# IMPROVING THE COLD TOLERANCE OF FALSE CODLING MOTH, *THAUMATOTIBIA LEUCOTRETA*, FOR BETTER PERFORMANCE IN A STERILE INSECT RELEASE PROGRAMME

A thesis submitted in the fulfilment of

the requirements for the degree of

**MASTER OF SCIENCE**

of

**RHODES UNIVERSITY**

by

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June 2016

# ABSTRACT

The false codling moth (FCM), *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae) is a major pest of citrus and other important crops in Sub-Saharan Africa. The introduction of a sterile insect technique (SIT) programme for FCM in South Africa has proven to be very effective in the control of FCM. The objective was to flood citrus orchards with large numbers of sterile males resulting in a ratio of at least 10 sterile to 1 wild moth, increasing the probability of a female moth mating with a sterile male. This target is often achieved and the programme is generally successful, however there are some challenges regarding this programme. The mass rearing envir

onment, artificial diet, handling methods and irradiation have an impact on sterile insect quality as environmental differences between the rearing facility and field, influences the insect phenotype and competitiveness. This is evident as wild male moths can theoretically actively fly at a temperature of 12°C and laboratory-reared sterilized moths, due to the radiation treatment, appear unable to fly below 20°C. As a consequence, sterile males are out-competed by wild males during the cooler months of the year. This is detrimental to the SIT programme as FCM do not undergo diapause, meaning they are active during winter and will still reproduce. Therefore, to maximize the effect of the SIT programme, it is vital to increase the flight ability of mass reared sterile males at lower temperatures. Various studies have shown that by adding cryoprotectants to the basic laboratory diets increases the cold tolerance of certain insects and thus may allow them to be mobile at lower temperatures, however it imperative that any chemical used to augment the commercial diet of the insect has no negative effects on the insect physiology and development.

To investigate this detail for FCM, five generations of FCM were reared on diets augmented with various known insect cryoprotectants. These augmented FCM were subsequently used in experiments designed to determine firstly, if the cryoprotectants had a positive result on the cold tolerance of the FCM, and secondly, if they had any adverse effects on other physiological aspects such as duration of development. Laboratory trials indicated that the flight ability of male FCM was improved when larvae were reared on diets augmented with trehalose and cholesterol (with an average of 40 % of cholesterol and trehalose augmented males that flew at 15 °C where 0 % of the control flew). Results obtained during the field trials support the laboratory results as there was a significant increase in the number of trehalose augmented moths caught in the field during March and July (winter). Results also showed potential for cholesterol to be used as an additive. Other

important findings show that both cholesterol and trehalose have no negative impacts on developmental rate, pupal size, and egg production and viability. Trehalose was found to increase the pupal mass of male and female FCM, as well as the number of eggs laid per female. Cholesterol was found to increase developmental rate and the number of eggs laid.

The main findings of this study were that diet additives could improve the mass-rearing of FCM for SIT and the competitiveness of the males, especially at lower temperatures. However, the additives were expensive and cost could well be a constraint to the wide scale implementation of the new technology.

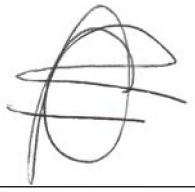
*To my family,*

*Henry, Jenny and Liam*

*Thank you all for your unconditional love and support*

# DECLARATION

The following thesis has not been submitted to any university other than Rhodes University, Grahamstown, South Africa. The work presented here is that of the author.



Date: 29/06/2015

# ACKNOWLEDGEMENTS


- My supervisor, Martin Hill and co- supervisor Sean Moore for their guidance, patience and constructive criticism. I take responsibility for a few of your grey hairs.
- Martin Villet for your statistical assistance as well as being a friend when times were tough.
- Julie Coetzee, for all your help with my statistics- I would be very lost without your guidance with this.
- Mellissa Peyper and Tanya Pretorius for all their help in the lab.
- Jeanne Van der Merwe for all those times I had no idea what I needed to do with paperwork and deliveries, you were very patient, thank you.
- Claire Love and Wayne Kirkman from CRI for all their help in the field.
- Craig Chambers from River Bioscience for all the times I bugged you for anything from egg sheets to email addresses, I really appreciate your help.
- Eugene Nepgen for help with papers and advice.
- Nevill Boersma, you have helped me so much with the sterilization part of my project and I know you took on a great deal of extra work helping me, I cannot say thank you enough.
- Kobus, the farm manager working on the citrus farm where my project was based. Thank you for your help and awesome 4X4 rides.
- A special thank you goes to my bursars, CRI, citrus academy THRIP and Rhodes University as without the funding I have received, I would not have been able to do my honours and master's degrees.
- Granny, you helped out so much and I know you would be proud, I wish you could enjoy this with me.
- Liam Richter- My love/ assistant. All those late nights when you helped me and never complained once! You are amazing and I doubt I would have been able to accomplish this without your help and support, thank you so much.
- To my parents, without you and your hard work I would never have been able to attend varsity. I thank you for all your guidance and support through the tough times, and laughter and celebration during the good times. I appreciate you guys every day.
- Finally I would like to give thanks to my heavenly father.

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## CHAPTER ONE



## CHAPTER TWO

# LIST OF ABREVIATIONS

ANOVA- Analysis of variance

ATP- Adenosine triphosphate

AW-IPM- area- wide integrated pest management

CM- codling moth

cm- centimetres

CrleGV-SA

DF- degrees of freedom

$dH_2O$- distilled water

dsRNA- double-stranded RNA

e.g.- example

EPN- entomopathogenic nematode

EPPO- European and Mediterranean Plant Protection Organization

EU- European Union

*et al.* - et alia (and others)

F1- first filial generation

F2- second filial generation

F3- third filial generation

F4- forth filial generation

FABI- Forestry and Agricultural Biotechnology Institute

FAO- Food and Agriculture Organization of the United Nations

FCM- false codling moth

g- grams

Gy- gray

Hz- hertz

i.e.- id est (namely)

IGR- insect growth regulators

INA- ice-nucleating agent

IPM- integrated pest management

Kg- kilogram

L- Litre

Mg- milligram

ml- millilitre

mm- millimetre

msec- millisecond

N- null

P1- parent generation

PBW- pink boll worm

PVC- Polyvinyl chloride

SA-DAFF

SCP- super cooling point

SIT- sterile insect technique

Spp. - species

THF- thermal hysteresis factors

*tra* gene- transformer gene

USDA- the United States Department of Agriculture

USA- United States of America

XSIT- X Sterile Insect Technique Pty. (Ltd)

ZAR- South African Rand

&- and

%- percentage

°C- degrees Celsius

±- standard error

+ve- positive

-ve- negative

# I
# GENERAL INTRODUCTION

## 1.1 FALSE CODLING MOTH, *THAUMATOTIBIA LEUCOTRETA*

### *1.1.1 Taxonomy and geographic distribution*

*Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae), commonly known as false codling moth (FCM) was first recorded as a pest on citrus by Fuller (1901) in Kwa-Zulu Natal, South Africa where he first identified it as the Natal codling moth. This identification of FCM as the Natal codling moth was due to the very similar appearance, development, behaviour and effects that FCM and the true codling moth (CM), *Cydia pomonella* (Linnaeus) (Lepidoptera: Tortricidae) had on fruit, albeit different fruit types. It was, again, later mistaken by Howard in 1909 who recorded it as *Enarmonia batrachopa*, commonly known as the orange codling moth (Stotter & Terblanche 2009). Only later was it commonly called false codling moth. The name false codling moth was justified due to its resemblance to the codling moth. Subsequently, it was taxonomically described for the first time by Meyrick (1912) as *Argyroploce leucotreta* (Newton 1998; Razowski 2000). Clarke (1958) then moved it to the genus *Cryptophlebia*, naming it *Cryptophlebia leucotreta*. It was again re-classified as *Thaumatotibia leucotreta* by Komai (1999) and this is, currently, its taxonomic status.

FCM is thought to have originated in Sub-Saharan Africa, and is endemic to all countries in Africa south of the Sahara Desert (Annecke & Moran 1982). However, it is mostly confined to the hot tropics and sub tropics. Some of the countries, including those not found in Africa, that have recorded established populations of FCM are Angola, Benin, Burkina Faso, Burundi, Cameroon, Cape Verde, Central African Republic, Chad, The Democratic Republic of Congo, Eritrea, Ethiopia, Gambia, Ghana, Israel, Ivory Coast, Kenya, Madagascar, Malawi, Mali, Mauritius, Mozambique, Niger, Nigeria, Réunion, Rwanda, St. Helena, Senegal, Sierra Leone, Somalia, South Africa, Sudan, Swaziland, Tanzania, Togo, Uganda, Zambia and Zimbabwe. It has even made its way to Israel (Jack 1916; Gunn 1921; Meyrick 1912; Bredo 1933; Thompson 1946; Hepburn 1947; Stofberg 1954; Pearson 1958; Sweeney 1962; Catling 1969; CIBC 1984; Mück 1985; Wysoki 1986; Moore 2002; EPPO 2013).

It was first found in Israel in 1984 on macadamia nuts and in 2003, it was still present but with a limited distribution, on cotton and castor bean (EPPO 2003). Recent information indicates that it is still found in the coastal area between Ashdod and Hadera (Opatowski, pers. comm. 2012). In 2009, FCM was detected in the Netherlands on glasshouse *Capsicum chinense*, and was consequently eradicated (EPPO 2010). The moth has also been occasionally noticed by lepidopterists in several Northern European countries such as the Netherlands, Sweden, Ireland and the UK (Svensson 2002; Huisman & Koster 2000; Langmaid 1996; Knill-Jones 1994; Karnoven 1983), however, it is unlikely that these moths came from established populations and were most likely imported with produce from Africa (Bradley *et al.* 1979; Karvonen 1983).

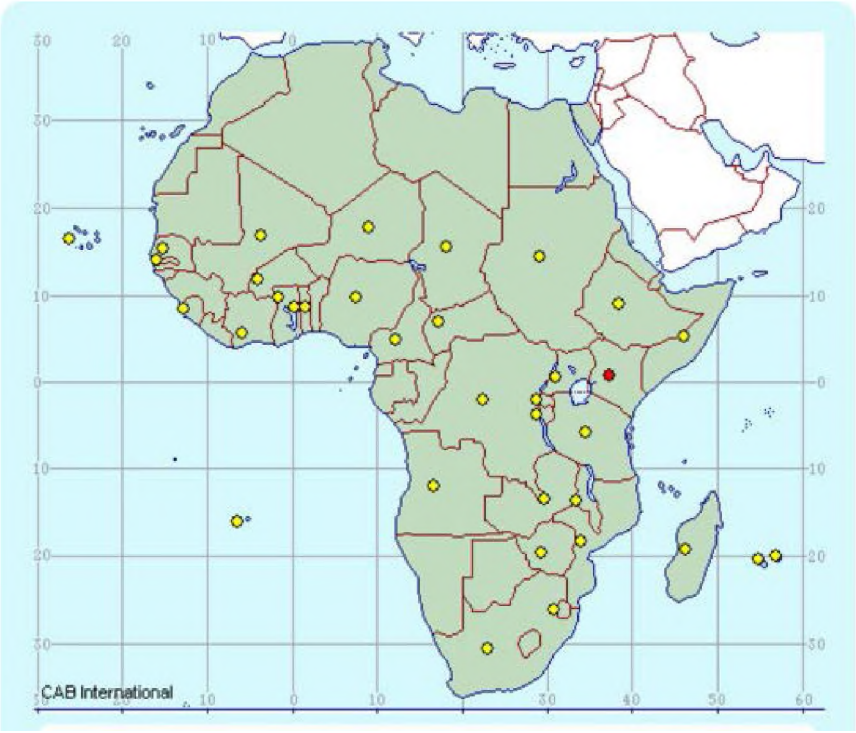



**Figure 1.1:** Geographic distribution of FCM in Africa and neighbouring islands. Dots indicate the presence of the pest as of 2006 (retrieved online at www.cdfa.ca.gov)

### *1.1.2 Life history and host plants*

Feeding and development of larvae can affect fruit development at any stage, causing premature ripening and fruit drop (Schwartz & Kok 1976; USDA 1984; Newton 1988, 1989; Begemann & Schoeman 1999), and they are even capable of developing in hard green fruit (Catling & Aschenborn 1974). Once a fruit is damaged, it becomes vulnerable to fungal organisms and scavengers (Newton 1989). The complete life cycle of FCM ranges from 45 days to 100 days under natural conditions, depending on the seasonal conditions (Stofberg

1954) with as many as six generations per year succeeding on oranges in South Africa (Newton 1998).

*Egg*- Eggs are laid individually, in large numbers and are hemispherical in shape with a granulated surface (Figure 1.2) (Catling & Aschenborn 1974). The eggs are usually deposited on the surface of the host fruit over irregular intervals throughout the female's life. On average, a single female will lay approximately 100 eggs over her life time, however under ideal temperatures, that number may reach 800 (Stibick 2006). They are translucent in colour however, appear cream to white when first laid. This then changes to pink/ red and eventually grey/ black as the development of the organism progresses. The time taken to egg hatch is directly dependant on temperature, where higher temperatures correlate with an increase in developmental rate (Daiber 1980). The period from oviposition to hatching may range from 9-14 days with hatching occurring at any time of the day. Sizes of the egg vary slightly, however the average size is 1 mm in diameter (Daiber 1979a)

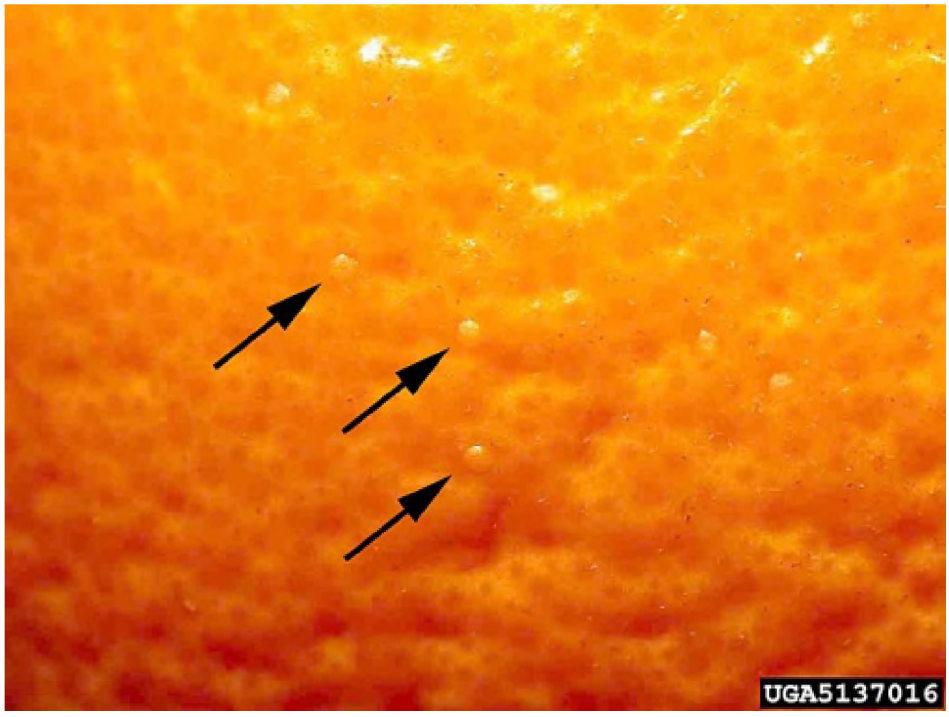



**Figure 1.2:** Eggs of the false codling moth (retrieved online at idtools.org)

*Larvae*- Once hatched from the eggs, the larvae enter the host fruit through an injury hole that they create. This results in a discoloration around the wound site, an important identifying feature when looking for FCM infested fruit (Daiber 1979b; Newton & Crause 1990). Once the larvae are inside the pulp they begin to feed. As the larvae get older, they move towards the centre of the fruit. While young, the larvae are a creamy/ white colour with

a black head. Once mature, the larvae exit the fruit through frass filled exit holes where they then drop to the ground on silken threads (Stofberg 1954). By this stage the larvae are approximately 10 - 15mm in length and pink/red in colour (Figure 1.3).

**Figure 1.3:** Fifth instar larva of the false codling moth (retrieved online at ukmoths.org.uk)

*Pupae*- In the pupal stage, the FCM larvae spin a white/ cream coloured cocoon in the soil. The length of this stage is both temperature and gender dependant whereby warmer periods are correlated with a more rapid rate of emergence, and cooler temperatures reduce the process to a slower rate. Male moths require between 13 and 47 days to reach maturity and females require between 11 and 39 days. Pupae are cream coloured and soft at first but then harden and darken as they mature (Figure 1.4). Pupae are sensitive to cold temperatures and rainfall when young (Daiber 1989). The pupae emerge out of the cocoon just before the adult emerges out of the pupal casing. When this occurs, the pupal case stays attached to the cocoon (Myburgh & Bass 1969).

**Figure 1.4:** False codling moth pupae (retrieved online at www.riverbioscience.co.za/electron.html)

*Adult*- Adult FCM are small and inconspicuous (Figure 1.5). They are inactive during the day, where they take refuge in shaded portions of the host plant, and are only active during portions of the night. The male lifespan is between 14 and 57 days, whereas females may live between 16 and 70 days. Adults have patterned wings of 1.25 to 2 cm in a variation of colours, including grey, brown, black, and orange-brown. Females communicate with males via pheromones a few hours after dark, peaking at five hours and then decreasing thereafter until sunrise (Blomefield 1978; Bestmann *et al.* 1988).

False codling moth is found on a wide array of plants including cultivated crops and indigenous host plants (Table 1.1).

**Figure 1.5:** Adult false codling moth (retrieved online at www.export.biocontrol.ch)

**Table 1.1:** Relevant wild and cultivated (in bold) plants that are documented as hosts for false codling moth

| Common name | Species name | Family name | Reference |
|---|---|---|---|
| **Asparagus** | ***Asparagus crassicladus*** | **Asparagaceae** | Kirkman & Moore 2007 |
| **Avocado** | ***Persea americana*** | **Lauraceae** | Erichsen & Schoeman 1992; Grové *et al.* 2000 |
| **Carambola** | ***Averrhoa carambola chayote*** | **Oxalidaceae** | Grové *et al.* 2000 |
| Caster bean | *Ricinus communis* | Euphorbiaceae | Hamburger *et al.* 2001 |
| **Citrus** | ***Citrus spp.*** | **Rutaceae** | Newton 1988, 1989, 1998 |
| **Coffee** | ***Coffea arabica, Coffea spp.*** | **Rubiaceae** | EPPO 2013 |
| **Cotton** | ***Gossypium hirsutum*** | **Malvaceae** | Reed 1974; Hamburger *et al.* 2001 |
| **Egg plant** | ***Solanum melongena*** | **Solanaceae** | EPPO 2013 |

| | | | |
|---|---|---|---|
| **Grape** | ***Vitis spp.*** | **Vitaceae** | EPPO 2013 |
| **Guava** | ***Psidium guyajava*** | **Myrtaceae** | Gunn 1921 |
| Jakkalsbessie | *Diospyros mespiliformis* | Ebenaceae | Stofberg 1939 |
| **Litchi** | ***Litchi chinensis*** | **Sapindaceae** | Newton & Crause 1990 |
| **Loquat** | ***Eribotrya japonica*** | **Rosaceae** | EPPO 2013 |
| **Macadamia nut** | ***Macadamia ternifolia*** | **Proteaceae** | Wysoki 1986 |
| **Maize** | ***Zea mays*** | **Poaceae** | Reed 1974 |
| **Mango** | ***Mangifera indica*** | **Anacardiaceae** | Javaid 1986 |
| **Nectarine** | ***Prunus persica var. nucipersica*** | **Rosaceae** | Blomefield 1989 |
| **Oak** | ***Quercus spp.*** | **Fagaceae** | Anderson 1986; Stotter 2009 |
| **Peach** | ***Prunus persica*** | **Rosaceae** | Blomefield 1989; Daiber 1976 |
| **Pepper/ pimento** | ***Capsicum spp.*** | **Solanaceae** | Collingwood *et al.* 1981; Fritsch 1988 |
| **Persimmon** | ***Diospyros spp.*** | **Ebanaceae** | Stotter 2009 |
| **Plum** | ***Prunus spp.*** | **Rosaceae** | Blomefield 1989 |
| **Pomegranate** | ***Punica granatum*** | **Lythraceae** | Wohlfarter *et al.* 2010 |
| **Rose** | ***Rosa spp.*** | **Rosaceae** | EPPO 2013 |
| **Sorghum** | ***Sorghum spp.*** | **Poaceae** | Reed 1974 |
| **Star apple** | ***Chrysophyllum cainito*** | **Sapotaceae** | EPPO 2013 |
| **Sugar cane** | ***Saccharum officinarum*** | **Poaceae** | EPPO 2013 |
| Suurprium/ large sour plum | *Ximenia caffra* | Olacaceae | Stofberg 1939 |
| Waterbessie | *Syzygium cordatum* | Myrtaceae | EPPO 2013 |
| Wild custard apple | *Annona senegalensis* | Annonaceae | Stofberg 1939 |
| Wild plum | *Harpephyllum caffum* | Anacardiaceae | Willers 1979 |

### *1.1.3 Economic damage due to false codling moth*

Citrus trees were first introduced into South Africa as far back as 1654 with the first recorded sales of oranges having taken place in the Cape and the former Transvaal in the latter half of the 19th century. In 1907, citrus fruit was first exported to Europe (Moore 1962). By the 1960s South Africa was ranked amongst the top five fresh citrus exporters in the world as it was, at the time, exporting over half of all fresh citrus produced in the southern hemisphere (Mather 2002). In 1995 the citrus industry in South Africa exported 43 million cartons of citrus with a gross value of ZAR1.6 billion (FABI 1998). By 2006, the South African citrus industry was the second biggest in the world (FAO: Citrus Fruit Annual Statistics 2006). The largest export markets at this stage were Europe, Japan, China and the USA. In 2008 South Africa exported 89 million cartons, which generated ZAR5.1 billion (Edmonds, pers. comm). In terms of gross value, during the 2012- 2013 production season, the citrus industry was the third largest horticultural industry after deciduous fruits and vegetables and contributed R8.3 billion to the total gross value of South Africa's agricultural production (CGA 2014).

In the past, the FCM has had a significant impact on the production of citrus. Fruit losses as a result of FCM attacks have ranged from below 2% to as high as 90% (Newton 1998) with certain citrus varieties being more susceptible to FCM attack than others. For example Navel oranges are favoured as host plants for FCM (Schwartz 1978; Love *et al.* 2014) whereas others, such as lemons, are not suitable hosts (Stotter 2009). Long term citrus crop losses in Nelspruit during 1971 were recorded as 24-45 fruit per tree (Newton 1998) and by 2004, Moore reported annual losses due to FCM attack, in excess of ZAR100 million to the South African citrus industry (Moore *et al.* 2004). This was mainly a consequence of pre-harvest fruit loss caused by the internal feeding of the larvae, and as a product of post-harvest decay of fruit (Moore *et al.* 2004).

Besides the negative impact FCM has on the production of citrus in South Africa, there is another major concern. False codling moth is a recognized phytosanitary pest for certain export markets (Carpenter *et al.* 2004). Countries importing citrus from South Africa are imposing stricter regulations regarding its importation, usually requiring cold-sterilisation of fruit en route to market as an FCM disinfestation protocol (SA-DAFF 2014). These stricter regulations result in higher costs and a greater rejection of fruit, which contributes to a further loss of revenue for the South African citrus industry (Moore 2002; Nepgen 2014). Despite

recorded interceptions in importing countries such as the United States of America (USA), the European Union (EU), China and Japan, FCM has not established in these countries (Botha *et al.* 2004).

### *1.1.4 Current control tactics*

#### *1.1.4.1 Monitoring*

False codling moth is effectively monitored through the use of a pheromone based trapping system. Yellow delta traps or PVC pipe traps are hung in a tree on the upwind side of the orchard (as male moths fly against the wind in order to detect the females), and each has a dispenser loaded with synthetic female pheromone used to lure the males, and a sticky floor placed at the bottom of the trap. One trap should be used for every 4 to 6 hectares of citrus. Male moths are then attracted to the pheromone source and on entering the trap are caught on the sticky floor inserted. Most monitoring of pests is used to develop thresholds for action, however due to the phytosanitary status of FCM, corrective measures are essential to be applied regardless of the population levels found in traps and fruit inspection points (Moore 2011). Trap data can be used as an early warning management tool, but not directly for control of FCM (Hofmeyr 2003), as well as to determine the correct timing of application, prioritising of orchards and comparisons between seasons (Moore 2011).

As an added measure, dropped fruit should be dissected and inspected weekly for FCM infestation from the beginning of January until harvest. This is not a practical reflection on present levels of FCM as fruit drop only occurs a few weeks after an increase in moth activity and larval infestation (Hofmeyr 2003). However, data gathered from fruit drop analysis can be used as an indication of the extent of the FCM status in a specific orchard and consequently the resultant risk for post-harvest infestation (Moore 2011).

#### *1.1.4.2 Orchard sanitation*

Orchard sanitation is the removal of damaged and infested fruit, both from the orchard floor and fruit still remaining in the trees. Moore (2011) states that the removal of larvae and rotting fruit from orchards is the foundation of FCM control in citrus. This is verified as in certain areas, on average 75% of false codling moth larvae can be removed by simple weekly orchard sanitation (Moore & Kirkman 2009). Regular weekly orchard sanitation should commence over the whole season, ensuring that there are no fruit left in the orchards after harvesting that could potentially be a source of FCM infestation for the following season

(Stofberg 1954; Hofmeyr 2003). It is recommended that sanitation be conducted more frequently in warm summer months, due to the temperature dependant development life cycle of FCM (Moore 2011). All fruit collected from the orchard during sanitation events should be destroyed to ensure no larvae escape and continue their development through to adulthood (Hepburn 1947).

#### *1.1.4.3 Biological control*

Biological control relies on natural mechanisms such as predation, parasitism, herbivory and/ or reproduction (Flint *et al.* 1998). Suppression of FCM populations by biological means has proven successful for a number of different parasitoids, pathogens and predators.

*Augmentation*- Augmentation involves the supplemental release of natural enemies, in order to boost the naturally occurring population. There are many naturally occurring enemies for FCM in South Africa. Several species of flies and wasps are parasitoids of FCM larvae and can play a major role in suppression of FCM populations. In the Eastern Cape, the wasp *Agathis bishopi* (Nixon) (Hymenoptera: Braconidae) parasitizes up to 40% of FCM larvae (Gendall *et al.* 2006). One of the most popular natural enemies of FCM is T*richogrammatoidea cryptophlebiae* (Nagaraja) (Hymenoptera: Trichogrammatidae), referred to as trichogramma. These insects are minute parasitoid wasps native to Southern Africa. Trichogramma are egg parasitoids, which ensures that the developing larvae are destroyed inside the egg before penetration and fruit injury can occur (Carpenter *et al.* 2004). An augmentative biological control programme using trichogramma is underway in South Africa (Newton & Odendaal 1990; Newton 1998).

*Sterile insect technique* - One of the most popular examples of biological control using genetics is the sterile insect technique whereby insects are mass reared and sterilized with radiation treatment and then released into the wild in order to mate with wild females resulting in nonviable eggs (Dyck *et al.* 2005; Vreysen *et al.* 2007). The sterile insect technique will be discussed in greater detail later in this chapter.

#### *1.1.4.4 Mating disruption*

Mating disruption controls FCM by preventing mating and as a result suppresses egg-laying on fruit. Lures with large amounts of synthetic female sex pheromone are distributed over a large area of the orchard in order to confuse males to such an extent that they are unable to find females for mating (Hofmeyr 2003; Carde & Minks 1995). Isomate and Checkmate are two registered mating disruption products. Isomate distributes the synthetic pheromone into the orchard as an aerosol via thin polyethylene tube dispensers (Moore 2011). Checkmate is a pheromone containing capsule suspension formulation and is applied as a foliar spray (Moore 2011).

#### *1.1.4.5 Microbial control*

Malan *et al.* (2011) performed bioassays which demonstrated promise with entomopathogenic nematodes (EPNs) as a possible biological control agent for soil life stages of FCM. The EPN that demonstrate promise are *Heterohabditis bacteriophora* (Rhabditida: Heterorhabtidae) and *Steinernema yirgalemense* (Rhabditida: Steinernematidae). Other natural enemies include a virus that is now available for commercial use, *Cryptophlebia leucotreta granulovirus* (CrleGV-SA) (Moore *et al.* 2015). Investigations into entomopathogenic fungi are currently underway with various isolates being collected from soil samples from various farms within the Eastern Cape Province, South Africa (Goble *et al.* 2010; Coombes *et al.* 2015).

#### *1.1.4.6 Chemical control*

At present, there are six products that are registered for the control of FCM on citrus: Alsystin (triflumuron), Nomolt (teflubenzuron), Cypermethrin, Meothrin (fenpropathrin), Delegate (Spinetoram) and Coragen (Rynaxapyr) (EPPO 2013). Alsystin and Nomolt are chitin synthesis inhibitors that target the eggs and so aim to disrupt embryonic development and prevent hatching. Results from laboratory trials indicate good residual action for the suppression of egg hatch in addition to a prominent reduction in fruit fall from Navel orange trees (Hofmeyr 1984). However, FCM has developed a resistance to Alsystin and Nomolt in the Western Cape (Hofmeyr & Pringle 1998) and Mpumulanga (Moore 2002). Adding to the resistance, Alsystin has detrimental effects on the egg parasitoid, *Trichogrammatoidea chryptophlebia* (Hattingh & Tate 1997), and therefore is not compatible in an integrated pest management (IPM) programme. Alternatively, cypermethrin and Meothrin are synthetic

pyrethroids. These chemicals have an extensive effect on FCM including an inhibitory effect on the female's egg-laying ability, as well as a direct contact and residual activity on eggs (Hofmeyer 1983). Pyrethroids are classified as broad spectrum insecticides that are toxic to a wide range of beneficial insects and as a result may cause repercussions for California red scale (*Aonidiella aurantii*, Maskell 1879), mealybug species (Family: Pseudococcidae, Heymons 1915) and soft brown scale (*Coccus hesperidium*, Linnaeus 1758) (Moore 2011) as well as reduce the numbers of natural enemies in the area (EPPO 2013).

