, partial sequence (98%)	HM171926.1 - *Microbacterium* sp. Z5 16S ribosomal RNA gene, partial sequence (99%)
	HM303366.1 - Uncultured bacterium clone ncd819f12c1 16S ribosomal RNA gene, partial sequence, Comamonas sp. N19-3 16S ribosomal RNA gene, partial sequence (99%)	EU879962.1 - *Microbacterium laevaniformans* strain NML 16S ribosomal RNA gene, partial sequence (98%)
		HM171926.1 - *Microbacterium* sp. Z5 16S ribosomal RNA gene, partial sequence (97%)
0.5	EU346911.1 - *Leucobacter chironomi* strain MM2LB 16S ribosomal RNA gene, partial sequence (97-98%)	HQ113217.1 - *Microbacterium paraoxydans* strain CL-9.11a 16S ribosomal RNA gene, partial sequence (98%)
	HQ246280.1 - *Enterobacter* sp. 7A18S4 16S ribosomal RNA gene, partial sequence (97%)	HM171926.1 - *Microbacterium* sp. Z5 16S ribosomal RNA gene, partial sequence (98%)
		GQ504012.1 - *Leucobacter* sp. NAL101 16S ribosomal RNA gene, partial sequence (99%)
		HM171926.1 - *Microbacterium* sp. Z5 16S ribosomal RNA gene, partial sequence (98%)
		GQ504012.1 - *Leucobacter* sp. NAL101 16S ribosomal RNA gene, partial sequence (96%)
1	HM820111.1 - Uncultured bacterium clone nby372d11c1 16S ribosomal RNA gene, partial sequence (99%)	HM171926.1 - *Microbacterium* sp. Z5 16S ribosomal RNA gene, partial sequence (98%)
		HQ113217.1 - *Microbacterium paraoxydans* strain CL-9.11a 16S ribosomal RNA gene, partial sequence (98%)
		EU346911.1 - *Leucobacter chironomi* strain MM2LB 16S ribosomal RNA gene, partial sequence (99%)
10	FJ652595.1 - *Pseudomonas fluorescens* strain PSY-11 16S ribosomal RNA gene, partial sequence, AY514432.1 - *Serratia marcescens* strain N1.8 16S ribosomal RNA gene, partial sequence(98%)	HM171926.1 - *Microbacterium* sp. Z5 16S ribosomal RNA gene, partial sequence (98%)
	AJ704545.1 - *Chryseobacterium meningosepticum* partial 16S rRNA gene, strain ATCC 51720 (99%)	HQ113217.1 - *Microbacterium paraoxydans* strain CL-9.11a 16S ribosomal RNA gene, partial sequence (98%)

	AJ704542.1 - *Chryseobacterium meningosepticum* partial 16S rRNA gene, strain ATCC 13255 (100%)	
30	EU302858.1 - *Serratia marcescens* strain DAP33 16S ribosomal RNA gene, partial sequence (98%)	GU481093.1 - *Chryseobacterium* sp. RBT 16S ribosomal RNA gene, partial sequence (96%)
	J870662.1 - *Chryseobacterium* sp. pp2f 16S ribosomal RNA gene, partial sequence (99%)	HQ113217.1 - *Microbacterium paraoxydans* strain CL-9.11a 16S ribosomal RNA gene, partial sequence (99%)
		FJ870662.1 - *Chryseobacterium* sp. pp2f 16S ribosomal RNA gene, partial sequence (99%)
100	HM003215.1 - *Delftia tsuruhatensis* strain WYLW2-1 16S ribosomal RNA gene, partial sequence (99%)	EF035134.1 - *Serratia marcescens* strain N4-5 16S ribosomal RNA gene, partial sequence (99%)
	HM003215.1 - *Delftia tsuruhatensis* strain WYLW2-1 16S ribosomal RNA gene, partial sequence (99%)	AJ704545.1 - *Chryseobacterium meningosepticum* partial 16S rRNA gene, strain ATCC 51720 (99%)
		EU302852.1 - *Serratia marcescens* strain DAP27 16S ribosomal RNA gene, partial sequence (99%)
		EF035134.1 - *Serratia marcescens* strain N4-5 16S ribosomal RNA gene, partial sequence (98%)
		AM942043.1 - *Ochrobactrum anthropi* partial 16S rRNA gene, strain PH-03 (99%)

Table: 7.33. Bacterial identities found in Asian *Aedes aegypti* larvae and water samples at the different chlortetracycline concentrations (0-100 µg ml^{-1}) and grown on nutrient agar supplemented with 50 µg ml^{-1} of chlortetracycline (percent identity).

Chlortetracycline concentration (µg ml^{-1})	Water	Larvae
0	AJ704545.1 - *Chryseobacterium meningosepticum* partial 16S rRNA gene, strain ATCC 51720 (99%)	AJ704545.1 - *Chryseobacterium meningosepticum* partial 16S rRNA gene, strain ATCC 51720 (98-99%)
	AJ704545.1 - *Chryseobacterium meningosepticum* partial 16S rRNA gene, strain ATCC 51720 (99%)	AJ704545.1 - *Chryseobacterium meningosepticum* partial 16S rRNA gene, strain ATCC 51720 (99%)
	EF426425.1 - *Elizabethkingia meningoseptica* strain 2.5 16S ribosomal RNA gene (99%)	DQ298759.1 - Bacterium 7C2 16S ribosomal RNA gene, partial sequence (99%)
	EF426425.1 - *Chryseobacterium meningosepticum* partial 16S rRNA gene, strain ATCC 51720 (99%)	AJ704542.1 - *Chryseobacterium meningosepticum* partial 16S rRNA gene, strain ATCC 13255 (100%)
	EF426425.1 - *Elizabethkingia meningoseptica* strain 2.5 16S ribosomal RNA gene, partial sequence (99%)	DQ298759.1 - Bacterium 7C2 16S ribosomal RNA gene, partial sequence (100%)
		AJ704542.1 - *Chryseobacterium meningosepticum* partial 16S rRNA gene, strain ATCC 13255 (99%)
0.1	AJ704545.1 - *Chryseobacterium meningosepticum* partial 16S rRNA gene, strain ATCC 51720 (99%)	FJ652595.1 - *Pseudomonas fluorescens* strain PSY-11 16S ribosomal RNA gene, partial sequence AY514432.1 - *Serratia marcescens* strain N1.8 16S ribosomal RNA gene, partial sequence (99%)
	FP929040.1 - *Enterobacter cloacae* subsp. cloacae NCTC 9394 draft genome and uncultured bacteria (97-98%)	AJ704545.1 - *Chryseobacterium meningosepticum* partial 16S rRNA gene, strain ATCC 51720 (99%)
	FJ816020.1 - *Elizabethkingia meningoseptica* strain G3-1-08 16S ribosomal RNA gene, partial sequence (99%)	HM171926.1 - *Microbacterium* sp. Z5 16S ribosomal RNA gene, partial sequence(98%)
0.5	FJ816020.1 - *Elizabethkingia meningoseptica* strain G3-1-08 16S ribosomal RNA gene, partial sequence (99%)	FJ816020.1 - *Elizabethkingia meningoseptica* strain G3-1-08 16S ribosomal RNA gene, partial sequence (99%)
	AB244457.1 - *Enterobacter cloacae* gene for 16S rRNA, partial sequence, strain: An20-1 (98%)	GU481093.1 - *Chryseobacterium* sp. RBT 16S ribosomal RNA gene, partial sequence (99%)
	GU481093.1 - *Chryseobacterium* sp. RBT 16S ribosomal RNA gene, partial sequence (99%)	AJ704545.1 - *Chryseobacterium meningosepticum* partial 16S rRNA gene, strain ATCC 51720 (98%)
		AB244457.1 - *Enterobacter cloacae* gene for 16S rRNA, partial sequence, strain: An20-1 (98%)
		GU481093.1 - *Chryseobacterium* sp. RBT 16S ribosomal RNA gene, partial sequence (98%)
1	AJ704545.1 - *Chryseobacterium meningosepticum* partial 16S rRNA gene, strain ATCC 51720 (99%)	HM171926.1 - *Microbacterium* sp. Z5 16S ribosomal RNA gene, partial sequence (98%)
	AJ704545.1 - *Chryseobacterium meningosepticum* partial 16S rRNA gene, strain ATCC 51720 (99%)	AB244457.1 - *Enterobacter cloacae* gene for 16S rRNA, partial sequence, strain: An20-1 (98%)
	AJ704545.1 - *Chryseobacterium meningosepticum* partial 16S rRNA gene, strain ATCC 51720 (98%)	FJ405359.1- *Microbacterium* sp. GE1017 16S ribosomal RNA gene, partial sequence (97%)
		HM171926.1 - *Microbacterium* sp. Z5 16S ribosomal RNA gene, partial sequence (99%)
		HM171926.1 - *Microbacterium* sp. Z5 16S ribosomal RNA gene, partial sequence (97%)
10	AJ704545.1 - *Chryseobacterium meningosepticum* partial 16S rRNA gene, strain ATCC 51720 (99%)	HM171926.1 - *Microbacterium* sp. Z5 16S ribosomal RNA gene, partial sequence (98%)
	GU180606.1- *Elizabethkingia meningoseptica* strain EKMS1 16S ribosomal RNA gene, partial sequence (94%)	HQ113217.1 - *Microbacterium paraoxydans* strain CL-9.11a 16S ribosomal RNA gene, partial sequence (99%)

