# The influence of sirtuin-controlled circadian rhythmicity and diet on lifespan in the honey bee, *Apis mellifera*

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I hereby declare that this thesis is based on work conducted by myself and has not contributed to any other degree. Reference to ideas and work of others has been specifically acknowledged.

## Abstract

This thesis explores the role of sirtuin controlled circadian rhythmicity and diet on honey bee longevity. By manipulating the circadian period of newly emerged honey bees and using qPCR to measure the expression of metabolic and circadian genes, we attempt to understand the effect of non-24 h circadian periods on longevity. We found evidence that altering the circadian period to 20 h or 28 h resulted in reduced longevity and a 1.6-fold increase in mortality, disruption to circadian clockwork and observed that this effect was not additive with that of a high-EAA diet, suggesting a potential shared pathway (Chapter 3).

Rapamycin is an inhibitor of the *mTOR* pathway and has been shown to extend lifespan across species. We investigate the effect of rapamycin on longevity and appetite in the honey bee. We also observe the effects of rapamycin in combination with high-EAA diets. We found no evidence of any life extending effects and in some cases a reduction in longevity as well as polyphagic behaviour common in metabolic diseases (Chapter 4).

Methylation plays an important role in sirtuin controlled mediation of lifespan, however the study of methylation in insects is time consuming and costly due to low global methylation levels. In this study we aim to test the viability and suitability of common methods of measuring global and site-specific methylation changes. We determine that methylation levels were too low to effectively measure with generic ELISA-kits in the honey bee and this also made site specific analysis for circadian genes challenging (Chapter 5).

In the final chapter we analyse the effect of methionine on longevity and behaviour in the honey bees. By varying the dietary methionine content and analysing key metabolic genes *FOXO* and *Sir2* we attempt to determine the mechanisms controlling these effects. We found that a high methionine diet reduced lifespan, appetite and lead to upregulation of *FOXO* but not *Sir2* (Chapter 6).

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# List of abbreviations

| | |
|---|---|
| **µl** | Microliter |
| **10-HAD** | p10-Hydroxy-2-decenoic acid |
| **5-mC** | 5-Methylcytosine |
| **ADP** | Adenosine diphosphate |
| **ALS** | Amyotrophic Lateral Sclerosis |
| **AMP** | Adenosine monophosph |
| **AMPK** | AMP-activated kinase |
| **ANOVA** | Analysis of variance |
| **ATG** | Autophagy related protein |
| **ATP** | Adenosine triphosphate |
| **bgm** | Bubblegum gene |
| **bsDNA** | Bisulfite converted DNA |
| **C** | Carbohydrate |
| **CCD** | Colony Collapse Disorder |
| **cDNA** | Complementary DNA |
| **Clk** | Clock protein |
| **Coxreg** | Cox regression |
| **CP** | Circadian period |
| **CpG** | Cytosine-phosphate-guanine |
| **Cry2** | Cryptochrome-2 |
| **$C_t$** | $C_{threshold}$ value |
| **CYC** | Cycle protein |
| **DD** | Continuous darkness |
| **DI** | Deionised |
| **DL** | Dark:light |
| **DMGs** | Differentially methylated genes |
| **DNA** | Deoxyribonucleic acid |
| **DNase** | Deoxyribonuclease |
| **DNMT1** | DNA (cytosine-5)-methyltransferase 1 |
| **DR** | Dietary restriction |
| ***dTIM2*** | Drosophila Timeout |
| **EAA** | Essential amino acid |
| **ELISA** | Enzyme-linked immunosorbent assay |
| **FFA** | Free fatty acids |
| ***FOXO/A*** | Forkhead Box |
| **g** | Grams |
| **G** | Gravity |
| **Gnmt** | Glycine N-methyltransferase |

| | |
|---|---|
| **GSK- 3b** | Glycogen synthase kinase 3 beta |
| **H** | Hours |
| **$H_2O$** | Water |
| **hESCs** | Human embryonic stem cells |
| **HR** | Hazard ratio |
| **IGF** | Insulin-like growth factors |
| **IIS** | Insulin and insulin-like growth factor pathway |
| **JH** | Juvenile hormone |
| **L** | Litre |
| **LAN** | Light at night |
| **LD** | Light:dark |
| **LL** | Continuous light |
| **LSD** | Fisher's Least Significant Difference |
| **M** | Molar |
| **MBD4** | Methyl-CpG Binding Protein 4 |
| **MEDCs** | More economically developed countries |
| **MEFs** | Mouse embryonic fibroblasts |
| **Met-O** | Methionine sulfoxide |
| **min** | Minutes |
| **mitROS** | Mitochondrial ROS |
| **ml** | Millilitres |
| **mm** | Millimetres |
| **mRNA** | Messenger RNA |
| **Msrs** | Methionine sulfoxide reductase system |
| **mtDNA** | Mitochondrial DNA |
| ***mTOR*** | Mechanistic target of rapamycin |
| **MTORC1/2** | Mammalian target of rapamycin complex ½ |
| **NAD+** | Nicotinamide adenine dinucleotide |
| **NC** | Negative control |
| **ng** | Nanograms |
| **ºC** | Celsius |
| **OD** | Optica density |
| **PC** | Positive control |
| **PCR** | Polymerase chain reaction |
| **PER** | Period protein |
| **PKB** | Protein kinase B |
| **RFU** | Reflective fluorescence units |
| **RH** | Relative humidity |
| **RICTOR** | Rapamycin-insensitive companion of mTOR |
| **RNA** | Ribonucleic acid |

| | |
|---|---|
| **RNAi** | RNA interference |
| **RNase** | Ribonuclease |
| **ROS** | Reactive oxygen species |
| **RP49** | Ribosomal protein 49 |
| **RSK** | Ribosomal protein S6 kinase |
| **RTqPCR** | Real-time quantitative PCR |
| **s** | Seconds |
| **SAM** | S-adenosyl-methionine |
| **SCN** | Suprachiasmatic nucleus |
| **SGG** | Shaggy protein |
| **Sir2** | Sir2 histone deacetylase, transcript variant 1 |
| **siRNA** | Small interfering RNA |
| **Sirt1** | Sirtuin-1 |
| **TERT** | Telomerase reverse transcriptase |
| **Tim1** | Timeless protein 1 |
| **TIR** | Taste receptor type 1 |
| **TP** | Time point |
| **TSC2** | Tuberous Sclerosis Complex 2 |
| **VG** | Vitellogenin |
| **WGBS** | Whole-genome bisulfite sequencing |
| **ΔΔCt** | Delta Delta Ct |
| **µM** | Micromolar |
| **τ** | Tau |

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# 1.0 General Introduction

## 1.1 **The role of the honey bee in pollination**

The honey bee (*Apis mellifera*) is responsible for the pollination of a wide range of agricultural and naturally occurring plant life. As much as 75% of all agricultural crop produce is at least partially dependant on insect pollination (Hiyama and Hiyama, 2007), of which honey bees are a crucial component (Shay and Wright, 2000).

Since 1950 the global population has more than tripled (Powers *et al.*, 2009), and is projected to reach 11 billion by 2100 (Fargnoli *et al.*, 1990). This increase in population puts tremendous pressure on agriculture to produce enough food to feed an ever-growing population. This pressure has led to the adoption of techniques such as monoculture, pesticide use and destruction of naturally occurring habitats harbouring wild plants to maximise space and efficiency, all of which are known to be harmful to honey bee populations (Harrison et al., 2009; Bishop et al., 2010; Shavlakadze et al., 2018).

Honey bee populations worldwide are currently suffering from a phenomenon known as Colony Collapse Discord (CCD). This multifactorial problem is categorised by the abandonment of the hive by adult bees in the early spring, leaving capped brood and food stores behind and results in the loss of the hive. The US alone lost 5.81% of its total productive colonies in 2007 (Moskalev and Shaposhnikov, 2010). It is hypothesised that a combination of increased pesticide use, nutrient poor diets, parasites and climate change create pressure on colonies, which leave them vulnerable to disease, thus contributing to CCD (Cota *et al.*, 2006). One theory on the cause of CCD proposes that honey bees prematurely age during the winter months and then in the spring, when it becomes warm enough to fly, are biologically predispositioned to forage, leaving no nurses left to care for the hive (Howitz *et al.*, 2003).

Honey bees show unusual physiological and behavioural plasticity which make them potentially useful models for the study of ageing. Despite being genetically identical, honey bee queens can live years longer than worker honey bees, a factor which is thought to be controlled epigenetically via diet during development (Kanfi *et al.*, 2012). To protect global food production, it is important that we understand how diet and environment influence honey bee ageing and how that information can be used to improve honey bee health. There are also possibilities that such insights might be beneficial in the study of human health.

## 1.2 **What is ageing?**

Before investigating the role of sirtuin-controlled circadian rhythmicity and diet on longevity in honey bee, it is necessary to consider the concepts of aging introduced here in a better studied system, in this case humans. While humans are living longer and the process has been studied for almost as long as the practice of "medicine" has existed, we still have no complete picture of the causes of ageing. While the sentiment proposed by Medawar (1952) in his inaugural lecture in 1952 that ageing was "an unsolved problem in biology" is no longer quite true, it is still one of the less well understood topics of the last century. To better understand the concept of aging, it is first important to define what we consider as "ageing". From some perspectives ageing could refer to the accumulation of experience or the development of infants through to adults, however these definitions are not the subject of this research. Biologically speaking, the definition of "ageing" is understandably narrower but still not straight-forward. This is partly because the mechanisms, which drive the ageing process are not fully understood but also because there is no universally agreed upon definition of what "ageing" actually is.

The father of the free-radical theory of ageing, Denham Harman's definition is that "ageing is the result of the accumulation of changes in the body which occur with the passing of time and which cause the increase in the probability of disease and death of the individual" (Vina *et al.*, 2007)). Strehler and Mildvan (1960) defined ageing as "the inherent process(es) whereby organisms exhibit a gradual change in their physical, chemical, or physiological properties after reproductive maturity. These changes result in a gradual increase in the probability of death in the organism's normal environment". Rose (1994) provided one of the most widely recognised definitions "Ageing is a progressive decline in physiological function, leading to an age-dependent decrease in rates of survival and reproduction". When concerns for the generalisation of the use of "progressive" are taken into account (Flatt, 2012) then this definition is a suitable starting point to work from.