Delegate and Coragen were registered for use against FCM in 2011 and have a comparable efficiency with a usual pest reduction between 50- 60 % when applied correctly (Moore & Kirkman 2011). These products have favorable eco- toxicology profiles when compared with the other chemical control options, making them compatible with IPM strategies (EPPO 2013).

### *1.1.5 Resistance to chemical control and the evolution of IPM strategies*

Control of insect pests on citrus still relies on insecticide use because, not only is the presence of phytosanitary pests a major influence on how much fruit is exportable, but the cosmetic integrity of the crop is equally essential for export. This reliance on pesticides has resulted in citrus exports to the EU becoming further restricted by the lowering of maximum residue limits of certain insecticides (Grout & Stephen 2005). This is mainly due to the EU's campaign to reduce the amount of unnecessary and harmful chemical pesticides used (Mather 1999) and, to a lesser degree, the modern consumer demand for organically produced foods, which prohibits the use of chemical pesticides (Zehnder *et al.* 2007).

Before the use of modern pesticides, pesticides used in citrus production in South Africa had a very limited residual action. These pesticides included: lime sulphur, resin wash, nicotine sulphate and sulphur dust. Modern insecticides have been used for the control of insect pests on citrus since the 1940s and until 1948 citrus pests were controlled significantly during the annual fumigation of trees using the highly toxic gas, hydrogen cyanide (Annecke & Moran 1982). After 1948, fumigation was replaced by organophosphate insecticides which were quicker and easier to apply (Bedford 1998).

Chemical pesticides are often relatively inexpensive and highly effective with results often noted immediately. When using pesticides, it is vital to consider what other impacts/ non-target effects that may occur when using that specific chemical; the impact it has on

natural enemies and other insect communities, and the development of pesticide resistance in both target pest and non-target pest species (Chagnon *et al.* 2015). An example of these nontarget effects was the resurgence of secondary pests such as soft brown scale and citrus red mite, *Panonychus citri*, which occurred mainly as a consequence of the toxic effect that organophosphate sprays had on the natural enemies (Annecke & Moran 1982). As a solution to the increase in these pest species at the time, the cycle of imprudent spraying of these chemical insecticides continued, which eventually led to insecticide resistance. With this chemical resistance on the rise, farmers were prompted to use new insecticides such as chlorpyrifos, mercaptothion and methidathion (Bedford 1998).The use of stronger pesticides to control resistant pests resulted in a 'pesticide treadmill' whereby the insect pests became resistant to the 'new' insecticides and as a result, more insecticides were needed. This unsustainable use of pesticides, as well as the high cost involved with multiple sprays meant that growers were forced to switch to an IPM approach and a narrow range of petroleum oils and insect growth regulators (IGR) replaced organophosphates (Bedford 1998; Morse *et al.* 2007). Chemical control has been the favoured form of control in South Africa for the past 100 years with biological control being essentially overlooked. In 1998, Bedford suggested that "the only way to avoid pesticide-induced outbreaks of pests is to strive towards integrated control". The term "integrated control" refers to a form of pest management that uses the combined effect of the valuable aspects of both chemical and biological control, therefore allowing for the control of insect pests with minimal non-target effects on natural enemy populations while maintaining longer lasting pest suppression without pesticide resistance (Orr 2003).

## 1.2 THE STERILE INSECT TECHNIQUE

### *1.2.1 Development of the sterile insect technique*

The sterile insect technique (SIT) is a method of biological control whereby insects are mass reared and the males are sterilized, usually with radiation treatment. These sterile males are then mass released into a wild population. The males then mate with the wild females which then produce infertile eggs, reducing the next generation's population. This is repeated for multiple generations until control is achieved (Knipling 1968; Dyck *et al.* 2005).

Genetic studies on how to induce sterility in insects have been conducted many times. Studies performed by Serebrovskii in the 1930s and 1940s focused on *Drosophila melanogaster* (Meigen) (Diptera: Drosophilidae), commonly known as the vinegar fly.

Serebrovskii had the intention to advance Soviet agriculture by using chromosomal translocations to cause partial inherited sterility for the suppression of the *Drosophila* pest (Serebrovskii 1940). These studies supported the principles of Mendelian genetics as he ultimately established the extent to which sterility would appear as an inherited trait. He also suggested releasing only male insects to avoid the problem of a temporary increase in the breeding population of the pest (Curtis 1968). Another study performed by Vanderplank at a tsetse fly field research station in Tanzania resulted in the discovery of a method to induce sterility by crossing two fly species, *Glossina swynnertoni* (Austen) and *Glossina morsitans* (Westwood) (Diptera: Glossinidae), creating hybrids (Vanderplank 1948). This discovery led to near elimination of *G. swynnertoni* in Tanzania (Vanderplank 1948).

The sterile insect technique (SIT) was first devised into a programme by Edward Knipling and Raymond Bushland in the USA in an effort to control the new world screwworm *Cochliomyia hominovorax* (Coquerel) (Diptera: Calliphoridae (Klaasen & Curtis 2005). Herman Muller had a role to play in the creation of the SIT programme. In the 1920s Muller had made the discovery in which he used a dentist's X- ray machine to induce sterility in the vinegar fly (Muller 1928). This way of producing sterility in insects was further researched by Bushland and Hopkins (1953) where they found that males were more sensitive to radiation treatment than females. They then proceeded to perform the first small scale radiation and release of screwworm pupae in the 1940s on Sanible Island, Florida (Bushland & Hopkins 1953).

### *1.2.2 Sterile insect technique programmes worldwide*

One of the best known and most successful SIT programmes in the world is that for New World Screwworm in the USA. This pest preys on warm blooded animals, particularly cattle. Edward Knipling and Raymond Bushland were the scientists who managed the programme. Knipling was based at the United States Department of Agriculture (USDA) and predominantly researched the mating behaviour of the New World Screwworm, particularly the sexual aggressiveness of the male screwworm, as well as the reluctance of the female to mate more than once in her life time (Knipling 1955, 1979). During his studies, he realised the potential for population suppression through the sterilisation and release of vast numbers of the insects (Knipling 1955) and soon after developed a mathematical model that determined the probability of eradication as long as a significantly high ratio of sterile to fertile screwworms was maintained over numerous consecutive generations (Barclay 2005;

Klassen 2005). In 1936, Bushland had developed a means to culture screwworm on ground meat, a major breakthrough in rearing an obligate parasite without its live host animal. This allowed for the first step in the programme, i.e. mass rearing (Melvin & Bushland 1936). A trial release was conducted and soon after, in 1957, the livestock owners in Florida persuaded the state legislature and the United States Congress to provide funds to initiate a control programme (Lindquist 1963). The New World screwworm has since been eradicated from the United States and Mexico, and efforts to eradicate it from all of Central America to Panama are on-going.

Another very successful SIT programme involves *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae), commonly known as pink bollworm (PBW) in the San Joaquin Valley of California (Staten *et al.* 1993). This cooperative grower-state-federal effort began in 1968 where sterile PBW adults, produced at the PBW rearing facility in Phoenix, Arizona, have been released each day of the cotton-growing season on approximately one million hectares of cotton. This programme has demonstrated success in preventing the high populations of PBW occurring in the neighbouring regions of southern California, Arizona, and northern Mexico and from becoming established in the San Joaquin Valley (Staten *et al.* 1993).Sterile insect technique programmes have since been applied worldwide in an attempt to control many other insect species however, only some of these programmes have shown success whilst others are still underway or have been aborted due to a lack of success (Table 1.2).

**Table 1.2**: Insect pest species for which the sterile insect technique has been used, is currently being used or is being developed **(**Brown & AliNiazee 1978; Seligman *et al.* 1990; Orankanok *et al.* 2007; Vreysen *et al.* 2009; Barnes *et al*. 2015; Hofmeyr *et al.* 2015).

| **Target Insect** | **Species name** | **Location** | **Outcome** |
|---|---|---|---|
| Screwworm | *Cochliomyia hominivorax* (Coquerel) | Curaçao, USA, Mexico, Puerto Rico, US Virgin Islands, Guatemala, Belize, Libya, Costa Rica, Panama | Eradicated |
| Mediterranean fruit fly | *Ceratitis capitate* (Wiedemann) | Italy, Peru, Mexico, USA (California), Israel, South Africa | Eradicated from several areas |
| Melon fly | *Bactrocera cucurbitae* (Coquillett) | Japan | Eradicated |
| Oriental fruit fly | *Bactrocera dorsalis* (Hendel) | Rota, Hawaii and Thailand | Reduced infestation and damage to fruit |
| Onion fly | *Delia antiqua* (Meigen) | Netherlands | Not viable |
| Mexican fruit fly | *Anastrepha ludens* (Loew) | USA/ Mexico | Started to eradicate then continued as a containment programme |
| Cherry fruit fly | *Trypeta cingulate* (Loew) | Switzerland | Substantial reduction in cherry infestation rates and subsequent progeny production |

| | | | |
|---|---|---|---|
| Pink bollworm | *Pectinophora gossypiella* (Saunders) | USA | Prevented the spread of bollworm to surrounding areas |
| Codling moth | *Cydia pomonella* (Linnaeus) | Canada, USA, Europe, South Africa | Eradication achieved in localized areas and population suppression in other areas of Canada |
| Gypsy moth | *Lymantria dispar dispar* (Linnaeus) | USA | Unknown |
| False codling moth | *Thaumatotibia leucotreta* (Meyrick) | South Africa | Population suppression in some areas |
| Tsetse flies | *Glossina spp* | United Republic of Tanzania, Nigeria and Uganda | Suppressed |
| Boll weevil | *Anthonomus grandis* (Boheman) | USA | Population suppressed below detection levels |
| Western Encephalitis Mosquito | *Culex tarsalis* (Coquillett) | USA | No population reduction |
| Asian tiger mosquito | *Aedes albopictus* (Skuse) | Reunion Island | Two fold reduction of wild populations fertility |
| Sweet potato weevil | *Cylas formicarius* (Fabricius) | | Eradicated |

| | | | |
|---|---|---|---|
| New world malaria mosquito | *Anopheles albimanus* (Wiedemman) | El Salvador | Target population reduced by 97% |
| Cockchafer | *Melolontha hippocastani* (Fabricius) | Switzerland | Target populations reduced by up to 100% |
| Yellow fever mosquito | *Aedes aegypti* (Linnaeus) | Kenya | Partial sterility |
| Southern house mosquito | *Culex quinquefasciatus* (Say) | India | Up to 90% sterile eggs |
| House mosquito | *Culex pipiens* (Linnaeus) | France | Population reduced |

The sterile insect technique has given farmers the ability to control certain insects that threaten livestock, fruit, vegetable, and fibre crops. It has also been celebrated for its environmentally friendly approach whereby it uses no noxious chemicals, leaves no residues, and is entirely species specific, thus having no non- target effects (Dyck *et al.* 2005).

### *1.2.3 Sterile insect technique programme for false codling moth in South Africa*

The first preliminary investigation of the SIT technology for the control and/or suppression of FCM in South Africa was conducted in Citrusdal, Western Cape Province. It was found that there was a reduction in fruit fall in Navel orange orchards by 94.4% (Hofmeyr & Hofmeyr 2004). After the trial investigation, an SIT programme was commercialised in 2007 by X Sterile Insect Technique Pty. (Ltd) (XSIT). The target was to flood citrus orchards with large numbers of sterile male moths resulting in a ratio of 1 wild moth to 10 sterile moths. The first commercialised programme was for the release of sterile insects over 1500 hectares of citrus in Citrusdal and over a period of three years, there was a reduction of feral male FCM populations in the SIT area with 3-, 8- and 10-fold reductions, respectively when compared to populations in the non-SIT areas (Hofmeyr *et al.* 2015). As a result of this significant reduction in the wild moth population, further trials were conducted in the Sunday's River Valley, Eastern Cape and Letsitele, Limpopo. Results for the Eastern Cape were impressive with an 80% reduction in Navel fruit infestation whereas results

obtained from Limpopo were poor (Hofmeyr & Hofmeyr 2010). Subsequently, expansion in the Eastern Cape was inevitable and was further driven by the increasing phytosanitary risk of FCM present in fruit destined for EU markets. In 2014, the SIT programme in the Eastern Cape is releasing sterile moths over 3400 hectares of citrus (Nepgen 2014).

### *1.2.4 Integrated pest management and SIT*

IPM is one of several major strategies for coping with pest problems (Kogan 2000). Various IPM strategies have evolved in response to the various challenges presented by the many different pest species (Klassen 2005). Area-wide (AW) IPM has been used in efforts to control pest species for many years and also has ancient roots in coping with locust plagues and vector-borne diseases (Klassen 2000). Lindquist (2000) wrote about several important ways in which AW-IPM differs from standard pest management of local pest populations. Firstly, it focuses on the preventive control of pest populations throughout the area, whereas standard strategies focus particularly on defending the valued commodity, such as a crop, livestock and/ or people, from the direct attack of pests. Secondly, it involves multi-year planning, and an organization committed solely to implementing the IPM programme. The conventional strategy, however, is straight forward and involves only minimal future planning as it is implemented by individual producers. Lastly, AW-IPM utilizes advanced technologies, as well as cultural practices, whereas the standard strategy tends to rely on tactics and tools that can be reliably implemented by non-specialists, otherwise known as 'Low-tech' (Lindquist 2000, 2001).

Area-wide IPM programmes are able to take advantage of specialized methods of insect control that are not effective when used on an individual farm-by-farm basis. These include certain programmes of mass releases of parasitoids, semiochemicals, mating inhibitors, large-scale trap cropping and treatment of hosts on public lands and in private gardens (Klaasen 2005). Area-wide IPM is also better able to capture the benefits of wild natural enemies (Knipling 1992) and it enables many producers to combine resources to enable them to utilize technologies and expertise that are usually too expensive for individual producers (Klaasen 2005).

In order for an IPM strategy to work effectively and efficiently with a SIT programme, the selectivity and efficiency of available strategies should be evaluated and used to benefit the SIT programme. For example, the use of a selective insecticide benefits SIT by increasing the sterile: wild ratio. In addition, a release of both a pest-specific parasitoid and

sterile insects is likely to be mutually beneficial (Klaasen 2005). When the economic threshold of the pest is fairly high, several strategies that have cumulative effects against these dense populations may be combined to give a much more reliable suppression result than from a single method (Knipling 1979).

### *1.2.5 Disadvantages*

*Species specificity*- Under most circumstances, a species specific programme in the control of an insect pest is ideal. However, in some cases there is more than one species that needs to be controlled, such as the tsetse fly in Africa, where there are 22 species (Jordan 1994). This means that an SIT programme needs to be separately implemented for each species and hence becomes impractical where these species occur together (Robinson & Hendrichs 2005).

*Sexing*- Rendón *et al.* (2004), explained that an SIT programme can often be made much more effective if only males are mass-reared and released. However, in certain species of insects, it is hard to differentiate between males and females although this can be easily performed on some species such as FCM Medfly and screwworm, albeit possibly not practical. In species where sexing is not possible, there are two alternative measures one can take to ensure the release of male insects only. The first means is targeted towards killing females and the second is transforming putative female zygotes into males. Both measures require conditionality to ensure maintenance of colonies (Robinson & Hendrichs 2005). For fruit flies, a female killing system has been constructed using a tetracycline repression system (Heinrich & Scott 2000). In the Mediterranean fruit fly mass production units, researchers are manipulating a sex- determining gene, the *transformer (tra*) gene. It has been the target for transforming females into males by injecting double-stranded RNA (dsRNA) for part of the *tra* gene into embryos (Pane *et al.* 2002).

*Radiation-* In most cases radiation affects the health of the male insects by reducing the competitiveness of sterile insects and as a consequence these sterilized insects are at a disadvantage when competing for females, particularly during the cooler months of the year. When developing a SIT programme, an optimum radiation dose must be identified for that particular species and strain (Mehta & Parker 2006).

*Mass rearing*- The artificial environment where these insect species are mass reared provides a tremendous challenge for the insects to adapt to the artificial conditions. Adapting

to the artificial diet as well as the growth room conditions (e.g. temperature and humidity) may, in some cases, reduce the fitness of the insects. Mass rearing protocols generally follow the principle of large cycling colonies, whereby a portion of the offspring from the production culture is used in SIT programmes and the other portion is put back into the cultures to maintain the production colony. This will inevitably, in time, result in an accumulation of highly selected genotypes and this might significantly compromise the quality and competitiveness of the insects once released into the field (Miyatake & Haraguchi 1996; Briceño & Eberhard 2002).

*Transport*- Nepgen (2014) found that transport via road significantly impacted the flight ability and longevity of mass reared, irradiated male and female FCM, as when delivered for release the insects are chilled (to limit activity) and placed in insulated cooler boxes in high numbers. It was also found that emergence delayed by shipping pupae under hypoxia in a low-oxygen atmosphere, resulted in a reduction in the number of insects if shipping time was prolonged (Dowell *et al*. 2005).

*Cost*- One of the major disadvantages of using this technique is the cost of producing such a large number of sterile insects and is often not viable in poorer countries. All insects that are mass-reared are sustained on an artificial diet. The larval diet of many species is an expensive part of the mass-rearing costs, and logistical problems are presented if the diet includes constituents that are poorly defined, with consequential difficulties in confirming their quality. Two other important costs to consider are: the costs involved when larvae are separated from the diet where they are then allowed to pupate; and as the radioactive source declines, it must be recharged, which is a very complicated and expensive procedure (Robinson & Hendrichs 2005). Another important aspect pertaining to cost is that SIT has a non- discriminatory approach and so there is no possibility for specific cost determination for any individual orchard, farm or season- if SIT is indeed necessary at the time, it is simply performed and the cost acquired.

*Possibility of resistance*- With the increase in SIT use to suppress pest populations there is a concern about the development of resistance. This is a result of the SIT programmes having a rather long-term plan, which may provide opportunity for natural selection to select individuals that may, in some way, differentiate between a sterile male and a wild male (Itô *et al*. 2003).

### *1.2.6 Female sterility and its role in SIT, particularly for FCM*

The role of sterile females that are also released alongside males into the field during mass release efforts has huge implications for certain SIT programmes. For many species of insect, the mass release of sterile females into the field would have detrimental effects to not only the programme, but humans too. This is evident for species whose females cause damage through oviposition inside of the fruit or whose females feed on their prey. A considerable amount of research has been done to develop genetic sexing strains for such species allowing for mass rearing facilities to dispose of females before release. An example of a successful sexing strain development is for that of the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann). Genetic sexing strains based on the temperature sensitive lethal (tsl) mutation are being used to produce sterile male flies for large scale SIT programmes of this pest. This use of male-only strains allows for an increase in the overall efficiency of the technique (Caceres 2002). However, there are many other SIT programmes where the developments of male-only sexing strains are necessary, but not possible. *Aedes albopictus* (Linnaeus) is a vector of a number of severe arthropod-borne diseases which affect humans including dengue fever, yellow fever and chikungunya (Benedict *et al.* 2007; Gasperi *et al.* 2012; Bonizzoni *et al.* 2013). Consequently, there is a crucial need to control this mosquito species and SIT has great potential to aid in this (Zhang *et al.* 2015). However, released female mosquitoes are capable of feeding on people and in turn transmitting diseases, therefore the most critical step for this SIT programme is sex separation (Gilles *et al.* 2014). A genetic sexing strain is not available for *Ae. albopictus*, however there is a difference in pupal body size between males and females which allows for the majority of the female pupae to be separated from the males through a Fay-Morlan separator (Fay & Morlan 1959; Focks 1980). This is not ideal in large scale operations as millions of male adult mosquitoes are released into the field each time and even a small percentage of remaining females would mean that thousands of females are released, thus reducing the effectiveness of the programme on population suppression, and increasing the abundance of potential disease vectors (Zhang *et al.* 2015).

Fortunately, not all SIT programmes require the development of such a strain as it is not warranted because the species biology does not pose a threat to the programme or to humans. Examples of such species include the cactus moth, codling moth and false codling moth. However, the role of females in these SIT programmes has been considered. There could be positive consequences where the mass release of sterile females could result in a

'sperm-sink' as wild males are attracted to and mate with the sterile females ultimately having a positive effect on population suppression in the field, or there could be negative consequences where the 'sperm-sink' is seen in the sterile male population as they may be attracted to the sterile females released alongside them. The sex ratio for FCM is 1:1 which results in 50% of released FCM being females. This may have huge implications for the FCM SIT programmes worldwide as they may play a role in the overall effectiveness of the programme, be it positive or negative.

## 1.3 PHYSIOLOGY OF INSECT COLD SENSITIVITY

### *1.3.1 The effects of low temperatures on insects*

Unlike most other animals that are able to generate their own heat internally (endothermic), insects rely on external sources to provide heat for everyday functions (ectothermic). Thus, insects during winter must tolerate freezing or rely on other mechanisms to avoid freezing. Loss of enzymatic function and eventual freezing due to low temperatures threatens the maintenance of these organisms during winter. Because of this threat, insects have evolved a number of strategies to deal with the problems posed by the colder winter temperatures, a predicament they would otherwise not survive. The general ranges for various activities differ from species to species (Lee & Denlinger 1991); however a general guide to how insects respond can be seen in Table 1.3.

There are many ways in which temperature can affect the development and functioning of an insect. The first effect temperature has is on metabolic rate is that the metabolic rate gradually decreases with decreasing temperature even in immobile insects in cold shock (Resh & Cardé 2003). The second effect temperature has is on insect size. Adult insects are generally smaller in body size if the larvae are reared at high temperatures. A good example of this is that the females of *Bicyclus* butterflies are larger when reared at 20°C than when reared at 27°C. Furthermore, females laid larger eggs when they were reared or acclimatized for 10 days at lower temperatures compared to higher temperatures (Fischer *et al.* 2004). The third effect temperature has is on developmental rate. Developmental rate is null at the lower temperature threshold, and increases with an increase in temperature and eventually levels off at optimum temperatures after which it decreases rapidly as the higher temperature threshold is approached (Roy *et al.* 2002). Finally, cold injury and death are extreme consequences of being exposed to cold temperatures for a prolonged period of time,

or from sudden exposure to extreme temperatures. This inhibits mobility and in turn results in a chill coma or chill shock which is manifested in a complete arrest in movement (Findson *et al*. 2014).

**Table 1.3**: Insect responses to low temperatures (general ranges of activity). Adapted from Lee & Denlinger (1991)

| Physiological response | Min temperature (°C) | Max temperature (°C) |
|---|---|---|
| Fly | 0 | >20 |
| Walk | -5 | >20 |
| Chill coma | -5 | 10 |
| Chill shock | -20 | 5 |
| Freeze avoidance | -65 | 0 |
| Freeze tolerance | <-70 | 0 |

### *1.3.2 Mechanisms promoting insect survival at low temperatures*

Insects exposed to sub-zero temperatures are at the risk of ice formation in the cells and to a small poikilotherm, the management of body water becomes a critical issue (Hallman & Denlinger 1998). There are many ways in which insects survive the cold and prevent chill injury and they are organized into two categories, freeze avoidance and freeze tolerance.

*Freeze avoidance*- In the freeze avoidance category, insects may implement behavioural, physiological and/or biochemical adaptations to prevent intra- and extracellular freezing.

Behavioural adaptations include finding a suitable microhabitat with a thermal buffer, such as underground nests where they subsequently enter a form of diapause or torpor, depending on the severity of the cold temperatures (Marchand 1996). Migration is another behavioural adaption to cold temperatures where insects may migrate to warmer areas. A classic example of mass insect migration is the monarch butterfly which migrates from as far as Canada through to Mexico and southern California (Urquhart & Urquhart 1976). Communal living is another form of adapting to the cold, whereby they congregate in large clusters in order to maintain suitable temperatures. This is more commonly seen in social insects such as bees (Southwick & Heldmaier 1987).

Physiological adaptations include those initiated by the insect and include changes in the density of the wax covering the cuticle (Duman 2001). Some insects, particularly from the order Lepidoptera and Hymenoptera, increase thoracic hair density as a form of insulation (Southwick 1985).

Supercooling involves both behavioural and biochemical adaptions and is defined as the process by which water cools below its freezing point without changing phase into a solid due to the lack of a nucleation source and/or the accumulation of cryoprotective compounds (Lee & Denlinger 1991). In the initial phase of seasonal cold hardening, ice-nucleating agents (INAs) such as food particles, dust particles and bacteria, in the gut or intracellular compartments of freeze avoidant insects have to be removed or inactivated. This is the behavioural component of this strategy, as the removal of ice-nucleating material from the gut is achieved by a cessation of feeding (Olsen & Duman 1997), emptying the gut and removing lipoprotein ice nucleators from the haemolymph (Neven *et al.* 1986). The biochemical adaptations during supercooling include the synthesis and accumulation of cryoprotectants such as polyols and sugars, which reduce the lethal freezing temperature of the body (Pfister & Storey 2006). Though not all freeze avoidant insects produce polyols, most hibernating insects produce thermal hysteresis factors (THFs) which act directly with the ice crystals by adsorbing to the developing crystals in order to inhibit their growth and reduce the chance of lethal freezing.

*Freeze tolerance*- As all insects are ectothermic, they are vulnerable to freezing. Freeze tolerance in insects refers to the ability of certain insect species to survive ice formation within their tissues. These specific types of insects are considered the most cold-hardy species (Bale 1996). In most animals, intra- and extracellular freezing results in severe tissue damage, following in death and/ or extensive injury. Because of this risk, insects have evolved several freeze-tolerant strategies to limit tissue damage by controlling where, when, how and to what extent ice forms (Ramlov 2000). In contrast to freeze avoiding insects, freeze tolerant insects limit supercooling and rather initiate the freezing of their body fluids at relatively high temperatures. This is achieved through inoculative freezing, the production of certain INA proteins, crystalloid compounds, and/or microbes (Lee & Costanzo 1998). Freeze tolerance has evolved multiple times in insects and can be found in the orders; Orthoptera, Blattodea, Diptera, Coleoptera, Lepidoptera and Hymenoptera (Sinclair *et al.* 2003).

### *1.3.3 Cryoprotectants found in insects*

There are two main types of cryoprotective compounds found in insects- low molecular mass compounds (polyols and sugars) and high molecular mass compounds (THPs) (Block *et al*. 1990). Regardless of an insect's capacity to survive freezing, most cold acclimated insects have increased levels of low molecular weight polyols, sugars or amino acids (Fields *et al*. 1998). Polyols and sugars act together in lowering the whole body super cooling point (SCP) of the insect. Polyols have an important cryoprotective function in stabilising proteins and enzymes against cold denaturation; they also promote desiccation resistance by increasing the proportion of unfrozen water. The most common compounds found in insects are, but not limited to, glycerol, mannitol, sorbitol, ribitol, erythritol, threitol, trehalose, sucrose, fructose and ethylene glycol (Block *et al.* 1990; Storey & Storey 1991; Fields *et al*. 1998). The various types of cryoprotectants found in insects vary greatly from species to species and this can be seen when comparing the larvae of the Antarctic midge, *Belgica antarctica*, and the gallfly, *Eurosta solidaginis.* The Antarctic midge contains erythritol, glucose and trehalose, whereas the gallfly contains glycerol, sorbitol and trehalose (Block *et al*. 1990).

High molecular mass compounds are proteinaceous in character and are recognized as an antifreeze mechanism whereby the freezing point of the insect is depressed, and the melting point temperature remains the same. Examples of high molecular mass compounds that are well known in insect cryobiology are the amino acids, proline and alanine (Block *et al*. 1990; Fields *et al.* 1998).

### *1.3.4 Improving the cold tolerance of an insect using cryoprotectants*

Various studies have shown that adding cryoprotectants to basic laboratory diets increases the cold tolerance of certain insects (Hendrix & Salvucci 1998; Shreve *et al*. 2007; Kostal *et al*. 2012). Improving the cold tolerance of FCM may be done in a similar way, via augmenting the basic diet by adding cryoprotectants such as proteins and alcohol- sugars. In a study done by Kostal *et al*. (2012), the cold tolerance of the drosophilid fly, *Chymomyza costata*, was increased. The fly was raised on a diet to which the cryoprotein, proline was added. The proline was fed at two quantities, which were called pro10 (10 mg proline/ 1 g diet) and pro50 (50 mg proline/ 1 g diet). There was also a study by Shreve *et al.* (2007) where membrane cholesterol levels of *Drosophila melanogaster* were increased by raising the larvae on a cholesterol-augmented diet which then improved the cold tolerance. Another

study done by Hendrix and Salvucci (1998) showed that sorbitol accumulation provided a thermo- and osmo-protection mechanism in white flies and that mannitol played a similar role in aphids. Munyiri and Ishikawa (2005) stated that trehalose is the principle sugar circulating in the haemolymph of most insects and it serves as an immediate source of glucose for tissue metabolism.

## 1.4 INSECT FLIGHT

### *1.4.1 Fuel utilisation in energy production*

Flight in insects involves an extremely high energy demand (Conley & Lindstedt 1998). As a result, metabolic rate is extremely high during flight with an increase of 50-200 fold when undergoing the transition from rest to flight; such factorial scopes are unprecedented for both vertebrate flight and other forms of arthropod locomotion (Dudley 2000). This is an enormous increase, as small mammals running at maximum speed and flying birds only see a 7- 14 fold increase in activity (Beenakkers *et al.* 1984).