	AY335554.1 - *Enterobacter aerogenes* strain HK 20-1 16S ribosomal RNA gene, partial sequence (99%)	HM171926.1 - *Microbacterium* sp. Z5 16S ribosomal RNA gene, partial sequence (98%)
	AJ704545.1 - *Chryseobacterium meningosepticum* partial 16S rRNA gene, strain ATCC 51720 (98%)	HM171926.1 - *Microbacterium* sp. Z5 16S ribosomal RNA gene, partial sequence (99%)
30	AJ704545.1 - *Chryseobacterium meningosepticum* partial 16S rRNA gene, strain ATCC 51720 (99%)	FJ870662.1 - *Chryseobacterium* sp. pp2f 16S ribosomal RNA gene, partial sequence (97-99%)
		EF035134.1 - *Serratia marcescens* strain N4-5 16S ribosomal RNA gene, partial sequence (98%)
100	AJ704545.1 - *Chryseobacterium meningosepticum* partial 16S rRNA gene, strain ATCC 51720 (99%)	EF035134.1 - *Serratia marcescens* strain N4-5 16S ribosomal RNA gene, partial sequence (99%)
	AJ704545.1 - *Chryseobacterium meningosepticum* partial 16S rRNA gene, strain ATCC 51720 (99%)	GU481093.1 - *Chryseobacterium* sp. RBT 16S ribosomal RNA gene, partial sequence (98%)
	EF035134.1 - *Serratia marcescens* strain N4-5 16S ribosomal RNA gene, partial sequence (99%)	FJ652595.1 - *Pseudomonas fluorescens* strain PSY-11 16S ribosomal RNA gene, partial sequence EU302856.1 - *Serratia marcescens* strain DAP31 16S ribosomal RNA gene, partial sequence (91%)
	FJ870662.1 - *Chryseobacterium* sp. pp2f 16S ribosomal RNA gene, partial sequence (99%)	FJ662869.1 - *Serratia nematodiphila* strain P36 16S ribosomal RNA gene, complete sequence FJ662868.1 - *Serratia marcescens* strain P32 16S ribosomal RNA gene, complete sequence (99%)
		FJ662869.1 - *Serratia nematodiphila* strain P36 16S ribosomal RNA gene, complete sequence FJ662868.1 - *Serratia marcescens* strain P32 16S ribosomal RNA gene, complete sequence (99%)

Table: 7.34. Bacterial identities found in Mexican *Aedes aegypti* larvae and water samples at the different chlortetracycline concentrations (0-100 µg ml^{-1}) (percent identity).

Chlortetracycline concentration (µg ml^{-1})	Water	Larvae
0	EU346911.1 - *Leucobacter chironomi* strain MM2LB 16S ribosomal RNA gene, partial sequence (98%)	EU879962.1 - *Microbacterium laevaniformans* strain NML 16S ribosomal RNA gene, partial sequence (97%)
		GU180606.1 - *Elizabethkingia meningoseptica* strain EKMS1 16S ribosomal RNA gene, partial sequence (98%)
		EF035134.1 - *Serratia marcescens* strain N4-5 16S ribosomal RNA gene, partial sequence (99%)
		EU879962.1 - *Microbacterium laevaniformans* strain NML 16S ribosomal RNA gene, partial sequence (99%)
		GU180606.1 - *Elizabethkingia meningoseptica* strain EKMS1 16S ribosomal RNA gene, partial sequence (98%)
0.1	EU302852.1 - *Serratia marcescens* strain DAP27 16S ribosomal RNA gene, partial sequence (99%)	EU879962.1 - *Microbacterium laevaniformans* strain NML 16S ribosomal RNA gene, partial sequence (98%)
	GU180606.1 - *Elizabethkingia meningoseptica* strain EKMS1 16S ribosomal RNA gene, partial sequence (98%)	EU879962.1 - *Microbacterium laevaniformans* strain NML 16S ribosomal RNA gene, partial sequence (97%)
	AJ704541.1 - *Chryseobacterium meningosepticum* partial 16S rRNA gene, strain ATCC 13254 (99%)	EU879962.1 - *Microbacterium laevaniformans* strain NML 16S ribosomal RNA gene, partial sequence (97%)
	HM063035.1 - *Microbacterium* sp. CRRI-13 16S ribosomal RNA gene, partial sequence (96%)	EU879962.1 - *Microbacterium laevaniformans* strain NML 16S ribosomal RNA gene, partial sequence (98%)
	EU879962.1 - *Microbacterium laevaniformans* strain NML 16S ribosomal RNA gene, partial sequence (97%)	EU346911.1 - *Leucobacter chironomi* strain MM2LB 16S ribosomal RNA gene, partial sequence (98%)
	EF035134.1 - *Serratia marcescens* strain N4-5 16S ribosomal RNA gene, partial sequence (99%)	AM040493.1 - *Leucobacter iarius* 40 16S rRNA gene, type strain 40T (99%)
0.5	HM159984.1 - *Ochrobactrum* sp. OTU29 16S ribosomal RNA gene, partial sequence (98%)	EU302852.1 - *Serratia marcescens* strain DAP27 16S ribosomal RNA gene, partial sequence (99%)
	HM587796.1 - Delftia sp. MV01 16S ribosomal RNA gene, partial sequence (99%)	AB244483.1 - *Arthrobacter woluwensis* gene for 16S rRNA, partial sequence, strain: limp 5-2 (98%)
	FJ816020.1 - *Elizabethkingia meningoseptica* strain G3-1-08 16S ribosomal RNA gene, partial sequence (98%)	GU180606.1 - *Elizabethkingia meningoseptica* strain EKMS1 16S ribosomal RNA gene, partial sequence (99%)
	GU180606.1 - *Elizabethkingia meningoseptica* strain EKMS1 16S ribosomal RNA gene, partial sequence (98%)	EF035134.1 - *Serratia marcescens* strain N4-5 16S ribosomal RNA gene, partial sequence (99%)
1	HQ436416.1 - *Elizabethkingia* sp. dS13-11 16S ribosomal RNA gene, partial sequence (98%)	FJ652595.1 - *Pseudomonas fluorescens* strain PSY-11 16S ribosomal RNA gene, partial sequence EU302855.1 - Serratia marcescens strain DAP30 16S ribosomal RNA gene, partial sequence (98%)
	FJ816020.1 - *Elizabethkingia meningoseptica* strain G3-1-08 16S ribosomal RNA gene, partial sequence (99%)	AJ704541.1 - *Chryseobacterium meningosepticum* partial 16S rRNA gene, strain ATCC 13254 (99%)
	FJ816020.1 - *Elizabethkingia meningoseptica* strain G3-1-08 16S ribosomal RNA gene, partial sequence (99%)	GQ351502.1 - *Serratia marcescens* strain N80 16S ribosomal RNA gene, partial sequence (99%)
	AJ704541.1 - *Chryseobacterium meningosepticum* partial 16S rRNA gene,	GQ165511.1 - Bacterium S119(2009) 16S ribosomal RNA gene, partial sequence (99%)

	strain ATCC 13254 (99%)	
	AJ704541.1 - *Chryseobacterium meningosepticum* partial 16S rRNA gene, strain ATCC 13254 (99%)	GU180606.1 - *Elizabethkingia meningoseptica* strain EKMS1 16S ribosomal RNA gene, partial sequence (98%)
		GU272355.1 - *Microbacterium* sp. LP2ME 16S ribosomal RNA gene, partial sequence (98%)
10	HQ436416.1 - *Elizabethkingia* sp. dS13-11 16S ribosomal RNA gene, partial sequence (98%)	EU931562.1 - *Klebsiella pneumoniae* subsp. pneumoniae strain ZFJ-7 16S ribosomal RNA gene, partial sequence (98%)
	FJ816020.1 - *Elizabethkingia meningoseptica* strain G3-1-08 16S ribosomal RNA gene, partial sequence (98%)	EU302852.1 - *Serratia marcescens* strain DAP27 16S ribosomal RNA gene, partial sequence (99%)
	GU183606.1 - Uncultured bacterium clone NMG46 16S ribosomal RNA gene, partial sequence (97%)	HQ436416.1 - *Elizabethkingia* sp. dS13-11 16S ribosomal RNA gene, partial sequence (98%)
	GU180606.1 - *Elizabethkingia meningoseptica* strain EKMS1 16S ribosomal RNA gene, partial sequence (97%)	GU180606.1 - *Elizabethkingia meningoseptica* strain EKMS1 16S ribosomal RNA gene, partial sequence (98%)
	AJ704541.1 - *Chryseobacterium meningosepticum* partial 16S rRNA gene, strain ATCC 13254 (98%)	HQ436416.1 - *Elizabethkingia* sp. dS13-11 16S ribosomal RNA gene, partial sequence (98%)
		HQ436416.1 - *Elizabethkingia* sp. dS13-11 16S ribosomal RNA gene, partial sequence (97%)
30	FJ652595.1 - *Pseudomonas fluorescens* strain PSY-11 16S ribosomal RNA gene, partial sequence EU302855.1 - *Serratia marcescens* strain DAP30 16S ribosomal RNA gene, partial sequence (98%)	EU302852.1 - *Serratia marcescens* strain DAP27 16S ribosomal RNA gene, partial sequence (95%)
		EU302852.1 - *Serratia marcescens* strain DAP27 16S ribosomal RNA gene, partial sequence (97%)
100	GQ351502.1 - *Serratia marcescens* strain N80 16S ribosomal RNA gene, partial sequence (97%)	EU931562.1 - *Klebsiella pneumoniae* subsp. pneumoniae strain ZFJ-7 16S ribosomal RNA gene, partial sequence (99%)
	GQ351502.1 - *Serratia marcescens* strain N80 16S ribosomal RNA gene, partial sequence (99%)	AB548592.1 - *Mycobacterium massiliense* gene for 16S ribosomal RNA, partial sequence, strain: A1 (99%)
	EU879962.1 - *Microbacterium laevaniformans* strain NML 16S ribosomal RNA gene, partial sequence (97%)	EU879962.1 - *Microbacterium laevaniformans* strain NML 16S ribosomal RNA gene, partial sequence (98%)
	EU879962.1 - *Microbacterium laevaniformans* strain NML 16S ribosomal RNA gene, partial sequence (98%)	EU879962.1 - *Microbacterium laevaniformans* strain NML 16S ribosomal RNA gene, partial sequence (98%)
	AF287752.1 - *Microbacterium* sp. oral strain C24KA 16S ribosomal RNA gene, partial sequence (97%)	EU879962.1 - *Microbacterium laevaniformans* strain NML 16S ribosomal RNA gene, partial sequence (99%)

Table: 7.35. Bacterial identities found in Mexican *Aedes aegypti* larvae and water samples at the different chlortetracycline concentrations (0-100 µg ml^{-1}) on chlortetracycline supplemented agar plates (percent identity).