## 1.3 **Theories and purpose of ageing**

From an evolutionary point of view ageing makes little sense in the abstract. When an individual dies, it loses the ability to pass on its genes and so should be in the most literally sense "counter-productive". Some organisms such as symmetrically dividing bacteria are capable of, under favourable conditions, negligible senescence (Coelho *et al.*, 2013). Even larger organisms like cnidarians show no signs of ageing and maintain telomere length

between divisions (Martínez, 1998) and non-clonal organisms like lobsters show increased fertility with age and no signs of epigenetic ageing (Klapper *et al.*, 1998), so it is clearly not a biological impossibility.

As Dobzhansky (1973) said (paraphrasing the Jesuit priest Pierre Teilhard de Chardin), in one of the most quoted essay titles in science; "Nothing in Biology Makes Sense Except in the Light of Evolution", so there must be an explanation for such a trait being so widely present across almost all life-forms yet discovered. One of the co-discoverers of the theory of evolution, Russel Wallace was one of the first to address this issue in 1889

*"When one or more individuals have provided a sufficient number of successors, they themselves, as consumers of nourishment in a constantly increasing degree, are an injury to those successors. Natural selection therefore weeds them out, and in many cases favours such races as die almost immediately after they have left successors." (Weissman, 1889)*

Wallace proposed that ageing was a biological necessity and that from an evolutionary point of view it is better for a species that was composed of more, smaller and younger animals with easier to achieve metabolic requirements than fewer, older individuals who would be less likely to meet their metabolic needs and so be more vulnerable to changes in conditions, leading to death (Travis, 2004).

One of the more modern interpretations on the evolutionary purpose of ageing is the "disposable soma" theory (Jiang *et al.*, 2007). This theory suggests that ageing occurs because of a limited availability of resources (or 'soma') within the cell, which leads to a trade-off between processes that increase longevity such as deoxyribonucleic acid (DNA) maintenance and cell repair and processes necessary for growth and reproduction. This trade-off leads to cell damage and eventual cell death.

### 1.4 **Ageing and healthspan**

Across the globe life-expectancy is increasing and particularly in more economically developed countries (MEDCs), the proportion of the population which is over 65 has increased significantly. While undoubtably a positive thing, this has posed challenges to both the social security net (Tinker, 2002) and health care services (Jones, 2016) in providing for the elderly in later life. While suggesting the solution to the problems of people living longer is to invest in ways to further extend lifespan may sound counter-intuitive, investing in methods of increasing lifespan which also increase health span is not. It is only when people

live longer but do not see a slowdown in ageing that problems arise. This "avoiding the fate of Tithonus" (Richardson, 2013), the Trojan mythological figure who was granted immortality by Zeus but not eternal youth, resulting in unending suffering, is a critical factor in any intervention. It is not simply enough to delay death, the ageing process itself must be slowed and the years of which a person is healthy and mobile extended to see maximum benefits.

To improve health span alongside lifespan it is vitally important to understand the mechanisms and processes which drives our descent into old age. These mechanisms are many in number and not yet fully understood, but significant funding has been invested to unravel and explore the many potential underlying pathways that could be sources of clinical intervention. There is continual debate on the priority and importance of which areas should be researched and as many opinions as there are options, but some efforts have been made to seriously categorise significant targets.

### 1.5 **The Nine hallmarks of ageing**

López-Otín *et al.* (2013) states that there are "nine tentative hallmarks that represent common denominators of aging in different organisms… These hallmarks are: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication." These hallmarks of aging are discussed below.

#### 1.5.1 ***Genomic instability***

Since as early as the 1950's it has been thought that genomic damage acquired over time is a primary driver in the ageing process. The Free Radical theory of ageing was first proposed by Harman (1956) as an attempt to explain where this accumulation of damage within the cells could occur. He suggested (and later refined) a theory where unstable reactive oxygen species (ROS) produced by metabolic processes in the cell cause oxidative damage to the tissues (including the genetic material) over time. It has since become clear that ROS are not the only source of damage to the integrity and stability of genomic DNA (Hoeijmakers, 2009), and that they are continuously under attack by physical, chemical, and biological agents, as well as DNA replication errors and reactive oxygen species.

### 1.5.2 ***Telomere attrition***

One specific case of accumulated DNA damage might play a crucial role in the genetic determination of ageing. The end of each chromosome contains a repetitive stretch of nucleotides known as a telomere. These telomeres cap the chromosome and prevent deterioration or conjoining of chromosomes (Jiang *et al.*, 2007).

The discovery that DNA polymerases lacks the ability to replicate completely the terminal ends of DNA molecules lead to a new theory on the causes of ageing (Olovnikov, 1973). This deficiency in DNA polymerase leads to the progressive and cumulative loss of telomere caps from chromosomes. This can be repaired by telomerase reverse transcriptase (TERT) but this enzyme is only expressed in some stem cells (Hiyama and Hiyama, 2007). It was proposed that because of this telomeric shortening, most eukaryotic cells have a strict limit on the number of replications possible before apoptosis occurs, known as the Hayflick limit (Shay and Wright, 2000).

### 1.5.3 ***Epigenetic alterations***

Lifespan is determined by several factors both genetic and environmental. It is clear that heritable genetics plays an important role in ageing, accounting for up to 25% of measurable longevity (Herskind *et al.*, 1996). What makes up the other 75% comes from external or variable factors. One of the key ways in which the environment shapes ageing is through epigenetics. From the Greek ‘epi’ meaning ‘on top of’, epigenetics is a catch-all term for all processes which result in heritable changes in gene expression that are not the result of modifications to the genome (Holliday, 2006). These can be conformational changes to the supporting structure of DNA via histone modification (Turner, 2000), non-coding RNA (Mattick and Makunin, 2006) or direct reversible-modification of the bases that form DNA, the most common of which is methylation (Razin and Riggs, 1980). DNA methylation provides a method by which the basic and stable genomic sequence does not need to change for dynamic and flexible changes to proteogenesis (Boyes and Bird, 1991). This happens because the methylation state can determine if an exon is ‘read’ or not, allowing for the transcription of different proteins to be turned on or off depending on cell sate (Bird, 1986).

DNA methylation is the addition of a methyl group ($-CH_3$) to the position 5 carbon in the base of a DNA molecule (Cheng *et al.*, 2011). Of the 4 base pairs that make up genomic DNA, methylation occurs by far most frequently in cytosine, specifically when cytosine is followed by guanine along the 5’-3’ base sequence (Hattori and Ushijima, 2011). These

dinucleotides are known as CpG (cytosine-phosphate-guanine) sites. These sites are not spread uniformly and display certain patterns, tending to aggregate at the 5' end of a gene; these aggregations of CpG sites are known as a 'CpG island' (Larsen *et al.*, 1992). This methylation of cytosine by DNA methyltransferases to form 5-methylcytosine has dramatic consequences on the expression of genomic DNA. CpG islands which are hypermethylated are often associated with genes that are transcriptionally repressed whereas genes following CpG islands which are hypomethylated are usually transcriptionally activated (Deaton and Bird, 2011). Methylation in the promoter region silences genes by inhibiting the binding of transcription factors or by recruitment of a protein with a methyl-CpG binding domain (MBD), which then form a complex with histone-deacetylases, condensing the histones (Illingworth and Bird, 2009).

DNA methylation is an essential process in development, some but not all of its responsibilities include X-chromosome inactivation (Allen *et al.*, 1992), tissue-specific gene expression (Christensen *et al.*, 2009), as well as silencing of imprinted genes from the parents (Li *et al.*, 1993). Changes to the methylome have a significant stochastic component as well as being vulnerable to environmental modification (Feinberg and Irizarry, 2010). These influences are felt most strongly during development and early life, where conditions can have a large impact on future health and lifespan. This is also true of parents, the health of a parent methylome plays a key role in determining the epigenetic makeup of the offspring (Sharp *et al.*, 2015). Methylation can also change with age. Large scale increases in methylation have been observed across the genome in humans, while local demethylation in promoter regions of genes is a notable feature of some age-related diseases (van Otterdijk *et al.*, 2013).

Because they are controlled by outside factors, epigenetic alterations to genomic material are not permanent. This reversibility is the reason they are a potential target for therapeutic interventions to increase life and healthspan. Changes to the methylome are distinct features of many age-related diseases in humans such as Alzheimer's (Prasad and Jho, 2019), various cancers (Irizarry *et al.*, 2009; Fang *et al.*, 2011; Cheong *et al.*, 2016) and diabetes (Thongsroy *et al.*, 2017) and there is significant evidence to suggest that modifying the DNA methylation profile might go some way to alleviating the effects of some of these. It has been proposed that the life-extending effects of dietary restriction (DR) are at least in part due to the Sirt1-mediated maintenance of DNA methylation patterns (Wakeling *et al.*, 2009). DR has shown to reduce the instances of age associated diseases in organisms such as rats, mice and

primates (Kang *et al.*, 1998; Guo *et al.*, 2002; Colman *et al.*, 2014) in which it extends lifespan (Fontana and Klein, 2007) but also extends lifespan in mice which are disease-free at the time of death (Shimokawa *et al.*, 1993). This is in keeping with the disease specific and general age-related changes in DNA methylation patterns.

Aside from epigenetic drift, there are other ways in which the methylome can change over time. One of the most important is changes in the physiological state of the cell. Oxidative stress is known to result in stimulation of methyl-CpG binding protein 4 (MBD4) activity and, in conjunction with DNA (cytosine-5)-methyltransferase 1 (DNMT1), mediates methyl-DNA repression thus protecting mammalian cells from oxidative damage (Laget *et al.*, 2014). This is especially important during development, where gestation conditions can radically alter the epigenetic profile and subsequent life and healthspan of an individual (Fernandez-Twinn and Ozanne, 2006). Methylome profiling of centenarians in humans and their offspring showed a delay in the global hypomethylation that occurs as we age, as well as a subsequent delay in age-related diseases (Gentilini *et al.*, 2013). It is clear that maintenance of the methylome plays an important part in the regulation of both life and health span and is one potential route for alleviating the effects of age-related declines in health.