Adenosine triphosphate (ATP) is the most common energy key to all forms of biological work and is essential for muscle contraction and is known to regulate its own production (Sacktor l975; Buton *et al.* 2004). Newsholme and Start (1973) suggest that only small amounts of ATP are stored in the muscle cells with only enough ATP to sustain contractions for 100 msec. Any continued contractions of these muscles are only able to occur because of other substances i.e. fuels. These fuels yield their bonded energy to the production of ATP when oxidised. The storage potential for fuels in the muscle cells is far greater than that of ATP. However, most insects have fuel reserves stored elsewhere which can, when needed, be mobilised and used to produce ATP. Two of the most common storage sites for these fuels are the haemolymph and fat bodies (Stokes & Morgan 1981).

Muscle metabolism can be based on several different fuels, the proportions of which vary according to the insect species and the degree of flight activity (Candy *et al.* 1997). There are three categories of insects that are grouped in respect to the major fuel substrates utilized by their muscles when generating ATP. The first group is insects which generate their energy from carbohydrates, the second group is insects which generate their energy using carbohydrates and lipids, and the last group generates energy from specific amino acids, with the main one being proline (Stokes & Morgan 1981).

### *1.4.2 Carbohydrates and insect flight*

The physiologically significant carbohydrate substrates available for mobilisation as fuels for insect muscles are glycogen, glucose and trehalose (Bedford 1977; Stokes & Morgan 1981). Carbohydrates are the only source of fuel substances that allow ATP production under anaerobic conditions and while this is an inefficient way to produce ATP, certain insect muscles derive energy in this manner. Most insect muscles, however respire aerobically and thus are not subject to hypoxic or anoxic conditions (Stokes & Morgan 1981). There are two limiting factors in using carbohydrates as fuels for ATP production in insect muscle. The first factor is that carbohydrates yield less ATP than other fuels as the energy content of a carbohydrate is low on a per mole basis and the maximum amount of ATP which can be formed is 39 moles ATP per mole of glycogen oxidised (Stokes & Morgan 1981). The second limiting factor is that carbohydrates cannot be stored in significant quantities in the insect muscle and as a result the muscle needs to be continuously supplied with additional carbohydrates when supporting intense activity (Beenakkers 1969; Stokes & Morgan 1981). In most species of Hymenoptera and Diptera, carbohydrates act as the predominant fuel substrate for flight, whereas in many species of Lepidoptera and Orthoptera, carbohydrates are used in conjunction with lipids. An example of insects that utilise carbohydrates whilst flying are blow flies, *Phormia regina*, and although the stored glycogen in the muscle is depleted after about 10- 15 minutes of flight, these flies continue to metabolise glycogen stored in the fat body and the gut (Beenakkers *et al.* 1984; Stokes & Morgan 1981). Stored carbohydrates are mainly supplied to the flight muscles in the form of trehalose, the principle sugar found in insect haemolymph. Trehalose in the haemolymph is replenished from glycogen stores found in the fat body of an insect, the main storage area for metabolic reserves (Beenakkers *et al.* 1984).

### *1.4.3 Lipids and insect flight*

There are two distinct types of insects that utilise lipids as a fuel for energy – those that undergo prolonged flight during events such as migration, and those which do not feed during the adult life stage (Stokes & Morgan 1981). A lipid droplet consists of a core of neutral lipid molecules, made up of triglyceride and cholesterol esters, which are surrounded by a monolayer of phospholipid, cholesterol and a matrix of proteins (Arrese & Soulages 2010). There are significant advantages for insects using lipids as a form of metabolic fuel. For one, the isocaloric content of a lipid molecule is less voluminous than that of a

carbohydrate molecule with the physiological combustion value being double that of a carbohydrate. This allows for a more compact energy source which is an important factor for migrating insects and insects which depend on food reserves stored as a larva. The second advantage is that the oxidation of lipids yields roughly twice the amount of water as a by-product than that produced by carbohydrates. This is a great benefit to insects during migration (Stokes & Morgan 1981).

In earlier studies it was thought that lipids were the primary source of fuel for flight in many insect species, particularly lepidopterans (Zebe 1954; Dudley 2000). This, however, is an exception as some species which continue to feed on carbohydrates as adults, as well as those known for prolonged flights, utilise carbohydrates in the initial stage of flight and then later switch to lipid use. This two-fuel system has been studied extensively, especially in the migratory locust, *Locusta migratoria,* whereby it uses glycogen in the flight muscles and then trehalose from the haemolymph. This is thought to be the main source of energy during early flight and as the trehalose levels decrease during flight, the lipid level is increased by the introduction of diglycerides from the lipid stores in the fat body. The only disadvantage in using lipids as a metabolic fuel is that lipids are considerably more difficult to mobilize than carbohydrates, but since carbohydrates are rapidly utilised, continued muscular activity can only be prolonged when supported by other energy reserves, namely lipids (Stokes & Morgan 1981).

### *1.4.4 Proline and insect flight*

Proline utilisation used as a fuel in the production of energy is becoming increasingly recognised as an important adaptation for flight in many insect species with large amounts found in both the haemolymph and flight muscles of some insects. The role of protein as a metabolic fuel may be different for different insect species, ranging from providing intermediates of the citric acid cycle to acting as the primary energy substrate for insect flight (Beenakkers *et al.* 1984). One of the insects most renowned to utilise proline as a metabolic fuel is the tsetse fly, *G. morsitans*, whereby proline serves as an energy reserve for the production of ATP (Nation 2008; Stokes & Morgan 1981). This use of proline in tsetse flies is thought to be an adaption to feeding on mammalian blood which contains proline. Other non-blood sucking insects, such as dipterans and hymenopterans, also utilise proline, however these are only contained in small amounts and are used during the early stages of flight. There are no reports of proline metabolism in any non-flight muscles in insects (Stokes

& Morgan 1981). Proline metabolism only releases up to 14 mols of ATP per mol of protein, which is significantly less than ATP produced via carbohydrates (Nation 2008). However, Bursell (1981) concluded that after considering factors such as yield of metabolic energy, solubility and amount of nitrogen production, proline is the most suitable of all the potential amino acids when it comes to using amino acids as metabolic fuels.

## 1.5 PROJECT PROPOSAL

### *1.5.1 Problem statement*

An SIT programme for the control and/or suppression of FCM in citrus was implemented in South Africa in 2007 (Nepgen 2014). The objective was to flood citrus orchards with large numbers of sterile males resulting in a ratio of at least 10 sterile to 1 wild moth, increasing the probability of a female moth mating with a sterile male (Hofmeyer & Hofmeyer 2004). This target is often achieved and the programme is generally successful. However, there are some challenges regarding the SIT programme. Some of the foremost problems include those created during the mass-rearing and radiation procedures. The mass rearing environment, artificial diet, handling methods and irradiation have an impact on sterile insect quality (Calkins & Parker 2005) as environmental differences between the rearing facility and field, influence the insect phenotype and competitiveness. This is evident where wild male moths can theoretically continue to actively fly down to a temperature of 12°C and laboratory-reared sterilized moths, due to the radiation treatment (Bloem *et al.* 2003), appear to be inactive below 20°C. As a consequence, sterile males are out-competed by wild males during the cooler months of the year. This is detrimental to the SIT programme, as FCM does not undergo diapause, meaning moths are active during winter and will still reproduce (Boardman *et al.* 2012). Therefore it is vital to increase the cold tolerance of the sterile males in order to maximize the effect of the SIT programme, even in the cooler months. Various studies have shown that by adding cryoprotectants to basic laboratory diets the cold tolerance of certain insects can be increased (Hendrix & Salvucci 1998; Shreve *et al.* 2007; Kostal *et al.* 2012). Improving the cold tolerance of FCM, increasing the fuel available for flight, or developing a technique that encompasses both might be achieved in a similar way, via augmenting the basic diet with cryoprotectants and fuels such as proteins and alcohol-sugars.

### *1.5.2 Objectives*

The fundamental purpose of this study is summarized in the title of this thesis with the ultimate goal to improve the activity of sterile male FCM at low temperatures, and then to incorporate this into the current SIT programme for FCM in South Africa. The SIT programme for FCM in South Africa has been generally successful however, it can still be greatly improved, especially concerning the flight ability of sterile males and ultimately their performance.

The first objective was to determine if and what cryoprotectants and/or fuels increase the flight ability of FCM under various temperatures in the laboratory. This was achieved by adding various cryoprotectants and fuels to the larval diet. The cryoprotectants tested were proline, sorbitol, mannitol, trehalose, glycerol, glucose, honey and cholesterol.

The second objective is to determine if there were any other physiological effects brought about by the additives in the various diets and how this may affect FCM and their future generations (other than flight ability). This is specifically important to the mass rearing of FCM for the SIT programme as it was vital to understand the effects that the additive might have on future generations if it were to be added to the commercial diet of FCM. It was important so as to ensure that the mass production of FCM was not hindered in any way. Particular aspects of interest include pupal mass, number of eggs laid and hatched per female egg hatch and developmental rate.

The third objective of this study was to implement the findings from the laboratory trials regarding FCM activity into field trials during different seasons: autumn, winter and spring. This was important as the laboratory results were preliminary and helped to identify the most effective additives which would then be used in field trials. Field trials were necessary as they gave a more realistic result on what can be expected regarding recaptures, as an indication of flight ability, if the additives were to be used commercially.

# II
# FCM DIET MANIPULATION AND ITS EFFECT ON FITNESS AND FLIGHT IN A CONTROLLED ENVIRONMENT

## 2.1 INTRODUCTION

Since its implementation in 2007, the sterile insect technique (SIT) programme for FCM in South Africa has shown great success (Barnes *et al.* 2015). Recent success of this programme is that of the Gamtoos River Valley where an SIT programme for FCM was implemented in 2014. Before the implementation of this programme, there was an average of 2.8 males caught in traps on a weekly basis, and within a week after implementation, the number of wild males caught per trap was reduced to less than one (N. Boersma pers. comm. 2015). The competitiveness of the laboratory reared FCM is greatly reduced due to various factors involved in the SIT process. These include, but are not limited to, the mass rearing procedure, radiation treatment and transportation of these insects over long distances (Bloem *et al.* 2003; Calkins & Parker 2005; Nepgen *et al.* 2015). Aspects of the mass-rearing procedure that have an impact on sterile insect quality include environmental and climatic differences between the rearing facility and the field, artificial diet as well as handling methods (Calkins & Parker 2005). Mellanby (1939) reported that individuals of numerous insect species (including *Blatta orientalis, Cimex lectularius*, and *Rhodnius prolixus*), which were acclimatised to warm temperatures, became immobilized at higher chill-coma temperatures than individuals of the same species that were acclimatised to colder conditions. Such may be the case for laboratory reared FCM which are reared at favourable temperatures of 27 °C. One of the most significant factors in reducing the fitness of FCM is the radiation treatment used to sterilize the males. As the radiation dose increases, sterility increases, however, quality and competitiveness decrease (Calkins & Parker 2005) and as Lepidoptera are relatively radio-resistant compared with other insects, they require a radiation dose between 150- 200 Gy to induce 100% sterility (Bloem *et al.* 2003) which is unfavourably high. This reduction in the competitiveness of reared laboratory FCM is evident in their lack of activity during the cooler months of the year whereby, wild moths are active down to a temperature between 10- 15 °C which is significantly lower than the laboratory reared males

(Stotter & Terblanche 2009) which have been known to only active down to a temperature of 20 °C (M. Hill. pers. comm. 2015).

Since FCM is present throughout the year in South African citrus orchards (Stofberg 1954; Begemann & Schoeman, 1999) and has no true diapause (Terblanche *et al.* 2014), it is suggested that low-temperature tolerance is crucial for their activity and survival during the low-temperature conditions commonly encountered by this species in the wild, particularly during the cooler winter months. This is dissimilar to codling moth whereby temperature and photoperiod work interactively to regulate diapause (Singh & Ashby 1986), thus suggesting that FCM has a greater tolerance to low temperatures and is also likely to have a lower minimum threshold regarding flight activity than that of the CM (Stotter & Terblanche 2009). A study performed by Stotter & Terblanche (2009) found that FCM lacks the ability to undergo rapid cold hardening, which suggests that this species has a limited ability to regulate its thermal tolerance over short timescales. It is unclear whether it was the laboratory-reared individuals that had lost their ability to rapidly cold harden relative to wild individuals, or because the naturally occurring conditions necessary to prompt a hardening response are not simulated when FCM is reared under laboratory conditions (Stotter & Terblanche 2009).

Since laboratory reared FCM have a reduced competitiveness and do not undergo rapid cold hardening, artificially improving the cold tolerance of these FCM may be an effective way to improve activity, and subsequently competitiveness at cooler temperatures. Improving the cold tolerance of an insect has been achieved in various studies (Hendrix and Salvucci 1998; Shreve *et al.* 2007; Kostal *et al.* 2012). Many of the common cryoprotectants found in insects are also known insect fuels, therefore by increasing the cryoprotectant levels in an insect, it is possible that it might have a twofold effect and increase the amount of fuel available for locomotion. For instance, Munyiri and Ishikawa (2005) stated that trehalose is not only a known cryoprotectant, but is also the principle blood- sugar circulating in the haemolymph of most insects and it functions as an immediate source of glucose for tissue metabolism. Insect are also able to generate their energy from carbohydrates, or from specific amino acids with the main one being proline, which is also a known cryoprotectant (Stokes & Morgan 1981).

The aim of the study reported in this chapter was to determine if certain larval diet additives (known insect cryoprotectants) could improve FCM male flight at lower

temperatures in a controlled environment. Thus, FCM was examined under laboratory conditions to determine which diet supplement might improve the flight competitiveness of individuals at cooler temperatures without having any negative adverse effects on key quality parameters such as developmental rate, pupal size as well as egg lay and viability, and ultimately improve sterile male FCM activity in the field as part of the SIT programme.

## 2.2 MATERIALS AND METHODS

### *2.2.1 Dosage response test*

Egg sheets comprising of wax paper and FCM eggs were obtained from a culture held at River Bioscience, Addo, South Africa. The egg sheets were cut into equal sizes possessing similar amounts of eggs (between 200- 300). These were surface sterilized by dipping them briefly (± 2 seconds either side) in a 25% formaldehyde (37% stock) solution. After sterilization, they were then placed individually into already prepared glass jars (height: 122.5 mm; Diameter: 73.5 mm; capacity: 352 ml) containing a laboratory diet for FCM (Moore *et al.* 2014), which was also mixed with various relevant additives. The FCM diet was bulk prepared by mixing the following together: maize meal (2000 g), wheat germ (200 g), Brewer's yeast (100 g), milk powder (36.5 g), nipagen (15 g) and ascorbic acid (6.5 g).

Individual jars of FCM diet were prepared by mixing 45g of the bulk diet with the relevant additive in a honey jar (350 ml capacity). The chemicals chosen to preform dosage response tests on were: 1) proline; 2) cholesterol; 3) sorbitol; 4) mannitol; 5) trehalose; 6) glucose 7) glycerol and; 8) ethyl glycol. The medium dosage for each chemical was determined by looking at literature where these chemicals have been added to the laboratory diets of other insects (Hendrix and Salvucci 1998; Shreve *et al.* 2007; Kostal *et al.* 2011; Kostal *et al.* 2012; Metwally *et al.* 2012). A higher dosage and a lower dosage were tested. This resulted in each chemical being tested at three dosages; low, medium and high. The dosages used for each chemical were as follows: proline 900 mg, 2250 mg & 3150 mg; sorbitol 900 mg, 1800 mg & 2700 mg; mannitol 450 mg, 900 mg & 3150 mg; ethyl glycol 1000 mg, 1500 mg & 2000 mg; trehalose 900 mg, 2250 mg & 3150 mg; glycerol 4500 mg, 6750 mg & 9000 mg; glucose 2700 mg, 3600 mg & 4500 mg; cholesterol 80 mg, 120 mg & 200 mg.

Each dosage of each chemical was added to 45g of the bulk diet and placed in a honey jar (350 ml capacity). Then 45ml of $dH_20$ was added to the augmented diet and allowed to

saturate through to the bottom of the diet. The jars (Figure 2.1) were subsequently closed with a cotton wool stopper and placed into an autoclave for 20 minutes at 121°C. The jars were then left to cool and inoculated with the eggs. Once the jars were prepared, they were all placed into a controlled environment room (CE room) at 27°C and the FCM were left to feed and develop until they pupated in the cotton wool. Each dosage for each chemical was repeated 3 times. Developmental time in days was recorded for each jar.

### *2.2.2 Development on augmented diets*

This process was similar to that of the dosage response test whereby egg sheets comprising of wax paper and FCM eggs were obtained from a culture held at River Bioscience, Addo, South Africa. The egg sheets were cut into equal sizes possessing similar amounts of eggs (between 200- 300). These were surface sterilized by dipping them briefly (± 2 seconds either side) in a 25% formaldehyde (37% stock) solution. After sterilization, they were then placed individually into already prepared glass jars (height: 122.5 mm; Diameter: 73.5 mm; capacity: 352 ml) containing a laboratory diet for FCM (Moore *et al.* 2014).

Individual jars of FCM diet were prepared by mixing 45g of the bulk diet with the final dosage of the relative additive in a honey jar (350 ml capacity), and then adding 45ml of $dH_20$ which was allowed to saturate through to the bottom of the diet. The jars (Figure 2.1) were subsequently closed with a cotton wool stopper and placed into an autoclave for 20 minutes at 121°C. The jars were then left to cool and inoculated with the eggs. Once the jars were prepared, they were all placed into a controlled environment room (CE room) (Figure 2.2) at 27°C and the FCM were left to feed and develop until they pupated in the cotton wool, after which the cotton wool stoppers were removed and then the pupae removed from the cotton wool stoppers. These pupae were then used in numerous other experiments. The developmental rate of the larvae was recorded for each jar as the amount of time taken in days for a single larva to pupate and then to eclose.

The additives chosen were cholesterol (200 mg/ jar), proline (3150 mg/ jar), sorbitol (2700 mg/ jar), mannitol (3150 mg/ jar), glycerol (9000 mg/ jar), honey (9000 mg/ jar), trehalose (3150 mg/ jar) and glucose (2700 mg/ jar). The laboratory FCM diet was augmented with the chosen additives, three times for each (three jars) and with three control jars, this resulted in a total of 27 jars. The final dosage for each additive was taken from the

dosage response test. In Some cases where dosage had no significant difference on the developmental rate of the FCM, the highest dosage was chosen. This was purely to allow for a greater amount of the additive to be spread throughout the basic larval diet ensuring optimal coverage.


**Figure 2.1:** Honey jar prepared with augmented diet inoculated with eggs and closed with a cotton wool stopper.

**Figure 2.2**: Prepared jars in a CE room at Rhodes University.

### *2.2.3 Pupal weight and fecundity*

Once all the pupae were removed from the cotton wool, they were separated into females and males. This was achieved by counting the number of segments on the pupa starting from below the wing sheath to the bottom end of the pupa. In males, the total number of segments is 4, whereas with the females it is 3 (Figure 2.3) (C. Chambers, pers. comm. 2015). Twenty females and 20 males from each treatment were weighed with a micro-scale ($\geq$ 0.000 g) in a balance room and then subsequently paired. The pairs were formed by putting a male and female from the same treatment together, this making 10 pairs of FCM from each treatment, and 90 pairs all together.

Each pair was then placed into a fertility chamber lined with wax paper and then placed in a CE room set at 27°C. The pairs were left to eclose, mate and the females, lay eggs. They were then left in the fertility chambers for a period of one week as the incubation time for eggs in the summer is 6- 8 days (Newton 1998). During that week the moths were watered once a day to ensure their survival. At the end of the week, the wax paper was removed and the total number of eggs counted. The eggs on the wax paper were left for a further 4 days to allow the newly laid eggs a chance to hatch. After 4 days, the number of eggs that did not hatch was counted under a dissecting microscope.

The fertility chambers (Figure 2.4) were created using 20 ml plastic vials. A hole was made in the centre of the lid of the vial with a heated piece of metal. The hole was then closed with a small cotton wool stopper which also acted as a water source as drops of water were placed onto the cotton wool using 3 ml pipettes.


**Figure 2.3:** Male (left) and female (right) FCM pupae (www.hantsmoths.org.uk).

**Figure 2.4**: Fertility chamber lined with wax paper

### *2.2.4 Flight ability*

From the remaining pupae that had been sexed, 60 males from each treatment were divided into 3 groups of 20 which were used in flight tests at three different temperatures: 15°C, 20°C and 27°C. The groups of 20 pupae were contained in 20 ml plastic vials which had a layer of cotton wool placed at the bottom to protect and cushion the pupae until they

were close to their eclosion time (which was noted by the colouration of the pupae; pupae that are close to eclosion are dark brown in colour). Once the majority of the pupae in each vial were close to eclosion, the vial was opened and placed into a flight chamber along with two cotton wool balls saturated with water. Once the pupae were placed into the flight chambers, the chambers were sealed with airtight lids and the moths were left for a two week period which allowed them time to eclose and fly out of the flight chamber. After the given time, the number of moths that remained in the flight chamber was recorded and the percentage of males flown for each treatment under each temperature was documented.

The flight chambers (Figure 2.5) were designed according to the standard chambers used at River Bioscience, Addo, South Africa (C. Chambers, pers. comm. 2015). A 10 mm hole was drilled near the top of the side of an Addis® black storage box (height: 25 cm; length: 50 cm; breadth: 36 cm; capacity: 45 L). Once the hole was made, a black PVC pipe (12 cm long by 1 cm wide) was put through the hole and secured with clear silicone glue. The black pipe was placed in such a way that the FCM were not able to crawl up the side and out the pipe (Figure 2.6).

**Figure 2.5:** Flight chamber with arrow showing exit hole on the exterior.

**Figure 2.6:** Position of black PVC pipe (highlighted by the arrow) on the interior of the flight chamber ensuring moths do not leave the chamber by crawling.

### *2.2.5 Multiple generation trials*

Once enough pupae were reserved for the various trials mentioned above, all of the remaining pupae (males and females) were placed into oviposition chambers (Figure 2.6). The oviposition chambers were created using plastic cups lined with wax paper. The top of the cups were closed off with a mesh held by an elastic band. A ball of cotton wool, saturated with water was placed on top of the mesh and re-wet every second day. The pupae were allowed to eclose, mate and the females lay eggs on the wax paper. After a week, the wax paper with the eggs was removed and cut into smaller pieces with approximately the same number of eggs on each piece. These were then placed onto three already prepared jars of diet for each chemical. The process of preparing the diets and sterilizing the egg sheets before inoculation was done according to the same procedure as mentioned above (Section 2.2.1). This, along with the various tests, was conducted for five generations including the parent generation.


**Figure 2.7:** Lay chambers for FCM (Note: cotton wool ball was not in place for picture)

### *2.2.6 Statistical analysis*

All data were tested for normality and it was found that all were not normally distributed. However, an ANOVA is rather robust and not highly sensitive to moderate deviations from normality. Simulation studies, using a variety of non-normal distributions, have shown that the false positive rate is not greatly affected by this violation of the assumption (Glass *et al.* 1972, Harwell *et al.* 1992, Lix *et al.* 1996). Because of the robust nature of an ANOVA, the developmental rate on various dosages (dosage response test), mean developmental rate, weight (females and males) and fecundity per female (number of eggs laid and not hatched) of FCM reared on various diets were each tested using an ANOVA and the post- hoc Tukey test. A Pearson product-moment correlation coefficient was used to assess the relationship between the number of eggs laid per female and female pupal weight. All of the above statistics were conducted using Statistica version 13 (StatSoft, Inc. 2015). For flight ability, a log linear chi-squared test was performed in R version 3.2.2 (R Core Team 2015).

## 2.3 RESULTS

### *3.3.1 Dosage response tests*

Pupation time of FCM reared on the control diet had a mean of 10.33 ± 0.34 days taken from inoculation to pupation and 14.33 ± 0.68 days taken from pupation to eclosion at a controlled temperature of 27 °C. The only significant differences seen between different dosages of the same chemical are for ethyl glycol and glycerol. For ethyl glycol the differences were between the low and medium to high dosages where the medium and high dosages resulted in the death of the colony, whereas the low dosage resulted in a mean developmental time of 13.33 ± 1.2 days to pupation and 17.67 ± 0.68 days to eclosion. For glycerol the significant difference between the dosages was seen in the time taken to eclosion for FCM reared on low and high dosages. The FCM reared on a low dosage of glycerol had a mean developmental time of 12.67 ± 0.34 days taken from inoculation to eclosion whereas FCM reared on the high dosage had a mean developmental time of 15.67 ± 1.22 days. All three dosages of cholesterol significantly decreased the developmental time (increasing the developmental rate) to pupation when compared to the control (low $P = 0.165$; medium $P = 0.0166$; high $P = 0.0166$) with an average time to pupation of 8 ± 0.0 days across all three dosages. The high dosage of ethyl glycol significantly increase developmental time (decreasing developmental rate) ($P = 0.0005$) with an average of 13.33 ± 1.20 days to pupation (Figure 2.8 A). When compared with the control cholesterol significantly decreased the developmental time to eclosion of FCM for all three dosages (low $P = 0.0004$; medium $P = 0.0052$; high $P = 0.0012$) with a mean of 10.6 ± 0.42 days taken to eclosion across all three dosages. The high dosage of ethyl glycol significantly increase developmental time ($P = 0.0052$) with an average of 17.67 ± 0.68 days to eclosion. Trehalose also significantly decreased developmental time to eclosion for all three dosages (low $P = 0.0004$; medium $P = 0.0004$; high $P = 0.0052$) with a mean of 10.5 ± 0.42 days taken to eclosion across all three dosages. The medium dosage of sorbitol significantly decreased developmental time ($P = 0.021$) with a mean of 11.3 ± 0.34 days taken to eclosion (Figure 2.8 B).

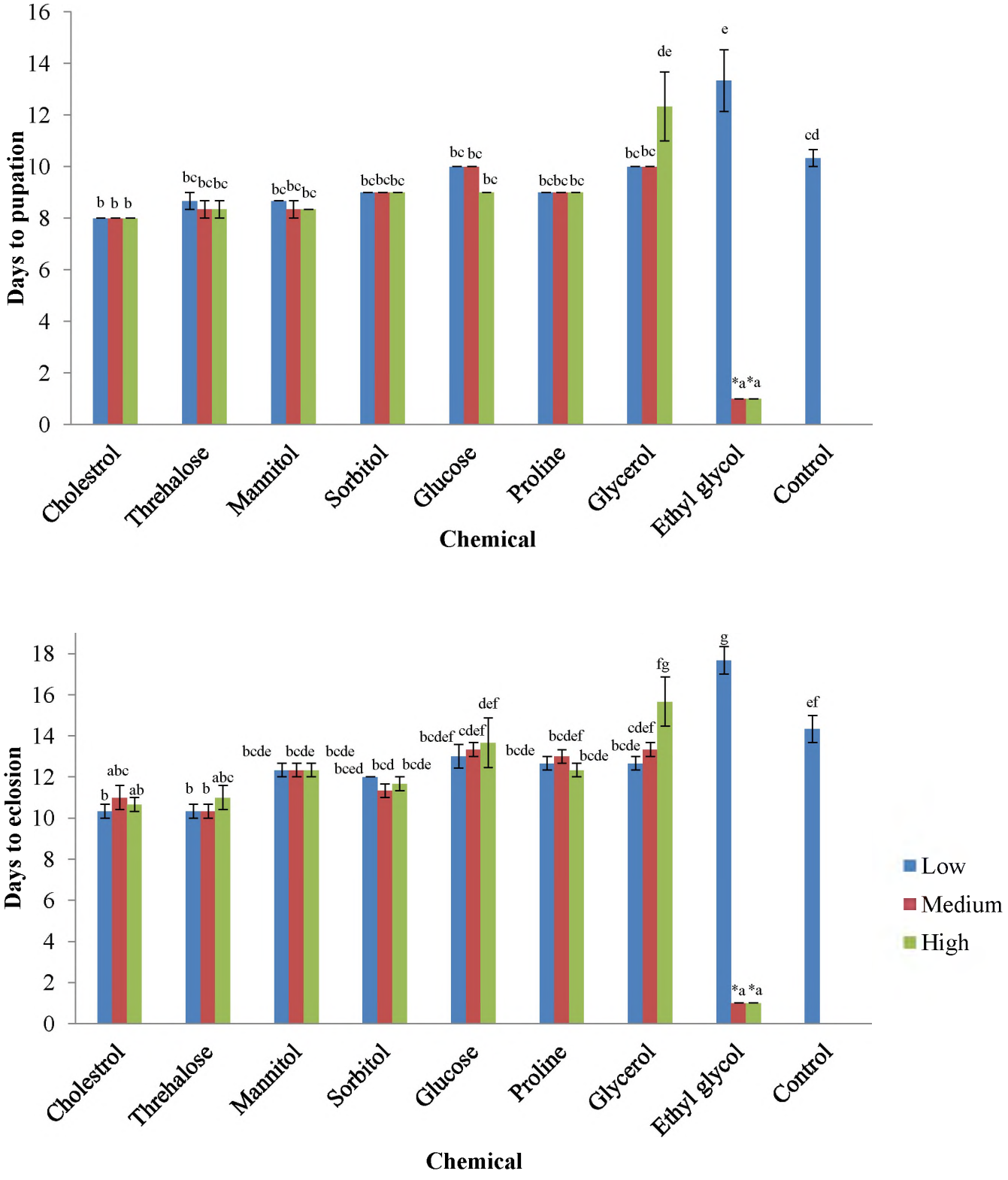



**Figure 2.8**: Dosage response test depicting developmental rate of FCM reared on various augmented diets at three different dosages, recorded as the time taken from inoculation to A) pupation and then B) eclosion at a constant temperature of 27 °C. Bars with different letters denote significant differences (Tukey HSD test $P < 0.05$).