Chlortetracycline concentration (µg ml^{-1})	Water	Larvae
0	EF035134.1 - *Serratia marcescens* strain N4-5 16S ribosomal RNA gene, partial sequence (99%)	HQ436416.1 - *Elizabethkingia* sp. dS13-11 16S ribosomal RNA gene, partial sequence (98%)
	EU302852.1 - *Serratia* marcescens strain DAP27 16S ribosomal RNA gene, partial sequence (98%)	HQ436416.1 - *Elizabethkingia* sp. dS13-11 16S ribosomal RNA gene, partial sequence (98%)
	EU302852.1 - *Serratia* marcescens strain DAP27 16S ribosomal RNA gene, partial sequence (98%)	HM130055.1 - *Elizabethkingia meningoseptica* strain S3 16S ribosomal RNA gene, partial sequence (99%)
	EF440614.1 - *Delftia tsuruhatensis* strain WXZ-1 16S ribosomal RNA gene, partial sequence (96%)	GQ504012.1 - *Leucobacter* sp. NAL101 16S ribosomal RNA gene, partial sequence (97%)
	EF035134.1 - *Serratia marcescens* strain N4-5 16S ribosomal RNA gene, partial sequence (99%)	EU302852.1 - *Serratia marcescens* strain DAP27 16S ribosomal RNA gene, partial sequence (98%)
	EF035134.1 - *Serratia marcescens* strain N4-5 16S ribosomal RNA gene, partial sequence (98%)	EF035134.1 - *Serratia marcescens* strain N4-5 16S ribosomal RNA gene, partial sequence (99%)
0.1	HM771025.1 - *Leucobacter* sp. INBio2553H 16S ribosomal RNA gene, partial sequence (99%)	FJ816020.1 - *Elizabethkingia meningoseptica* strain G3-1-08 16S ribosomal RNA gene, partial sequence (98%)
		DQ311007.1 - *Agromyces* sp. Xb-3 16S ribosomal RNA gene, partial sequence (98%)
		AF316618.1 - *Mycobacterium* fuerth 16S ribosomal RNA gene, partial sequence (99%)
0.5	Samples and PCR completed, sequencing did not work	AF316618.1 - *Mycobacterium* fuerth 16S ribosomal RNA gene, partial sequence (99%)
1	Samples and PCR completed, sequencing did not work	Samples and PCR completed, sequencing did not work
10	HQ436416.1 - *Elizabethkingia* sp. dS13-11 16S ribosomal RNA gene, partial sequence (98%)	AF535159.1 - *Microbacterium laevaniformans* LA 16S ribosomal RNA gene, complete sequence (92%)
	EU931562.1 - *Klebsiella pneumoniae* subsp. pneumoniae strain ZFJ-7 16S ribosomal RNA gene, partial sequence (96%)	HM159984.1 - *Ochrobactrum* sp. OTU29 16S ribosomal RNA gene, partial sequence (99%)
	FJ816020.1 - *Elizabethkingia meningoseptica* strain G3-1-08 16S ribosomal RNA gene, partial sequence (99%)	AB363526.1 - Uncultured bacterium gene for 16S ribosomal RNA, partial sequence, clone: 3-4-9 (95%)
	EU931562.1 - *Klebsiella pneumoniae* subsp. pneumoniae strain ZFJ-7 16S ribosomal RNA gene, partial sequence (98%) Klebsiella pneumoniae subsp. pneumoniae strain ZFJ-7 16S ribosomal RNA gene, partial sequence	
30	EU302852.1 - *Serratia marcescens* strain N4-5 16S ribosomal RNA gene, partial sequence (98%)	
	EF035134.1 - *Serratia marcescens* strain N1.6 16S ribosomal RNA gene, partial sequence (98%)	
	EF035134.1 - *Serratia marcescens* strain N1.6 16S ribosomal RNA gene, partial	

	sequence (97%)	
100	GQ351502.1 - *Serratia marcescens* strain N80 16S ribosomal RNA gene, partial sequence FJ652595.1 - *Pseudomonas fluorescens* strain PSY-11 16S ribosomal RNA gene, partial sequence (98%)	EU536491.1 - Uncultured bacterium clone nbt214f11 16S ribosomal RNA gene, partial sequence (99%)
	FJ652595.1 - *Pseudomonas fluorescens* strain PSY-11 16S ribosomal RNA gene, partial sequence EF035134.1 - *Serratia marcescens* strain N4-5 16S ribosomal RNA gene, partial sequence (99%)	GQ351502.1 - *Serratia marcescens* strain N80 16S ribosomal RNA gene, partial sequence (98%)
		EU302852.1 - *Serratia marcescens* strain DAP27 16S ribosomal RNA gene, partial sequence (98%)
		AY514432.1 - *Serratia marcescens* strain N1.8 16S ribosomal RNA gene, partial sequence (98%)

7.5. 454 pyrosequencing of bacteria in Mexican *Aedes aegypti* larvae, female adults and male adults with and without 30 µg ml^{-1}chlortetracycline

Table: 7.36. 454 Sequencing results of bacterial identities for larvae Mexican *Aedes aegypti*

Number of reads	% Identity	Accession number	Identity	Lineage
31731	100.0	EU879962	*Microbacterium laevaniformans* strain NML 16S ribosomal RNA gene, partial sequence	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Microbacteriaceae*; Microbacterium.*
1101	100.0	EU714377	*Microbacterium paraoxydans* strain 76 16S ribosomal RNA gene, partial sequence	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Microbacteriaceae*; Microbacterium.*
552	100.0	AM040493	*Leucobacter iarius* 40 16S rRNA gene, type strain 40T	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Microbacteriaceae; *Leucobacter*
59	96.0	EU717745	*Microbacteriaceae* bacterium ACTS123 16S ribosomal RNA gene, partial sequence	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Microbacteriaceae*; Microbacterium.*
49	97.8	DQ490450	*Microbacteriaceae* bacterium KVD-unk-03 16S ribosomal RNA gene, partial sequence	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Microbacteriaceae*; Microbacterium.*
48	96.3	EU879962	*Microbacterium laevaniformans* strain NML 16S ribosomal RNA gene, partial sequence	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Microbacteriaceae*; Microbacterium.*
10	97.0	DQ490450	*Microbacteriaceae* bacterium KVD-unk-03 16S ribosomal RNA gene, partial sequence	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Microbacteriaceae*; Microbacterium.*
10	99.6	AB244483	*Arthrobacter woluwensis* gene for 16S rRNA, partial sequence, strain: limp 5-2	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Micrococcaceae*; Arthrobacter*
1	97.8	AJ247194	*Asticcacaulis excentricus* partial 16S rRNA gene for 16S ribosomal RNA, strain DSM 4724(T)	Bacteria; Proteobacteria; Alphaproteobacteria; Caulobacterales;Caulobacteraceae*; Asticcacaulis*
1	98.9	DQ814374	Uncultured bacterium clone aab67f12 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples
1	100.0	GU428943	Comamonadaceae bacterium oral taxon A82 clone SV044 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae
1	95.6	AB244483	*Arthrobacter woluwensis* gene for 16S rRNA, partial sequence, strain: limp 5-2	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Micrococcaceae*; Arthrobacter*
1	99.3	AB377177	Peptostreptococcaceae bacterium SK031 gene for 16S ribosomal RNA, partial sequence	Bacteria; Firmicutes; Clostridia; Clostridiales; Peptostreptococcaceae

Table: 7.37. 454 Sequencing results of bacterial identities for male adult Mexican *Aedes aegypti*

Number of reads	% Identity	Accession number	Identity	Lineage
26723	100	GU180606	*Elizabethkingia meningoseptica* strain EKMS1 16S ribosomal RNA gene, partial sequence	Bacteria; Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae*; Elizabethkingia*
2024	100	DQ365580	*Erwinia persicina* strain GS04 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae*; Erwinia*
14	100	HM342703	Uncultured bacterium clone ncd1034b08c1 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples
10	98.8	AJ001190	*Erwinia persicinus* 16S rRNA gene, strain LMG 2691	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae*; Erwinia*
5	100	HM057713	Uncultured beta proteobacterium clone D8W_30 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Betaproteobacteria; environmental
5	100	GQ360067	*Acinetobacter* sp. pp2a 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Moraxellaceae; *Acinetobacter*
3	100	EU714377	*Microbacterium paraoxydans* strain 76 16S ribosomal RNA gene, partial sequence	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Microbacteriaceae*; Microbacterium*
3	100	NR_025643	*Jeotgalicoccus halotolerans* strain YKJ-101 16S ribosomal RNA, partial sequence	Bacteria; Firmicutes; Bacillales; *Jeotgalicoccus*
3	91.2	NR_025917	Acetivibrio cellulolyticus strain CD2 16S ribosomal RNA, partial sequence	Bacteria; Firmicutes; Clostridia; Clostridiales; Ruminococcaceae*; Acetivibrio*
2	100	AM040493	*Leucobacter iarius* 40 16S rRNA gene, type strain 40T	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales;Micrococcineae; Microbacteriaceae*; Leucobacter*
2	100	HM329229	Uncultured bacterium clone ncd957e02c1 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples
2	100	FJ859692	*Sphingobacterium spiritivorum* strain BIHB 346 16S ribosomal RNA gene, partial sequence	Bacteria; Bacteroidetes; Sphingobacteria; Sphingobacteriales;Sphingobacteriaceae*; Sphingobacterium*
2	99.6	GU429487	Beta proteobacterium oral taxon B96 clone ST047 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Betaproteobacteria
2	100	FM875873	*Leucobacter tardus* partial 16S rRNA gene, strain B2-50	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Microbacteriaceae*; Leucobacter*
2	100	HM332804	Uncultured bacterium clone ncd1061e06c1 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples
2	97.8	AJ247194	*Asticcacaulis excentricus* partial 16S rRNA gene for 16S ribosomal RNA, strain DSM 4724(T)	Bacteria; Proteobacteria; Alphaproteobacteria; Caulobacterales; Caulobacteraceae*; Asticcacaulis*
2	100	GQ157223	Uncultured bacterium clone 16slp101-1f11.w2k 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples
2	97.4	AY822552	Bacterium PBA-1-4 16S ribosomal RNA gene, partial sequence	