### 1.5.4 ***Loss of proteostasis***

Ageing and diseases associated with ageing often result in a loss of the ability to maintain homeostasis of proteins within the cell known as "proteostasis" (Powers *et al.*, 2009). Many of the mechanisms by which the cell identifies and fixes or destroys misfolded proteins, such as the response of the heat-shock family of proteins are depressed with age (Fargnoli *et al.*, 1990).

Eukaryotes have many defences that enable them to survive, grow and reproduce when presented with environmental challenges. These challenges include nutrient deprivation, oxidative stress, and chemical stressors that disrupt cytoplasmic balance or cause a loss of proteostasis (Madeo *et al.*, 2015). One important method of repairing and preventing cellular damage from the environment is autophagy (Neufeld, 2010). Autophagy is the intracellular controlled degradation and recycling of defective or unnecessary cellular components such as damaged organelles or long-lived proteins (Cuervo *et al.*, 2005b). Autophagy is especially important in cells that are depleted of nutrients, as it provides vital resources to enable survival in such circumstances (Lum *et al.*, 2005).

While there are several forms of autophagy, including micro and chaperone-mediated, the primary form in the cell is macroautophagy (referred to as autophagy from this point onwards). This phylogenetically conserved catabolic pathway delivers unwanted cytoplasmic components to the lysosomal membrane for enzymatic breakdown (Straus, 1954). This process involves the engulfment of defective or redundant organelles or proteins and their surrounding cytoplasm into double-membraned vesicles known as autophagosomes. These autophagosomes then deliver the contents to the lysosomes. The autophagosomes fuses membranes with the lysosome, which use enzymes and an acidic pH to degrade the vesicle contents (De Duve and Wattiaux, 1966). This process provides the cells with a course of free fatty acids (FFA) and amino acids, which can be recycled to synthesise new proteins or metabolised to replenish (ATP) stocks when cells are depleted of nutrients (i.e. during starvation) (Klionsky and Emr, 2000). Autophagy is controlled by Autophagy-related (ATG) proteins, whose genes are highly conserved and present in various forms in almost all eukaryotes such as yeast, mammals and insects (Meijer *et al.*, 2007), including the honey bee (Crozier and Crozier, 1993).

It is this failure in autophagy and subsequent build-up of damaged or superfluous organelles and macromolecules that is a hallmark of aged cells and degenerative diseases (Ravikumar *et al.*, 2005). This failure to remove misfolded proteins leading to aggregates is a defining symptom in age related diseases like Parkinson's (Trojanowski *et al.*, 1998), Alzheimer's (Hashimoto *et al.*, 2003), amyotrophic lateral sclerosis (ALS) (Watanabe *et al.*, 2001), Huntington's (Wyttenbach *et al.*, 2000) and amyloidosis (Koo *et al.*, 1999). Indeed, there is hope that there could be a common therapeutic target for all such similar diseases (Bucciantini *et al.*, 2002). It has been proposed that this is the method by which DR, which stimulates autophagy and leads to "cleaner" cells, extends lifespan (Cuervo et al., 2005).

In most circumstances, when resources are abundant, autophagy proceeds at a relatively slow rate. It is not until external sources of nutrients and their markers (specifically amino-acids and glucose) are depleted that this background rate increases, allowing cells to recycle nutrients providing a crucial resource in times of shortage (Ohsumi, 2014). One such example of this is the induction of autophagy due to amino-acid deprivation in perfused rat liver (Mortimore and Schworer, 1977); this suggests that it may not be general caloric restriction that extends lifespan but specific nutrient restriction (Min and Tatar, 2006; Grandison *et al.*, 2009). As such, eukaryotic cells can only use autophagy to remove faulty organelles during times of fasting or nutrient depletion. This is a key problem in degenerative diseases which

feature dysregulation in autophagy pathways. Autophagy is the only method by which the cell can rid itself of defective mitochondria and other damaged organelles so dysfunction in these pathways leads to potentially damaging build-ups (Ding and Yin, 2012).

Hansen *et al.* (2008) investigated the relationship between DR, autophagy and life-extension in the nematode *C. elegans*. As well as finding that inhibiting genes required for autophagy attenuated the life-extending effects of DR, they also found that transcription factor PHA-4/FOXA is required for autophagy to take place, suggesting that changes in gene expression are required for DR to stimulate autophagy. While autophagy is crucial in the life-extension associated with DR, it also requires other transcription factors like DAF-16/FOXO to make use of the recycled material to create new structures and lengthen lifespan. When nematodes are subject to DR, as well as siRNA to knockdown *ATG* genes, the life-extending effects are absent (Jia and Levine, 2007).

In untreated cells and organisms lysosomal activity declines with age, leading to impaired autophagy (Cuervo and Dice, 2000). In rats, the rate of autophagy is directly linked to remaining life expectancy and inversely correlates with the age-associated build-up of lipids like dolichol in the liver, which damages the composition and stability of cell membranes (Kalén *et al.*, 1989). Both the decline in autophagy and build-up of harmful metabolic products is inhibited by DR (Marino *et al.*, 1998) in a dose (Cavallini *et al.*, 2002) and time (Dolfi *et al.*, 2003) dependent manner. Alzheimer's in humans is the most common age dependent disease with a high economic burden, afflicting over 40% of those aged 80 and over. As previously stated this disease is characterised by a build-up of proteins in the neurons called amyloid-β plaques. This build up is thought to be associated with aberrant autophagy and a general decline in autophagic activity (Funderburk *et al.*, 2010). It is also thought that autophagy could play a role in limiting DNA damage and chromosomal instability (Mathew *et al.*, 2007).

### 1.5.5 ***Deregulated nutrient sensing***

The cell has multiple systems that measure and regulate metabolic pathways to maintain homeostasis. The four key protein groups in this process are insulin-like growth factors (IGF-1), mechanistic target of rapamycin (mTOR), sirtuins and AMP-activated kinase (AMPK).

mTOR is a collection of two protein complexes called mTORC1 and mTORC2. Its primary responsibility is the sensing of amino acids and nutrient abundance within the cell. Because of this, it plays an important role in the synthesis of new molecules within the cell

(anabolism) (Cota *et al.*, 2006). *mTOR* activity increases with age and attenuating this with inhibitors like rapamycin result in extended longevity across a range of model organisms including mice (Komarova *et al.*, 2012; Neff *et al.*, 2013), rats (Kolosova *et al.*, 2012; Singh *et al.*, 2017), *Drosophila* (Zid *et al.*, 2009) and the roundworm *Caenorhabditis elegans (Lee et al., 2006)*.

IGF-1 works in conjunction with insulin to detect glucose as part of the "insulin and insulin-like growth factor" (*IIS*) pathway. This pathway plays an important role in anabolism. The relationship between the *IIS* pathway and ageing is not well understood. While attenuation of the pathway leads to extended longevity in some models, in naturally ageing organisms the *IIS* pathway activity decreases with age (Bishop *et al.*, 2010).

Sirtuins are a family of nicotinamide adenine dinucleotide ($NAD^+$) dependent histone deacetylase proteins which control access to the DNA by facilitating or denying spatial access for enzymes like RNA polymerase to attach to the DNA, through modification of lysines on the histone surface (Sauve *et al.*, 2006). When nutrient levels are low, $NAD^+$ levels increase, which are sensed by sirtuins. This causes them to modify the histones into a more tightly wrapped configuration and forcing it into a less transcriptionally active state, as well as helping to moderate catabolic metabolism. Upregulation of some sirtuins has been shown to have positive effects on survival (Howitz *et al.*, 2003; Kanfi *et al.*, 2012), although results are mixed (Smith *et al.*, 2007).

AMPK senses AMP adenosine monophosphate (AMP) and adenosine diphosphate (ADP), the precursors to ATP, which are scarce when nutrient levels within the cell are high, but increase with nutrient depletion. Like Sirtuins, *AMPK* plays an important role in the catabolic pathway and upregulation of this pathway is found in individuals with DR enhanced lifespans (Stenesen *et al.*, 2013).

### 1.5.6 ***Mitochondrial dysfunction***

The free radical theory of ageing suggests that production of ROS over the lifetime of the cell results in deterioration of the mitochondria contained within. This deterioration results in an acceleration in the rate at which ROS are produced, which further speeds up the decline in mitochondrial functionality (Pinto and Moraes, 2015). Recent discoveries however have complicated this picture. Work on *C. elegans* with abnormally high ROS production has shown increased longevity compared to their wild-type counterparts (Doonan *et al.*, 2008) and studies on mice have shown no negative effects of increased ROS on ageing and no

protective benefits from antioxidants (Pérez *et al.*, 2009). Hekimi *et al.* (2011) have attempted to synthesise these two concepts into a single theory, suggesting that in a similar fashion to AMP or $NAD^+$, ROS acts as a stressor signal in aged cells to activate compensatory mechanisms, but once the concentration of ROS exceeds a threshold the negative effects begin to outweigh the ability of the cell to counteract them.

A further three hallmarks of ageing are laid out by López-Otín, these being cellular senescence, stem cell exhaustion and altered intercellular communication. These hallmarks are not discussed in this work as they are beyond the scope of the project.