*dead cultures

### *2.3.2 Development on augmented diets*

Parent generation (P1) FCM reared on the control diet had a mean developmental time of 25.3 ± 0.3 days taken from inoculation to eclosion at a controlled temperature of 27 °C, and there was no significant difference between the P1 and subsequent generations (F1-F4) of FCM reared on the control diet. For the P1 generation, honey and glycerol significantly increased the developmental time (decreasing the developmental rate) of FCM ($P < 0.0001$ for both) with a mean of 30.3 ± 0.33 days taken to eclosion for honey and 29.7 ± 0.33 days taken to eclosion for glycerol. Cholesterol significantly decreased the developmental time (increasing the developmental rate) of FCM ($P < 0.0001$) with a mean of 22 ± 0.58 days taken to eclosion (Figure 2.9 A). The same trend can be seen in the F1 generation, however, results show that proline ($P = 0.03$) and mannitol ($P < 0.0001$) also significantly decreased developmental time with a mean of 22.33 ± 0.89 days taken to eclosion for proline and 19.33 ± 0.33 days taken to eclosion for mannitol (Figure 2.9 B). In the F3 generation the same trend was observed, however, proline had no significant difference on the developmental time of FCM, whereas glucose showed that it significantly increased developmental time ($P = 0.001$) with a mean of 26.67 ± 0.33 days taken to eclosion (Figure 2.9 C). For generations F3 and F4 a similar trend was observed for both generations whereby mannitol and cholesterol significantly decreased developmental time and glycerol and honey significantly increased developmental time (Figure 2.9 D and E). See Appendix 2 for complete ANOVA univariate results.

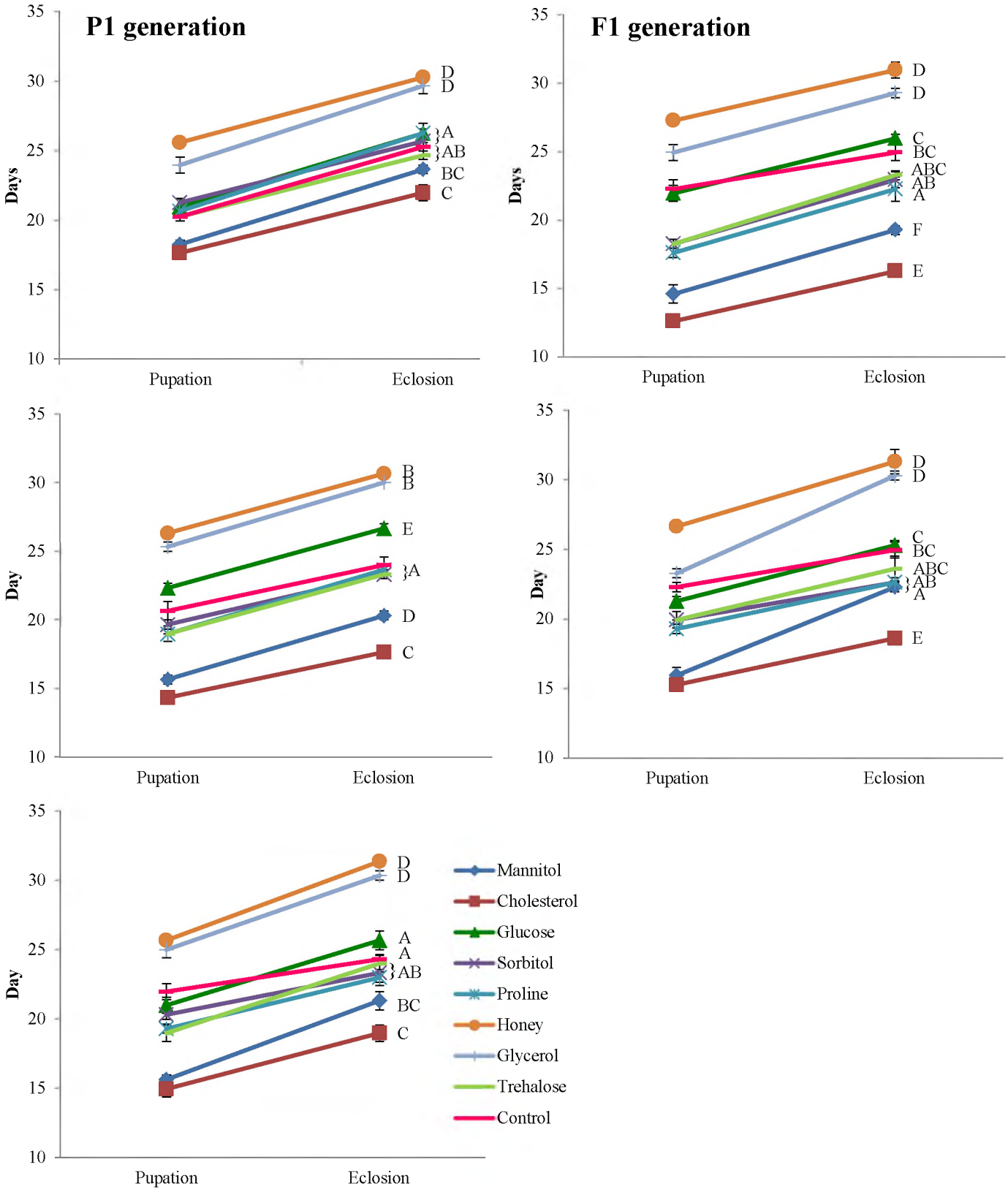



**Figure 2.9:** Developmental rate of multiple generations of FCM reared on various augmented diets, recorded as the time taken from inoculation to pupation and then eclosion at a constant temperature of 27 °C. Points with different letters denote significant differences (Tukey HSD test $P < 0.05$).

### *2.3.3 Pupal weight*

Female P1 generation FCM reared on the control diet had a mean pupal mass of 24.3 ± 1 mg at a controlled temperature of 27 °C. The mean pupal mass for male P1 generation FCM was 16.7 ± 0.87 mg with no significant difference found between the P1 and subsequent generations for both female and male weight. For all five generations, only moths reared on trehalose augmented diets showed a significant increase in female pupae weight (P1, P = 0.03; F1 generation, P = 0.048; F2 generation, P = 0.026; F3 generation, P = 0.012; F4 generation, P = 0.017) with a mean weight of 29.61 ± 1.03 mg for the P1 generation, 28.86 ± 0.90 for the F1 generation, 29.03 ± 0.90 for the F2 generation, 29.03 ± 1.01 for the F3 generation and 29.43 ± 1.11 for the F4 generation (Figure 2.10). Pupal weight for male FCM was significantly increased when larvae were reared on trehalose augmented diets. This trend was recorded in all five generations for moths reared on trehalose augmented diets (P1 generation, P = 0.005; F1 generation, P = 0.008; F2 generation, P = 0.014; F3 generation, P = 0.001; F4 generation, P = 0.002) with a mean weight of 21.5 ± 0.99 mg for the P1 generation, 21.7 ± 1.11 mg for the F1 generation, 21.2 ± 0.90 mg for the F2 generation, 21.52 ± 0.80 mg for the F3 generation and 21.74 ± 0.89 mg for the F4 generation. For the P1, F3 and F4 generations, mannitol also significantly increased the weight of male FCM pupae (P1 generation, P = 0.03; F3 generation, P = 0.009; F4 generation, P = 0.028) with a mean weight of 20.8 ± 0.83 mg for the P1 generation, 20.76 ± 0.55 mg for the F3 generation and 20.73 ± 0.72 mg for the F4 generation. Significant differences were found between most of the males and females reared on the same augmented diet. However, there is no significant difference for males and females reared on honey augmented diets in the P1 generation, proline augmented diets for the F1 generation, honey, proline and cholesterol for the F2 generation, proline and glycerol in the F3 generation and honey, proline and glycerol in the F4 generation (Figure 2.10). See appendix 3 for complete ANOVA univariate results regarding female weight and appendix 4 regarding male weight.

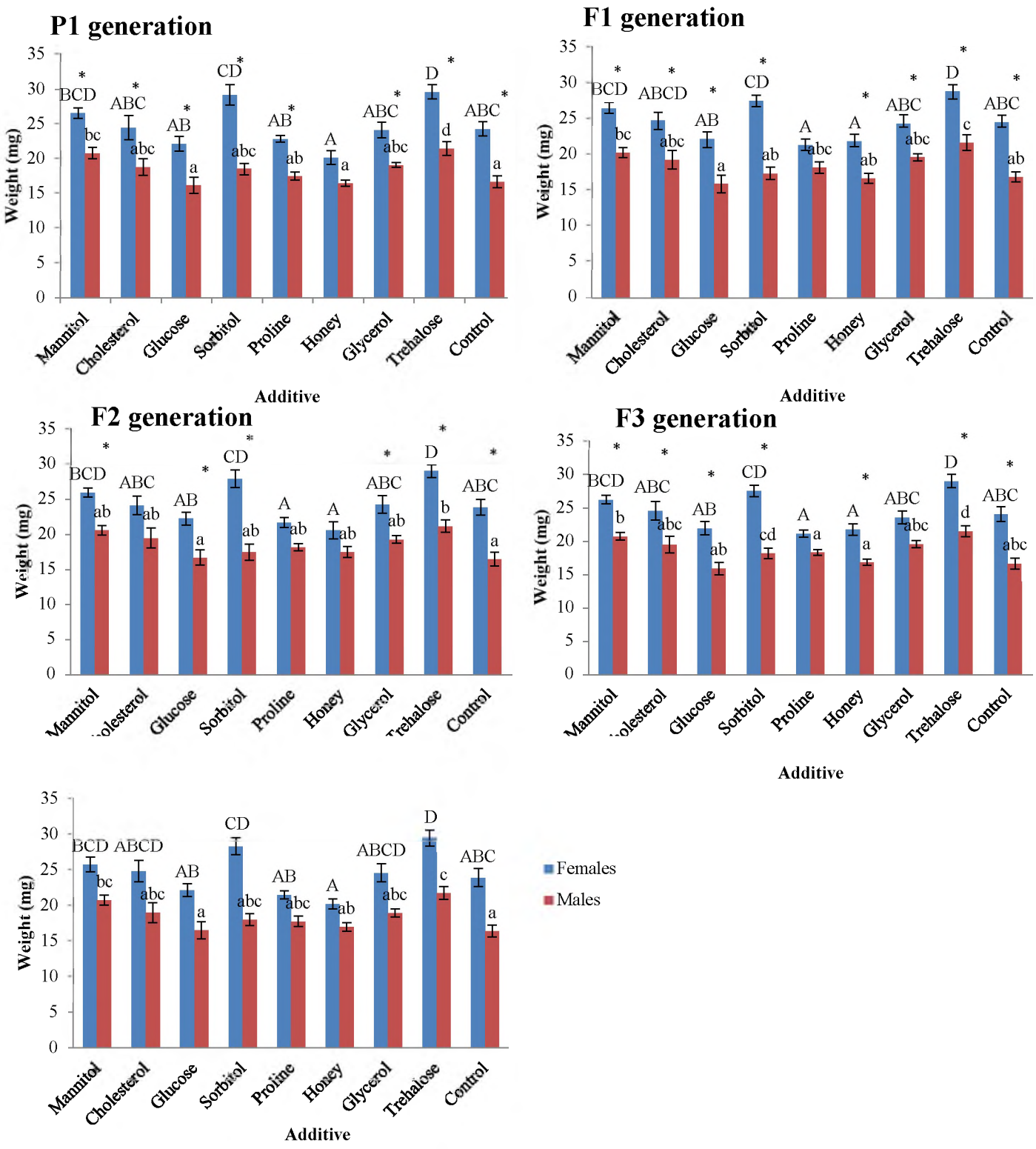



**Figure 2.10:** Weight of male and female FCM pupae reared on various augmented diets, at a constant temperature of 27 °C. Bars with different upper case letters denote significant differences between females whereas bars with different lower case letters denote significant differences between males (Tukey HSD test $P < 0.05$).

* Denotes significant difference between males and females of the same treatment (additive).

### *2.3.4 Fecundity*

P1 generation FCM reared on the control diet had a mean number of eggs laid per female of 218.5 ± 12.76 at a controlled temperature of 27 °C. The mean number of eggs not hatched per female for the P1 generation was 29.7 ± 2.88 eggs with no significant difference found between the P1 and subsequent generations for the number of eggs laid as well as not hatched. All five generations of female moths reared on trehalose augmented diets laid significantly more eggs ($P < 0.0001$ for all five generations) with a mean number of eggs laid per female of 367.8 ± 15.8 for the P1 generation, 371. 5 ± 13.4 eggs for the F1 generation, 375.2 ± 14.2 eggs for the F2 generation, 374.5 ± 15.9 eggs for the F3 generation and 385.1 ± 17 eggs for the F4 generation. Cholesterol significantly increased the number of eggs laid per female for the first four generations (P1 generation, $P = 0.003$; F1 generation, $P = 0.002$; F2 generation, $P = 0.005$; F3 generation, $P = 0.01$) with a mean of 303 ± 11.7 eggs for the P1 generation, 300.1 ± 9.5 eggs for the F1 generation, 304.6 ± 11.5 eggs for the F2 generation and 291.3 ± 3.1 eggs for the F3 generation. Sorbitol significantly increased the number of eggs laid per female for the P1 and F3 generations (P1 generation, $P = 0.031$; F3 generation, $P = 0.009$) with a mean of 287.5 ± 19.1 eggs for the P1 generation and303.5 ± 11.3 eggs for the F3 generation. Female FCM reared on proline augmented diets laid significantly more eggs per female for generations F1, F2 and F3 (F1 generation, $P = 0.04$; F2 generation, = P 0,031; F3 generation, $P = 0.05$) with a mean number of eggs per female of 282.6 ± 19.3 eggs for the F1 generation, 292.7 ±19.2 eggs for the F2 generation, 291.3 ± 3.1 eggs for the F3 generation. There was no significant increase or decrease in the number of eggs that did not hatch per female for all five generations. See appendix 5 for complete ANOVA univariate results regarding the number of eggs laid per female, and appendix 6 regarding the number of eggs not hatched.

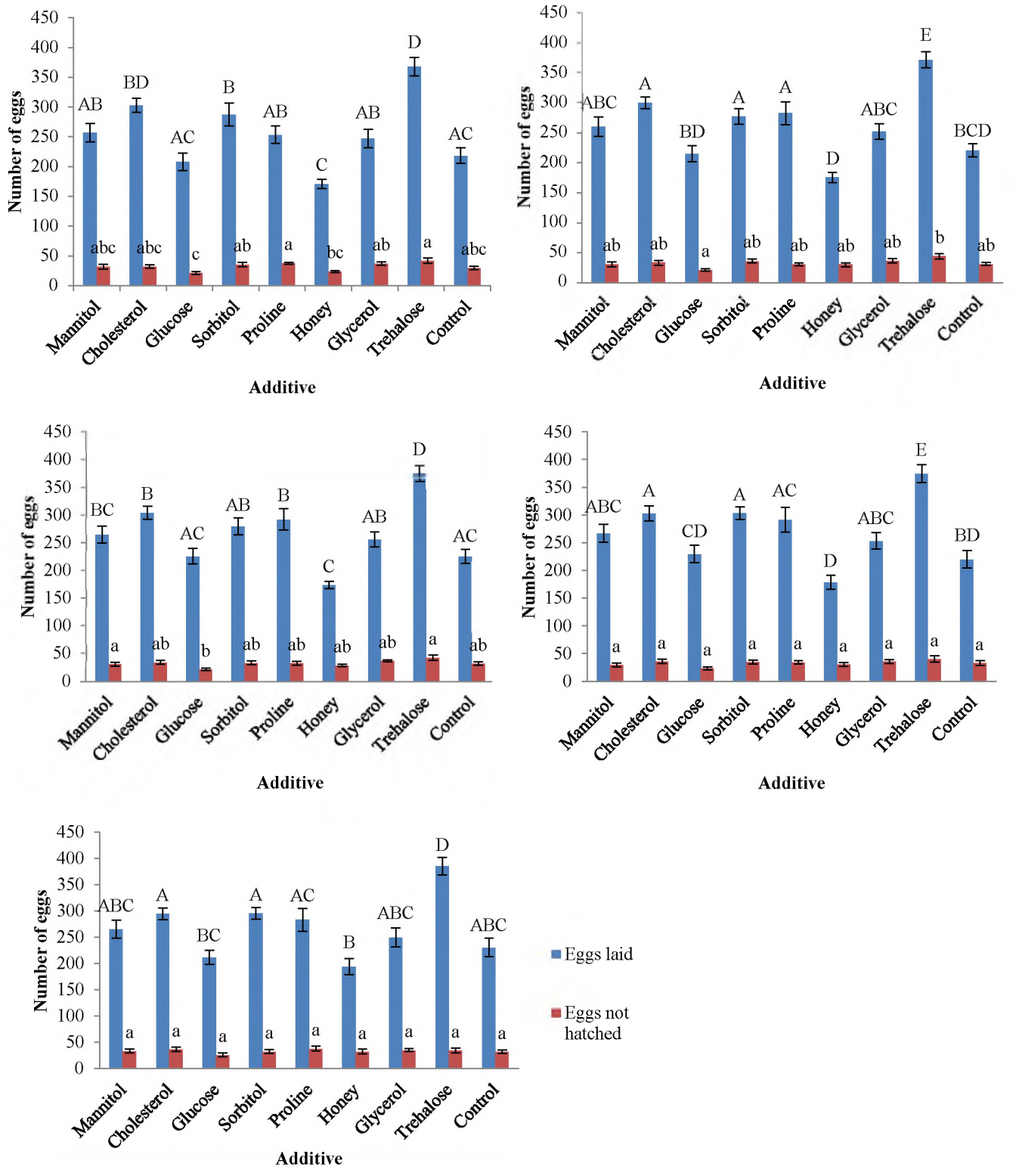



**Figure 2.11:** Number of eggs laid as well as not hatched per female FCM reared on various augmented diets, recorded at a constant temperature of 27 °C. Bars with different upper case letters denote significant differences between the number of eggs laid per female whereas bars with different lower case letters denote significant differences between the amount of eggs that did not hatch (Tukey HSD test $P < 0.05$).

### *2.3.5 Relationship between pupal weight and fecundity*

There was a positive correlation between the amount of eggs laid per female and female pupal weight for all five generations. There was a strong correlation seen in the P1, F3 and F4 generations (P1 generation, $r = 0.791$, $n = 90$, $P < 0.0001$; F3 generation, $r = 0.635$, $n = 90$, $P < 0.0001$; F4 generation, $r = 0.673$, $n = 90$, $P < 0.0001$), and a weaker correlation in the F1 and F2 generations (F1 generation, $r = 0.550$, $n = 90$, $P < 0.0001$; F2 generation, $r = 0.534$, $n = 90$, $P < 0.0001$).

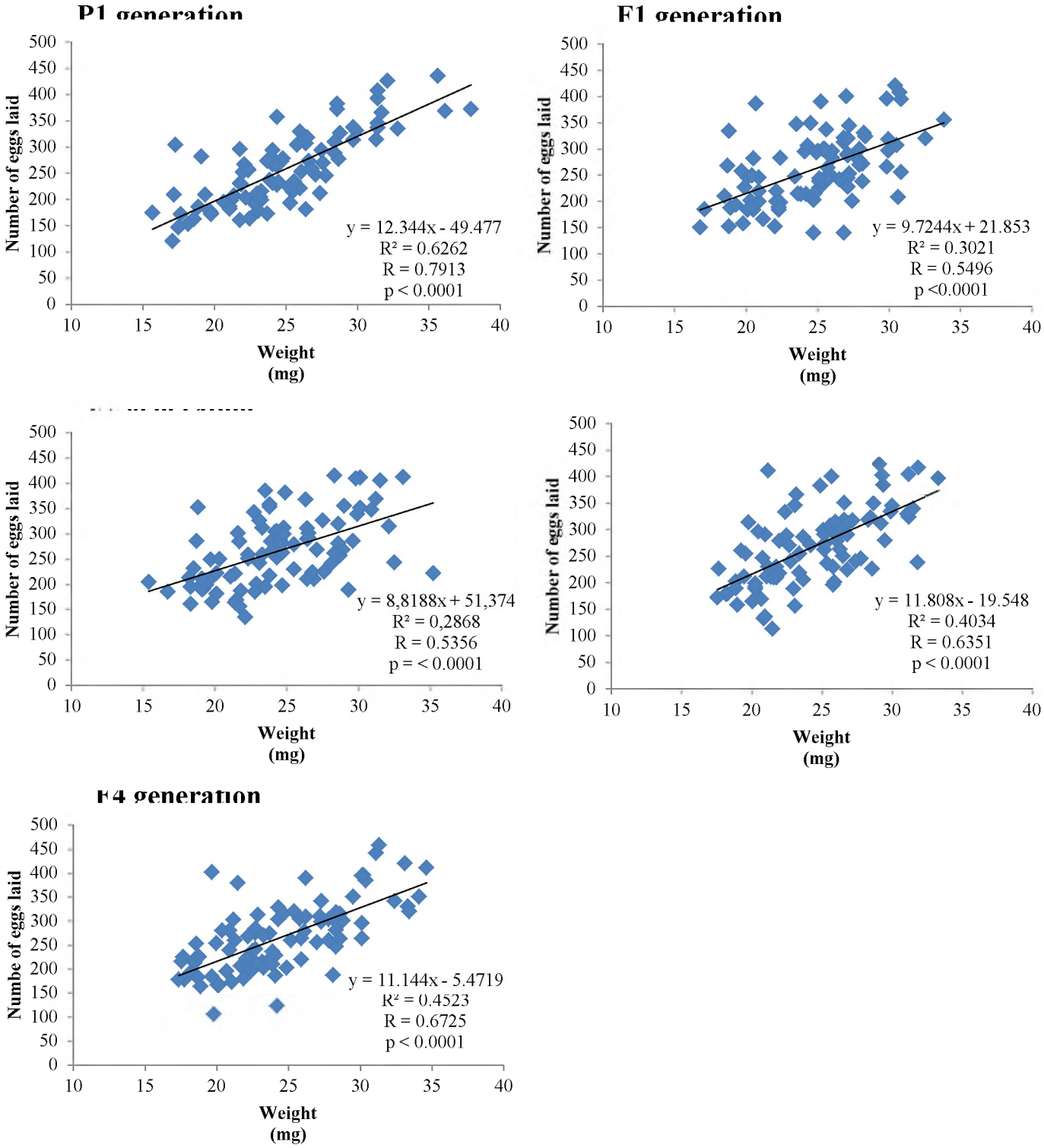
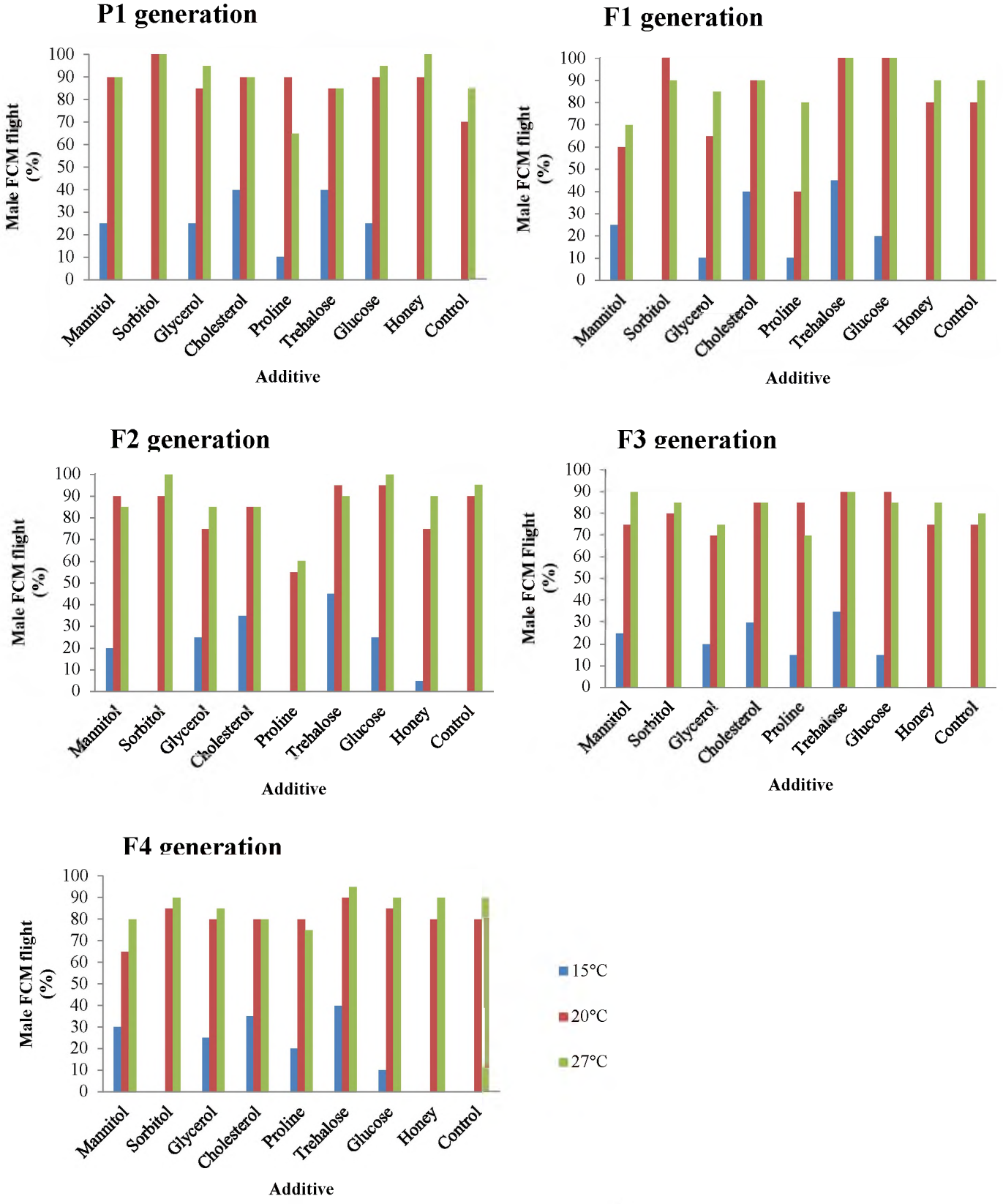



**Figure 2.12**: Scatter plots showing the amount of eggs laid per female versus female pupal weight with correlation coefficients for multiple generations reared on various augmented diets.

### *2.3.6 Flight ability*

Males of the P1 generation FCM reared on the control diet had a percentage flight of 0 % at 15 °C, 70 % at 20 °C, and 85 % at 27 °C. There was some variation between the P1 and subsequent generations of FCM reared on the control diet with flight at 20 °C reaching 90 % and flight at 27 °C reaching 95 %; however, the 0 % flight at 15 °C was consistently found in all subsequent generations. Flight was increased at 15 °C for moths reared on diets augmented with mannitol, glycerol, glucose (with 25 % flight), cholesterol, trehalose (with 40 % flight) and proline (with 10 % flight). Sorbitol and honey had no effect on flight at 15 °C for all five generations except for the F2 generation where moths reared on diets augmented with honey had a 5 % increase in flight. Moths reared on diets augmented with trehalose and cholesterol had the greatest increase in flight at all five generations, with trehalose increasing flight by up to 45 % in the F1 and F2 generations, and cholesterol increasing flight by up to 40 % in the P1 and F1 generations (Figure 2.13). Even though differences are evident on the graphs, chi square tests revealed no significant differences for any of the five generations (Table 2.1).




erent temperatures (15 °C, 20 °C and 27 °C) after larvae were reared on various augmented diets.

**Table 2.1:** Chi square test results for the number of male FCM that flew at different temperatures

| | | $X^2$ | DF | P value |
|---|---|---|---|---|
| P1 generation | Likelihood ratio | 17.13818 | 15 | 0.3106712 |
| | Pearson | 13.31040 | 15 | 0.5783362 |
| F1 generation | Likelihood ratio | 22.00452 | 15 | 0.1076854 |
| | Pearson | 20.38383 | 15 | 0.1576978 |
| F2 generation | Likelihood ratio | 15.21941 | 15 | 0.4357310 |
| | Pearson | 10.79622 | 15 | 0.7669019 |
| F3 generation | Likelihood ratio | 16.87182 | 15 | 0.3265845 |
| | Pearson | 12.84563 | 15 | 0.6142200 |
| F4 generation | Likelihood ratio | 23.19067 | 15 | 0.08017464 |
| | Pearson | 18.14796 | 15 | 0.25496691 |

## 2.4 DISCUSSION

The effects that diet additives had on the developmental rate, pupal weight, female fecundity, and male flight ability of FCM was tested in a controlled environment (Table 2.2). To date, the ability to artificially improve the cold tolerance of an insect that is freeze-intolerant has only been achieved in a handful of studies (Hendrix & Salvucci 1998; Shreve et al. 2007; Kostal et al. 2012). No studies have previously attempted to improve the cold tolerance in FCM albeit critical for management by means of SIT. In addition to the necessity to improve the cold tolerance of sterile FCM, it is vital to consider how an increase in these cryoprotectants may affect other aspects of the insect's physiology. This is important because if these cryoprotectants are to be used in mass rearing facilities as part of an SIT programme, the quality of the cultures should not be compromised.