2	99.6	GU642046	Uncultured bacterium clone RW6702 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples
2	98.4	DQ365580	*Erwinia persicina* strain GS04 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae*; Erwinia*
2	94.5	EU305584	Uncultured *Pedobacter* sp. clone 3-C 16S ribosomal RNA gene, partial sequence	Bacteria; Bacteroidetes; Sphingobacteria; Sphingobacteriales;Sphingobacteriaceae*; Pedobacter*; environmental samples
1	100	EF433462	*Devosia* sp. IPL18 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Hyphomicrobiaceae*; Devosia*
1	99.6	GQ891705	*Caulobacter leidyia* strain X 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales; Sphingomonadaceae
1	92.31	AY193185	Uncultured candidate division OD1 bacterium clone DA23 16S ribosomal RNA gene, partial sequence	Bacteria; candidate division OD1; environmental samples
1	97.8	DQ801310	Uncultured bacterium clone RL388_aao93g08 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples
1	93.0	AB480775	*Erwinia tasmaniensis* gene for 16S ribosomal RNA, partial sequence, strain: Acj 211	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae*; Erwinia*
1	97.4	EF667911	Uncultured Bdellovibrionales bacterium clone Hv(lab)_1.20 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Deltaproteobacteria; Bdellovibrionales; environmental samples
1	97.0	NR_024786	*Delftia tsuruhatensis* strain T7 16S ribosomal RNA, partial sequence >gi|17974274|dbj|AB075017.1| *Delftia tsuruhatensis* gene for 16S rRNA, partial sequence	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae*; Delftia*
1	98.2	AB271045	*Devosia ginsengisoli* gene for 16S rRNA, partial sequence	Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Hyphomicrobiaceae*; Devosia.*
1	99.6	GU727800	Uncultured bacterium clone A196 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples
1	97.0	FJ828944	*Variovorax* sp. enrichment culture clone 13.4 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales;Comamonadaceae*; Variovorax;* environmental samples
1	100	HM267307	Uncultured bacterium clone ncd212e06c1 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples
1	98.5	AB294175	*Alkalibacterium olivapovliticus* gene for 16S rRNA, partial sequence, strain: NCIMB 13710	Bacteria; Firmicutes; Lactobacillales; Carnobacteriaceae;*Alkalibacterium*
1	100	HM269911	Uncultured bacterium clone ncd257e09c1 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples
1	92.3	GQ250436	*Xanthomonas* sp. MJ10 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae*; Xanthomonas*
1	100	AB195767	Aquatic bacterium R1-B35 gene for 16S ribosomal RNA, partial sequence	

1	100	EF469609	*Pseudacidovorax intermedius* strain CC-CC21 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae*; Pseudacidovorax*
1	97.4	AY162048	Alpha proteobacterium PI_GH2.1.D7 small subunit ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Alphaproteobacteria.
1	89.4	AY468464	*Chryseobacterium* sp. FRGDSA 4034/97 16S ribosomal RNA gene, partial sequence	Bacteria; Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae*; Chryseobacterium*

Table: 7.38. 454 Sequencing results of bacterial identities for male adult Mexican *Aedes aegypti* treated with 30 µg ml^{-1} of chlortetracycline

Number of reads	% Identity	Accession Number	ID Name	Lineage
21392	98.9	FJ784637	*Raoultella* sp. Z2NS-91 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae*; Raoultella*
4867	100	GU180606	*Elizabethkingia meningoseptica* strain EKMS1 16S ribosomal RNA gene, partial sequence	Bacteria; Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae*; Elizabethkingia*
1687	100	HM057713	Uncultured beta proteobacterium clone D8W_30 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Betaproteobacteria; environmental samples
65	98.4	GQ284560	*Serratia marcescens* strain A2.4bii 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae*; Serratia*
49	91.1	FN297940	Uncultured Enterobacteriales bacterium partial 16S rRNA gene, clone CAR-W23r-C4	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; environmental samples
44	98.1	GU640749	Uncultured bacterium clone RW5405 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
30	93.7	GU410540	*Achromobacter xylosoxidans* clone FH043 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales;Alcaligenaceae*; Achromobacter*
22	99.5	HM130059	*Elizabethkingia meningoseptica* strain S7 16S ribosomal RNA gene, partial sequence	Bacteria; Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae*; Elizabethkingia*

19	89.9	FJ184330	Uncultured soil bacterium clone B5_4 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples.
17	96.7	GU180606	*Elizabethkingia meningoseptica* strain EKMS1 16S ribosomal RNA gene, partial sequence	Bacteria; Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae*; Elizabethkingia*
11	98.8	GU180606	*Elizabethkingia meningoseptica* strain EKMS1 16S ribosomal RNA gene, partial sequence	Bacteria; Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae*; Elizabethkingia*
6	97.0	FJ607964	Uncultured *Serratia* sp. clone LF8 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae*; Serratia;* environmental samples
6	95.6	DQ304115	*Streptomyces* sp. DA01013 16S ribosomal RNA gene, partial sequence	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Streptomycineae; Streptomycetaceae*; Streptomyces*
5	92.4	AB274281	*Enterobacter sakazakii* gene for 16S rRNA, partial sequence, strain: HT011	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae*; Cronobacter*
4	100	FJ859692	*Sphingobacterium spiritivorum* strain BIHB 346 16S ribosomal RNA gene, partial sequence	Bacteria; Bacteroidetes; Sphingobacteria; Sphingobacteriales; Sphingobacteriaceae*; Sphingobacterium*
2	97.4	GU180606	*Elizabethkingia meningoseptica* strain EKMS1 16S ribosomal RNA gene, partial sequence	Bacteria; Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae*; Elizabethkingia*
2	88.0	GQ284560	*Serratia marcescens* strain A2.4bii 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae*; Serratia.*
2	92.7	HM130059	*Elizabethkingia meningoseptica* strain S7 16S ribosomal RNA gene, partial sequence	Bacteria; Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae*; Elizabethkingia*
1	95.6	GQ284560	*Serratia marcescens* strain A2.4bii 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae*; Serratia.*
1	95.6	EU834233	*Pseudomonas* sp. RZ 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae*; Pseudomonas*

1	95.6	DQ417330	*Pseudomonas fluorescens* strain 3B 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae*; Pseudomonas*

Table: 7.39. 454 Sequencing results of bacterial identities for female adult Mexican *Aedes aegypti*

Number of reads	% Identity	Accession Number	Identity	Lineage
458	100	GU180606	*Elizabethkingia meningoseptica* strain EKMS1 16S ribosomal RNA gene, partial sequence	Bacteria; Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae*; Elizabethkingia*
44	100	DQ365580	*Erwinia persicina* strain GS04 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae*; Erwinia*
39	98.9	FJ784637	*Raoultella* sp. Z2NS-91 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae*; Raoultella*
2	100	AB238051	Uncultured bacterium gene for 16S rRNA, partial sequence, clone: 16S-KM-B-42	Bacteria; environmental samples
2	98.2	GQ500800	Uncultured bacterium clone MACA-EFT33 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples
2	98.2	AB252934	Uncultured alpha proteobacterium gene for 16S rRNA, partial sequence, clone: 225	Bacteria; Proteobacteria; Alphaproteobacteria; environmental samples
2	98.1	GQ348782	Uncultured alpha proteobacterium clone SHAB715 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Alphaproteobacteria; environmental
2	92.3	GQ988711	Uncultured bacterium clone FW_C02fw20 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples
2	92.3	GQ988710	Uncultured bacterium clone FW_H06fw32 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples
2	100	AJ536464	*Melosira varians* chloroplast 16S rRNA gene, strain p107	Eukaryota; stramenopiles; Bacillariophyta; Coscinodiscophyceae; Coscinodiscophycidae; Melosirales; Melosiraceae*; Melosira*
2	99.3	EF580977	Uncultured bacterium clone CM132 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples

2	99.6	EU850384	Uncultured *Rhodocyclaceae* bacterium clone F5 16S ribosomal RNA gene, partial sequence	Bacteria; Proteobacteria; Betaproteobacteria; Rhodocyclales; Rhodocyclaceae; environmental samples
1	99.6	GU902766	Uncultured bacterium clone PP254-b02 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples
1	100	FN691990	*Pseudomonas* sp. NR6 partial 16S rRNA gene, strain NR6	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae*; Pseudomonas*
1	93.0	GQ339139	Uncultured bacterium clone IS-32 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples
1	100	AY957928	Uncultured bacterium clone B3NR69D13 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples
1	100	EU580492	Uncultured bacterium clone D44 39 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples
1	96.0	AY989122	Uncultured soil bacterium clone L1A.8D09 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples
1	100	HM251894	Uncultured bacterium clone ncd22a07c1 16S ribosomal RNA gene, partial sequence	Bacteria; environmental samples
1	99.3	AY947930	Uncultured Bacteroidetes bacterium clone IRD18D04 16S ribosomal RNA gene, partial sequence	Bacteria; Bacteroidetes; environmental samples

7.6 Application for RIDL®

7.6.1 Introduction

The RIDL® technique involves the release of male mosquitoes into the wild. Successful field trials in Grand Cayman involved the release of 3.3 million male mosquitoes into the wild over a period of 6 months (www.newscientist.com, http://www.oxitec.com/wp-content/uploads/2010/11/Oxitec-MRCU-press-release.pdf). Only male mosquitoes are released therefore, mosquitoes need to be sexed before release.

Mosquitoes can be sexed at the pupal stage. Mosquito pupae differ by the shape of the genital lobe or by the size (females tend to be larger than males). The only methods used to sex the pupae are: the use of a light microscope and sexing by hand and secondly, using a grid which separates the pupae by size with a 97% success rate (Sharma *et al*., 1972). These methods work well but only to a certain scale of rearing. For a RIDL® control program millions of pupae will have to be sexed and the above methods are far too labour intensive for this. Therefore new and faster methods of sorting males from females are required.

A female L4-pupae marker was proposed as a method to sort males and females to allow an easy identification of females. The L4-pupae larvae can then be sorted so that only male pupae emerge, potentially saving time on sorting larvae from pupae and then sexing the pupae.