### 1.6 **Interventions to extend lifespan**

DR, limiting the energy intake to 50-70% of individuals allowed to feed *ad libitum,* without compromising essential nutrients, was shown to have a life extending effect as early as 1935 (McCay *et al.*, 1935). By limiting growth through DR McCay and colleagues were able to extend lifespan in rats to almost twice the expected level without any pharmacological input. Since this seminal paper, similar effects have been shown in other rodents such as mice (Weindruch *et al.*, 1986) as well as primates (Colman *et al.*, 2009) and model organisms including yeast (Jiang *et al.*, 2000), nematodes (Lakowski and Hekimi, 1998) and the fruit fly *Drosophila melanogaster* (Partridge *et al.*, 2005). Not only has DR been shown to have general life-extending properties but it is also known to delay the onset of several age-related diseases like cancer (Fernandes *et al.*, 1976; Engelman *et al.*, 1990), diabetes (Johnson *et al.*, 1997) and neurodegenerative disease (Duan and Mattson, 1999; Zhu *et al.*, 1999), increasing further its interest to researchers.

Ageing is a complex process involving many components and so the mechanisms of action of DR have been the subject of extensive debate. One mode of action supported by experimental data is the Oxidative Damage Attenuation Hypothesis: that DR plays a part in reducing or preventing the damage caused by metabolic products such as ROS. It has been shown that DR reduces the accumulation of oxidative damage in proteins (Youngman *et al.*, 1992), lipids (Ward *et al.*, 2005) and genetic material (Sohal *et al.*, 1994), supporting the basis for this theory. It has been suggested that a reduction in metabolic rate due to DR is responsible for a decrease in the rate of production of ROS and as such a reduction in damage (Sohal *et al.*, 1994). This does not hold true in all cases however, as DR has shown to extend lifespan in rodents even without decreasing the metabolic rate (Duffy *et al.*, 1991; McCarter and Palmer, 1992) and consequently additional or alternative mechanisms must come into play.

## 1.7 **Circadian clockwork**

The circadian clock is the mechanism by which internal processes are synchronised with external cues to produce a diurnal rhythm. This is crucial in allowing an organism to anticipate repetitive environmental events and so time their behaviour to the most opportune time. This system is divided into two sections: a central oscillator like the suprachiasmatic nucleus (SCN) which in predominantly controlled by external factors such as the cycle of light and dark, and a cellular clock controlled by the endocrine system. Hormones released from the SCN into the blood can reach cells all over the body synchronizing anatomically unconnected processes (Bloch *et al.*, 2013).

Circadian rhythms are defined as biological rhythms that have three distinct properties: a) they persist, or ‘‘free-run’’, with a period of about 24h without external input; b) they can be reset or ‘entrained’ by external cues; c) they have a stable cycle duration across a wide range of physiologically relevant temperatures. This ‘temperature compensation’ is important because most chemical reactions are accelerated when temperature is increased and energy is added to the system. To compensate for this specific mechanisms and enzymes are required.

In eukaryotic cells this rhythm is controlled by a group of proteins, which form a “clock”. The positive arm of this clock are the proteins CLOCK and BMAL1, which form a heterodimer and bind with DNA to promote the translation of CRY2 and PER. These proteins form the “negative limb” of the clock. As CRY2 and PER levels accumulate throughout the “day” they reach a threshold upon which they disrupt the CLOCK:BMAL1 dimer and inhibit their own transcription, “resetting the clock” **(Figure 1.1)**.

While external, physical cues are the dominant Zeitgeber in the SCN, for these peripheral cellular clocks they are predominantly metabolic. The synthesis of heme (one of the core components of haemoglobin) reciprocally regulates the circadian clock (Yin *et al.*, 2007) and restriction of food availability uncouples the peripheral cellular clocks from the central clock in the SCN (Stokkan *et al.*, 2001).

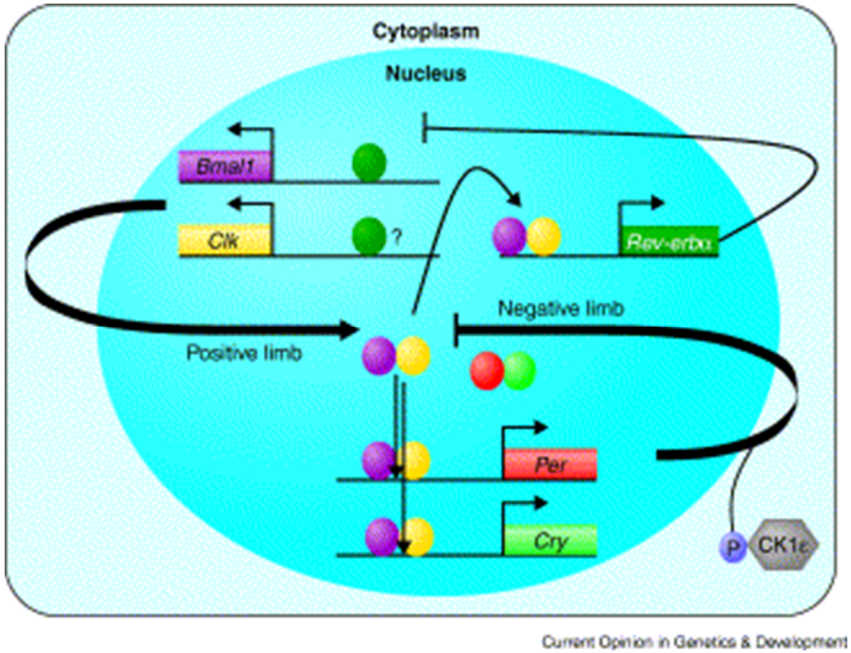


**Figure 1.1** The positive and negative limb of the mammalian circadian clock. Bmal1 and Clock (Clk) form a dimer in the nucleus which upregulates the expression of both *Per* and *Cry* genes. The presence of Per and Cry proteins in the nucleus forms the negative limb of the clock, they inhibit both their own expression by disrupting the dimer and the expression of Bmal1 indirectly (Albrecht and Eichele, 2003).

### 1.7.1 ***Honey bee circadian clock***

A key characteristic of eusocial animals is a division of labour based on certain factors. In honey bees this determination is based on sex and age. Female bees take up the vast majority of the work within the hive. Male drones primarily concern themselves with mating and helping to control hive temperature. Female roles are divided by age, with 'nurses' maintaining managing the interior of the hive (including the brood), while older 'foragers' collect nectar and pollen from plants and flowers (Robinson, 1992). This division of labour is physiologically based; forager's diets change fundamentally prior to first flight abandoning lipids and amino acids almost entirely (Van der Steen, 2015). Workers show a decrease in lipid deposits before their first foraging flights, meaning these changes are not driven by the highly demanding physiological process of flying. Lipid stores are also not essential for nurse behaviour, reversal of foraging behaviour is possible without replacing lipid stores (Toth and Robinson, 2005) and bees that are forced to forage precociously show many of the same physiological states as regular foragers (Toma et al., 2000). This division of labour is socially regulated by the primer pheromone Ethyl oleate, which is transferred via trophallaxis. Because of this, it is possible for bees to speed up, slow down, or reverse their maturation state in response to the needs of the colony (Leoncini et al., 2004).

One of the key differentiating factors between nurses and foragers is the onset of circadian rhythm development sometime between 1 and 3 weeks post-eclosure (Spangler, 1972; Toma *et al.*, 2000) **(Figure 1.2)**. Honey bee larvae require care around the clock which means that nurses must match these demands to care for them. Foragers however, must time their foraging trips when nectar levels are highest and use visual cues like the waggle dance (Wenner *et al.*, 1967) or sun-compass (Lindauer, 1960) to navigate, which requires a sophisticated and robust circadian clock (von Frisch, 1968; Lehmann *et al.*, 2011).

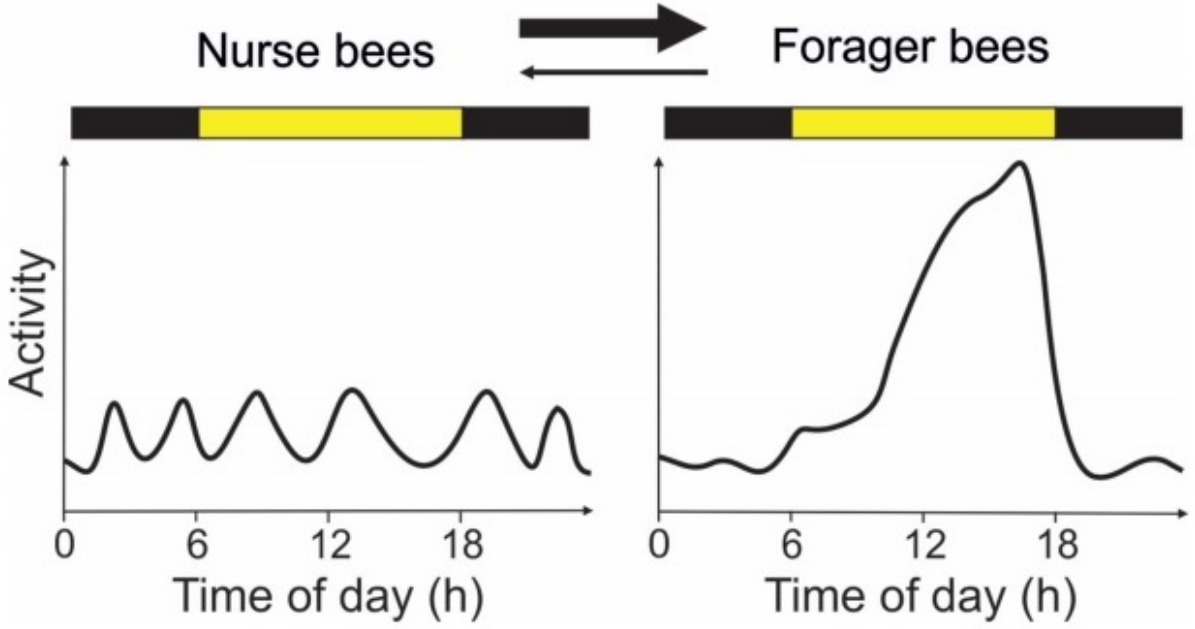


**Figure 1.2** The activity patterns of newly emerged workers compared to older workers undertaking foraging duties (Beer and Bloch, 2020)

The drivers behind this onset of circadian rhythmicity are not fully understood. Work by Bloch *et al.* (2002) showed that despite their being significant differences in the onset of circadian rhythmicity between different sourced colonies, Juvenile Hormone (JH) analogs like methoprene and allatectomy had no effect on onset of rhythmicity or the free-running period (tau) of rhythmic locomotor behaviour. Increases in JH levels are responsible for the degeneration of the hypopharyngeal gland and halt in vitellogenin synthesis (Rutz *et al.*, 1976), both key markers in the transition between arrhythmic nurses and strongly diurnal foragers. There are however some factors known to play a role in circadian rhythmicity in the honey bee. The circadian protein Period (PER) is expressed at higher levels in honey bees that exhibit foraging behaviour (whether naturally or forced to precociously) than either normally aged nurses or older 'reverted' nurses (Bloch et al., 2001).