**Table 2.2**: Summary of changes in developmental rate, pupal mass, egg production and flight ability as means for multiple (five) generations of FCM reared on various augmented diets. Increases (+ve), decreases (-ve) or no difference (N) are indicated relative to the control. All differences are indicated as statistically significant with the exception of flight ability which is an observed difference due to the nature of the trial and analysis.

| **Diet supplement** | **Life History effect** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **Development rate** | **Pupa mass** | | **Egg production** | | **Flight ability** | | |
| | | **Female** | **Male** | **Laid** | **Not hatched** | **15 °C** | **20 °C** | **27 °C** |
| Mannitol | +ve | N | +ve | N | N | +ve | N | N |
| Sorbitol | N | N | N | N | N | -ve | N | N |
| Glycerol | -ve | N | N | N | N | +ve | N | N |
| Cholesterol | +ve | N | N | +ve | N | +ve | N | N |
| Proline | +ve | N | N | +ve | N | +ve | N | N |
| Trehalose | N | +ve | +ve | +ve | N | +ve | N | N |
| Glucose | N | N | N | N | N | +ve | N | N |
| Honey | -ve | N | N | N | N | -ve | N | N |

In this study it was shown that the augmentation of the basic laboratory diet for FCM played an important role in altering the developmental rate of the FCM cultures. FCM reared on diets augmented with trehalose and cholesterol significantly increased developmental rate for all five generations. FCM reared on glycerol and honey augmented diets had a significantly decreased developmental rate for all five generations. As a decrease in developmental rate is not an ideal quality for mass rearing, honey and glycerol were rejected as potential additives to diets for FCM at mass rearing facilities. The increase in developmental rate of FCM reared on cholesterol augmented diets may be as a result of the increase in the amount of cholesterol available for lipid droplet formation, as the level of nutrient reserves accumulated in the fat body modulates several important aspects of the insect's life, such as the rate of insect growth (Mirth & Riddiford 2007). Fat bodies coordinate insect growth, metamorphosis or reproduction by storing or releasing components central to these events (Arrese & Soulages 2010) and the insect fat body is a known store of energy reserves, made up of many cells known as adipocytes. Insect adipocytes are able to

store a great amount of lipid reserves by means of cytoplasmic lipid droplets (Arrese & Soulages 2010). These individual lipid droplets consist of a nucleus of neutral lipids (triglyceride and cholesterol esters) enclosed by a monolayer of phospholipid and cholesterol, into which specific proteins are implanted or peripherally allied (Brasaemle 2007; Bickel *et al.* 2009). McKennis (1947) states that cholesterol serves as a growth factor for certain lower animals, particularly insects and that dietary cholesterol has been shown to be a potent growth promoting substance. The physiological role of trehalose during insect development is rather unclear with little literature available. Matsuda *et al.* (2015) performed a study on larvae of *Drosophila* spp. where they examined larvae with a mutant trehalose-synthesizing enzyme (*Tps1)* which prevented the larvae from producing trehalose. It was found that the Tps1 mutant larvae failed to grow on a low-sugar diet and exhibited severe growth defects on a low-protein diet. This study demonstrated the critical role of trehalose during development in *Drosophila* spp. and may be the reason why an increase in trehalose may result in an increase in developmental rate.

The trehalose augmented diet was the only diet to significantly increase the weight of male and female FCM pupae for all five generations. Moths reared on trehalose augmented diets also produced significantly more eggs for all five generations. The overall number of eggs laid per female correlated positively with the pupal weight. This has been noted in many other studies which state that the fecundity of females increases with female body size (Honěk 1993; Botto-Mahan & Medel 2007; Berger *et al.* 2008). A). With regard to the direct effect of trehalose on the size of the FCM, a study performed by Foster (2009), showed that haemolymph trehalose concentration of *Heliothis virescens* moths may influence glycolysis in gland cells and thus, the levels of cytosolic citrate and acetyl-CoA used in pheromone biosynthesis. The study also showed that the increase in pheromone levels in mated females facilitated further mating and increased fecundity. Another study performed by Kaspi & Yuval (2000) found that protein and sugar fed males of the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) who had access to water and apples after 4 days of feeding on protein and sugar, or sugar alone, were significantly more likely to copulate than their starved competitors who had access to water alone, emphasising the necessity for sugar in a diet.

In this study, there was no significant difference in the number of eggs that did not hatch which is an important finding if trehalose is to be used as part of the commercial diet as it is important to ensure that the number of non-viable eggs is not increased. Results also showed a considerable increase in male FCM flight at 15 °C, under laboratory conditions,

when moths were reared on trehalose and cholesterol augmented diets. Trehalose is a major substrate used for insect flight by both long-term and short-term flyers (Van der Horst *et al.* 1980; Elliott *et al.* 1984). Long-term flyers such as locusts (Van der Horst *et al.* 1980) and mosquitoes (Kaufmann & Briegel 2004; Kaufmann & Brown 2008), begin flying by using trehalose and after some time switch to lipids. FCM falls into the short-term flyers category which is known to use mostly trehalose as their fuel for flight (Arrese & Soulages 2010). As previously stated, trehalose is the main blood sugar circulating in the haemolymph of insects (Wyatt & Kalf, 1957; Thompson 2003) and it serves as an immediate source of glucose for tissue metabolism (Munyiri & Ishikawa 2005). The conversion of carbohydrates such as trehalose to lipid in the insect fat body is well documented (Bailey 1975; Inagaki & Yamashita 1986; Arrese & Soulages 2010).Trehalose is important for insect activity as it acts as an energy store (the traditional role ascribed for trehalose), a cryoprotectant, reducing the supercooling point of some freeze-avoiding insects which enables the them to survive under cooler conditions, and a protein stabilizer during osmotic and thermal stress (Thompson 2003). The overwintering survival of CM is positively associated with the accumulation of trehalose (Khani *et al.* 2007; Yin *et al.* 2006; Boardman *et al.* 2012). If trehalose had to have a similar effect on the overwinter survival rate of FCM, it would allow the FCM more opportunity to fly by increasing the number of nights endured.

Under laboratory conditions, trehalose and cholesterol augmented diets appear to be the most promising diets that may improve the cold tolerance and/or activity of FCM at lower temperatures. The physiological parameters tested show the ability of FCM to develop on these diets without any hindrance to developmental rate, weight and the number of eggs laid per female. This is of importance for the mass rearing aspect of the SIT programme as it is essential to confirm that adding a new chemical to the commercial laboratory diet of FCM won't have negative effects on any aspect of the mass rearing component, but this laboratory trial needs to be validated in the field (Chapter 3).

# III
# FIELD TRIALS: STERILE MALE FCM FLIGHT ABILITY

## 3.1 INTRODUCTION

An essential requirement for the success of an SIT programme is ensuring that sterile males are fit enough to be competitive with wild males and ultimately, be successful at finding wild females (Hendrichs *et al.* 2002; Enkerlin 2005). Calkins and Parker (2005) stated that the cornerstone of SIT is the ability of released sterile males to compete for a female and this should be assessed and quantified to ensure that the goal of SIT is maintained. In the past, effort has been focussed on numbers, while insect quality has been generally overlooked or marginalized and for many years, SIT was recognized as a "numbers game" (Calkins & Parker 2005). This meant that if a programme began to fail, the remedy was to increase the rate of sterile insect release, and only when a programme failed completely was the quality of the sterile insects doubted (Calkins & Parker 2005). Mass rearing and release methods existing for the current FCM SIT programme in South Africa attempt to maintain the insect's competitiveness whilst allowing for optimal production at minimal costs and in minimal time. However, there are still many aspects to the SIT programme procedures that have a negative effect on insect quality and consequently, competitiveness.

When exposed to artificial rearing conditions for a prolonged period of time, key behavioural and physiological traits undergo change. These were characterized by Ochieng'-Odero (1994) as acclimatization, selection, and domestication with examples of such traits being fecundity, pre-oviposition period, courtship rituals, flight ability, oviposition, development rate, pheromone production, response to pheromone, eye morphology, visual sensitivity, metabolic rate, and ability to resist stress (Mangan 1992). These changes in the various traits are a result of the very different conditions that the insects are exposed to in the lab versus the natural conditions they have adapted to in the wild. However, the competitiveness of mass-reared strains may be retained by practicing an SIT programme that houses insect colonies under "relaxed" conditions with the regular replacement of mass-

reared strains therefore minimizing the selection of undesirable traits, and maximizing the genetic variation (Leppla *et al.* 1983, McInnis *et al.* 1985, McInnis *et al.* 2002).

Radiation can have a significant effect on the quality of the mass-reared male insects; as the radiation dose increases, sterility increases, however quality and competitiveness decrease (Calkins & Parker 2005). This is evident as using radiation to induce sterility, significantly reduced the amount of recaptured mass-reared codling moth in field experiments, most likely by reducing their general flight and dispersal ability (Judd & Gardiner 2006). Complete sterility might not always be required and is essentially unfavourable for some species, as certain insects may be, to an extent, regarded as radio-resistant (Lachance & Graham 1984). Therefore, by using high amounts of radiation it may critically compromise their competitiveness in the field which is a dilemma for insects such as lepidopterans, as they require high doses of radiation to become fully sterile. It is therefore imperative to sterilize the insects using the lowest amount of radiation possible to improve their ability to disperse, fecundity and sperm competitiveness (North 1975; Carpenter *et al.* 1997; Bloem *et al.* 2001). A method to induce sterility at lower doses for relatively radio-resistant insects may be achieved by employing the genetic phenomenon known as inherited sterility. This is whereby the parent generation is exposed to sub-sterilizing doses and are then either inbred or outcrossed with fertile counterparts, subsequently resulting in their offspring (F1 generation) showing a higher level of sterility than their parents (Carpenter *et al.* 2005).

The aim of this chapter was to take the results obtained in chapter 2 regarding diet augmentation and flight ability, and apply this to the field. This is in order to determine if diet additives could improve FCM male flight at lower temperatures in one of their natural environments, a citrus orchard.

## 3.2 MATERIALS AND METHODS

### *3.2.1 Rearing and sterilization of FCM*

All FCM used in the field trials were reared, irradiated and delivered from XSIT (Pty) Ltd, Citrusdal, Western Cape. Glass jars containing trehalose (17500 mg/ 250 g diet), cholesterol (1111 mg/ 250 g diet) or no additive (control), at the same dose as the laboratory trials, were added to 250 g of commercial FCM diet and then saturated with $dH_2O$ allowing the diet to comprise of 48-50 % moisture. Control moths were fed a diet containing Calco Oil

Red® (Royce International, Sarasota, Florida, U.S.A.), which stains the guts pink whereas the moths reared on the augmented diet had no gut stain, allowing for easy differentiation between the two in the field. Subsequently, the jars were placed in an autoclave for 15 minutes at 120 °C and once cooled they were inoculated with egg sheets under a laminar flow cabinet. Before inoculation, the egg sheets containing approximately 1200 eggs which were washed in a 25% formaldehyde (37% stock) solution. The jars were then incubated in CE rooms at 25 °C on a twelve hour day/night cycle. After 13 days the glass jars were opened and the larvae were allowed to exit the jar where they were pupated in pupation boards. After seven days the pupae were placed in a laminar flow cabinet at 25 °C. As soon as the FCM emerged, they were captured and packed into petri dishes. They were then cooled down to 8 °C and sterilised at 160 Gy. After sterilization they were packed at 6 °C and transported to Addo in cooler boxes as immobilized adults at 6- 10 °C. Once the FCM arrived at Addo, Eastern Cape, the cooler boxes were then packed directly into a cold room which was maintained at a temperature of 4-5 °C for approximately 2- 3 hours until collected for release.

### *3.2.2 Release site*

All three trials were conducted on Sackville Farm, Addo, Eastern Cape, 33°31'57.06"S and 25°39'03.07"E (Figure 3.1). The release site was a lemon orchard and was therefore not included in the commercial SIT programme, as lemons are not a suitable host for FCM (Moore *et al.* 2015), thus minimising contamination of traps with both wild and sterile released FCM. The site was completely surrounded by other lemon orchards, which were surrounded by natural bush, with the nearest farm utilizing the commercial SIT programme 850 m away. The pesticidal spray programme for the orchard consisted of only two spray events for the trial period from March through to October 2015. Delegate® (active ingredient: 250 g/kg Spinetoram; Dow AgroSciences, Australia) was sprayed on the 19 March and on the 13 October the orchard was sprayed with a combination of Cyperfos® (active ingredients: 450 g/L Chlorpyrifos and 50 g/L Cypermethrin; Plaaskem (PTY) Ltd, Witfield) and Bendazid® (active ingredient: 50 g/L Carbendazim Plaaskem (PTY) Ltd. However as these products were applied at least a week before the FCM releases, there would have been no effect on recaptures of released irradiated FCM. A study done by Nepgen (2014) showed that these chemicals only have an effect on FCM up to seven days after application.


**Figure 3.1:** Sackville farm, Addo, where releases were conducted. Green blocks containing 10 rows of trees in each, form part of release area while the orange rows highlighted, indicate the rows of trees where individual control traps were placed

### *3.2.3 FCM release and monitoring*

Trials were performed for three separate field trials: March, July and October. Release and monitoring efforts were replicated for each trial.

Moths collected in Addo were weighed in petri dishes (diameter: 100 mm; height: 15 mm) on a micro-scale ($\geq$ 0.000 g) to ensure that there was an equal number of control moths to trial moths (FCM reared on augmented diets). The petri dishes, containing the newly irradiated male and female FCM (estimated 1:1 male: female) were transported to the release site inside cooler boxes containing Poly-ice® packs (Xymech (Pty) Ltd, Centurion) (Figure 3.2) which maintained the moths in an immobilized state during their transport. Upon arrival at the release site, 36 yellow delta traps containing CHEMPAC® FCM lures and sticky floors (Chempac (Pty) Ltd, Simondium) were set up (Figure 3.3). Thirty of the traps were used for the recapture portion of the trial whereas six were used as control traps placed on the northern side of the orchard (marked as orange in figure 3.1) five trees north of the road boundary, thus allowing the recapture results to be amended for any contamination from wild or commercially released sterile FCM. The 30 traps for recapture evaluation were placed in three groups of 10 rows each with a single row of lemon trees in between each group of 10 rows. Each trap was placed on the southern side of the orchard, in the fifth tree down from

the perimeter (marked as green in Figure 3.1), on the eastern side of the tree in order for the prevailing south-easterly wind to expedite pheromone distribution into the orchard (Hofmeyr 2003). The traps were suspended 1.5 m above the ground, in the outer foliage of the tree. Branches and leaves situated around the traps were removed to ensure unobstructed air flow as well as free and unhindered access for the FCM.

It is important to note that for the first (March/ autumn) trial, newly irradiated FCM reared on control and trehalose augmented diets were released. The second (July/ winter) trial was conducted in the same manner, except that pupae were used during releases instead of adults. The pupae were in the process of eclosing with the presence of several adults on collection, thus it was observable that they would eclose in the following day or two after release. For the third trial (October/ spring), moths reared on control and cholesterol augmented diets were released and the trials were conducted in the same manner as the previous two.

**Figure 3.2:** Sterile FCM retained in petri dishes kept cool by Poly-ice® packs

**Figure 3.3:** The setup of a yellow delta trap with a CHEMPAC® FCM lure and sticky pad

The moths were evenly released (similar numbers per row) by hand (Figure 3.4) for all 30 rows, from a position 15 m down the row, north of the traps. The traps were then left for a week, after which the sticky floors and pheromone lures were removed and the number of FCM per trap counted. The moths were identified by crushing the thorax and abdomen and noting the colour of the guts (Figure 3.5). New sticky floors and FCM pheromone lures were placed in the traps at the beginning of each trial. In the case of the second field trial where pupae were released, the pupation boards housing the pupae where cut into 30 equal sized pieces for each treatment and placed in plastic vials which were taped to the centre branches of each tree using masking tape (Figure 3.6).

Temperature, particularly night temperature between 19:00 and 02:00 (Nepgen 2014) (due to the nocturnal nature of adult FCM (Stofberg 1954)), was considered a key factor influencing FCM activity and consequently the flight ability of sterile and wild FCM. Two electronic data loggers were placed centrally between the branches of a tree located in the orchard to monitor and express any variation in temperature.

**Figure 3.4:** Release of FCM by hand. The control was handled by one person and the test (either trehalose or cholesterol) was handled by another.

**Figure 3.5:** Crushed FCM recaptured on the sticky floors showing the pink stained gut of a control FCM (circled by green), and the standard brown/ orange gut of an unstained trehalose augmented FCM (circled by purple).

**Figure 3.6:** FCM pupae boards containing irradiated FCM in plastic vials taped to the fifth tree in every row.

### *3.2.4 Statistical analysis*

A t-test was done as a direct comparison between the number of control and augmented FCM recaptured to determine if a significant difference exists in the flight ability between the two independent samples. The average monthly temperature and night temperature for the three trial months at the orchard was tested using an ANOVA and the post- hoc Tukey test. Temperature data were found to be normally distributed. The above statistics were done using Statistica version 13 (StatSoft, Inc. 2015).

## 3.3 RESULTS

### *3.3.1 FCM recaptures*

The number of control moths recaptured during the trials performed in March and July were similar with a mean of 0.42 ± 0.12 FCM found per trap for March, and 0.47 ± 0.12 FCM found per trap for July. During the October trial no control FCM were recaptured in any traps including the border traps. The number of trehalose augmented moths caught in March was slightly higher than that in July with a mean of 2.06 ± 0.39 trehalose augmented FCM recorded per trap for March, and 1.40 ± 0.27 trehalose augmented FCM recorded per trap for July. The mean number of cholesterol augmented moths recaptured in October was 0.72 ±

0.81. Commercially released sterile FCM were only recorded in border traps during the March field trial and were identified by the Calco Oil Red® dye in their gut; however, wild FCM were found in border traps during the March and October field trials and not the July trial. Significant differences were found between the number of control and augmented FCM recaptured for all three trials; March p=0.0002, July p=0.003 and October p=0.0001 (Table 3.1).

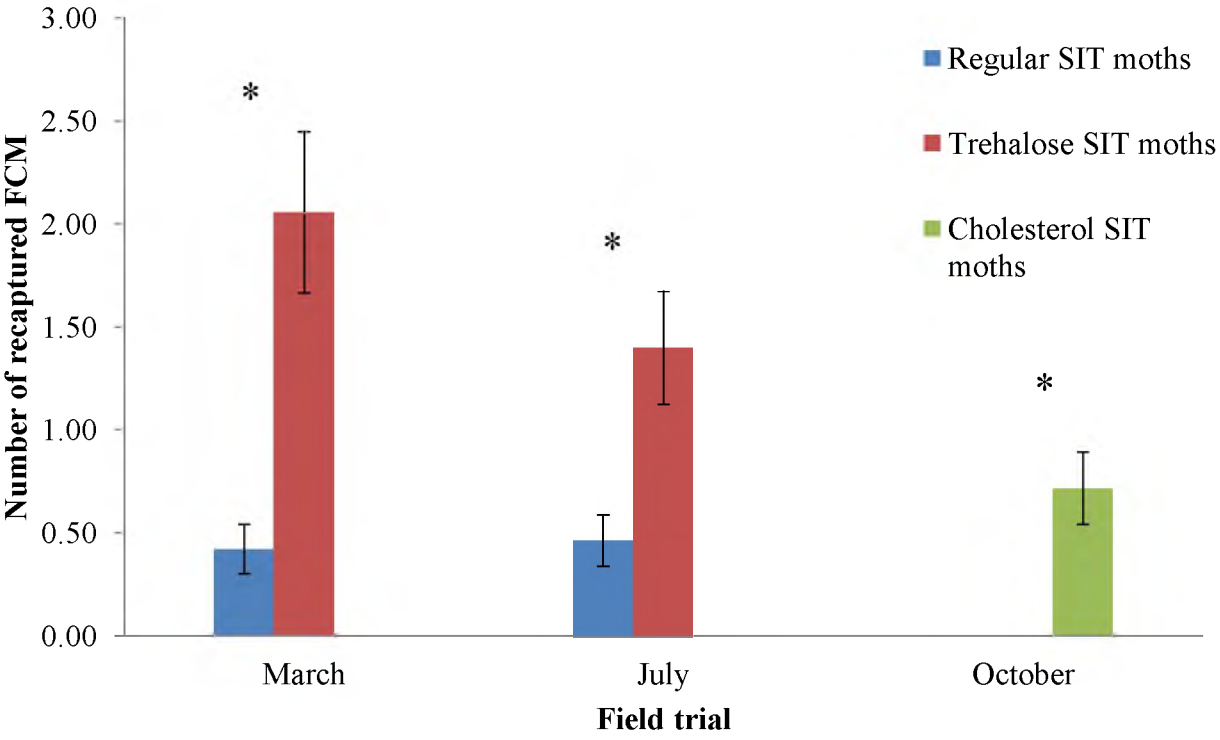



**Figure 3.7:** The number of recaptured FCM reared on the normal larval diet (control) and on two augmented diets, recorded as the mean number of FCM caught per trap.
* denote significant differences (t-test).

**Table 3.1:** Results of the t-test comparison between the flight ability (recaptures) of control and diet-augmented FCM during three field trials.

| | t-value | DF | p-value (two tailed) |
|---|---|---|---|
| March (Autumn) | 3.980 | 58 | 0.0002 |
| July (Winter) | 3.104 | 58 | 0.003 |
| October (Spring) | 4.068 | 58 | 0.0001 |

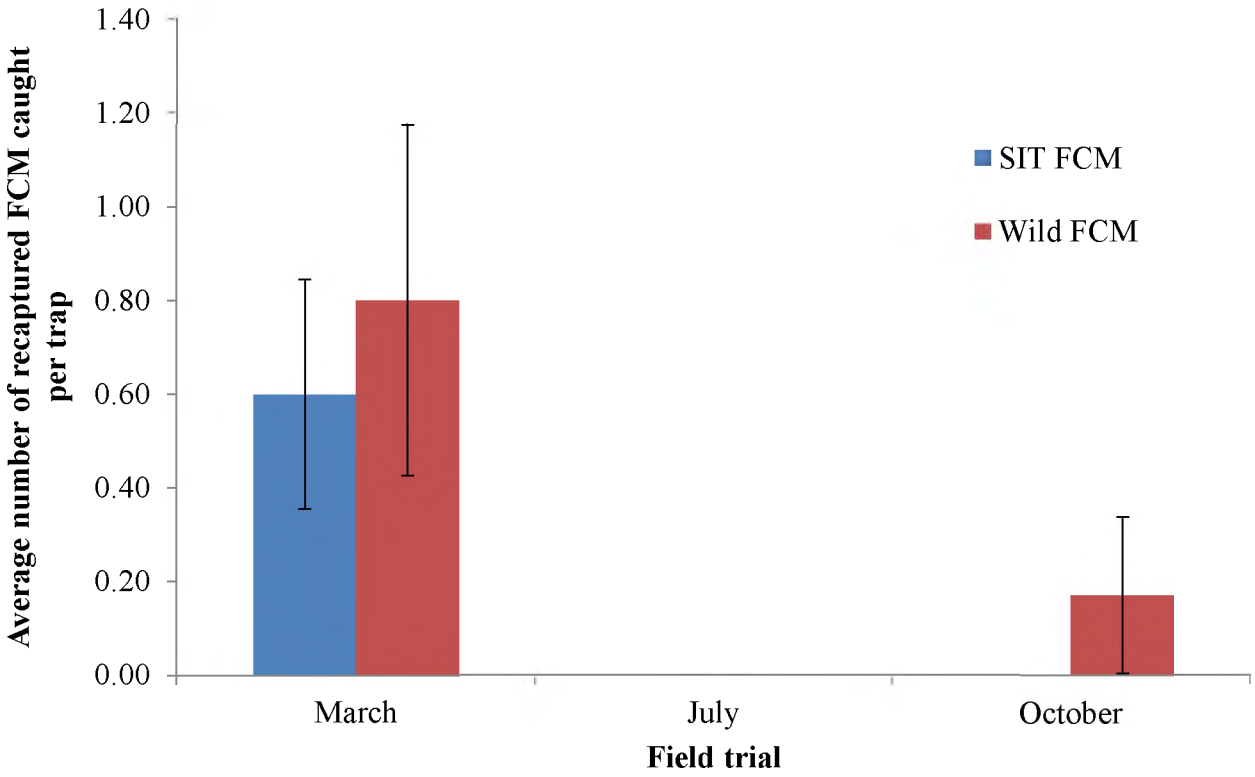



**Figure 3.8:** The number of wild and commercial released SIT FCM caught in border traps recorded as the mean number of moths caught per trap.

### *3.3.1 Temperature data*

The highest average monthly temperature was recorded for the month of October with a mean of 21.65 ± 0.76 °C, and the lowest monthly temperature was recorded for the month of July with a mean of 15.18 ± 0.61 °C. The month with the highest recorded night time temperature was March with a mean of 18.96 ± 1.32 °C and the month with the lowest recorded night time temperature was July with a mean of 14.53 ± 0.60 °C. See appendix 7 for complete ANOVA results

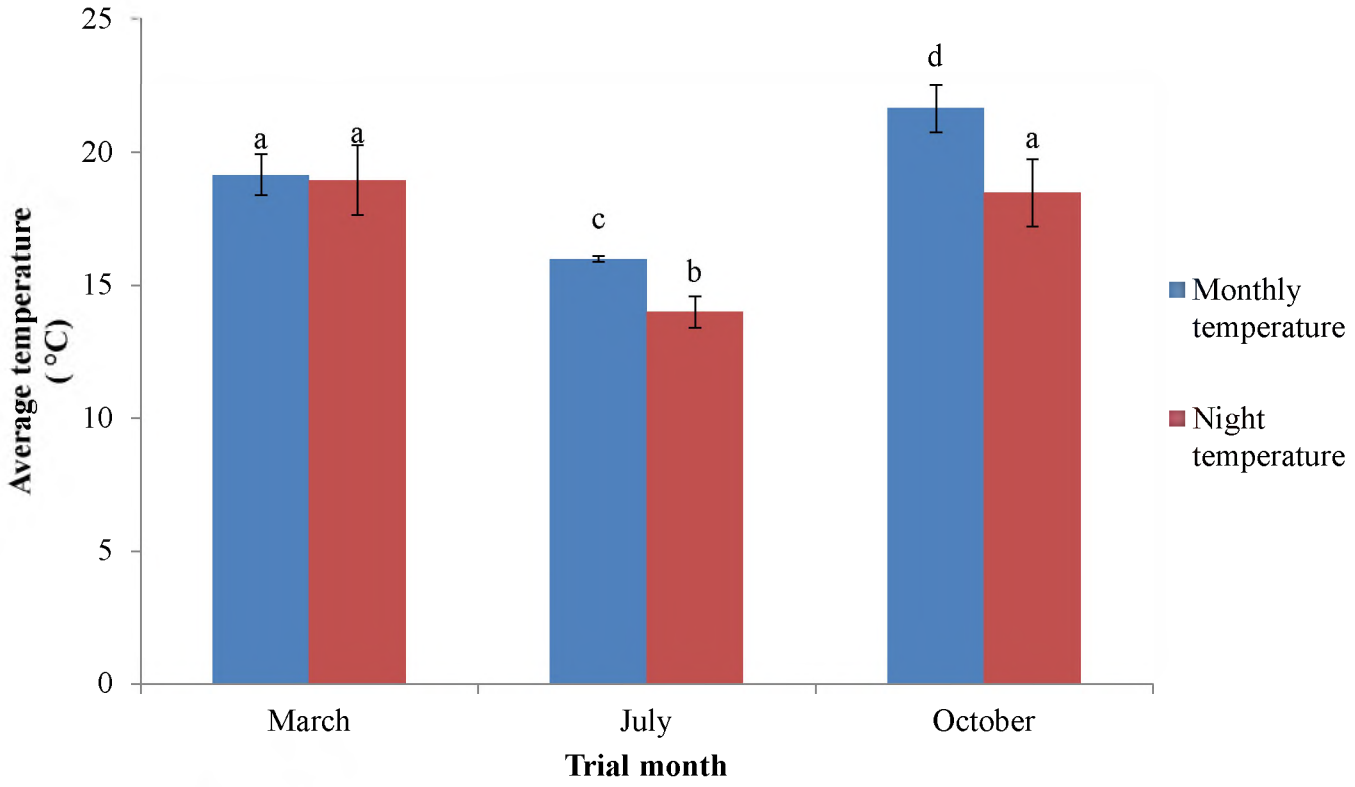



**Figure 3.9:** Average temperatures and average night temperatures (from 19:00 to 02:00) for the months of March (autumn), July (winter) and October (spring). Bars with different lower case letters denote significant differences between males (Tukey HSD test $P < 0.05$).

## 3.4 DISCUSSION

It is evident that SIT will remain a highly effective control method, on the condition that wild and mass reared colonies are compatible and competitive both sexually and behaviourally (Hendrichs *et al.* 2002). Certain parameters necessary for reared and sterilized males to be compatible and competitive in the field include successful emergence, sufficient mobility, mating competitiveness, mating compatibility with a wild female, successful transfer of sperm and accessory gland fluids, and fair survival (Calkins & Parker 2005). In this chapter the effects of adding known cryoprotectants and fuels on an important aspect of insect quality i.e. flight ability was assessed.

Flight ability is an important aspect of an insect's competitiveness in the field (Calkins & Parker 2005) and thus should be of the utmost importance in ensuring good quality sterile moths within an SIT programme. By being active at low temperatures (such as night time and during the winter months), it is presumed that FCM has some sort of rapid cold hardening response. This is apparent as in the Citrusdal region peak moth catches occur between May and June (Stotter & Terblanche 2009). However, a study, by Stotter &

Terblanche (2009) found that adult FCM appear to not employ a rapid cold hardening response in order to cope with the cooler conditions, and therefore they suggest that FCM may have developed an increased basal low-temperature tolerance without a short-term plastic response over evolutionary timescales. It is important to note that the lack of ability for rapid cold hardening of FCM during their study might simply be an outcome of the incomplete combination of necessary cold-hardening pre-treatments (Stotter & Terblanche 2009).