To create a female-specific L4 marker, the female-specific splicing region for the Actin-4 gene was used. The *Aedes* Actin-4 gene was shown to be expressed in only female *Aedes aegypti* (Muñoz *et al*., 2004; Fu *et al*., 2010). The *Actin-4* gene is expressed in the indirect flight muscles in females (Muñoz *et al*., 2004). This female-specific protein is generated by sex-specific splicing; the intron found between exons 1 and 2 is completely spliced in females (Fu *et al*., 2010). However, in males the intron is partially spliced out producing a second version that is 244 bp longer (Figure: 7.1) (Fu *et al*., 2010). This 244 bp region contains stop codons and therefore, prevents the expression of the Actin-4 gene in males (Fu *et al*., 2010). When the intron is completely removed there are no stop codons present and therefore the expression of the Actin-4 continues and the protein is produced (Fu *et al*., 2010). DsRed was used as the marker with the promoter Hr5IE1

(immediate-early promoter) which is a ubiquitous promoter. Therefore, the DsRed fluorescence should be expressed in females only. Transformed mosquitoes were identified by expression of cyan fluorescence in the eyes and anal papillae.

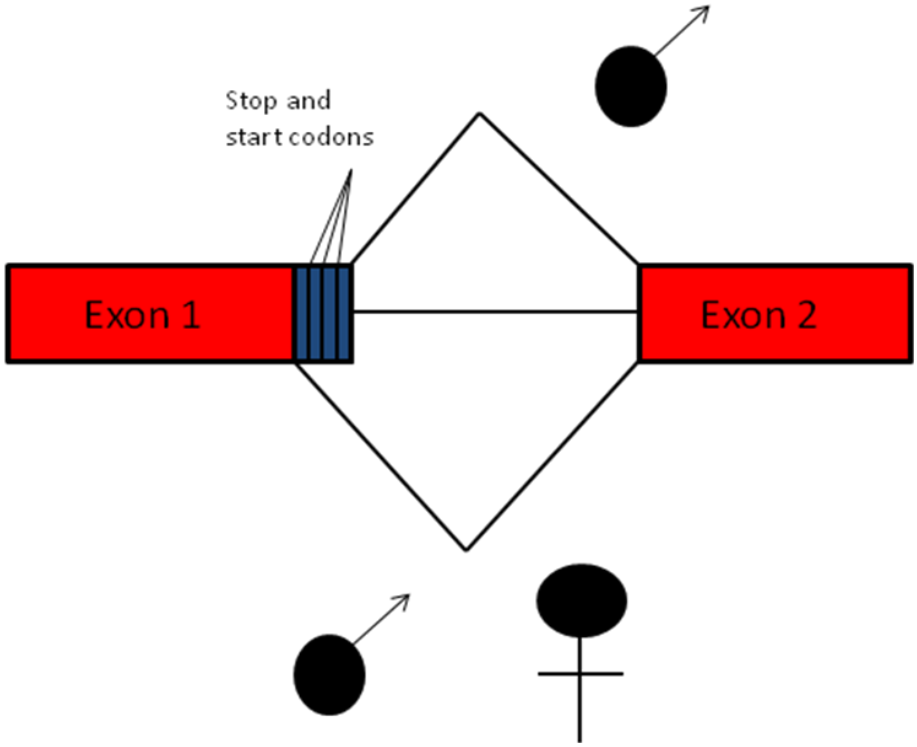
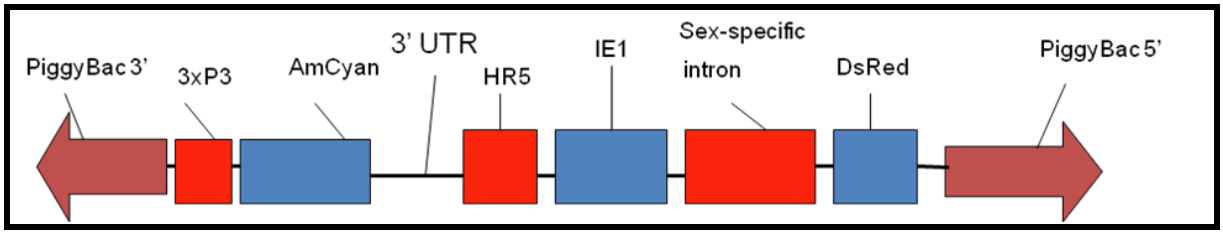


Figure: 7.1. Illustration of the splicing region for the Actin-4 gene. In males the intron is only partially spliced out, whereas in females and in some males the intron is completely removed (Fu *et al*., 2010).

7.6.2 Methods: Production and analysis of transgenic mosquitoes – Female-specific marker for mass rearing

A female specific marker (OX4272) was created (designed by Guoliang Fu), for sex separation during mass rearing of *Aedes aegypti*. This plasmid (OX4272) will allow all over body DsRed expression in females only at the L4-pupae stage (Figure: 7.2).

a)



b)

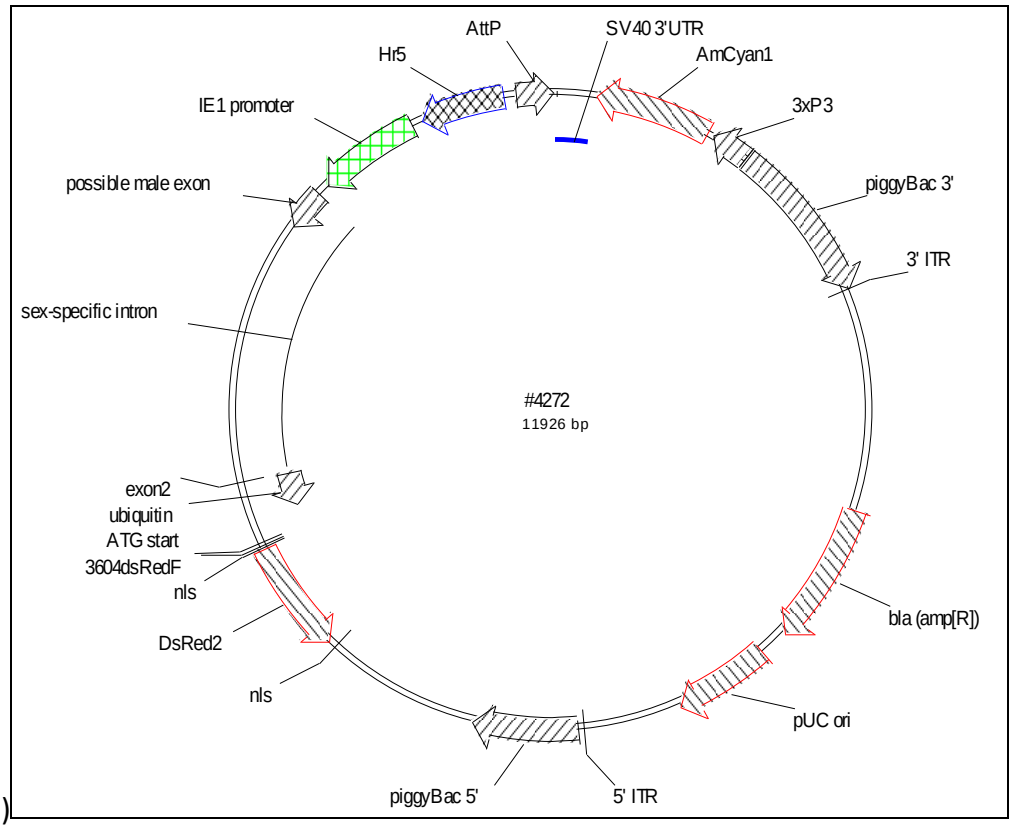


Figure: 7.2. a) Summary of the DNA produced to create female-specific expression of DsRed in wild-type Asian *Aedes aegypti*. DsRed expression under the control of the HR5IE1 promotor with spliced intron of AeAct4, the plasmid also contains an AmCyan fluorescent marker under the control of the 3XP3 promoter resulting in expression in the optic nerve b) the plasmid of OX4272.

7.6.2.1 Plasmid Construction

The 3 PCR reactions of DsRed, HR5IE1 and 3xP3AmCyan involved the primers Table: 7.40. PCR amplification of DsRed (from OX3604, Figure: 7.3) and HR5IE1 (from OX3778, Figure: 7.4) was performed in 50 µl of a mix containing 1X *Herculase* polymerase buffer, 2 mM $MgCl_2$, 0.3 mM of each deoxynucleoside triphosphate, 0.33 µM of each primer, 1 µL of template DNA and 0.025 U of *Herculase* II Fusion DNA polymerase (Stratagene, Agilent Technologies). The PCR mixtures were incubated for 70 seconds at 94 °C, followed by 2 cycles of 30 seconds at 58 °C, 68 °C for 60 seconds, 2 cycles of 10 seconds at 94 °C, 30 seconds at 55 °C, 68 °C for 60 seconds and 20 cycles of 10 seconds at 94 °C, 30 seconds at 52 °C and 60 seconds at 68 °C with a final incubation of 6 minutes at 68°C. The predicted sizes were 0.9 and 1.5 kb for DsRed and HR5IE1, respectively.

PCR amplification of 3xP3AmCyan from OX3604 (Figure: 7.3) was performed in 50 µl of a mix containing 1X *Taq* DNA polymerase buffer, 2 mM $MgCl_2$, 0.3 mM of each deoxynucleoside triphosphate, 0.33 µM of each primer, 1 µL of template DNA and 0.025 U of *Taq* DNA polymerase (New England Biolabs). The PCR mixtures were incubated for 70 seconds at 94 °C, followed by 2 cycles of 30 seconds at 58 °C, 68 °C for 90 seconds, 2 cycles of 10 seconds at 94 °C, 30 seconds at 55 °C, 68 °C for 90 seconds and 20 cycles of 10 seconds at 94 °C, 30 seconds at 52 °C and 90 seconds at 68 °C with a final incubation of 6 minutes at 68 °C. The predicted size was 1.632kb.

Table: 7.40. Primers used to amplify the sequences required in the female-specific marker.