The molecular control of circadian rhythm has been extensively studied in *Drosophila melanogaster* (Konopka and Benzer, 1971; Helfrich-Förster, 1995; Rutila et al., 1998). Their clock genes are, however, significantly different to mammals. While the *Drosophila* circadian clock is dependent on *Tim1* to function and respond to light cues, this gene has not been conserved during mammalian evolution and the only orthologue produced is in the SCN, which is in fact not a true orthologue of *Tim1* but of drosophila timeout (*dTIM2*) which has no function in circadian control in *Drosophila* (Koike et al., 1998). In mammals, the role of Tim1 is replaced by cryptochromes (CRY) (Van Der Horst et al., 1999; Vitaterna et al., 1999), which are active in the mouse without the presence of light, but not in *Drosophila* (Kume et al., 1999).

Honey bees however are not identical to *Drosophila*, work by Rubin et al. (2006) explored the honey bee circadian clockwork extensively and built on their previous identification of *Per* by identifying, clock (*Clk*), cry and *CYC/BMAL*. The honey bee, independently, has lost the *Tim1* orthologue like mammals and the function of the other genes is closer to that of the mouse than *Drosophila*. For these reasons they propose that the honey bee may in fact provide an improved model system for the study of circadian rhythms in the future.

### 1.7.2 ***The molecular basis of circadian rhythms***

There are a number of factors known to play a role in circadian rhythmicity in the honey bee. The circadian protein period (PER) is expressed at higher levels in honey bees that exhibit foraging behaviour (whether naturally or forced to precociously) than either older reverted-nurses or naturally aged nurses (Bloch et al., 2001). *Per* mRNA levels are higher during the hours of darkness and shows cycling in both young and mature honey bees (Toma et al.,

2000; Bloch et al., 2003; Bloch et al., 2004). Likewise, the number of Per-immunoreactive neurones is higher in foragers than in nurses (Bloch et al., 2003).

In mammals, the central control of the circadian clock is regulated by the SCN, which not only produces “clock” genes but also acts on the pineal gland to release melatonin during the dark phase of the circadian period. Like PER, melatonin has been shown to exist in a cyclical manner throughout the day in honey bees and be present in higher levels based on age and role within the hive (Yang et al., 2007). It has been proposed that melatonin acts on the SCN to modulate clock gene production in mammals, since melatonin injections in the Syrian hamster *Mesocricetus auratus* decrease the levels of PER1 in the SCN (Vriend and Reiter, 2015). As melatonin is produced at night this explains why *Per* mRNA levels peak early during the dark phase of the cycle.

## 1.8 **Research rationale**

Honey bees are severely pressured by changes in the environment and CCD is a risk to global food security. The honey bee is a crucial part of food production and also shows distinct similarities to mammals in their circadian clockwork and metabolic pathways. Because of this similarity it may be able to provide insights into human circadian and dietary control of ageing, with potential to provide a new target for interventions to improve health and lifespan. This study aims to help better understand the interaction between circadian rhythmicity and diet on honey bee longevity.

### 1.8.1 ***Research hypothesis***

It is hypothesised that;

1. Exposure to non-24 h circadian cycles will cause honey bees to be subject to constant, daily re-entrainment which will cause disruption to key circadian and metabolic genes (*CRY2*, *PER2*, *CYC*, *CLK* and *Sir2*). This disruption will negatively influence over-all fitness and survival and further disruption to metabolic pathways will have a reduced effect on longevity.

2. Rapamycin positively influences the longevity of honey bees on both high and low concentrations of essential amino acids (EAAs)

3. Methylation sites within key circadian and metabolic genes (*CRY2*, *PER2*, *CYC*, *CLK* and *Sir2*) will be differentially methylated based on age and rearing conditions.

4. High levels of dietary methionine reduce longevity and increases *Sir2* and *Foxo* expression.

### 1.8.2 ***Project aim***

The overall aim of the present project is to investigate the role of sirtuin-controlled circadian rhythmicity and diet on the lifespan of honey bee.

### 1.8.3 ***Project objectives***

This project seeks to:

1. Investigate the effects of rearing newly emerged honey bees in non-24 h circadian cycles on survival and expression of key circadian genes (*CRY2*, *PER2*, *CYC*, *CLK* and *Sir2*) (Chapter 3).

2. Investigate if honey bees fed a diet high in EAAs on non-24 h circadian cycles will lead to an increase in mortality compared to those with unaltered circadian cycles (Chapter 3).

3. Determine if global changes to DNA methylation in the honey bee can be detected by a standard ELISA kit (Chapter 4).

4. Identify potential CpG sites in circadian and metabolic genes *CRY2*, *PER2*, *CYC*, *CLK* and *Sir2* for MiSeq analysis and if those CpG sites are differentially methylated in bees of different ages and roles (Chapter 4).

5. Determine if the mTOR inhibitor, Rapamycin, fed as part of a prolonged liquid diet alters lifespan and appetite of the newly-emerged honey bee *A. mellifera* (Chapter 5).

6. Determine how varying the concentration of methionine in the diet of newly emerged honey bees affects longevity and expression of *FOXO* and *Sir2* (Chapter 6).

# 2.0 General Materials and Methods

## 2.1 **General honey bee husbandry**

### 2.1.1 ***Insect sourcing***

For all experiments conducted at Newcastle University (Newcastle, UK) *Apis Mellifera Buckfast* hybrids were sourced from managed hives on the roof of the Ridley Building. This strain was chosen for its stability in the climate of Northern England (Olszewski *et al.*, 2012). Frames containing approximately 1600 unhatched broods were removed from 1 of 2 hives in the summer (May-September) of 2014 and 2015. These brood frames were then stored in climate control chambers at 34ºC, 12:12 light:dark (L:D) and 70% relative humidity (RH) in specially designed wooden cages with mesh panels for ventilation (275mm X 440mm X 140mm). Newly emerged individuals were collected from the frame every 24 hours to ensure age matching and allocated to housing.

### 2.1.2 ***Insect housing***

Brood was allowed to emerge over a period of 24 h and then emptied into a plastic refuse bag where they were transferred with plastic vials into custom made Perspex housing cages (110mm X 200mm X 60mm) **(Figure 2.1)** in cohorts of 25 and stored in identical conditions to the brood box. Workers were caged without queens, as their introduction is shown to alter free-running rhythms of worker bees (Moritz and Sakofski, 1991). The housing cages consisted of a Perspex base with two sliding panels on either side that allow for easy access to add or remove animals. At either end two round holes allow the insertion of feeding tubes containing the experimental diets where the bees were allowed to feed *ad libitum*. Above the two diet tubes at one end of the box was a fifth tube filled with water to ensure that the bees could drink without being forced to consume the experimental diets; this enabled them to regulate the temperature of the cages, as they would in the hive. A layer of absorbent paper was placed in the bottom of each cage to collect any spillage.

**Figure 2.1** Newly emerged honey bees were transferred to Perspex housing cages within 24 h of hatching. Each cage contained either 12 or 25 bees, which were allowed to feed freely from the feeding tubes at all times. When individuals died the sliding side panels moved up to allow easy removal and to prevent build-up of harmful waste.

2.1.3 ***Insect diet***

All diets were prepared before the start of the experiment and decanted into 50 ml falcon tubes, which were frozen at -20 $^{o}$C until required to prevent spoilage. The feeding tubes consisted of 2 ml Eppendorf tubes with 4 equally spaced, 3 mm diameter holes drilled into it along the upper surface. The tubes were taped in place from the outside to prevent the bees turning the tubes while feeding and the diet being lost.

All tubes in the survival and collection boxes contained the same 1M sucrose diet with the exception of the fifth tube which contained tap water to allow the bees to meet their mineral and hydration requirements without being forced to consume the diet. Halfway through the 'light' section of a cycle all tubes were replaced with tubes containing fresh diet; dead bees were removed and recorded. On days 6, 7 and 8, as well as 13, 14 and 15 consumption was measured, by recording the weight of the filled tube before it was inserted and then the weight either 20, 24 or 28 h later. All treatments had 10 identical controls without bees which were measured at the same periods to control for evaporation. An average of the three days for both weight change of the diet tube and weight change of the evaporation tube was then taken. Total daily consumption was measured as:

$$\left[ \frac{\text{Tube weight change - mean evaporation weight change}}{\text{Cycle length}} \right] \text{X } 24$$

2.1.4 ***The interaction between diet and circadian period***

Alongside 1M sucrose, some trials were run with either high essential amino acid (EAA) or low EAA content. All diets were made with a base of 1M sucrose solution which either contained a 1:10 ratio of the 10 essential amino acids to carbohydrate (EAA:C) or 1:500 EAA:C. These ratios were calculated on a molar–molar basis **(Table 2.1)**. Essential amino acids were as described for honey bees (de Groot, 1953) as methionine, tryptophan, arginine, lysine, histidine, phenylalanine, isoleucine, threonine, leucine and valine. Due to the small quantities of EAAs needed in the 1:500 solutions, a 1:10 stock was made and the diluted down to 1:500 with 1M sucrose.