However, results from this study show conflicting results to those obtained by Stotter and Terblanche (2009) as no wild males caught in the border traps for the winter month of July, and with the highest number of wild males caught during the trial period occurring in March. It was also found that both treatments of sterile males (control and trehalose) were recaptured during the July field trial. These results regarding the recaptured control moths were unanticipated as the minimum threshold for sterile male activity is stipulated as higher than that of the wild males with the flight of irradiated FCM generally ceasing at temperatures below 20 °C (M. Hill pers. comm. 2015). As the maximum night time temperature for July was 17. 59 °C indicating that non-augmented control sterile FCM are able to fly at a temperature of at least 17.59 °C, meaning that the minimum temperature for SIT FCM flight should be revaluated in the field. However, the low numbers caught are probably indicative of the limited number of night hours during which temperatures were sufficiently warm for flight. There was only one night during the week-long trial, which exceeded 16 °C. The number of moths reared on trehalose augmented diets caught in the traps during March and July was significantly higher than the number of control moths recaptured, suggesting that there has been some improvement in the cold tolerance of those FCM as they were able to fly during cooler nights when the control FCM could not. Results also showed that there were no control FCM found in the trial traps, as well as no commercially released FCM found in the border traps for the spring month of October. This too is unexpected as the average night time temperature for October is not significantly different to that of March, with both months reaching favourable temperatures. These results, whereby no control and commercially released FCM were recaptured in October, could be due to the quality of the moths released. Studies have shown that the temperature tolerance of an insect is not a fixed characteristic and that lethal temperatures are determined by a host of factors that may vary over a range of timescales, both within and amongst species. An insect's temperature tolerance may be affected by its thermal history, either within its lifetime or its parental lifetime (Crill *et al.* 1996; Chown & Nicolson 2004). Therefore, it is possible

that the temperature tolerance of FCM could be altered and may ultimately allow for increased flight at lower temperatures. Trehalose and cholesterol are known insect cryoprotectants that may aid in the cold tolerance of FCM. A study by Shreve *et al.* (2007) managed to enhance membrane cholesterol levels of *Drosophila melanogaster* by rearing larvae on a cholesterol augmented diet, and individuals that developed on the cholesterol augmented diet exhibited a greater inherent cold tolerance. In a study done on four temperate species of adult *Drosophila (D. subauraria, D. triauraria, D. rufa* and *D. lutescens*), it was found that trehalose levels rose in early winter and gradually declined thereafter suggesting that low temperatures facilitated trehalose synthesis in these species (Kimura *et al.* 1997). Another study performed by Khani *et al.* (2007) shows that overwintering larvae of codling moth (CM) accumulated trehalose during winter where there was a threefold increase in trehalose content between larvae at the onset of diapause, and larvae in a fully developed diapause. Trehalose content was also found to be correlated with super cooling capacity, survival at low temperatures and chilling tolerance of CM, suggesting that trehalose may well play a role in the development of cold tolerance in the CM (Khani *et al.* 2007). In order to produce the large numbers of insects needed for an SIT programme, an artificial diet must be developed and proven adequate for the development of juvenile stages. However, when a large number of eggs are placed on a limited amount of diet and are confined to a smaller environment, the nutritional value of the diet may change (Calkins & Parker 2005). Therefore, by adding these various chemicals to the diets, it may be adding additional nutrition in the form of fuels for flight or cryoprotectants thus resulting in improved flight ability. The potential physiological reasons why trehalose and cholesterol may have an impact on the flight ability of FCM at cooler temperatures was discussed in chapter 2.

The thermal tolerance of laboratory reared FCM has major implications for the success of the SIT programme as a whole, as microclimate temperatures will influence many aspects regarding sterile insect fitness such as reproduction rate, longevity, survival and flight ability. All of these aspects consequently affect the ability of sterile insects to sufficiently compete for wild females in the field and thus, should be a focal point when looking to optimise the SIT programme for FCM. The general quality of FCM should be an essential part of the SIT programme, as it should for any SIT programme and will be discussed further in chapter 4.

# IV
# GENERAL DISCUSSION

## 4.1 INTRODUCTION

The ultimate aim of this study was to improve the competitiveness of sterile male FCM by improving their flight ability at cooler temperatures through diet manipulation without compromising other physiological aspects of insect quality such as developmental rate, fecundity and pupal mass. SIT is a complex programme that has many aspects to it that intertwine with one another, ensuring the continuation of the programme. Each aspect (e.g. insect quality, irradiation, and mass rearing and release techniques) needs to be optimized to ensure a high quality programme as the outcome (Robinson & Hendrichs 2005). Various studies have been performed to ensure optimisation of these different aspects, including mass rearing (Moore *et al.* 2014), transport (Nepgen *et al.* 2015) and release (Nepgen 2014) efforts, as well as attaining the optimal minimum dose of radiation needed for successful sterilization (Bloem *et al.* 2003). These components of the programme have been extensively studied and modified for the FCM SIT programme since its implementation. Studies such as these have opened doors to more specialised aspects of the programme, including sterile male competitiveness, particularly sterile male flight ability. Results from this study show that it is possible to improve the flight ability of sterile male FCM via diet manipulation without compromising other important physiological attributes of the moths.

## 4.2 THE IMPORTANCE OF COMPETITIVENESS, PARTICULARLY FLIGHT ABILITY, IN AN SIT PROGRAMME

Insect quality is a recognised scientific discipline and is a key factor in running and sustaining viable modern SIT programmes (Cohen 2001). However, the focus of large SIT programmes is usually on the number of insects reared and released. With this emphasis on insect numbers, insect quality may become secondary in importance and if a programme begins to fail, the general solution pursued may be to increase the sterile insect release rate in order to 'flood' the orchards with more sterile males. Thus it is vital to incorporate insect quality into an SIT programme to increase the chances of success (Hendrichs *et al.* 2005). This was the case in the beginning of the new world screwworm programme in the early 1970s in the south-western USA, whereby various strategies were continually replaced by

new ones in response to problems experienced with the programme over the 43 year campaign (Klassen 2005; Vargas-Terán *et al.* 2005). The debate concerning possible causes of these continuous failures of multiple strategies led to the implementation of regular strain renewal and the introduction of a quality control system (including optimizing rearing conditions and methods, and release efforts) used to continually monitor the quality of the sterile insect colony, resulting in success and ultimately improvement of the programme (Klassen 2005). This highlights the importance of insect quality in an SIT programme.

Important parameters of quality control that should be regarded when running an SIT programme include: egg hatch, larval developmental time, pupal size, percentage adult emergence, sex ratio, timing of eclosion, pheromone production and response, vision, longevity, startle activity, mating propensity, compatibility and competitiveness, re-mating and flight ability (Calkins & Parker 2005). Declines in key aspects regarding insect quality such as flight, dispersal, and survival due to rearing techniques and/ or irradiation have been comprehensively studied (Dame *et al.* 1969; Rajagopalan *et al.* 1973; Sharp 1976; Nelson & Milby 1980; Nakamori & Soemori 1981; Smith *et al.* 1981), with the flight ability of laboratory reared FCM being extensively reviewed in this study. In order for growth of this SIT programme to occur, focus needs to be on improvement of sterile male FCM flight ability, as the effectiveness of the programme is compromised by moths being physiologically unable to fly at low temperatures at which wild males are active. This is especially important for FCM, where control is needed late in the growing season, autumn and winter, when many of the cultivars are harvested.

## 4.3 EFFECT OF AUGMENTED DIETS ON STERILE MALE FCM FLIGHT ABILITY

Laboratory trials showed that trehalose and cholesterol improved the flight ability of sterile male FCM at 15°C. These chemicals, that showed promise under controlled conditions, were then used in augmented diets to determine if similar results could be obtained under natural conditions in the field. Field trial results showed a significant increase in the number of recaptured moths that were reared on either the trehalose or cholesterol augmented diets. Improving the cold tolerance of an insect via diet manipulation is not new and has been achieved in a handful of studies (Shreve *et al.* 2007; Kostal *et al.* 2012) however, preceding this study, has never been considered for SIT. In a study performed by Kostal *et al.* (2012), the cold tolerance of *Chymomyza costata* was increased by feeding the

larvae a diet augmented with the cryoprotectant, proline. Another study performed by Shreve *et al.* (2007) increased membrane cholesterol levels of *Drosophila melanogaster* by rearing the larvae on a cholesterol-augmented diet that then improved its cold tolerance.

The findings of this study have significant implications for the current status of sterile male FCM flight ability as, if this augmentation of the diet is to be practiced on a commercial scale, there may be no further issues around sterile males and their inability to compete during the colder months of winter, a time when many cultivars are harvested and therefore a time most critical to maintain control of FCM. This would then allow for the SIT programme to be more effective during the cooler months and with an increase in efficiency, there may be an increase in the positive response the programme would receive from growers and IPM managers. The willingness to try SIT as a means of FCM control would thus increase and, as the efficiency of SIT is enhanced in an area-wide approach, (Hendrichs & Robinson 2009). Furthermore, if FCM is able to be suppressed during winter, it will ensure that the pest inoculum in spring, at the start of the new season, will be low. Without this suppression, the inverse may be true, placing a greater pressure on the SIT programme during the new season. SIT cannot handle such pressure, as it is a negatively density dependent technology whereby control of low numbers is effective, and control of high numbers is very difficult as the ratio of sterile to wild becomes compromised (Alphey *et al.* 2010).

These results also serve as a milestone for not only FCM SIT but SIT as a whole, with more SIT programmes potentially researching the augmentation of certain diets in order to improve the cold tolerance and flight ability of the particular insect. Other current and even discontinued SIT programmes may be revitalised by improving the flight ability, and in turn, quality, of the insect in question. A study by Judd and Gardiner (2006) showed that irradiation has an indirect negative effect on pheromone response by reducing general flight activity and dispersal of CM and thus reducing its effectiveness in the field. Even in cases where the competitiveness of sterile males has not been compromised, it may be enhanced to not only meet the competitive nature of wild males, but surpass it. For example, in a study by Munhenga *et al.* (2011) the fitness between samples of wild and sterile laboratory reared *Anopheles arabiensis* was investigated under various laboratory conditions, including temperature. They found that the laboratory reared colony retained sufficient reproductive and physiological measures when compared to the wild males, meaning there was no significant reduction in fitness of the sterile individuals relative to their wild competitors. Therefore, by augmenting the laboratory diet with a chemical such as trehalose it may be

possible to improve the fitness of the males by improving their flight ability, even though the fitness of *An. arabiensis* was not compromised during mass rearing and/ or radiation efforts.

## 4.4 EFFECT OF AUGMETED DIETS ON THE BIOLOGY OF FCM

The laboratory trials undertaken showed that the flight ability of sterile male FCM can be improved by rearing the larvae on diets augmented with trehalose or cholesterol. Not only was the flight ability of FCM improved, but results also showed that the augmentation of diets with these chemicals had no negative effects on other important quality aspects of the insect's physiology (developmental rate, pupal mass and fecundity) and in fact improved some of these qualities. Diet is a crucial element in a mass rearing operation for an insect and designing a suitable diet for a specific insect species comes with the challenge of ensuring the nutritional needs of the insect are met in comparison to its natural diet (Cohen 2015). Diets are continually improved for all mass reared insects with a good example being that of the pyralid insect, *Chilo parfellus* (Swinhoe) whereby the laboratory diet has been adjusted at least 10 times from 1960 to 1985 (Pant *et al.* 1960; Dang *et al.* 1970; Lminarayana & Soto 1971; Moorty 1973; Siddiqui & Chatterji 1972; Siddiqui *et al.* 1977; Sharma & Sarup 1978; Seshu Reddy & Davies 1979; Taneja & Leuschner 1985). The first artificial diet used to rear *C. parrellus* included a combination of casein, glucose, salt mixture, yeast, choline chloride, cholesterol, cellulose, leaf factor, agar, methyl paraben and water (Pant *et al.* 1960). The major breakthrough in the mass rearing of *C. partellus* came was when the Kabuli gram based diet was introduced (Dang *et al.* 1970) as this diet had fewer and more readily available ingredients. This is the diet used the most, however it has been adjusted to either delete, add or change the quantity of one or other ingredients (Taneja & Nwanze 1990).

Diets augmented with trehalose significantly increased female and male FCM pupal weight and number of eggs produced per female. These results may have a major impact on future FCM mass production as well as commercial virus production. By increasing the number of eggs laid per female, it is possible to have a greater number of FCM produced without demanding an increase in the core colony, thereby saving space and cutting costs. Mediouni & Dhouibi (2007), found that the fecundity and fertility of the carob moth, *Ectomyelois ceratoniae* (Zeller), was lower in mass rearing conditions and that developments were needed to further improve performance of the moth under the mass rearing system. Augmenting the larval diet with trehalose resulted in larger $5^{th}$ instar FCM larvae, which is the life stage used for production of the *Cryptophlebia leucotreta*

granulovirus (Moore 2002). An increase in weight equates to an increase in size of larvae available for virus production, which should facilitate a greater production of virus per larva. These aspects of an increased number of eggs laid and increased pupal mass, may make it possible for smaller businesses to produce and sell the virus regionally allowing a greater range of people, particularly in the rural areas, access to the product. This not only will promote awareness and use of biological control, but will also contribute in local job creation, education, help build the local economy and promote area-wide SIT. The reduction in space necessary for FCM development may also allow for smaller businesses to incorporate egg parasitoid (*Trichogrammatoidea cryptophlebiae*) rearing and thus allow for local farmers to start implementing an IPM approach to FCM control.

Diets augmented with cholesterol also resulted in an increase in the number of eggs laid per female, thus allowing for the same possibility for a greater number of FCM produced in a smaller facility. Additionally, by augmenting the diets with cholesterol, results showed that there was a significant increase in developmental rate. This is an important factor when considering the output rate of sterile male FCM. If the developmental rate of commercially produced FCM is increased, it increases moth production and may allow for expansion of SIT to other regions. If expansion of the programme is not necessary at the time, increasing the developmental rate of FCM may be beneficial to research projects running on limited time and may allow for more generations to be produced in a year thus reducing laboratory research time.

A major drawback to the commercial use of trehalose is the cost. Cost is a very important factor when determining the viability of an SIT programme, as if the programme is too costly, it may not be realistic to continue with it. Such was the case with codling moth in South Africa, where the SIT programme was terminated in 2014 due to economic reasons (Barnes *et al.* 2015). The price of trehalose is exceptionally high and even with the increase in flight ability, number of eggs produced and weight, it appears that the results might not justify the expense. However, as Burton (1970) suggests, the more defined an artificial diet is, the more expensive a diet becomes and this raises the question; at what point does modifying a diet become uneconomical. Cholesterol, on the other hand, is much cheaper in relation to trehalose and smaller quantities are needed (160 mg cholesterol vs. 3150 mg trehalose per 45 g diet). If trehalose were to be added to the basic FCM diet, it would increase the price per 45 g diet by ZAR 133.88, whereas if cholesterol is added it would increase the price per 45 g diet by ZAR 17.90 (prices obtained from Sigma-Aldrich, May 2016). Even with cholesterol costing a great deal less than trehalose, an increase of ZAR17.90 is still not viable as in 2014

the cost to produce a single jar of FCM diet was calculated to be ZAR0.29. There is however, another cholesterol product available which may be tested and when bought in bulk it would increase the cost per 45 g jar by ZAR0.83. The only setback in using cholesterol in place of trehalose is that cholesterol does not seem to increase FCM pupal weight, and it does not result in as high a percentage flight at 15 °C. However, the difference was not significant. Alternatively, research may be conducted involving cheaper chemicals similar to trehalose, otherwise an arrangement may be agreed on with larger chemical companies such as, Merck, WhiteSci or Sigma-Aldrich for reasonable prices on bulk purchases.

## 4.5 THE FUTURE OF SIT IN SOUTH AFRICA

Outcomes of this study emphasized the importance of further research on diet manipulation for not only the FCM SIT programme, but other SIT programmes currently operating, or future programmes. The reduced flight ability of sterile male FCM could be resolved by utilising an improved diet containing trehalose and/ or cholesterol. The evolution of SIT in South Africa is inevitable as globally, consumers are advocating for a 'greener' approach to food production, elevating IPM strategies, including SIT programmes, to greater favour than standard chemical control methods.

Pursuits at improving sterile male performance show great promise for future implementation, with inventive proposals which include exposing sterile male insects to hormonal, nutritional, microbial, and semiochemical supplements (Robinson & Hendrichs 2005). Additionally, management of core colonies will be significantly improved in order to reduce the effects of colonization and genetic bottlenecks, and slowing down mass-rearing side-effects on key behavioural parameters that often result in a rapid depreciation of colony quality. Improvement and standardization of quality control protocols is a necessary step towards improved SIT, and should be implemented for both the product (the target insect), and the process as a whole (procedures used to rear, sterilize and release the sterile insect). Such a protocol has been implemented for the fruit fly SIT programme whereby an internationally agreed set of protocols is used to monitor the quality of the sterile insect (Robinson & Hendrichs 2005). More attention should be paid to quality as opposed to quantity, and the more expensive an insect is to produce, the more important it is that quality be maintained to ensure satisfactory results.

In addition to the future of SIT quality control, SIT in the immediate future should focus on improving the overall efficiency of the technique for existing programmes as well as

trying to implement an IPM approach that utilises SIT. The inevitable increase of globalization will predictably lead to an increase in the invasion of unwanted alien insect pests into new areas. The concept of using SIT against such pests should be a point of interest as this would ultimately prevent pest establishment. A Californian model for the prevention of Mediterranean fruit flies, *Ceratitis capitata*, has utilised a preventive approach, whereby sterile Mediterranean fruit flies are continuously released in areas which are considered high-risk for establishment due to the recurring introductions into the region (Dowell *et al.* 2000). The programme is considered successful. Attention also needs to be given to the cost effectiveness of known, as well as future aspects of individual IPM programmes. This involves all aspects of the technique from cutting costs of laboratory proceedings such as cage design and diet, to finding the most cost effective release method.

## 4.6 CONCLUSION

When examining the outcomes of this study, advancements can be made towards the improvement of the current SIT programme for FCM control in South Africa. Such findings are likely to be extensively studied and incorporated into the commercial SIT programme for FCM. Such results may also be used as a reference for other SIT programmes involving other insect species, to help improve the activity and subsequently, competitiveness of such insects.

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# APPENDIX 1

**Appendix 1a:** Univariate Tests of Significance for developmental rate during dosage test

| | | DF | F- value | p- value |
|---|---|---|---|---|
| Pupation | Dosage | 2 | 37.53 | < 0.0001 |
| | Chemical | 8 | 54.36 | < 0.0001 |
| | Dosage*Chemical | 16 | 39.97 | < 0.0001 |
| Eclosion | Dosage | 2 | 47.81 | < 0.0001 |
| | Chemical | 8 | 54.69 | < 0.0001 |
| | Dosage*Chemical | 16 | 43.51 | < 0.0001 |

**Appendix 1b:** p-values from the ANOVA performed for days taken to pupation during dosage test

| Cell no. | Dosage | Chemical | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Low | Control | | 0.3364 | 0.7542 | 0.7542 | 0.7542 | 0.0166 | 0.0004 | 1.0000 | 1.0000 |
| 2 | Low | Trehalose | 0.3364 | | 1.0000 | 1.0000 | 1.0000 | 0.9999 | 0.0002 | 0.7542 | 0.7542 |
| 3 | Low | Proline | 0.7542 | 1.0000 | | 1.0000 | 1.0000 | 0.9812 | 0.0002 | 0.9812 | 0.9812 |
| 4 | Low | Mannitol | 0.7542 | 1.0000 | 1.0000 | | 1.0000 | 0.9812 | 0.0002 | 0.9812 | 0.9812 |
| 5 | Low | Sorbitol | 0.7542 | 1.0000 | 1.0000 | 1.0000 | | 0.9812 | 0.0002 | 0.9812 | 0.9812 |
| 6 | Low | Cholesterol | 0.0165 | 0.9999 | 0.9812 | 0.9812 | 0.9812 | | 0.0002 | 0.0894 | 0.0894 |
| 7 | Low | Ethyl glycol | 0.0004 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | | 0.0002 | 0.0002 |
| 8 | Low | Glucose | 1.0000 | 0.7542 | 0.9812 | 0.9812 | 0.9812 | 0.0894 | 0.0002 | | 1.0000 |
| 9 | Low | Glycerol | 1.0000 | 0.7542 | 0.9812 | 0.9812 | 0.9812 | 0.0894 | 0.0002 | 1.0000 | |
| 10 | Medium | Control | 1.0000 | 0.3364 | 0.7542 | 0.7542 | 0.7542 | 0.0166 | 0.0004 | 1.0000 | 1.0000 |
| 11 | Medium | Trehalose | 0.0894 | 1.0000 | 0.9999 | 0.9999 | 0.9999 | 1.0000 | 0.0002 | 0.3364 | 0.3364 |
| 12 | Medium | Proline | 0.7542 | 1.0000 | 1.0000 | 1.0000 | 1.0000 | 0.9812 | 0.0002 | 0.9812 | 0.9812 |
| 13 | Medium | Mannitol | 0.3364 | 1.0000 | 1.0000 | 1.0000 | 1.0000 | 0.9999 | 0.0002 | 0.7542 | 0.7542 |
| 14 | Medium | Sorbitol | 0.7542 | 1.0000 | 1.0000 | 1.0000 | 1.0000 | 0.9812 | 0.0002 | 0.9812 | 0.9812 |
| 15 | Medium | Cholesterol | 0.0166 | 0.9999 | 0.9812 | 0.9812 | 0.9812 | 1.0000 | 0.0002 | 0.0894 | 0.0894 |
| 16 | Medium | Ethyl glycol | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 |
| 17 | Medium | Glucose | 1.0000 | 0.7542 | 0.9812 | 0.9812 | 0.9812 | 0.0894 | 0.0002 | 1.0000 | 1.0000 |
| 18 | Medium | Glycerol | 1.0000 | 0.7542 | 0.9812 | 0.9812 | 0.9812 | 0.0894 | 0.0002 | 1.0000 | 1.0000 |
| 19 | High | Control | 1.0000 | 0.3364 | 0.7542 | 0.7542 | 0.7542 | 0.0166 | 0.0004 | 1.0000 | 1.0000 |
| 20 | High | Trehalose | 0.0894 | 1.0000 | 0.9999 | 0.9999 | 0.9999 | 1.0000 | 0.0002 | 0.3364 | 0.3364 |
| 21 | High | Proline | 0.7542 | 1.0000 | 1.0000 | 1.0000 | 1.0000 | 0.9812 | 0.0002 | 0.9812 | 0.9812 |
| 22 | High | Mannitol | 0.7542 | 1.0000 | 1.0000 | 1.0000 | 1.0000 | 0.9812 | 0.0002 | 0.9812 | 0.9812 |
| 23 | High | Sorbitol | 0.7542 | 1.0000 | 1.0000 | 1.0000 | 1.0000 | 0.9812 | 0.0002 | 0.9812 | 0.9812 |
| 24 | High | Cholesterol | 0.0165 | 0.9999 | 0.9812 | 0.9812 | 0.9812 | 1.0000 | 0.0002 | 0.0894 | 0.0894 |
| 25 | High | Ethyl glycol | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 |
| 26 | High | Glucose | 0.7542 | 1.0000 | 1.0000 | 1.0000 | 1.0000 | 0.9812 | 0.0002 | 0.9812 | 0.9812 |
| 27 | High | Glycerol | 0.0894 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.9812 | 0.0166 | 0.0166 |

| Cell no. | Dosage | Chemical | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Low | Control | 1.0000 | 0.0894 | 0.7542 | 0.3364 | 0.7542 | 0.0166 | 0.0002 | 1.0000 | 1.0000 |
| 2 | Low | Trehalose | 0.3364 | 1.0000 | 1.0000 | 1.0000 | 1.0000 | 0.9999 | 0.0002 | 0.7542 | 0.7542 |
| 3 | Low | Proline | 0.7542 | 0.9999 | 1.0000 | 1.0000 | 1.0000 | 0.9812 | 0.0002 | 0.9812 | 0.9812 |
| 4 | Low | Mannitol | 0.7542 | 0.9999 | 1.0000 | 1.0000 | 1.0000 | 0.9812 | 0.0002 | 0.9812 | 0.9812 |
| 5 | Low | Sorbitol | 0.7542 | 0.9999 | 1.0000 | 1.0000 | 1.0000 | 0.9812 | 0.0002 | 0.9812 | 0.9812 |
| 6 | Low | Cholesterol | 0.0166 | 1.0000 | 0.9812 | 0.9999 | 0.9812 | 1.0000 | 0.0002 | 0.0894 | 0.0894 |
| 7 | Low | Ethyl glycol | 0.0004 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 |
| 8 | Low | Glucose | 1.0000 | 0.3364 | 0.9812 | 0.7542 | 0.9812 | 0.0894 | 0.0002 | 1.0000 | 1.0000 |
| 9 | Low | Glycerol | 1.0000 | 0.3364 | 0.9812 | 0.7542 | 0.9812 | 0.0894 | 0.0002 | 1.0000 | 1.0000 |
| 10 | Medium | Control |  | 0.0894 | 0.7542 | 0.3364 | 0.7542 | 0.0166 | 0.0002 | 1.0000 | 1.0000 |
| 11 | Medium | Trehalose | 0.0894 |  | 0.9999 | 1.0000 | 0.9999 | 1.0000 | 0.0002 | 0.3364 | 0.3364 |
| 12 | Medium | Proline | 0.7542 | 0.9999 |  | 1.0000 | 1.0000 | 0.9812 | 0.0002 | 0.9812 | 0.9812 |
| 13 | Medium | Mannitol | 0.3364 | 1.0000 | 1.0000 |  | 1.0000 | 0.9999 | 0.0002 | 0.7542 | 0.7542 |
| 14 | Medium | Sorbitol | 0.7542 | 0.9999 | 1.0000 | 1.0000 |  | 0.9812 | 0.0002 | 0.9812 | 0.9812 |
| 15 | Medium | Cholesterol | 0.0166 | 1.0000 | 0.9812 | 0.9999 | 0.9812 |  | 0.0002 | 0.0894 | 0.0894 |
| 16 | Medium | Ethyl glycol | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 |  | 0.0002 | 0.0002 |
| 17 | Medium | Glucose | 1.0000 | 0.3364 | 0.9812 | 0.7542 | 0.9812 | 0.0894 | 0.0002 |  | 1.0000 |
| 18 | Medium | Glycerol | 1.0000 | 0.3364 | 0.9812 | 0.7542 | 0.9812 | 0.0894 | 0.0002 | 1.0000 |  |
| 19 | High | Control | 1.0000 | 0.0894 | 0.7542 | 0.3364 | 0.7542 | 0.0166 | 0.0002 | 1.0000 | 1.0000 |
| 20 | High | Trehalose | 0.0894 | 1.0000 | 0.9999 | 1.0000 | 0.9999 | 1.0000 | 0.0002 | 0.3364 | 0.3364 |
| 21 | High | Proline | 0.7542 | 0.9999 | 1.0000 | 1.0000 | 1.0000 | 0.9812 | 0.0002 | 0.9812 | 0.9812 |
| 22 | High | Mannitol | 0.7542 | 0.9999 | 1.0000 | 1.0000 | 1.0000 | 0.9812 | 0.0002 | 0.9812 | 0.9812 |
| 23 | High | Sorbitol | 0.7542 | 0.9999 | 1.0000 | 1.0000 | 1.0000 | 0.9812 | 0.0002 | 0.9812 | 0.9812 |
| 24 | High | Cholesterol | 0.0166 | 1.0000 | 0.9812 | 0.9999 | 0.9812 | 1.0000 | 0.0002 | 0.0894 | 0.0894 |
| 25 | High | Ethyl glycol | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 1.0000 | 0.0002 | 0.0002 |
| 26 | High | Glucose | 0.7542 | 0.9999 | 1.0000 | 1.0000 | 1.0000 | 0.9812 | 0.0002 | 0.9812 | 0.9812 |
| 27 | High | Glycerol | 0.0894 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0166 | 0.0166 |

| Cell no. | Dosage | Chemical | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Low | Control | 1.0000 | 0.0894 | 0.7542 | 0.7542 | 0.7542 | 0.0166 | 0.0002 | 0.7542 | 0.0894 |
| 2 | Low | Trehalose | 0.3364 | 1.0000 | 1.0000 | 1.0000 | 1.0000 | 0.9999 | 0.0002 | 1.0000 | 0.0002 |
| 3 | Low | Proline | 0.7542 | 0.9999 | 1.0000 | 1.0000 | 1.0000 | 0.9812 | 0.0002 | 1.0000 | 0.0002 |
| 4 | Low | Mannitol | 0.7542 | 0.9999 | 1.0000 | 1.0000 | 1.0000 | 0.9812 | 0.0002 | 1.0000 | 0.0002 |
| 5 | Low | Sorbitol | 0.7542 | 0.9999 | 1.0000 | 1.0000 | 1.0000 | 0.9812 | 0.0002 | 1.0000 | 0.0002 |
| 6 | Low | Cholesterol | 0.0166 | 1.0000 | 0.9812 | 0.9812 | 0.9812 | 1.0000 | 0.0002 | 0.9812 | 0.0002 |
| 7 | Low | Ethyl glycol | 0.0004 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.9812 |
| 8 | Low | Glucose | 1.0000 | 0.3364 | 0.9812 | 0.9812 | 0.9812 | 0.0894 | 0.0002 | 0.9812 | 0.0166 |
| 9 | Low | Glycerol | 1.0000 | 0.3364 | 0.9812 | 0.9812 | 0.9812 | 0.0894 | 0.0002 | 0.9812 | 0.0166 |
| 10 | Medium | Control | 1.0000 | 0.0894 | 0.7542 | 0.7542 | 0.7542 | 0.0166 | 0.0002 | 0.7542 | 0.0894 |
| 11 | Medium | Trehalose | 0.0894 | 1.0000 | 0.9999 | 0.9999 | 0.9999 | 1.0000 | 0.0002 | 0.9999 | 0.0002 |
| 12 | Medium | Proline | 0.7542 | 0.9999 | 1.0000 | 1.0000 | 1.0000 | 0.9812 | 0.0002 | 1.0000 | 0.0002 |
| 13 | Medium | Mannitol | 0.3364 | 1.0000 | 1.0000 | 1.0000 | 1.0000 | 0.9999 | 0.0002 | 1.0000 | 0.0002 |
| 14 | Medium | Sorbitol | 0.7542 | 0.9999 | 1.0000 | 1.0000 | 1.0000 | 0.9812 | 0.0002 | 1.0000 | 0.0002 |
| 15 | Medium | Cholesterol | 0.0166 | 1.0000 | 0.9812 | 0.9812 | 0.9812 | 1.0000 | 0.0002 | 0.9812 | 0.0002 |
| 16 | Medium | Ethyl glycol | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 1.0000 | 0.0002 | 0.0002 |
| 17 | Medium | Glucose | 1.0000 | 0.3364 | 0.9812 | 0.9812 | 0.9812 | 0.0894 | 0.0002 | 0.9812 | 0.0166 |
| 18 | Medium | Glycerol | 1.0000 | 0.3364 | 0.9812 | 0.9812 | 0.9812 | 0.0894 | 0.0002 | 0.9812 | 0.0166 |
| 19 | High | Control |  | 0.0894 | 0.7542 | 0.7542 | 0.7542 | 0.0166 | 0.0002 | 0.7542 | 0.0894 |
| 20 | High | Trehalose | 0.0894 |  | 0.9999 | 0.9999 | 0.9999 | 1.0000 | 0.0002 | 0.9999 | 0.0002 |
| 21 | High | Proline | 0.7542 | 0.9999 |  | 1.0000 | 1.0000 | 0.9812 | 0.0002 | 1.0000 | 0.0002 |
| 22 | High | Mannitol | 0.7542 | 0.9999 | 1.0000 |  | 1.0000 | 0.9812 | 0.0002 | 1.0000 | 0.0002 |
| 23 | High | Sorbitol | 0.7542 | 0.9999 | 1.0000 | 1.0000 |  | 0.9812 | 0.0002 | 1.0000 | 0.0002 |
| 24 | High | Cholesterol | 0.0166 | 1.0000 | 0.9812 | 0.9812 | 0.9812 |  | 0.0002 | 0.9812 | 0.0002 |
| 25 | High | Ethyl glycol | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 |  | 0.0002 | 0.0002 |
| 26 | High | Glucose | 0.7542 | 0.9999 | 1.0000 | 1.0000 | 1.0000 | 0.9812 | 0.0002 |  | 0.0002 |
| 27 | High | Glycerol | 0.0894 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 |  |