Sequence (Size of band)	Forward Primer DNA sequence (5'-3')	Reverse Primer DNA sequence (5'-3')
HR5IE1 (1.5 kb)	AAATGCTTTACGAGTAGAATTCTACGCGT AAAACAC	CGCGTTTGTTTGATCGCACGGTTC
DsRed (0.9 kb)	GTGATGGGAGATCCCACCCCACCCAAGA	ATGATCAGTTATCTAGACCCGGTGGATCTTA
3xP3AmCyan (1.6 kb)	TTGGTCTAGCGTGTTAATTAAGCGGTAA GATACATTGATGAG	TGAACATTGTCAGGCCGGCCCGCTCGCCCGGGG AACTAGTTCAA

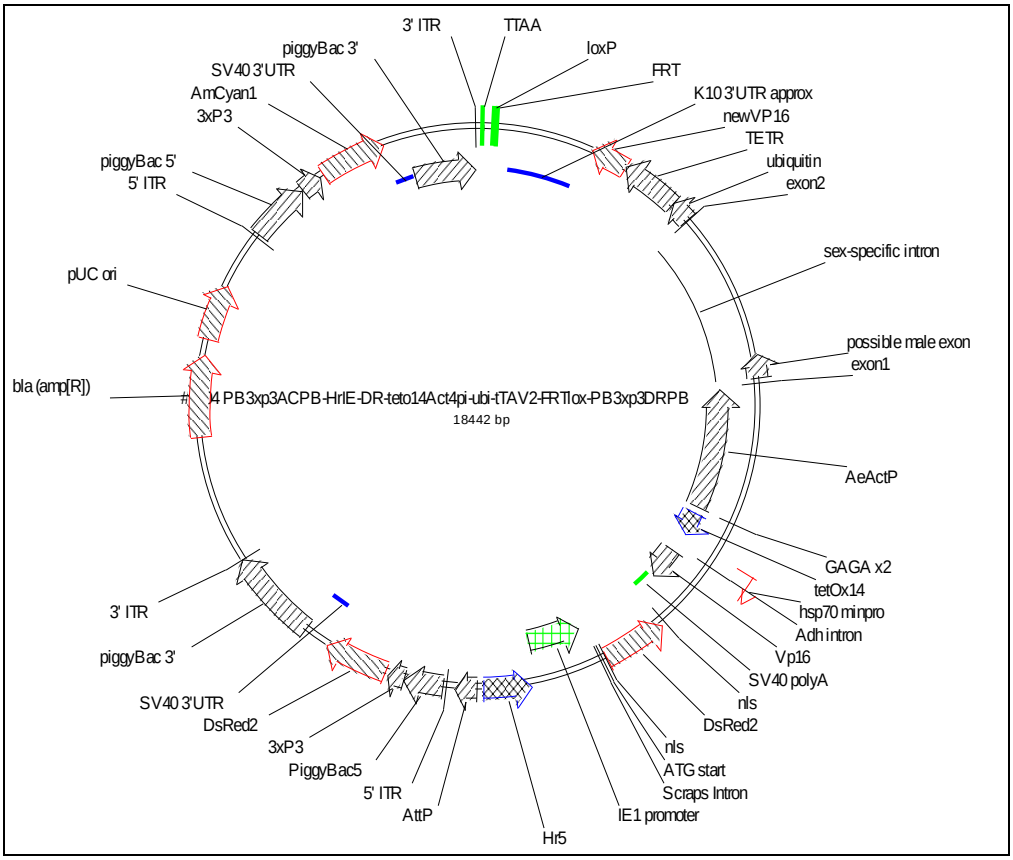


Figure: 7.3. The plasmid of OX3604.

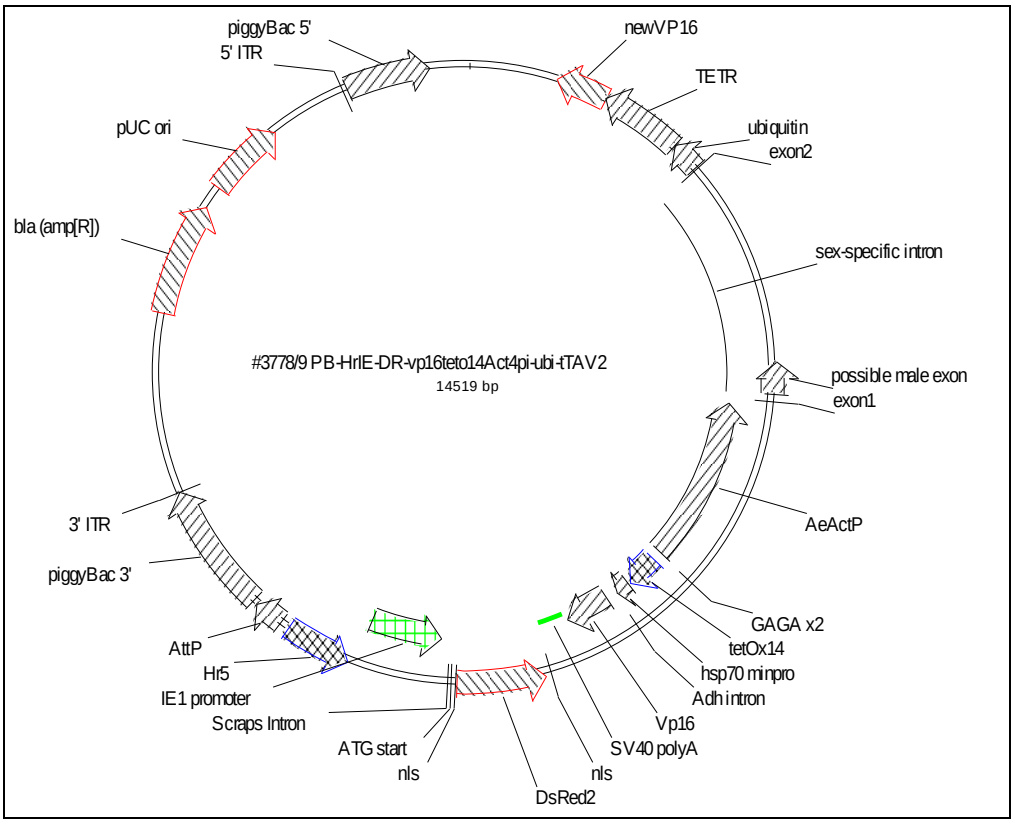


Figure: 7.4. The plasmid of OX3778.

The OX4272 PB(3xP3-AmCyan-HR5-IE1-Actin4intron-DsRed) construct was made by modifying OX3778 PB(HRIE-DR-vp16-TetO-Act4Pi-ubi-tTav2) (Figure: 7.4) by removing Ttav2. The Ttav2 was removed using *PmlI* and *XbaI* (New England Biolabs), amplified DsRed was then inserted into the *PmlI*/*XbaI* site to create OX3778 PB(HRIE-DR-vp16-TetO-AeActPi-ubi-DsRed). HRIE-DR-VP16-TetO-AeActP was removed from OX3778 PB(HRIE-DR-vp16-TetO-AeActPi-ubi-DsRed) using *PacI* and *RsrII* and HR5IE1 was inserted into the *PacI*/*RsrII* site to create OX3778 PB(HR5IE1-Act4i-ubi-DsRed). Amplified 3xP3-AmCyan and OX3778 PB(HR5IE1-Act4i-ubi-DsRed) were then digested with *PacI* and *NgomIV* and 3xP3-AmCyan was inserted into the *PacI*/*NgomIV* site of OX3778 PB(HR5IE1-Act4i-ubi-DsRed) to create OX4272 PB(3xP3-AmCyan-HR5IE1-Act4intron-ubi-DsRed).

All PCR and digest reactions were run on 0.8% agarose gel at 120V for 30 minutes and visualized using ethidium bromide under UV light. Digestion bands and PCR reactions were purified using QIAquick Gel extraction kit (Qiagen, Crawley, West Sussex, UK) or using QIAquick PCR purification kit (Qiagen, Crawley, West Sussex, UK) eluted in 15 µl and 30 µl of elution buffer, respectively.

Products were ligated together using T4 DNA ligase (New England Biolabs) and cloned into XL-10-Gold Ultra-competent Cells (Stratagene, Agilent Technologies). Bacterial colonies were PCR screened and individual colonies picked for screening were also used to inoculate LB broth with 100 µg ml^{-1} ampicillin and grown in a shaking incubator for several hours at 37 °C. Six positive colonies were selected, 20 µl of the positive inoculate was used to inoculate 3 ml of LB with 100 µg ml^{-1} ampicillin. The bacteria were grown overnight at 37 °C in a shaking incubator.

DNA was extracted from 1 ml of the cultures by using the GeneJet^{TM} Plasmid Miniprep kit (Fermentas, York, UK) and eluting in 50 µl of elution buffer. Digestion of the plasmids was used to determine the correct insertion. Positive digestions were sequenced using GATC Biotech (Germany).

Bacteria expressing the final construct were used to inoculate 3 ml of LB with 100 µg ml^{-1} of ampicillin. The cultures were grown for 8 hours at 37 °C at 200 rpm, these cultures were then was used to inoculate 250 ml of LB with 100 µg ml^{-1} of ampicillin and grown overnight at 37 °C at 200 rpm. DNA was extracted from 1 ml of the culture by using the

GeneJet™ Plasmid Miniprep kit and eluting in 50 µl of elution buffer and 1 ml was removed for a glycerol stock. DNA was extracted from the remaining bacterial cells using the Endofree Plasmid Maxiprep kit (Qiagen, Crawley, West Sussex, UK) and eluting in 50 µl of endotoxin-free water.

Data was analysed using VectorNTi (Invitrogen).