**Table 2.1** The composition of 1 litre of all diets used in the circadian experiments. All diets were 1M sucrose with either no EAAs added or a molar-molar ratio of 1:10 EAA:C or 1:500 EAA:C. Therefore the actual ratio of each individual amino acid in solution was either 1:100 EAA:C or 1:5000 EAA:C.

| | Sucrose only | 1:10 | 1:500 |
|---|---|---|---|
| Sucrose | 342.3g | 342.3g | 342.3g |
| Methionine | 0g | 1.49g | 0.030g |
| Tryptophan | 0g | 2.04g | 0.041g |
| Arginine | 0g | 1.74g | 0.035g |
| Lysine | 0g | 1.46g | 0.029g |
| Histidine | 0g | 1.55g | 0.031g |
| Phenylalanine | 0g | 1.65g | 0.033g |
| Isoleucine | 0g | 1.31g | 0.026g |
| Threonine | 0g | 1.19g | 0.024g |
| Leucine | 0g | 1.31g | 0.026g |
| Valine | 0g | 1.17g | 0.023g |

The high and low EAA treatments were treated in exactly the same way as the sucrose only treatments. Every day at the midpoint of the 'light' cycle tubes of diet were replaced with tubes containing fresh solution and dead bees were removed and recorded. In the high EAA diets where large quantities of waste built up, any still-wet waste on the base of the housing chamber was removed with a paper towel.

## 2.2 **Molecular analysis**

### 2.2.1 ***Nucleic acid preparation***

Honey bees were euthanized in their housing at -80 $^{o}$C, frozen and transferred to 50 ml falcon tubes for storage. Once ready for analysis the head was separated from the thorax and abdomen. Material was then immediately snap frozen with liquid nitrogen and ground into powder with a pre-cooled pestle and mortar. Crushed material for both head and body was then stored separately at -80 $^{o}$C until extraction.

### 2.2.2 ***RNA extraction***

For RNA extraction, samples were removed from the -80 $^{o}$C storage and allowed to defrost at room temperature for 5 min. Once defrosted, the samples were treated with 1 ml of TRIzol (*Invitrogen*) solution and incubated for 5 min. After incubation 0.2 ml of chloroform was added and the mixture shaken vigorously for 15 s and allowed to stand for a further 2-3 min at room temperature. The mixture was centrifuged for 15 min at 4 $^{o}$C and 12000 G. The aqueous layer was then transferred to a new tube were Trizol was added and a second extraction took place to ensure cleanliness of the sample. Isopropanol (0.5 ml, Sigma-Aldrich) was added to the extract as well as 2.5 µl of a co-precipitant (2.5 µl Glycoblue, *Ambion*) to help visualise the pellet, and then stored at -80 $^{o}$C for 24 h.

The extract was then defrosted at room temperature for 10 min, centrifuged at 12000 G again for 10 min in a centrifuge pre-cooled to 4$^{o}$C and the was supernatant removed. The pellet was then washed with 1 ml 75% ethanol and centrifuged at 7500 G for 5 min. The wash was performed a second time before the wash was removed and the pellet allowed to air dry before resuspension in 10 µl of $H_2O$. The RNA yield was determined using a Nanodrop spectrophotometer ND-1000. Optical density ratios were measured for both 260/280 and 260/230.

2.2.3 ***DNase treatment***

To remove any DNA contamination an RNase-free DNase treatment kit (Promega) was applied to 4.5 µg of extracted RNA as per the manufacturers instructions. The final mixture of 4.5 µl of DNase, 2 µl of reaction buffer and a combination of RNA and $H_2O$ up to a final volume of 20 µl was incubated at 37 $^oC$ for 35 min before a stop solution was added to terminate the reaction. The solution was then incubated for a further 10 min at 65 $^oC$ before being frozen at -80 $^oC$.

2.2.4 ***Reverse transcription***

First strand cDNA synthesis from the RNA was conducted with iScript™ cDNA Synthesis Kit (*BioRad*). Briefly, 2 µg of RNA was treated with 8 µl of 5x iScript reaction mix, 2 µl of iScript reverse transcriptase and brought up to 40 µl with RNase free $H_2O$. The mixture was primed at 25 $^oC$ for 5 min before being held for 20 min at 46 $^oC$ for reverse transcription before a 1 min inactivation step at 90 $^oC$. The cDNA was then stored at -20 $^oC$.

2.2.5 ***Primers***

Primers were manually designed to *CLOCK, Cryptochrome-2* (*Cry 2*), *Period* (*PER*) and and *Sir2* (see **Table 2.2**). *RP49* was used as the as reference gene as in Robertson and Wanner (2006).

**Table 2.2 PCR primers**. Sequences for both forward and reverse primers are in the 5'-3' direction and subscript numerals refer to positions within the sequences deposited under the stated Genbank accession numbers.

| **Gene** | **Gene ID** | **Sequence** $_{\text{(position in sequence)}}$ **5'-3'** | **Product length** |
|---|---|---|---|
| *Sir2 histone deacetylase, transcript variant 1* (*Sir2*) | XM_003251332.1 | $_{2100}$gttgtaatagtgttgacagtcacgaagac$_{2119}$ | 184 |
| | | $_{2274}$tctgaatatacttctgctccagg$_{2252}$ | |
| *Ribosomal protein S8* (*Rps8*) | NM_001011604.3 | $_{376}$acgaggtgcgaaactgactgaagc$_{399}$ | 182 |
| | | $_{557}$cacgaccgcactgtccaggt$_{538}$ | |
| *Apis mellifera B-actin* | AB023025 | tgccaacactgtcctttctg | 155 |
| | | agaattgacccaccaatcca | |
| *Apis mellifera clock (Clk)* | XM_394233.4 | $_{1971}$caagacgaattgcaac$_{1987}$ | 143 |
| | | $_{2094}$catacaatgccaccaacac$_{2113}$ | |
| *Apis mellifera cry2 (CRY2)* | NM_001083630.1 | $_{969}$gatcgtatgcaaggtaatcc$_{988}$ | 106 |
| | | $_{1057}$gatttccctggatcgatg$_{1084}$ | |
| *Apis mellifera Period (PER)* | NM_001011596.1 | $_{3563}$gcagtgatagtggtagcag$_{3581}$ | 158 |
| | | $_{3701}$ctcaagttgtatcttgccag$_{3720}$ | |

### 2.2.6 ***Real-time quantitative PCR***

Real-time quantitative PCR was performed with a BioRad CFX Connect thermal cycler using 10µl reactions set up in 96-well format. A mixture of containing 7 µl of $H_2O$, 1 µl iTaq™ Universal SYBR® Green (*BioRad*), 0.5 µM of each forward and reverse primer and 1 µl of cDNA (diluted to 1:2) or a water sample for negative controls was placed into each well. After denaturing for 5 min at 95 °C, 40 cycles were carried out using the following parameters: 95 °C (10 s); 55 °C (10 s) and 72 °C (15 s).

## 2.3 **Statistical Methods**

The amount of food consumed was analysed with generalized linear models using SPSS (IBM SPSS Statistics 23). The impact of diet and circadian period on survival was analysed using a Cox regression (Coxreg) analysis with circadian period as the covariate to calculate the hazard ratio (HR). The death of a bee was coded as an event and was entered as the dependent variable. Comparisons between groups were evaluated using the 'indicator' contrasts in SPSS.

The raw results of the mRNA expression levels measured with RT-qPCR were converted using the Delta Delta Ct (ΔΔCt) method (Livak and Schmittgen, 2001) against the reference genes. Individual timepoints were compared using t-tests (Excel 2016). A standard curve was run for each gene in order to confirm amplification efficiencies for each primer.

Consumption rate was analysed using SPSS (IBM SPSS Statistics 23) with either day or circadian period as a main effect in a univariate ANOVA.

## 2.4 **Pilot studies for optimisation of honey bee assays**

### 2.4.1 ***Housing of newly emerged honey bees affects survival***

The possibility of individually housing newly emerged bees was explored and a pilot study was conducted to measure any changes in survival rate for this method of housing. Individuals were stored in 50ml falcon tubes with a hole drilled into the cap to allow for insertion of a feeding tube as described previously and maintained at 12:12 L:D, 34 ºC and 70% RH. Honey bees in individual housing showed significant differences in their 14-day survival based, on their housing conditions **(Figure 2.2)**. Newly emerged honey bees placed in individual tubes suffered significantly higher mortality than those kept in cohorts of 25 (Coxreg, housing, HR = 11.076, [95 % CI (confidence interval) 3.379–36.301], $P < 0.005$) across the 14 days of the experiment. On this basis the use of individual housing for newly emerged honey bees was rejected for subsequent studies measuring survival.

### 2.4.2 ***Honey bees show seasonal differences in survival***

In standard experimental conditions and a standard 24 h circadian cycle honey bees had a x 3.3 higher chance of death during the earlier months of the summer while fed a diet of 1M sucrose relative to those who were collected later in the summer (Coxreg, Season, HR = 3.306, [95 % CI 2.686–4.070], $P < 0.005$) **(Figure 2.3)**.