**Appendix 1c:** p-values from the ANOVA performed for days taken to eclosion during dosage test

| Cell no. | Dosage | Chemical | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Low | Control | | 0.0003 | 0.8230 | 0.5101 | 0.2272 | 0.0003 | 0.0052 | 0.9774 | 0.8230 |
| 2 | Low | Trehalose | 0.0003 | | 0.2272 | 0.5101 | 0.8230 | 1.0000 | 0.0002 | 0.0771 | 0.2272 |
| 3 | Low | Proline | 0.8230 | 0.2272 | | 1.0000 | 1.0000 | 0.2272 | 0.0002 | 1.0000 | 1.0000 |
| 4 | Low | Mannitol | 0.5101 | 0.5101 | 1.0000 | | 1.0000 | 0.5101 | 0.0002 | 1.0000 | 1.0000 |
| 5 | Low | Sorbitol | 0.2272 | 0.8230 | 1.0000 | 1.0000 | | 0.8230 | 0.0002 | 0.9995 | 1.0000 |
| 6 | Low | Cholesterol | 0.0003 | 1.0000 | 0.2272 | 0.5101 | 0.8230 | | 0.0002 | 0.0771 | 0.2272 |
| 7 | Low | Ethyl glycol | 0.0052 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | | 0.0002 | 0.0002 |
| 8 | Low | Glucose | 0.9774 | 0.0771 | 1.0000 | 1.0000 | 0.9995 | 0.0771 | 0.0002 | | 1.0000 |
| 9 | Low | Glycerol | 0.8230 | 0.2272 | 1.0000 | 1.0000 | 1.0000 | 0.2272 | 0.0002 | 1.0000 | |
| 10 | Medium | Control | 0.0003 | 1.0000 | 0.2272 | 0.5101 | 0.8230 | 1.0000 | 0.0002 | 0.0771 | 0.2272 |
| 11 | Medium | Trehalose | 0.0003 | 1.0000 | 0.2272 | 0.5101 | 0.8230 | 1.0000 | 0.0002 | 0.0771 | 0.2272 |
| 12 | Medium | Proline | 0.9774 | 0.0771 | 1.0000 | 1.0000 | 0.9995 | 0.0771 | 0.0002 | 1.0000 | 1.0000 |
| 13 | Medium | Mannitol | 0.5101 | 0.5101 | 1.0000 | 1.0000 | 1.0000 | 0.5101 | 0.0002 | 1.0000 | 1.0000 |
| 14 | Medium | Sorbitol | 0.0214 | 0.9995 | 0.9774 | 0.9995 | 1.0000 | 0.9995 | 0.0002 | 0.8230 | 0.9774 |
| 15 | Medium | Cholesterol | 0.0052 | 1.0000 | 0.8230 | 0.9774 | 0.9995 | 1.0000 | 0.0002 | 0.5101 | 0.8230 |
| 16 | Medium | Ethyl glycol | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 |
| 17 | Medium | Glucose | 0.9995 | 0.0214 | 1.0000 | 0.9995 | 0.9774 | 0.0214 | 0.0002 | 1.0000 | 1.0000 |
| 18 | Medium | Glycerol | 0.9995 | 0.0214 | 1.0000 | 0.9995 | 0.9774 | 0.0214 | 0.0002 | 1.0000 | 1.0000 |
| 19 | High | Control | 0.0003 | 1.0000 | 0.2272 | 0.5101 | 0.8230 | 1.0000 | 0.0002 | 0.0771 | 0.2272 |
| 20 | High | Trehalose | 0.0052 | 1.0000 | 0.8230 | 0.9774 | 0.9995 | 1.0000 | 0.0002 | 0.5101 | 0.8230 |
| 21 | High | Proline | 0.5101 | 0.5101 | 1.0000 | 1.0000 | 1.0000 | 0.5101 | 0.0002 | 1.0000 | 1.0000 |
| 22 | High | Mannitol | 0.5101 | 0.5101 | 1.0000 | 1.0000 | 1.0000 | 0.5101 | 0.0002 | 1.0000 | 1.0000 |
| 23 | High | Sorbitol | 0.0771 | 0.9774 | 0.9995 | 1.0000 | 1.0000 | 0.9774 | 0.0002 | 0.9774 | 0.9995 |
| 24 | High | Cholesterol | 0.0012 | 1.0000 | 0.5101 | 0.8230 | 0.9774 | 1.0000 | 0.0002 | 0.2272 | 0.5101 |
| 25 | High | Ethyl glycol | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 |
| 26 | High | Glucose | 1.0000 | 0.0052 | 0.9995 | 0.9774 | 0.8230 | 0.0052 | 0.0003 | 1.0000 | 0.9995 |
| 27 | High | Glycerol | 0.9774 | 0.0002 | 0.0214 | 0.0052 | 0.0012 | 0.0002 | 0.5101 | 0.0771 | 0.0214 |

| Cell no. | Dosage | Chemical | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Low | Control | 0.0003 | 0.0003 | 0.9744 | 0.5101 | 0.0214 | 0.0052 | 0.0002 | 0.9995 | 0.9995 |
| 2 | Low | Trehalose | 1.0000 | 1.0000 | 0.0771 | 0.5101 | 0.9995 | 1.0000 | 0.0002 | 0.0214 | 0.0214 |
| 3 | Low | Proline | 0.2272 | 0.2272 | 1.0000 | 1.0000 | 0.9774 | 0.8230 | 0.0002 | 1.0000 | 1.0000 |
| 4 | Low | Mannitol | 0.5101 | 0.5101 | 1.0000 | 1.0000 | 0.9995 | 0.9774 | 0.0002 | 0.9995 | 0.9995 |
| 5 | Low | Sorbitol | 0.8230 | 0.8230 | 0.9995 | 1.0000 | 1.0000 | 0.9995 | 0.0002 | 0.9774 | 0.9774 |
| 6 | Low | Cholesterol | 1.0000 | 1.0000 | 0.0771 | 0.5101 | 0.9995 | 1.0000 | 0.0002 | 0.0214 | 0.0214 |
| 7 | Low | Ethyl glycol | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 |
| 8 | Low | Glucose | 0.0771 | 0.0771 | 1.0000 | 1.0000 | 0.8230 | 0.5101 | 0.0002 | 1.0000 | 1.0000 |
| 9 | Low | Glycerol | 0.2272 | 0.2272 | 1.0000 | 1.0000 | 0.9774 | 0.8230 | 0.0002 | 1.0000 | 1.0000 |
| 10 | Medium | Control |  | 1.0000 | 0.0771 | 0.5101 | 0.9995 | 1.0000 | 0.0002 | 0.0214 | 0.0214 |
| 11 | Medium | Trehalose | 1.0000 |  | 0.0771 | 0.5101 | 0.9995 | 1.0000 | 0.0002 | 0.0214 | 0.0214 |
| 12 | Medium | Proline | 0.0771 | 0.0771 |  | 1.0000 | 0.8230 | 0.5101 | 0.0002 | 1.0000 | 1.0000 |
| 13 | Medium | Mannitol | 0.5101 | 0.5101 | 1.0000 |  | 0.9995 | 0.9774 | 0.0002 | 0.9995 | 0.9995 |
| 14 | Medium | Sorbitol | 0.9995 | 0.9995 | 0.8230 | 0.9995 |  | 1.0000 | 0.0002 | 0.5101 | 0.5101 |
| 15 | Medium | Cholesterol | 1.0000 | 1.0000 | 0.5101 | 0.9774 | 1.0000 |  | 0.0002 | 0.2772 | 0.2772 |
| 16 | Medium | Ethyl glycol | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 |  | 0.0002 | 0.0002 |
| 17 | Medium | Glucose | 0.0214 | 0.0214 | 1.0000 | 0.9995 | 0.5101 | 0.2272 | 0.0002 |  | 1.0000 |
| 18 | Medium | Glycerol | 0.0214 | 0.0214 | 1.0000 | 0.9995 | 0.5101 | 0.2272 | 0.0002 | 1.0000 |  |
| 19 | High | Control | 1.0000 | 1.0000 | 0.0771 | 0.5101 | 0.9995 | 1.0000 | 0.0002 | 0.0214 | 0.0214 |
| 20 | High | Trehalose | 1.0000 | 1.0000 | 0.5101 | 0.9774 | 1.0000 | 1.0000 | 0.0002 | 0.2272 | 0.2272 |
| 21 | High | Proline | 0.5101 | 0.5101 | 1.0000 | 1.0000 | 0.9995 | 0.9774 | 0.0002 | 0.9995 | 0.9995 |
| 22 | High | Mannitol | 0.5101 | 0.5101 | 1.0000 | 1.0000 | 0.9995 | 0.9774 | 0.0002 | 0.9995 | 0.9995 |
| 23 | High | Sorbitol | 0.9774 | 0.9774 | 0.9774 | 1.0000 | 1.0000 | 1.0000 | 0.0002 | 0.8230 | 0.8230 |
| 24 | High | Cholesterol | 1.0000 | 1.0000 | 0.2272 | 0.8230 | 1.0000 | 1.0000 | 0.0002 | 0.0771 | 0.0771 |
| 25 | High | Ethyl glycol | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 1.0000 | 0.0002 | 0.0002 |
| 26 | High | Glucose | 0.0052 | 0.0052 | 1.0000 | 0.9774 | 0.2272 | 0.0771 | 0.0002 | 1.0000 | 1.0000 |
| 27 | High | Glycerol | 0.0002 | 0.0002 | 0.0771 | 0.0052 | 0.0002 | 0.0002 | 0.0002 | 0.2272 | 0.2272 |

| Cell no. | Dosage | Chemical | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Low | Control | 0.0003 | 0.0052 | 0.5101 | 0.5101 | 0.0771 | 0.0012 | 0.0002 | 1.0000 | 0.9774 |
| 2 | Low | Trehalose | 1.0000 | 1.0000 | 0.5101 | 0.5101 | 0.9774 | 1.0000 | 0.0002 | 0.0052 | 0.0002 |
| 3 | Low | Proline | 0.2272 | 0.8230 | 1.0000 | 1.0000 | 0.9995 | 0.5101 | 0.0002 | 0.9995 | 0.0214 |
| 4 | Low | Mannitol | 0.2101 | 0.9774 | 1.0000 | 1.0000 | 1.0000 | 0.8230 | 0.0002 | 0.9774 | 0.0052 |
| 5 | Low | Sorbitol | 0.8230 | 0.9995 | 1.0000 | 1.0000 | 1.0000 | 0.9774 | 0.0002 | 0.8230 | 0.012 |
| 6 | Low | Cholesterol | 1.0000 | 1.0000 | 0.5101 | 0.5101 | 0.9774 | 1.0000 | 0.0002 | 0.0052 | 0.0002 |
| 7 | Low | Ethyl glycol | 0.0000 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0003 | 0.5101 |
| 8 | Low | Glucose | 0.0771 | 0.5101 | 1.0000 | 1.0000 | 0.9774 | 0.2272 | 0.0002 | 1.0000 | 0.0771 |
| 9 | Low | Glycerol | 0.2272 | 0.8230 | 1.0000 | 1.0000 | 0.9995 | 0.5101 | 0.0002 | 0.9995 | 0.0214 |
| 10 | Medium | Control | 1.0000 | 1.0000 | 0.5101 | 0.5101 | 0.9774 | 1.0000 | 0.0002 | 0.0052 | 0.0002 |
| 11 | Medium | Trehalose | 1.0000 | 1.0000 | 0.5101 | 0.5101 | 0.9774 | 1.0000 | 0.0002 | 0.0052 | 0.0002 |
| 12 | Medium | Proline | 0.0771 | 0.5101 | 1.0000 | 1.0000 | 0.9774 | 0.2272 | 0.0002 | 1.0000 | 0.0771 |
| 13 | Medium | Mannitol | 0.5101 | 0.9774 | 1.0000 | 1.0000 | 1.0000 | 0.8230 | 0.0002 | 0.9774 | 0.0052 |
| 14 | Medium | Sorbitol | 0.9995 | 1.0000 | 0.9995 | 0.9995 | 1.0000 | 1.0000 | 0.0002 | 0.2272 | 0.0002 |
| 15 | Medium | Cholesterol | 1.0000 | 1.0000 | 0.9774 | 0.9774 | 1.0000 | 1.0000 | 0.0002 | 0.0771 | 0.0002 |
| 16 | Medium | Ethyl glycol | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 1.0000 | 0.0002 | 0.0002 |
| 17 | Medium | Glucose | 0.0214 | 0.2272 | 0.9995 | 0.9995 | 0.8230 | 0.0771 | 0.0002 | 1.0000 | 0.2272 |
| 18 | Medium | Glycerol | 0.0214 | 0.2272 | 0.9995 | 0.9995 | 008230 | 0.0771 | 0.0002 | 1.0000 | 0.2272 |
| 19 | High | Control |  | 1.0000 | 0.5101 | 0.5101 | 0.9774 | 1.0000 | 0.0002 | 0.0052 | 0.0002 |
| 20 | High | Trehalose | 1.0000 |  | 0.9774 | 0.9774 | 1.0000 | 1.0000 | 0.0002 | 0.0771 | 0.0002 |
| 21 | High | Proline | 0.5101 | 0.9774 |  | 1.0000 | 1.0000 | 0.8230 | 0.0002 | 0.9774 | 0.0052 |
| 22 | High | Mannitol | 0.5101 | 0.9774 | 1.0000 |  | 1.0000 | 0.8230 | 0.0002 | 0.9774 | 0.0052 |
| 23 | High | Sorbitol | 0.9774 | 1.0000 | 1.0000 | 1.0000 |  | 0.9995 | 0.0002 | 0.5101 | 0.0003 |
| 24 | High | Cholesterol | 1.0000 | 1.0000 | 0.8230 | 0.8230 | 0.9995 |  | 0.0002 | 0.0214 | 0.0002 |
| 25 | High | Ethyl glycol | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 | 0.0002 |  | 0.0002 | 0.0002 |
| 26 | High | Glucose | 0.0052 | 0.0771 | 0.9774 | 0.9774 | 0.5101 | 0.0214 | 0.0002 |  | 0.5101 |
| 27 | High | Glycerol | 0.0002 | 0.0002 | 0.0052 | 0.0052 | 0.0003 | 0.0002 | 0.0002 | 0.5101 |  |

# APPENDIX 2

**Appendix 2a:** Univariate Tests of Significance for developmental rate

| | DF | F- value | p- value |
|---|---|---|---|
| Parent generation | 26 | 40.6607143 | < 0.0001 |
| F1 generation | 26 | 80.0119048 | < 0.0001 |
| F2 generation | 26 | 142.375 | < 0.0001 |
| F3 generation | 26 | 56.1521739 | < 0.0001 |
| F4 generation | 26 | 53.1041667 | < 0.0001 |

**Appendix 2b:** p-values from the ANOVA performed for developmental time

| **Parent** | Glycerol | Trehalose | Control | Mannitol | Cholesterol | Glucose | Sorbitol | Proline | Honey |
|---|---|---|---|---|---|---|---|---|---|
| Glycerol | | 0.000174 | 0.000192 | 0.000173 | 0.000173 | 0.000723 | 0.000230 | 0.000723 | 0.960640 |
| Trehalose | 0.000174 | | 0.960640 | 0.738985 | 0.006230 | 0.171820 | 0.738985 | 0.171820 | 0.000173 |
| Control | 0.000192 | 0.960640 | | 0.171820 | 0.000723 | 0.738985 | 0.999597 | 0.738985 | 0.000174 |
| Mannitol | 0.000173 | 0.738985 | 0.171820 | | 0.171820 | 0.006230 | 0.061047 | 0.006230 | 0.000173 |
| Cholesterol | 0.000173 | 0.006230 | 0.000723 | 0.171820 | | 0.000192 | 0.000341 | 0.000192 | 0.000173 |
| Glucose | 0.000723 | 0.171820 | 0.738985 | 0.006230 | 0.000192 | | 0.960640 | 1.000000 | 0.000230 |
| Sorbitol | 0.000230 | 0.738985 | 0.999597 | 0.061047 | 0.000341 | 0.960640 | | 0.960640 | 0.000177 |
| Proline | 0.000723 | 0.171820 | 0.738985 | 0.006230 | 0.000192 | 1.000000 | 0.960640 | | 0.000230 |
| Honey | 0.960640 | 0.000173 | 0.000174 | 0.000173 | 0.000173 | 0.000230 | 0.000177 | 0.000230 | |

| **F1** | Glycerol | Trehalose | Control | Mannitol | Cholesterol | Glucose | Sorbitol | Proline | Honey |
|---|---|---|---|---|---|---|---|---|---|
| Glycerol | | 0.000175 | 0.000437 | 0.000173 | 0.000173 | 0.002055 | 0.000174 | 0.000173 | 0.383058 |
| Trehalose | 0.000175 | | 0.383058 | 0.000878 | 0.000173 | 0.082957 | 0.999910 | 0.888164 | 0.000173 |
| Control | 0.000437 | 0.383058 | | 0.000177 | 0.000173 | 0.988205 | 0.189231 | 0.033786 | 0.000175 |
| Mannitol | 0.000173 | 0.000878 | 0.000177 | | 0.013248 | 0.000174 | 0.002055 | 0.013248 | 0.000173 |
| Cholesterol | 0.000173 | 0.000173 | 0.000173 | 0.013248 | | 0.000173 | 0.000173 | 0.000175 | 0.000173 |
| Glucose | 0.002055 | 0.082957 | 0.988205 | 0.000174 | 0.000173 | | 0.033786 | 0.005151 | 0.000191 |
| Sorbitol | 0.000174 | 0.999910 | 0.189231 | 0.002055 | 0.000173 | 0.033786 | | 0.988205 | 0.000173 |
| Proline | 0.000173 | 0.888164 | 0.033786 | 0.013248 | 0.000175 | 0.005151 | 0.988205 | | 0.000173 |
| Honey | 0.383058 | 0.000173 | 0.000175 | 0.000173 | 0.000173 | 0.000191 | 0.000173 | 0.000173 | |

| **F2** | Glycerol | Trehalose | Control | Mannitol | Cholesterol | Glucose | Sorbitol | Proline | Honey |
|---|---|---|---|---|---|---|---|---|---|
| Glycerol | | 0.000173 | 0.000173 | 0.000173 | 0.000173 | 0.000240 | 0.000173 | 0.000173 | 0.905146 |
| Trehalose | 0.000173 | | 0.905146 | 0.000427 | 0.000173 | 0.000240 | 1.000000 | 0.998657 | 0.000173 |
| Control | 0.000173 | 0.905146 | | 0.000192 | 0.000173 | 0.001221 | 0.905146 | 0.998657 | 0.000173 |
| Mannitol | 0.000173 | 0.000427 | 0.000192 | | 0.001221 | 0.000173 | 0.000427 | 0.000240 | 0.000173 |
| Cholesterol | 0.000173 | 0.000173 | 0.000173 | 0.001221 | | 0.000173 | 0.000173 | 0.000173 | 0.000173 |
| Glucose | 0.000240 | 0.000240 | 0.001221 | 0.000173 | 0.000173 | | 0.000240 | 0.000427 | 0.000176 |
| Sorbitol | 0.000173 | 1.000000 | 0.905146 | 0.000427 | 0.000173 | 0.000240 | | 0.998657 | 0.000173 |
| Proline | 0.000173 | 0.998657 | 0.998657 | 0.000240 | 0.000173 | 0.000427 | 0.998657 | | 0.000173 |
| Honey | 0.905146 | 0.000173 | 0.000173 | 0.000173 | 0.000173 | 0.000176 | 0.000173 | 0.000173 | |

| **F3** | Glycerol | Trehalose | Control | Mannitol | Cholesterol | Glucose | Sorbitol | Proline | Honey |
|---|---|---|---|---|---|---|---|---|---|
| Glycerol | | 0.000174 | 0.000210 | 0.000173 | 0.000173 | 0.000250 | 0.000173 | 0.000173 | 0.910101 |
| Trehalose | 0.000174 | | 0.699867 | 0.699867 | 0.000250 | 0.438431 | 0.910101 | 0.910101 | 0.000173 |
| Control | 0.000210 | 0.699867 | | 0.046781 | 0.000174 | 0.999936 | 0.108272 | 0.108272 | 0.000174 |
| Mannitol | 0.000173 | 0.699867 | 0.046781 | | 0.003208 | 0.019322 | 0.999936 | 0.999936 | 0.000173 |
| Cholesterol | 0.000173 | 0.000250 | 0.000174 | 0.003208 | | 0.000174 | 0.001360 | 0.001360 | 0.000173 |
| Glucose | 0.000250 | 0.438431 | 0.999936 | 0.019322 | 0.000174 | | 0.046781 | 0.046781 | 0.000176 |
| Sorbitol | 0.000173 | 0.910101 | 0.108272 | 0.999936 | 0.001360 | 0.046781 | | 1.000000 | 0.000173 |
| Proline | 0.000173 | 0.910101 | 0.108272 | 0.999936 | 0.001360 | 0.046781 | 1.000000 | | 0.000173 |
| Honey | 0.910101 | 0.000173 | 0.000174 | 0.000173 | 0.000173 | 0.000176 | 0.000173 | 0.000173 | |

| **F4** | Glycerol | Trehalose | Control | Mannitol | Cholesterol | Glucose | Sorbitol | Proline | Honey |
|---|---|---|---|---|---|---|---|---|---|
| Glycerol | | 0.000175 | 0.000178 | 0.000173 | 0.000173 | 0.000414 | 0.000174 | 0.000173 | 0.919095 |
| Trehalose | 0.000175 | | 0.999946 | 0.054116 | 0.000273 | 0.464801 | 0.992295 | 0.919095 | 0.000173 |
| Control | 0.000178 | 0.999946 | | 0.022894 | 0.000219 | 0.721337 | 0.919095 | 0.721337 | 0.000174 |
| Mannitol | 0.000173 | 0.054116 | 0.022894 | | 0.121818 | 0.000772 | 0.252944 | 0.464801 | 0.000173 |
| Cholesterol | 0.000173 | 0.000273 | 0.000219 | 0.121818 | | 0.000174 | 0.000772 | 0.001676 | 0.000173 |
| Glucose | 0.000414 | 0.464801 | 0.721337 | 0.000772 | 0.000174 | | 0.121818 | 0.054116 | 0.000192 |
| Sorbitol | 0.000174 | 0.992295 | 0.919095 | 0.252944 | 0.000772 | 0.121818 | | 0.999946 | 0.000173 |
| Proline | 0.000173 | 0.919095 | 0.721337 | 0.464801 | 0.001676 | 0.054116 | 0.999946 | | 0.000173 |
| Honey | 0.919095 | 0.000173 | 0.000174 | 0.000173 | 0.000173 | 0.000192 | 0.000173 | 0.000173 | |

# APPENDIX 3

**Appendix 3a:** Univariate Tests of Significance for female weight

| | DF | F- value | p- value |
|---|---|---|---|
| Parent generation | 89 | 7.71670563 | < 0.0001 |
| F1 generation | 89 | 7.41831011 | < 0.0001 |
| F2 generation | 89 | 7.0185949 | < 0.0001 |
| F3 generation | 89 | 7.99187875 | < 0.0001 |
| F4 generation | 89 | 7.64516008 | < 0.0001 |

**Appendix 3b:** p-values from the ANOVA performed for female weight

| **Parent** | Glycerol | Trehalose | Control | Mannitol | Cholesterol | Glucose | Sorbitol | Proline | Honey |
|---|---|---|---|---|---|---|---|---|---|
| Glycerol | | 0.025324 | 1.000000 | 0.845683 | 1.000000 | 0.940695 | 0.054679 | 0.996097 | 0.253322 |
| Trehalose | 0.025324 | | 0.033167 | 0.604672 | 0.044623 | 0.000469 | 0.999999 | 0.001896 | 0.000134 |
| Control | 1.000000 | 0.033167 | | 0.886893 | 1.000000 | 0.912391 | 0.069947 | 0.991789 | 0.210439 |
| Mannitol | 0.845683 | 0.604672 | 0.886893 | | 0.924591 | 0.140492 | 0.779962 | 0.336449 | 0.004250 |
| Cholesterol | 1.000000 | 0.044623 | 1.000000 | 0.924591 | | 0.871293 | 0.091493 | 0.983131 | 0.168238 |
| Glucose | 0.940695 | 0.000469 | 0.912391 | 0.140492 | 0.871293 | | 0.001104 | 0.999961 | 0.947043 |
| Sorbitol | 0.054679 | 0.999999 | 0.069947 | 0.779962 | 0.091493 | 0.001104 | | 0.004710 | 0.000138 |
| Proline | 0.996097 | 0.001896 | 0.991789 | 0.336449 | 0.983131 | 0.999961 | 0.004710 | | 0.761821 |
| Honey | 0.253322 | 0.000134 | 0.210439 | 0.004250 | 0.168238 | 0.947043 | 0.000138 | 0.761821 | |

| **F1** | Glycerol | Trehalose | Control | Mannitol | Cholesterol | Glucose | Sorbitol | Proline | Honey |
|---|---|---|---|---|---|---|---|---|---|
| Glycerol | | 0.030893 | 1.000000 | 0.802469 | 0.999991 | 0.821950 | 0.302797 | 0.425747 | 0.668064 |
| Trehalose | 0.030893 | | 0.047580 | 0.706283 | 0.081742 | 0.000263 | 0.987604 | 0.000141 | 0.000177 |
| Control | 1.000000 | 0.047580 | | 0.877192 | 1.000000 | 0.733980 | 0.393168 | 0.331534 | 0.563631 |
| Mannitol | 0.802469 | 0.706283 | 0.877192 | | 0.945830 | 0.053613 | 0.996850 | 0.008642 | 0.026065 |
| Cholesterol | 0.999991 | 0.081742 | 1.000000 | 0.945830 | | 0.598804 | 0.528482 | 0.225914 | 0.425747 |
| Glucose | 0.821950 | 0.000263 | 0.733980 | 0.053613 | 0.598804 | | 0.004983 | 0.999474 | 0.999999 |
| Sorbitol | 0.302797 | 0.987604 | 0.393168 | 0.996850 | 0.528482 | 0.004983 | | 0.000650 | 0.002124 |
| Proline | 0.425747 | 0.000141 | 0.331534 | 0.008642 | 0.225914 | 0.999474 | 0.000650 | | 0.999991 |
| Honey | 0.668064 | 0.000177 | 0.563631 | 0.026065 | 0.425747 | 0.999999 | 0.002124 | 0.999991 | |

| **F2** | Glycerol | Trehalose | Control | Mannitol | Cholesterol | Glucose | Sorbitol | Proline | Honey |
|---|---|---|---|---|---|---|---|---|---|
| Glycerol | | 0.055027 | 0.999999 | 0.972783 | 1.000000 | 0.904796 | 0.277906 | 0.737190 | 0.257880 |
| Trehalose | 0.055027 | | 0.025743 | 0.512552 | 0.041996 | 0.000762 | 0.998527 | 0.000299 | 0.000138 |
| Control | 0.999999 | 0.025743 | | 0.904796 | 1.000000 | 0.972783 | 0.160122 | 0.879064 | 0.412173 |
| Mannitol | 0.972783 | 0.512552 | 0.904796 | | 0.954439 | 0.261152 | 0.916257 | 0.127962 | 0.016336 |
| Cholesterol | 1.000000 | 0.041996 | 1.000000 | 0.954439 | | 0.936384 | 0.229629 | 0.795253 | 0.309695 |
| Glucose | 0.904796 | 0.000762 | 0.972783 | 0.261152 | 0.936384 | | 0.007898 | 0.999995 | 0.971762 |
| Sorbitol | 0.277906 | 0.998527 | 0.160122 | 0.916257 | 0.229629 | 0.007898 | | 0.002602 | 0.000266 |
| Proline | 0.737190 | 0.000299 | 0.879064 | 0.127962 | 0.795253 | 0.999995 | 0.002602 | | 0.997509 |
| Honey | 0.257880 | 0.000138 | 0.412173 | 0.016336 | 0.309695 | 0.971762 | 0.000266 | 0.997509 | |

| **F3** | Glycerol | Trehalose | Control | Mannitol | Cholesterol | Glucose | Sorbitol | Proline | Honey |
|---|---|---|---|---|---|---|---|---|---|
| Glycerol | | 0.004070 | 0.999994 | 0.570160 | 0.998267 | 0.954092 | 0.097949 | 0.702362 | 0.918127 |
| Trehalose | 0.004070 | | 0.012351 | 0.515241 | 0.038184 | 0.000171 | 0.974107 | 0.000135 | 0.000154 |
| Control | 0.999994 | 0.012351 | | 0.790588 | 0.999986 | 0.829538 | 0.211964 | 0.470964 | 0.756755 |
| Mannitol | 0.570160 | 0.515241 | 0.790588 | | 0.945490 | 0.052657 | 0.988570 | 0.010046 | 0.036649 |
| Cholesterol | 0.998267 | 0.038184 | 0.999986 | 0.945490 | | 0.595158 | 0.418433 | 0.248695 | 0.505324 |
| Glucose | 0.954092 | 0.000171 | 0.829538 | 0.052657 | 0.595158 | | 0.002828 | 0.999733 | 1.000000 |
| Sorbitol | 0.097949 | 0.974107 | 0.211964 | 0.988570 | 0.418433 | 0.002828 | | 0.000461 | 0.001826 |
| Proline | 0.702362 | 0.000135 | 0.470964 | 0.010046 | 0.248695 | 0.999733 | 0.000461 | | 0.999964 |
| Honey | 0.918127 | 0.000154 | 0.756755 | 0.036649 | 0.505324 | 1.000000 | 0.001826 | 0.999964 | |

| **F4** | Glycerol | Trehalose | Control | Mannitol | Cholesterol | Glucose | Sorbitol | Proline | Honey |
|---|---|---|---|---|---|---|---|---|---|
| Glycerol | | 0.057657 | 0.999963 | 0.997956 | 1.000000 | 0.819475 | 0.300616 | 0.554082 | 0.131463 |
| Trehalose | 0.057657 | | 0.016703 | 0.304086 | 0.086997 | 0.000451 | 0.998086 | 0.000189 | 0.000134 |
| Control | 0.999963 | 0.016703 | | 0.958186 | 0.999609 | 0.967821 | 0.122090 | 0.826048 | 0.318173 |
| Mannitol | 0.997956 | 0.304086 | 0.958186 | | 0.999667 | 0.347429 | 0.773892 | 0.151897 | 0.018047 |
| Cholesterol | 1.000000 | 0.086997 | 0.999609 | 0.999667 | | 0.727901 | 0.393804 | 0.446806 | 0.089797 |
| Glucose | 0.819475 | 0.000451 | 0.967821 | 0.347429 | 0.727901 | | 0.004841 | 0.999971 | 0.946656 |
| Sorbitol | 0.300616 | 0.998086 | 0.122090 | 0.773892 | 0.393804 | 0.004841 | | 0.001199 | 0.000174 |
| Proline | 0.554082 | 0.000189 | 0.826048 | 0.151897 | 0.446806 | 0.999971 | 0.001199 | | 0.996341 |
| Honey | 0.131463 | 0.000134 | 0.318173 | 0.018047 | 0.089797 | 0.946656 | 0.000174 | 0.996341 | |