7.6.2.2 Microinjection of Asian WT *Aedes aegypti* mosquito eggs

Asian WT *Aedes aegypti* mosquitoes were reared at 28 °C with 80% humidity. The mosquitoes were blood fed 4 days before egg collection. On the day of egg collection, damp filter papers (FisherBrand) were placed into cages and the cages placed in the dark. Eggs were allowed to mature and aligned for preparation for injections. The eggs were then stuck to a cover slip using double sided tape, left to desiccate for 1-2 minutes and covered with halocarbon oil. Eggs were injected (into the syncytial mass) with the injection solution (containing the 300 ng μl^{-1} of plasmid DNA, 700 ng μl^{-1} of helper mRNA, injection buffer [5 mM KCl and 0.1 mM NaH_2PO_4 pH 6.8] and water) using an Eppendorf microinjector. Injected eggs were placed in water and transferred into a humidity box stored at 28 °C with 80% humidity to allow for recovery. Eggs were hatched under a vacuum for 1 hour 3-5 days after injection with 2 droplets of Liquifry No 1 (Aquatics Warehouse). Surviving mosquitoes were back-crossed with Asian WT, females were blood fed and the resulting eggs were hatched and screened.

7.6.2.3 Genomic analysis of *Aedes aegypti* injected with OX4272 using PCR

Only the transformation marker (3XP3AmCyan) was observed in the injected mosquitoes, therefore molecular analysis was conducted to determine why the transformation marker was visualized but not the sex-specific expression of DsRed.

Two PCR reactions were performed each using 1 µl of genomic DNA (extracted using Nucleospin® Tissue kit) from male and female pupae and 24 µl of master mix.

The master mix for the first PCR reaction consisted of 1X DreamTaq Buffer (Fermentas), 7.5 pmole of the Act4intronF (5'-GAAGTTCTGATTCAGAACCATCTCTCATG-3') forward and SeqRed2R (5'-CGATGAACTTCACCTTGTAGATGAAG-3') reverse primer, 0.2 mM of each deoxynucleoside triphosphate and 1.5 U of *DreamTaq* ™ DNA polymerase (Fermentas). The second PCR reaction consisted of of 1X DreamTaq Buffer (Fermentas), 7.5 pmole of

the Act4intronF (5'-GAAGTTCTGATTCAGAACCATCTCTCATG-3') primer and Red2midR (5'-CCGTCCTCGAAGTTCATCACG-3') reverse primer, 0.2 mM of each deoxynucleoside triphosphate and 1.5 U of *DreamTaq* ™ DNA polymerase (Fermentas).

Both PCR mixtures were incubated for 60 seconds at 94 °C, followed by 3 cycles of 15 seconds at 94 °C, 40 seconds at 58 °C, 72 °C for 60 seconds, 3 cycles of 15 seconds at 94 °C, 40 seconds at 55 °C, 72 °C for 60 seconds and 35 cycles of 15 seconds at 94 °C, 45 seconds at 52 °C and 60 seconds at 72 ° C with a final incubation of 6 minutes at 72 °C.

7.6.2.4 Transcript analysis: Production of cDNA from RNA samples using SuperScript™ II RT (Invitrogen)

RNA was extracted from OX3604 and OX4272 (injected 10/2/09 and injected 13/2/09) male and female pupae using TRI Reagent® according to the manufacturer's instructions (Applied Biosystems). Samples were kept at -80 °C. The samples were treated with DNase to remove contaminating DNA from the samples. After DNase treatment, the mixture consisting of 500 μg ml^{-1} of Oligo (dT), 5 μg of RNA and 12.5 pmole of dNTPs (made up in sterile distilled water) was heated to 65 °C for 5 minutes and chilled to 4 °C. First-Strand Buffer (1X), 0.01M DTT and RNaseOUT™ (40 U μl^{-1}) were added and gently mixed and incubated at 25 °C for 2 minutes. Two hundred units of SuperScript™ II RT was added and mixed by pipetting. The sample was then incubated at 25 °C for 10 minutes, followed by incubation at 42 °C for 50 minutes and a heat inactivation step of 15 minutes at 70 °C. The cDNA was stored at -20 °C.

7.6.2.5. Transcript analysis: Determination of DsRed expression using reverse transcriptase PCR and sequencing (assisted by Andrea Miles, Oxitec, Ltd).

The RT-PCR reactions were performed using 1 μl of cDNA and 24 μl of Master mix. The master mix consisted of 1X DreamTaq Buffer (Fermentas), 7.5 pmole of the ActRsR (5'-GGTACAGTCGGACCGCCACCATGGAACCGAGGATAACGAGAAG-3') forward and Red2midR (5'-CCGTCCTCGAAGTTCATCACG-3') reverse primers, 0.2 mM of each deoxynucleoside triphosphate and 1.5 U of *DreamTaq* ™ DNA polymerase (Fermentas). The PCR mixtures were incubated for 60 seconds at 94 °C, followed by 3 cycles of 15 seconds at 94 °C, 40 seconds at 58 °C, 72 C° for 60 seconds, 3 cycles of 15 seconds at 94 °C, 40 seconds at 55 °C, 72 C° for 60 seconds and 35 cycles of 15 seconds at 94 °C, 45 seconds at 52 °C and 60

seconds at 72 ° C with a final incubation of 6 minutes at 72 °C. The predicted size was 1.1 kb.

Twenty micro litres of the PCR reactions were run on a 0.8% ethidium bromide agarose gel at 120V for 30 minutes.

The correct bands were extracted using Qiagen QIAquick Gel extraction kit and eluted in 10 µl of Buffer EB (Qiagen, Crawley, West Sussex, UK). Pjet 1.2/Blunt cloning vector (Fermentas) and the PCR product were ligated together using T4 DNA ligase (New England Biolabs) at RT for 5 minutes. The ligated product was then cloned into XL-10-Gold Ultra-competent Cells (Stratagene, Agilent Technologies). Bacterial colonies were PCR screened using the PjetFP2 (ATCAACTGCTTTAACACTTGTGC) forward primer and PjetRP2 (AAAGAAGAACATCGATTTTCCATG) reverse primer by randomly removing individual colonies and dipping into the PCR mixture consisting of: 1X DreamTaq Buffer, 5 pmole of forward and reverse primers, 0.2 mM of each deoxynucleoside triphosphate and 2.5 U of DreamTaq ™ DNA polymerase (Fermentas). The PCR mixtures were incubated for 60 seconds at 94 °C, followed by 2 cycles of 15 seconds at 94 °C, 45 seconds at 60 °C, 68 °C for 90 seconds, 2 cycles of 15 seconds at 94 °C, 45 seconds at 57 °C, 68 °C for 90 seconds and 24 cycles of 15 seconds at 94 °C, 45 seconds at 54 °C and 90 seconds at 68 ° C with a final incubation of 6 minutes at 68 °C. The predicted size was 1.3 kb.

Five micro litres of the PCR reactions were run on a 0.8% ethidium bromide agarose gel at 120V for 20 minutes.

Individual colonies picked for screening were also used to inoculate LB broth as described in section 7.6.2.1. DNA was extracted from 1 ml of the cultures by using the GeneJet^{TM} Plasmid Miniprep kit (Fermentas, York, UK) and eluting in 50 µl of elution buffer.

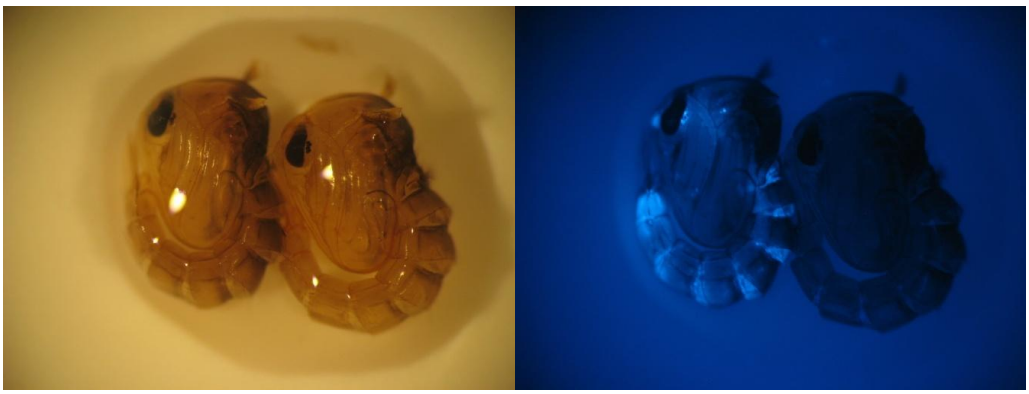
Positive digestions were sequenced using the PjetFP2 and PjetRP2 primers using GATC Biotech (Germany).

7.6.3 Results: Production of a Female-specific Marker in Asian *Aedes aegypti*

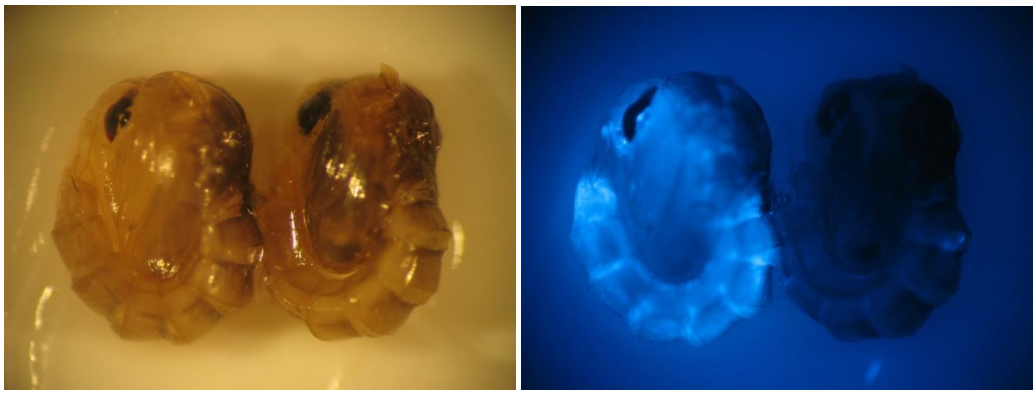
7.6.3.1 Injection of OX4272

Two independent transgenic lines were established using the construct OX4272 (Appendix: 7.6.2, Figure: 7.2). Approximately, 6000 eggs were injected and ~800 larvae hatched. Approximately 200 male and 500 female mosquitoes survived to adulthood to be crossed with wild-type Asian *Aedes aegypti*. The progeny (G1) were screened for the transformation marker with approximately 300 larvae expressing the 3XP3AmCyan marker. OX4272-1 showed eye and anal papillae expression of Cyan (Figure: 7.5) and OX4272-2 showed eye expression only. Expression was monitored daily through to pupae; but no female-specific marker expression was seen (Data not shown).

a) b)



c) d)



e) f) g)

Figure: 7.5. Expression of 3XP3AmCyan in OX4272-1 a) female pupae under white light b) female pupae using the filters for cyan fluorescence one pupa (left) showing the expression of 3XP3AmCyan c) male pupae under white light d) male pupae, one pupa (left) showing the expression of 3XP3AmCyan e) Larvae under normal view f) Larvae, one larva (left) showing the expression of 3XP3AmCyan g) Showing the expression of AmCyan in the optic nerve. *Courtesy of Amandine Collado, Oxitec Ltd*

7.6.3.2 Reverse transcriptase PCR analysis of transgenic OX4272

Molecular analysis of this line was conducted by extracting RNA and genomic DNA of pupae explained in Section: 7.6.2.3, 7.6.2.4 and 7.6.2.5. The genomic PCR amplifying the Actin4 intron and DsRed showed that the transgenic line was a real transgenic as the same sized band (700 bp) was observed in the original DNA (4272 Maxiprep) used for the injections (Figure: 7.6). However, no DNA was amplified in OX3604 which carried the same gene (Actin4 intron-DsRed), the amplified product was also greater than expected (700 bp).