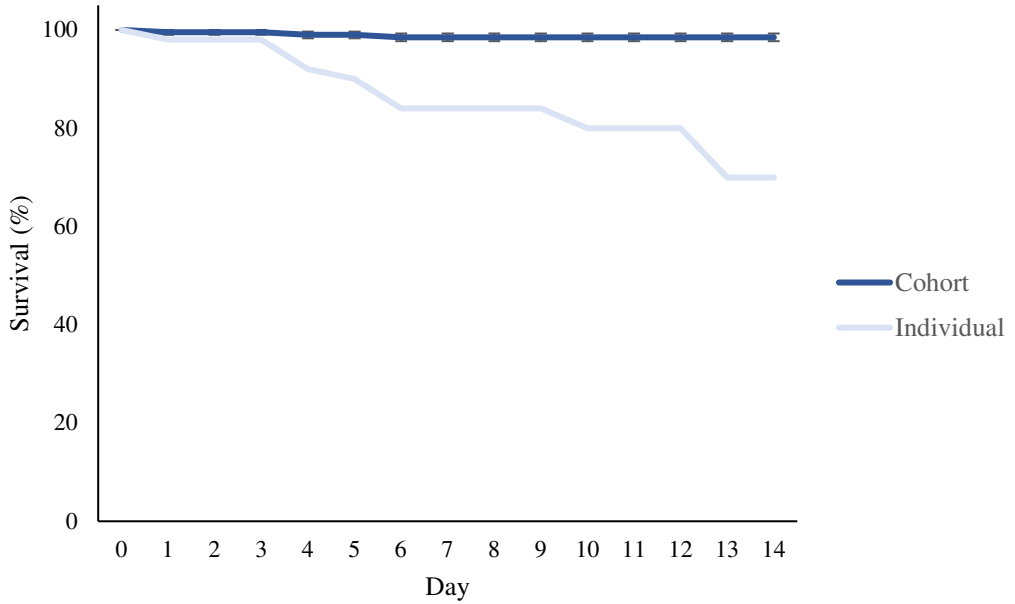


**Figure 2.2** The survival rate between newly emerged bees housed in individual tubes was significantly lower than the honey bees housed in Perspex cages in groups of 25 in the first 14 days post-emergence (Coxreg, housing, HR = 11.076, [95 % CI (confidence interval) 3.379–36.301], P < 0.005). Cohort N=10 boxes, individuals N=50.

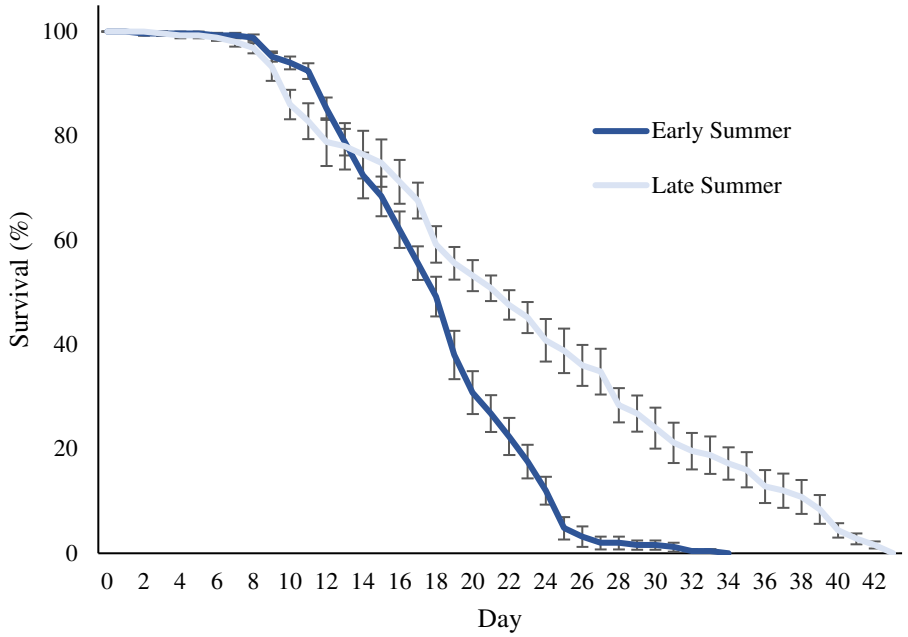


**Figure 2.3** Newly emerged honey bees collected from broodframes laid in the early months of the summer (May-June) were significantly shorter-lived under experimental conditions than those collected from frames of brood which were laid during the later stages of the summer (July-August) N=10 boxes of 25 bees each for both treatments.

### 2.4.3 ***Honey bee lifespan is dependent on the time of egg deposition***

The comparative study between the effects of altered day length in chapter 3 were carried out in two separate batches due to space limitations, meaning that both the 20 h and 28 h treatments were compared to their own 24 h controls several weeks apart. This is inherently problematic due to the nature of honey bee seasonality. It was important to compare the effects of this seasonality to help explain differences between the survival curves for seemingly identical control treatments between the 24 h controls. Our findings that honey bees live longer when they emerge later in the season is consistent with findings by Free and Spencer-Booth (1959), who found that workers in the peak of the season lived 4-6 weeks while others could live up to 12 at other points in the summer.

### 2.4.4 ***Honey bee lifespan is dependent on cohort size***

These pilot experiments provide strong evidence to the theory that honey bees kept in isolation do not survive as well as those kept in groups, more akin to their natural environment in the hive. This finding therefor influenced the experimental design of subsequent studies. Honey bees are highly social animals and have constant interactions with other members of the hive through visual cues like the waggle dance (Seeley, 2009), chemical cues such as the pheromone JH (Bloch *et al.*, 2002) as well as physical contact through behaviours like trophallaxis (Moritz and Kryger, 1994) and antennal contact (Nagari and Bloch, 2012).While there has been little work conducted regarding the suitability of individual housing for bees in longer term studies, and individual housing similar to that used in this pilot study has previously been used successfully in week-long behavioural studies (Toma *et al.*, 2000), findings from these pilot studies was enough for us to disregard individual housing in favour of more stable and proven cohort methods (Altaye *et al.*, 2010; Paoli *et al.*, 2014) for survival measures.

# Chapter 3. The interaction between diet composition and circadian period: Impacts on survival and circadian gene expression in the honey bee

## 3.1 **Introduction**

As early as the beginning of the 20th century von Buttel - Reepen (1900) showed that honey bees had the ability to "remember" time, an ability known as Zeitged-achtnis. Foragers can be entrained to forage for food at any time of the day and will "remember" even after the food source has been removed. This has been shown not just in colony foraging behaviour (Moore and Rankin, 1985), but in the locomotive behaviour of individually housed bees (Frisch and Aschoff, 1987). Work by Brady (1987) showed that this clock is not simply exogenous, but is in fact controlled endogenously by molecular clockwork. Not only is this clock capable of plasticity, it is also virtually unique in the insect kingdom in that it is "continuously consulted" giving the honey bee the ability to recognise the current point of its circadian cycle and adapt its behaviour (Pittendrigh and Minis, 1964). Not only this, but it is capable of sophisticated learning which combines both temporal and spatial information (Wahl, 1932). This allows bees to synchronise their foraging trips with the time when plants have the highest nectar levels, thus giving the forager maximum rewards for its efforts (Von Frisch, 1967).

This strong circadian rhythm is not true at all stages of the honey bee's lifespan, however. Newly emerged bees show no circadian rhythm at all for the first 8 days of life (Bloch *et al.*, 2002). The presence of the queen can also revert developed foragers to a free-running rhythm (Moritz and Sakofski, 1991). Many studies have found that regardless of age, foragers will show strong diurnal behaviour while those attending the queen will show none (Free *et al.*, 1992; Crailsheim *et al.*, 1996). Likewise precocious foragers have levels of the important clock gene *Period* (*Per*) similar to much older foragers (Toma *et al.*, 2000).

The recent discovery that bees share far more of their circadian machinery with mammals than that of other model insects, like *Drosophila,* is another advantage in their use as scientific models. The honey bee possesses no ortholog of timeless (*Tim1*) found in *Drosophila*, only the mammalian type. It is thought that the *Drosophila* circadian clockwork has elements that have been lost by both mammals and honey bees. Honey bees only have the mammalian type cryptochrome (*Cry-m*) as well as only a single ortholog for each of the other canonical "clock genes", unlike *Drosophila* (Rubin *et al.*, 2006). This makes the honey bee an interesting target for studying the effects of circadian rhythm.

### 3.1.1 *Manipulating circadian rhythms and entrainment*

Studies in the fruit fly *D. melanogaster* which manipulated the ratio between light and dark over a 24 h period from 16:8 (LD) to continuous light (LL) found that when the relative abundance of PER mRNA extracted from the whole head was measured against the reference gene RP49, less than 8 h of darkness in 24 h dampened the oscillations of PER mRNA. Furthermore with less than 6 hours of darkness relative abundance did not change significantly throughout the cycle (Qiu and Hardin, 1996).

Work by Bloch *et al.* (2004) found that exposing honey bees to light and allowing them to fly had no effect on PER expression in the brain of workers. These studies were however a comparison between access to light/dark and total darkness, which might not inhibit PER expression as the changes in expression are dependent on a sufficiently large dark phase for PER to "reset" which is present in both treatments.

Tau (τ), the endogenous free-running circadian period, is extended by both continuous light and continuous darkness in the silkworm *Bombyx mori*, which effectively reduced the number of cycles for each instar (Sailaja and Sivaprasad, 2010; Sailaja and Sivaprasad, 2011). Likewise, honey bees showed consistent lengthening of their τ when exposed to constant light and shortening when held in constant darkness. When entrained on a 16:8 LD cycle and then moved to the same cycle 4 h in advance bees were able to entrain within 2 days (Moore and Rankin, 1985). When given freedom to select their own ambient temperature from a gradient they will choose a higher temperature during their perceived day and a much lower one at night. When photoperiod is reversed from LD to DL this selection takes several days to reverse (Grodzicki and Caputa, 2012). There is clear evidence that photoperiod is an effective way of altering the honey bees' chronobiology, not just with regards to internal physiology but also external behaviours.

### 3.1.2 *The cost of clock desynchronization*

When tau and external time cues do not match up, either because they are not in temporal synchrony or because they are different lengths then the individual is subject to constant, daily re-entrainment. It has been proposed that this re-entrainment comes at significant physiological cost which could have a negative influence on overall fitness and survival (Withrow, 1959). *Drosophila* raised on 24 h 12:12 LD cycles which were then placed in either 21.5 h cycles or 27 h cycles showed significant increases in mortality compared to

those who remained on a 24 h cycle, with 27 h suffering the worst mortality on 3 of the 4 trials (Pittendrigh and Minis, 1972). This is true also in plants such as Arabidopsis (Dodd *et al.*, 2005) and mutant strains of mice where τ is not 24 h (Wyse *et al.*, 2010).

This work is complimented by observations made on the blowfly *Phormia terraenovae* which shows that when forced into a cycle longer (28 h) or shorter (20 h) than their natural freerunning period, survival is lower than when compared with a 24 h control (von Saint Paul and Aschoff, 1978). They propose that this is due to 'forced internal desynchronization' which, if experienced over long periods, could curtail lifespan.