# APPENDIX 4

**Appendix 4a:** Univariate Tests of Significance for male weight

| | DF | F- value | p- value |
|---|---|---|---|
| Parent generation | 89 | 4.81461063 | 0.0001 |
| F1 generation | 89 | 4.39720739 | 0.0002 |
| F2 generation | 89 | 3.35424908 | 0.002 |
| F3 generation | 89 | 6.1463684 | < 0.0001 |
| F4 generation | 89 | 4.23802507 | 0.0003 |

**Appendix 4b:** p-values from the ANOVA performed for male weight

| **Parent** | Glycerol | Trehalose | Control | Mannitol | Cholesterol | Glucose | Sorbitol | Proline | Honey |
|---|---|---|---|---|---|---|---|---|---|
| Glycerol | | 0.540272 | 0.607648 | 0.871706 | 1.000000 | 0.325323 | 0.999962 | 0.926252 | 0.446480 |
| Trehalose | 0.540272 | | 0.005502 | 0.999775 | 0.404236 | 0.001298 | 0.272212 | 0.034833 | 0.002501 |
| Control | 0.607648 | 0.005502 | | 0.030260 | 0.741710 | 0.999962 | 0.864492 | 0.999574 | 1.000000 |
| Mannitol | 0.871706 | 0.999775 | 0.030260 | | 0.766301 | 0.008000 | 0.618814 | 0.142527 | 0.014878 |
| Cholesterol | 1.000000 | 0.404236 | 0.741710 | 0.766301 | | 0.451868 | 1.000000 | 0.972576 | 0.585229 |
| Glucose | 0.325323 | 0.001298 | 0.999962 | 0.008000 | 0.451868 | | 0.607648 | 0.980345 | 1.000000 |
| Sorbitol | 0.999962 | 0.272212 | 0.864492 | 0.618814 | 1.000000 | 0.607648 | | 0.994020 | 0.736678 |
| Proline | 0.926252 | 0.034833 | 0.999574 | 0.142527 | 0.972576 | 0.980345 | 0.994020 | | 0.995103 |
| Honey | 0.446480 | 0.002501 | 1.000000 | 0.014878 | 0.585229 | 1.000000 | 0.736678 | 0.995103 | |

| **F1** | Glycerol | Trehalose | Control | Mannitol | Cholesterol | Glucose | Sorbitol | Proline | Honey |
|---|---|---|---|---|---|---|---|---|---|
| Glycerol | | 0.768807 | 0.457840 | 0.999872 | 1.000000 | 0.116790 | 0.755086 | 0.975189 | 0.383688 |
| Trehalose | 0.768807 | | 0.008349 | 0.965216 | 0.609887 | 0.000795 | 0.032634 | 0.149792 | 0.005729 |
| Control | 0.457840 | 0.008349 | | 0.183404 | 0.625627 | 0.998471 | 0.999952 | 0.979280 | 1.000000 |
| Mannitol | 0.999872 | 0.965216 | 0.183404 | | 0.997572 | 0.030540 | 0.417658 | 0.803670 | 0.142170 |
| Cholesterol | 1.000000 | 0.609887 | 0.625627 | 0.997572 | | 0.202191 | 0.881470 | 0.995514 | 0.546478 |
| Glucose | 0.116790 | 0.000795 | 0.998471 | 0.030540 | 0.202191 | | 0.960829 | 0.702302 | 0.999596 |
| Sorbitol | 0.755086 | 0.032634 | 0.999952 | 0.417658 | 0.881470 | 0.960829 | | 0.999596 | 0.999728 |
| Proline | 0.975189 | 0.149792 | 0.979280 | 0.803670 | 0.995514 | 0.702302 | 0.999596 | | 0.960829 |
| Honey | 0.383688 | 0.005729 | 1.000000 | 0.142170 | 0.546478 | 0.999596 | 0.999728 | 0.960829 | |

| **F2** | Glycerol | Trehalose | Control | Mannitol | Cholesterol | Glucose | Sorbitol | Proline | Honey |
|---|---|---|---|---|---|---|---|---|---|
| Glycerol | | 0.890823 | 0.406444 | 0.990253 | 1.000000 | 0.521911 | 0.881637 | 0.993433 | 0.893787 |
| Trehalose | 0.890823 | | 0.014170 | 0.999930 | 0.940659 | 0.024043 | 0.119058 | 0.359621 | 0.127819 |
| Control | 0.406444 | 0.014170 | | 0.055344 | 0.315671 | 1.000000 | 0.997138 | 0.918195 | 0.996243 |
| Mannitol | 0.990253 | 0.999930 | 0.055344 | | 0.997138 | 0.087043 | 0.315671 | 0.671465 | 0.332885 |
| Cholesterol | 1.000000 | 0.940659 | 0.315671 | 0.997138 | | 0.420964 | 0.809409 | 0.981312 | 0.825321 |
| Glucose | 0.521911 | 0.024043 | 1.000000 | 0.087043 | 0.420964 | | 0.999568 | 0.963018 | 0.999380 |
| Sorbitol | 0.881637 | 0.119058 | 0.997138 | 0.315671 | 0.809409 | 0.999568 | | 0.999783 | 1.000000 |
| Proline | 0.993433 | 0.359621 | 0.918195 | 0.671465 | 0.981312 | 0.963018 | 0.999783 | | 0.999859 |
| Honey | 0.893787 | 0.127819 | 0.996243 | 0.332885 | 0.825321 | 0.999380 | 1.000000 | 0.999859 | |

| **F3** | Glycerol | Trehalose | Control | Mannitol | Cholesterol | Glucose | Sorbitol | Proline | Honey |
|---|---|---|---|---|---|---|---|---|---|
| Glycerol | | 0.698163 | 0.166301 | 0.976144 | 1.000000 | 0.032138 | 0.937365 | 0.966076 | 0.246947 |
| Trehalose | 0.698163 | | 0.000916 | 0.998733 | 0.674399 | 0.000190 | 0.072844 | 0.100306 | 0.001724 |
| Control | 0.166301 | 0.000916 | | 0.009001 | 0.179936 | 0.999224 | 0.884711 | 0.826055 | 1.000000 |
| Mannitol | 0.976144 | 0.998733 | 0.009001 | | 0.970722 | 0.001060 | 0.328449 | 0.405402 | 0.016375 |
| Cholesterol | 1.000000 | 0.674399 | 0.179936 | 0.970722 | | 0.035655 | 0.946844 | 0.972152 | 0.264842 |
| Glucose | 0.032138 | 0.000190 | 0.999224 | 0.001060 | 0.035655 | | 0.495043 | 0.411177 | 0.995027 |
| Sorbitol | 0.937365 | 0.072844 | 0.884711 | 0.328449 | 0.946844 | 0.495043 | | 1.000000 | 0.946844 |
| Proline | 0.966076 | 0.100306 | 0.826055 | 0.405402 | 0.972152 | 0.411177 | 1.000000 | | 0.908880 |
| Honey | 0.246947 | 0.001724 | 1.000000 | 0.016375 | 0.264842 | 0.995027 | 0.946844 | 0.908880 | |

| **F4** | Glycerol | Trehalose | Control | Mannitol | Cholesterol | Glucose | Sorbitol | Proline | Honey |
|---|---|---|---|---|---|---|---|---|---|
| Glycerol | | 0.410953 | 0.556387 | 0.889250 | 1.000000 | 0.609662 | 0.998088 | 0.989709 | 0.829377 |
| Trehalose | 0.410953 | | 0.002252 | 0.996816 | 0.425905 | 0.002919 | 0.093105 | 0.055254 | 0.009449 |
| Control | 0.556387 | 0.002252 | | 0.027538 | 0.540394 | 1.000000 | 0.942651 | 0.980115 | 0.999961 |
| Mannitol | 0.889250 | 0.996816 | 0.027538 | | 0.898278 | 0.034444 | 0.446143 | 0.321888 | 0.089586 |
| Cholesterol | 1.000000 | 0.425905 | 0.540394 | 0.898278 | | 0.593723 | 0.997604 | 0.987902 | 0.817343 |
| Glucose | 0.609662 | 0.002919 | 1.000000 | 0.034444 | 0.593723 | | 0.960438 | 0.987902 | 0.999991 |
| Sorbitol | 0.998088 | 0.093105 | 0.942651 | 0.446143 | 0.997604 | 0.960438 | | 1.000000 | 0.996342 |
| Proline | 0.989709 | 0.055254 | 0.980115 | 0.321888 | 0.987902 | 0.987902 | 1.000000 | | 0.999571 |
| Honey | 0.829377 | 0.009449 | 0.999961 | 0.089586 | 0.817343 | 0.999991 | 0.996342 | 0.999571 | |

# APPENDIX 5

**Appendix 5a:** Univariate Tests of Significance for the number of eggs laid per female

| | DF | F- value | p- value |
|---|---|---|---|
| Parent generation | 89 | 15.9084543 | < 0.0001 |
| F1 generation | 89 | 17.9042131 | < 0.0001 |
| F2 generation | 89 | 16.6960083 | < 0.0001 |
| F3 generation | 89 | 13.4496415 | < 0.0001 |
| F4 generation | 89 | 12.2185978 | < 0.0001 |

**Appendix 5b:** p-values from the ANOVA performed for the number of eggs laid per female

| **Parent** | Glycerol | Trehalose | Control | Mannitol | Cholesterol | Glucose | Sorbitol | Proline | Honey |
|---|---|---|---|---|---|---|---|---|---|
| Glycerol | | 0.000134 | 0.892330 | 0.999933 | 0.159072 | 0.587743 | 0.581141 | 0.999998 | 0.009708 |
| Trehalose | 0.000134 | | 0.000132 | 0.000150 | 0.056296 | 0.000132 | 0.005799 | 0.000141 | 0.000132 |
| Control | 0.892330 | 0.000132 | | 0.630436 | 0.002875 | 0.999827 | 0.031262 | 0.742975 | 0.331152 |
| Mannitol | 0.999933 | 0.000150 | 0.630436 | | 0.388839 | 0.291147 | 0.861892 | 1.000000 | 0.002085 |
| Cholesterol | 0.159072 | 0.056296 | 0.002875 | 0.388839 | | 0.000512 | 0.997562 | 0.288588 | 0.000132 |
| Glucose | 0.587743 | 0.000132 | 0.999827 | 0.291147 | 0.000512 | | 0.006090 | 0.391840 | 0.678680 |
| Sorbitol | 0.581141 | 0.005799 | 0.031262 | 0.861892 | 0.997562 | 0.006090 | | 0.771738 | 0.000137 |
| Proline | 0.999998 | 0.000141 | 0.742975 | 1.000000 | 0.288588 | 0.391840 | 0.771738 | | 0.003716 |
| Honey | 0.009708 | 0.000132 | 0.331152 | 0.002085 | 0.000132 | 0.678680 | 0.000137 | 0.003716 | |

| **F1** | Glycerol | Trehalose | Control | Mannitol | Cholesterol | Glucose | Sorbitol | Proline | Honey |
|---|---|---|---|---|---|---|---|---|---|
| Glycerol | | 0.000132 | 0.770995 | 0.999958 | 0.229552 | 0.577084 | 0.918352 | 0.794850 | 0.003552 |
| Trehalose | 0.000132 | | 0.000132 | 0.000134 | 0.008830 | 0.000132 | 0.000235 | 0.000449 | 0.000132 |
| Control | 0.770995 | 0.000132 | | 0.480726 | 0.002256 | 0.999998 | 0.083832 | 0.040060 | 0.297489 |
| Mannitol | 0.999958 | 0.000134 | 0.480726 | | 0.484241 | 0.297489 | 0.993058 | 0.960752 | 0.000819 |
| Cholesterol | 0.229552 | 0.008830 | 0.002256 | 0.484241 | | 0.000833 | 0.952984 | 0.990871 | 0.000132 |
| Glucose | 0.577084 | 0.000132 | 0.999998 | 0.297489 | 0.000833 | | 0.037778 | 0.016729 | 0.480726 |
| Sorbitol | 0.918352 | 0.000235 | 0.083832 | 0.993058 | 0.952984 | 0.037778 | | 0.999999 | 0.000150 |
| Proline | 0.794850 | 0.000449 | 0.040060 | 0.960752 | 0.990871 | 0.016729 | 0.999999 | | 0.000137 |
| Honey | 0.003552 | 0.000132 | 0.297489 | 0.000819 | 0.000132 | 0.480726 | 0.000150 | 0.000137 | |

| **F2** | Glycerol | Trehalose | Control | Mannitol | Cholesterol | Glucose | Sorbitol | Proline | Honey |
|---|---|---|---|---|---|---|---|---|---|
| Glycerol | | 0.000133 | 0.831292 | 0.999957 | 0.285291 | 0.828759 | 0.957631 | 0.667553 | 0.002283 |
| Trehalose | 0.000133 | | 0.000132 | 0.000141 | 0.016571 | 0.000132 | 0.000336 | 0.002400 | 0.000132 |
| Control | 0.831292 | 0.000132 | | 0.555260 | 0.004752 | 1.000000 | 0.154527 | 0.030498 | 0.184001 |
| Mannitol | 0.999957 | 0.000141 | 0.555260 | | 0.562134 | 0.551824 | 0.998078 | 0.903322 | 0.000542 |
| Cholesterol | 0.285291 | 0.016571 | 0.004752 | 0.562134 | | 0.004674 | 0.944930 | 0.999584 | 0.000132 |
| Glucose | 0.828759 | 0.000132 | 1.000000 | 0.551824 | 0.004674 | | 0.152808 | 0.030061 | 0.185970 |
| Sorbitol | 0.957631 | 0.000336 | 0.154527 | 0.998078 | 0.944930 | 0.152808 | | 0.999368 | 0.000151 |
| Proline | 0.667553 | 0.002400 | 0.030498 | 0.903322 | 0.999584 | 0.030061 | 0.999368 | | 0.000133 |
| Honey | 0.002283 | 0.000132 | 0.184001 | 0.000542 | 0.000132 | 0.185970 | 0.000151 | 0.000133 | |

| **F3** | Glycerol | Trehalose | Control | Mannitol | Cholesterol | Glucose | Sorbitol | Proline | Honey |
|---|---|---|---|---|---|---|---|---|---|
| Glycerol | | 0.000145 | 0.847716 | 0.999490 | 0.392059 | 0.976148 | 0.375480 | 0.736632 | 0.029546 |
| Trehalose | 0.000145 | | 0.000132 | 0.000305 | 0.043735 | 0.000132 | 0.047087 | 0.009266 | 0.000132 |
| Control | 0.847716 | 0.000132 | | 0.461297 | 0.009670 | 0.999965 | 0.008878 | 0.045384 | 0.637800 |
| Mannitol | 0.999490 | 0.000305 | 0.461297 | | 0.791980 | 0.747579 | 0.776735 | 0.973587 | 0.004334 |
| Cholesterol | 0.392059 | 0.043735 | 0.009670 | 0.791980 | | 0.035352 | 1.000000 | 0.999852 | 0.000139 |
| Glucose | 0.976148 | 0.000132 | 0.999965 | 0.747579 | 0.035352 | | 0.032745 | 0.134741 | 0.353839 |
| Sorbitol | 0.375480 | 0.047087 | 0.008878 | 0.776735 | 1.000000 | 0.032745 | | 0.999784 | 0.000138 |
| Proline | 0.736632 | 0.009266 | 0.045384 | 0.973587 | 0.999852 | 0.134741 | 0.999784 | | 0.000198 |
| Honey | 0.029546 | 0.000132 | 0.637800 | 0.004334 | 0.000139 | 0.353839 | 0.000138 | 0.000198 | |

| **F4** | Glycerol | Trehalose | Control | Mannitol | Cholesterol | Glucose | Sorbitol | Proline | Honey |
|---|---|---|---|---|---|---|---|---|---|
| Glycerol | | 0.000134 | 0.996090 | 0.998833 | 0.566247 | 0.783281 | 0.539698 | 0.868324 | 0.298807 |
| Trehalose | 0.000134 | | 0.000132 | 0.000169 | 0.005182 | 0.000132 | 0.005884 | 0.000973 | 0.000132 |
| Control | 0.996090 | 0.000132 | | 0.848631 | 0.134529 | 0.996090 | 0.122973 | 0.365473 | 0.811736 |
| Mannitol | 0.998833 | 0.000169 | 0.848631 | | 0.933784 | 0.339907 | 0.922115 | 0.997338 | 0.064727 |
| Cholesterol | 0.566247 | 0.005182 | 0.134529 | 0.933784 | | 0.014954 | 1.000000 | 0.999882 | 0.001257 |
| Glucose | 0.783281 | 0.000132 | 0.996090 | 0.339907 | 0.014954 | | 0.013269 | 0.063281 | 0.997541 |
| Sorbitol | 0.539698 | 0.005884 | 0.122973 | 0.922115 | 1.000000 | 0.013269 | | 0.999795 | 0.001103 |
| Proline | 0.868324 | 0.000973 | 0.365473 | 0.997338 | 0.999882 | 0.063281 | 0.999795 | | 0.006685 |
| Honey | 0.298807 | 0.000132 | 0.811736 | 0.064727 | 0.001257 | 0.997541 | 0.001103 | 0.006685 | |

# APPENDIX 6

**Appendix 6a:** Univariate Tests of Significance for number of eggs not hatched per female

| | DF | F- value | p- value |
|---|---|---|---|
| Parent generation | 89 | 4.72014312 | 0.0001 |
| F1 generation | 89 | 3.30706475 | 0.0026 |
| F2 generation | 89 | 3.28115362 | 0.0027 |
| F3 generation | 89 | 1.54991196 | 0.1532 |
| F4 generation | 89 | 0.715201155 | 0.6774 |

**Appendix 6b:** p-values from the ANOVA performed for the n number of eggs not hatched per female

| **Parent** | Glycerol | Trehalose | Control | Mannitol | Cholesterol | Glucose | Sorbitol | Proline | Honey |
|---|---|---|---|---|---|---|---|---|---|
| Glycerol | | 0.974919 | 0.753807 | 0.954266 | 0.971490 | 0.012331 | 0.999975 | 1.000000 | 0.068201 |
| Trehalose | 0.974919 | | 0.140873 | 0.363442 | 0.420348 | 0.000390 | 0.852265 | 0.991027 | 0.002575 |
| Control | 0.753807 | 0.140873 | | 0.999918 | 0.999697 | 0.558155 | 0.937276 | 0.651537 | 0.900372 |
| Mannitol | 0.954266 | 0.363442 | 0.999918 | | 1.000000 | 0.262372 | 0.997051 | 0.908643 | 0.636176 |
| Cholesterol | 0.971490 | 0.420348 | 0.999697 | 1.000000 | | 0.219278 | 0.998816 | 0.937276 | 0.573837 |
| Glucose | 0.012331 | 0.000390 | 0.558155 | 0.262372 | 0.219278 | | 0.041691 | 0.007408 | 0.999595 |
| Sorbitol | 0.999975 | 0.852265 | 0.937276 | 0.997051 | 0.998816 | 0.041691 | | 0.999697 | 0.181275 |
| Proline | 1.000000 | 0.991027 | 0.651537 | 0.908643 | 0.937276 | 0.007408 | 0.999697 | | 0.044412 |
| Honey | 0.068201 | 0.002575 | 0.900372 | 0.636176 | 0.573837 | 0.999595 | 0.181275 | 0.044412 | |

| **F1** | Glycerol | Trehalose | Control | Mannitol | Cholesterol | Glucose | Sorbitol | Proline | Honey |
|---|---|---|---|---|---|---|---|---|---|
| Glycerol | | 0.800551 | 0.975252 | 0.947858 | 0.999311 | 0.056293 | 1.000000 | 0.961390 | 0.904369 |
| Trehalose | 0.800551 | | 0.169682 | 0.122464 | 0.385726 | 0.000399 | 0.777383 | 0.141247 | 0.086333 |
| Control | 0.975252 | 0.169682 | | 1.000000 | 0.999963 | 0.506870 | 0.980595 | 1.000000 | 0.999998 |
| Mannitol | 0.947858 | 0.122464 | 1.000000 | | 0.999674 | 0.605562 | 0.957197 | 1.000000 | 1.000000 |
| Cholesterol | 0.999311 | 0.385726 | 0.999963 | 0.999674 | | 0.248636 | 0.999577 | 0.999860 | 0.998282 |
| Glucose | 0.056293 | 0.000399 | 0.506870 | 0.605562 | 0.248636 | | 0.062797 | 0.563206 | 0.701720 |
| Sorbitol | 1.000000 | 0.777383 | 0.980595 | 0.957197 | 0.999577 | 0.062797 | | 0.968890 | 0.918584 |
| Proline | 0.961390 | 0.141247 | 1.000000 | 1.000000 | 0.999860 | 0.563206 | 0.968890 | | 1.000000 |
| Honey | 0.904369 | 0.086333 | 0.999998 | 1.000000 | 0.998282 | 0.701720 | 0.918584 | 1.000000 | |

| **F2** | Glycerol | Trehalose | Control | Mannitol | Cholesterol | Glucose | Sorbitol | Proline | Honey |
|---|---|---|---|---|---|---|---|---|---|
| Glycerol | | 0.935307 | 0.965748 | 0.914779 | 0.999572 | 0.024002 | 0.994824 | 0.988162 | 0.687371 |
| Trehalose | 0.935307 | | 0.288262 | 0.194676 | 0.628301 | 0.000422 | 0.462380 | 0.390930 | 0.068450 |
| Control | 0.965748 | 0.288262 | | 1.000000 | 0.999821 | 0.350479 | 0.999998 | 1.000000 | 0.999254 |
| Mannitol | 0.914779 | 0.194676 | 1.000000 | | 0.998076 | 0.477160 | 0.999907 | 0.999987 | 0.999955 |
| Cholesterol | 0.999572 | 0.628301 | 0.999821 | 0.998076 | | 0.118704 | 1.000000 | 0.999992 | 0.957090 |
| Glucose | 0.024002 | 0.000422 | 0.350479 | 0.477160 | 0.118704 | | 0.203862 | 0.254356 | 0.769980 |
| Sorbitol | 0.994824 | 0.462380 | 0.999998 | 0.999907 | 1.000000 | 0.203862 | | 1.000000 | 0.989855 |
| Proline | 0.988162 | 0.390930 | 1.000000 | 0.999987 | 0.999992 | 0.254356 | 1.000000 | | 0.995692 |
| Honey | 0.687371 | 0.068450 | 0.999254 | 0.999955 | 0.957090 | 0.769980 | 0.989855 | 0.995692 | |

| **F3** | Glycerol | Trehalose | Control | Mannitol | Cholesterol | Glucose | Sorbitol | Proline | Honey |
|---|---|---|---|---|---|---|---|---|---|
| Glycerol | | 0.995355 | 0.999909 | 0.964830 | 1.000000 | 0.379541 | 1.000000 | 1.000000 | 0.989389 |
| Trehalose | 0.995355 | | 0.919475 | 0.558068 | 0.996007 | 0.063716 | 0.984766 | 0.976348 | 0.691864 |
| Control | 0.999909 | 0.919475 | | 0.999201 | 0.999880 | 0.703511 | 0.999996 | 0.999999 | 0.999949 |
| Mannitol | 0.964830 | 0.558068 | 0.999201 | | 0.961417 | 0.971016 | 0.986451 | 0.991818 | 1.000000 |
| Cholesterol | 1.000000 | 0.996007 | 0.999880 | 0.961417 | | 0.368541 | 1.000000 | 0.999999 | 0.987988 |
| Glucose | 0.379541 | 0.063716 | 0.703511 | 0.971016 | 0.368541 | | 0.484416 | 0.533363 | 0.925190 |
| Sorbitol | 1.000000 | 0.984766 | 0.999996 | 0.986451 | 1.000000 | 0.484416 | | 1.000000 | 0.997101 |
| Proline | 1.000000 | 0.976348 | 0.999999 | 0.991818 | 0.999999 | 0.533363 | 1.000000 | | 0.998567 |
| Honey | 0.989389 | 0.691864 | 0.999949 | 1.000000 | 0.987988 | 0.925190 | 0.997101 | 0.998567 | |

| **F4** | Glycerol | Trehalose | Control | Mannitol | Cholesterol | Glucose | Sorbitol | Proline | Honey |
|---|---|---|---|---|---|---|---|---|---|
| Glycerol | | 1.000000 | 0.999840 | 0.999995 | 1.000000 | 0.788585 | 0.999928 | 0.999946 | 0.999840 |
| Trehalose | 1.000000 | | 0.999960 | 1.000000 | 0.999998 | 0.835010 | 0.999985 | 0.999795 | 0.999960 |
| Control | 0.999840 | 0.999960 | | 1.000000 | 0.998020 | 0.973472 | 1.000000 | 0.985963 | 1.000000 |
| Mannitol | 0.999995 | 1.000000 | 1.000000 | | 0.999795 | 0.931778 | 1.000000 | 0.996783 | 1.000000 |
| Cholesterol | 1.000000 | 0.999998 | 0.998020 | 0.999795 | | 0.658134 | 0.998861 | 1.000000 | 0.998020 |
| Glucose | 0.788585 | 0.835010 | 0.973472 | 0.931778 | 0.658134 | | 0.964745 | 0.491506 | 0.973472 |
| Sorbitol | 0.999928 | 0.999985 | 1.000000 | 1.000000 | 0.998861 | 0.964745 | | 0.990186 | 1.000000 |
| Proline | 0.999946 | 0.999795 | 0.985963 | 0.996783 | 1.000000 | 0.491506 | 0.990186 | | 0.985963 |
| Honey | 0.999840 | 0.999960 | 1.000000 | 1.000000 | 0.998020 | 0.973472 | 1.000000 | 0.985963 | |

# APPENDIX 7

**Appendix 7a:** Univariate Tests of Significance for night time and monthly temperature during the three trials

| | DF | F- value | p- value |
|---|---|---|---|
| Trial (month) | 2 | 242.81 | < 0.0001 |
| Temperature (monthly and night time) | 1 | 42.08 | < 0.0001 |
| Trial*Temperature | 1758 | 3.20 | 0.041109 |

**Appendix 7b:** p-values from the ANOVA performed for night time and monthly temperature during the three trials

| **Parent** | March (monthly) | March (night time) | July (monthly) | July (night time) | October (monthly) | October (night time) |
|---|---|---|---|---|---|---|
| March (monthly) | | 0.449305 | 0.000020 | 0.000020 | 0.000020 | 0.999951 |
| March (night time) | 0.449305 | | 0.000020 | 0.000020 | 0.000020 | 0.772918 |
| July (monthly) | 0.000020 | 0.000020 | | 0.000020 | 0.000020 | 0.000020 |
| July (night time) | 0.000020 | 0.000020 | 0.000020 | | 0.000020 | 0.000020 |
| October (monthly) | 0.000020 | 0.000020 | 0.000020 | 0.000020 | | 0.000024 |
| October (night time) | 0.999951 | 0.772918 | 0.000020 | 0.000020 | 0.000024 | |