Reverse trancriptase (RT) PCR was also conducted to confirm that Line 1 and 2 did not express DsRed. The sequence containing the splicing region (Actin4 intron) and DsRed was amplified. Two bands were observed when the RT-PCR was conducted, the top band (1 kb) was dominant and the bottom band was very faint (Figure: 7.7). Both these bands were cloned and sequenced and confirmed that the top band was the male-specific spliced transcript of DsRed and the second faint band was the female-specific transcript. Therefore, the DsRed expression was not observed in females as both males and females pre-dominantly expressed the male-specific transcript which meant that the DsRed gene was not expressed in male and female mosquitoes.

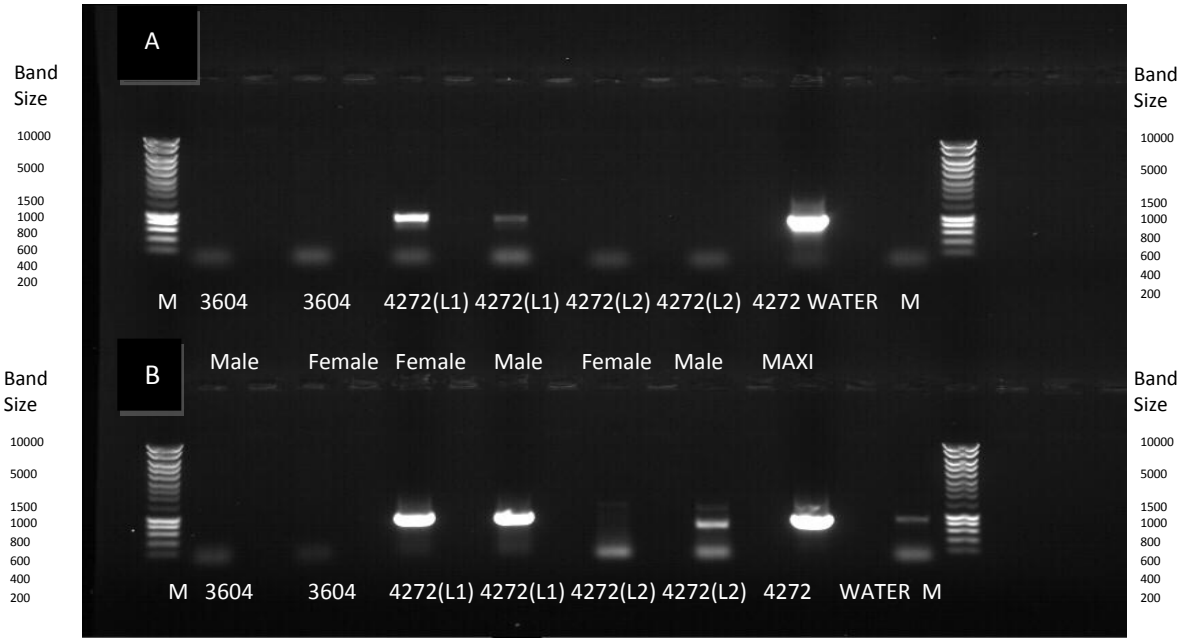


Figure: 7.6. Actin4 intron and DsRed detection by PCR using the primers Act4intron-F and SeqRed2R (PCR A) and Act4intron-F and Red2MidR (PCR B). Separation conditions 0.85% agarose gel, 1 X TAE using a separation voltage of 120. Size detection using 1kb DNA ladder (SMART).

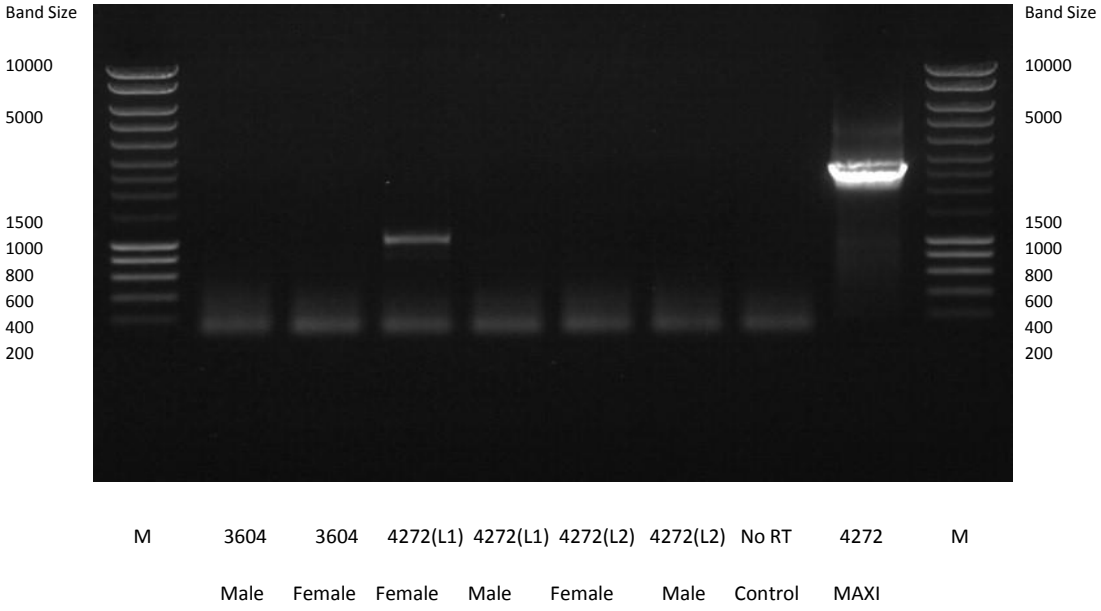


Figure: 7.7. DsRed expression detection using agarose gel electrophoresis. Separation conditions 0.85% agarose gel, 1 X TAE using a separation voltage of 120. Size detection using 1kb DNA ladder (SMART). Two bands were detected in 4272 (L1) Female after RT-PCR using the primers ActRsR and Red2MidR. Both fragments were gel extracted, cloned (pJET) and sequenced.

7.6.4 Discussion

The final aim of this thesis was to produce a successful female marker to allow for sex sorting during L4-pupae stage, however this proved unsuccessful at the time with transgenics only expressing the transformation marker. Unfortunately, the use of a female specific splicing region preceding the DsRed marker did not create female-specific expression and in fact, the partial splicing which occurs in male mosquitoes dominated. This result could be due to that fact that the insertion was incorrect or in the incorrect orientation, therefore an increase in the number of injections may increase the probability of obtaining a transgenic line which expresses DsRed in females.

An alternative sex-specific marker was also constructed and tested using the splicing region for the *Doublesex* (*dsx*) gene, a double-switch gene at the bottom of the sex-determination cascade that determines the differentiation of sexually dimorphic traits (Scali *et al*., 2005). These transcripts are sex-specifically spliced in male and females (Scali *et al*., 2005). This concept was used to create sex specific transcripts with DsRed expression under the Hr5IE1 promoter (Immediate early promoter 1) in male *Aedes aegypti*. While 2000 eggs were injected, no transgenic mosquitoes were produced either and no transformation was observed.

To obtain a suitable marker which will work successfully, alternative methods will need to be tested: an example is the use of the alcohol dehydrogenase gene/aldehyde dehydrogenase (Komitopoulou *et al*., 2004; Robinson and Van Heemart 1981; Robinson *et al*., 1986) to increase the tolerance of one sex to alcohol, the addition of alcohol would then eliminate one sex and leave the required sex.

This may be more successful if an alternative spicing region is used rather than *Doublesex* (*dsx*). This method could prove to be more advantageous when sex sorting if the female larvae die and do not require observations using fluorescence to determine the sex of the mosquito unlike the female-specific DsRed marker and a testis specific marker associated with the β-tubulin promoter (Smith *et al*., 2007). However, ethanol exposure may be costly and may impact the fitness of the male insects.

Until the successful transformation of *Aedes aegypti* to create a sex-specific marker, the only method available is to use a pupal sorter to separate sexes by size (females larger than males) (Sharma *et al*., 1972).

7.6.5 Conclusion

The unsuccessful generation of a female-specific marker in *Aedes aegypti* indicate that further attempts or alternative methods are required for sex-separation during mass rearing.

Abbreviations

AMPs – Antimicrobial peptides

ANCOVA - Analysis of covariance

ANOVA - Analysis of variance

dsx -Doublesex

GO- Gene Ontology

IMD – Immune deficiency

IL – Interleukin

KW – Kruskall Wallis test

MAPK – Mitogen activated protein kinase

MWU – Mann Whitney U test

PCR – polymerase chain reaction

PGRP – Peptidoglycan recognition protein

PI3K – Phosphoinositide 3 kinase

qRT-PCR – Quantitative reverse transcriptase polymerase chain reaction

RT-PCR – Reverse transcriptase PCR

TGFβ – Tumour growth factor β

TNFα – Tumour necrosis factor α

WT – Wild-type

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