This has been particularly prominent in the study of shift-workers and as such, carries important health considerations. Those with a genetic predisposition to internal desynchronization have been associated with various comorbidities such as sleep alteration, mood disorders and general fatigue (Reinberg and Ashkenazi, 2008) as well as more severe risks such as cardiovascular and renal disease (Martino *et al.*, 2008). It was suggested that on this basis, shift work might be significantly more detrimental to the health of some individuals more than others.

### 3.1.3 ***Circadian rhythm and ageing***

High-amplitude oscillations in circadian genes associated with a robust circadian rhythm are known to be depressed with age. When aged hamsters are given suprachiasmatic implants that restore these rhythms, longevity can be extended significantly (Hurd and Ralph, 1998). Circadian disruption has long been a known symptom of ageing (Bremner *et al.*, 1983; Weinert and Waterhouse, 2007). For example, aged rats which are subject to the same shifts in their LD as young rats fail to adapt to cycles as quickly, which results in more fragmented periods of physical activity with longer rests and an overall reduction in activity over 24 h compared to their younger counterparts, despite the same maximal intensity (Valentinuzzi *et al.*, 1997).

The negative health consequences associated with circadian rhythm dysfunction that comes with age are proposed to be in part down to an upregulation of the mechanistic target of rapamycin (mTOR) signalling pathway (Zheng and Sehgal, 2010). In *Drosophila,* the fly ortholog of *GSK- 3b*, *shaggy* (*SGG*) mTOR directly alters the circadian period of locomotor activity (Martinek *et al.*, 2001). Activation of the mTOR pathway is associated with increased rates of ageing (Johnson *et al.*, 2013) and degenerative diseases like Alzheimer's (Cai *et al.*, 2015). Using an *mTOR* inhibitor such as rapamycin extends lifespan in *Drosophila*, mice and yeast amongst others by up to 60% (Kapahi *et al.*, 2004; Alvers *et al.*, 2009; Harrison *et al.*, 2009). Studies in mice have shown that the mammalian *Cycle (CYC)* orthologue *BMAL1* is a

negative regulator of the *mTORC1* pathway in mice. *BMAL1*-/- mutants can see life-extending effects of 50% when treated with rapamycin, potentially in part due to strengthened circadian rhythm. As *mTOR* is a key integrator of upstream pathways such as insulin and amino acid regulation and plays an important role in diet and metabolism in liver, muscle and brown/white adipose tissues (Hay and Sonenberg, 2004). It is also dysregulated in metabolic disorders such as obesity and diabetes (Beevers *et al.*, 2006).

Sirtuins are one of the key components in the life extending properties of dietary restriction. It is SIRT1 activation in mammalian cells that extends survival when under dietary restriction (Cohen *et al.*, 2004) and sirtuin activators such as resveratrol mimic the life-extending and ageing delay in both simple metazoan (Wood *et al.*, 2004) and human cell lines (Morselli *et al.*, 2010). Sirtuins also play a role in metabolic signalling and obesity, Sirt6 ablation in mice leads to decreased somatic growth and increased weight (Schwer *et al.*, 2010).

For "clock" genes such as BMAL, CLOCK, Per and Cry2 to show high amplitude oscillations, SIRT1 must be present. SIRT1 in mice is a necessity for the deacetylation and degradation of PER2, which, if allowed to build up within the cell, leads to the repression of several key Per and Cry mRNAs (Asher et al.). This close connection becomes apparent when SIRT1 levels start to decrease, age-related decline in expression of SIRT1 in mice leads to a disrupted circadian rhythm and failure to adapt to new circadian cycles, while over-expression of SIRT1 protects against this.

Nakahata et al. (2008) propose that SIRT1 acts as a rheostat for the circadian clock, transducing metabolic signals such as NAD+ to the circadian pathways. They showed that CLOCK, BMAL1, and SIRT1 co-localize in a chromatin-associated regulatory complex as promoters of clock-controlled genes.

Dietary restriction is known to upregulate SIRT1 (Ma *et al.*, 2015) and this could play an important role in the downregulation of the mTOR pathway through BMAL1 as sirtuins are shown to regulate the CLOCK:BMAL complex through chromatin remodelling (Asher *et al.*, 2008; Nakahata *et al.*, 2008; Nakahata *et al.*, 2009). Paoli *et al.* (2014) showed that knocking down *Sir2* expression in honey bees reduces lifespan, as do diets high in essential amino acids (EAAs). Bees on low carbohydrate to EAA diets also had reduced *Sir2* mRNA levels at days 7 and 14 post-emergence. As the TORC1 complex is activated by amino acids (Zoncu *et al.*, 2011), they proposed that low levels of EAAs stimulate the expression of *Sir2* relative to bees fed sucrose alone, which extends lifespan, but are not high enough to activate *mTOR* as in the high C:EAA diets which decrease lifespan.

### 3.1.4 *Aims and hypothesis*

In this study we investigate the effect of rearing newly emerged honey bees in non-24 h circadian cycles and its impact on survival and expression of key circadian genes. Based on current literature I hypothesise this will cause them to be subject to constant, daily re-entrainment which will negatively influence over-all fitness and survival. Entrainment to a circadian period significantly different from their innate free-running period will cause disruption to key circadian genes: *Clock*, *Period* and *Sir2*. I hypothesise that this will result in a failure to match expression to environmental cues. We also investigate the effect of diet and circadian disruption to determine if they both act through a shared pathway. I propose that both of these mechanisms act through the mTOR pathway and because of this, additional mortality associated with either treatment will be absent or diminished.

## 3.2 **Materials and methods**

### 3.2.1 ***General maintenance, husbandry and survival techniques***

Brood was allowed to emerge over a period of 24 h and then collected in cohorts of 25 as described in chapter 2. Workers were caged without queens as their introduction is shown to alter free-running rhythms of worker bees (Moritz and Sakofski, 1991). At either end of the housing chambers 2 round holes allow the insertion of feeding tubes containing the experimental diets where the bees were allowed to feed *ad libitum*. Above the two diet tubes at one end of the box was a fifth tube filled with water to ensure that the bees had free access to water.

1 M sucrose was made up daily by dissolving 171.1g of sucrose in 500 ml of deionised (DI) water and all tubes were replaced with fresh diet at approximately the halfway point of the "day" part of the cycle. During the feeding process dead bees were removed and recorded to minimise disruption to the subjects. All individuals were kept at 34 $^{o}$C, 70% RH and a 12:12 LD cycle which was automatically controlled by the inbuilt software in the climate chambers. The experiment was terminated after 14 days and all surviving subjects euthanized in a -20$^{o}$C freezer.

Subjects for the preliminary study were collected from hives at ANU campus in February 2015 and stored in cohorts of 20. Animals were allowed free access to 1m sucrose solution throughout the entire study.

### 3.2.2 ***Circadian period manipulation and survival measurement***

Using the cohort method listed in 3.2.1 newly emerged bees were placed in Perspex housing cages in cohorts of 25. Ten cages were stored in one chamber set to the standard 24 h 12:12 LD conditions and 10 in either an extended 28 h 14:14 LD cycle or a shortened 20 h 10:10 LD cycle. Due to room number limitations, these experiments did not take place concurrently, a 24 h treatment took place alongside the 28 h treatment in May-June of 2015 and then a separate 24 h control was run alongside a 20 h treatment in July-August of 2015.

All diets were pre-made and provided as described in chapter 2. On days 6, 7 and 8, as well as 13, 14 and 15, consumption was measured by recording the weight of the filled tube before it was inserted and then the weight either 20, 24 or 28 h later. All treatments had 10 identical controls without bees which were measured at the same periods to control for evaporation. An average of the three days for both weight change of the diet tube and weight change of the evaporation tube was then taken. Total daily consumption was measured as:

$$\left[\frac{\text{Mean tube weight change - mean evaporation weight change}}{\text{Cycle length}}\right] \times 24$$

Consumption in the preliminary study was not measured and subjects were only exposed to 12:12 LD and 14:14 LD. Twenty individuals were exposed to the longer circadian period and 20 the shorter. After 19 complete 24 h cycles, a further 8 h was waited until both treatments were under opposite light conditions and then were quickly euthanised with liquid nitrogen and moved to -80 $^{o}$C storage.

3.2.3 ***Diet composition***

Alongside 1 M sucrose controls, 10 boxes for all 3 cycles were run with either high EAA:C or low EAA:C diets. All diets were made with a base of 1 M sucrose solution which either contained a 1:10 ratio of the 10-essential amino acid to carbohydrate (EAA:C) or 1:500 EAA:C. These ratios were calculated on a molar–molar basis **(Table 3.1)**. Essential amino acids were as described for honey bees by de Groot (1953) as methionine, tryptophan, arginine, lysine, histidine, phenylalanine, isoleucine, threonine, leucine and valine. Due to the small quantities of EAAs needed in the 1:500 solutions, a 1:10 stock was made and the diluted down to 1:500 with 1 M sucrose. The high and low EAA:C treatments were treated in exactly the same way as the sucrose only treatments. In the high EAA:C diets, where large quantities of faecal matter built up, any still-wet waste on the base of the housing chamber was removed with a paper towel.

**Table 3.1** The composition of 1 litre of all diets used in all experiments. All diets were 1 M sucrose with either no EAAs added or a molar-molar ratio of 1:5, 1:10 or 1:500 EAA:C. Therefore the actual ratio of each individual amino acid in solution was either 1:100 EAA:C or 1:5000 EAA:C.

| | Sucrose only | 1:5 | 1:10 | 1:500 |
|---|---|---|---|---|
| Sucrose | 342.3g | 342.3g | 342.3g | 342.3g |
| Methionine | 0g | 2.98g | 1.49g | 0.030g |
| Tryptophan | 0g | 4.08g | 2.04g | 0.041g |
| Arginine | 0g | 3.48g | 1.74g | 0.035g |
| Lysine | 0g | 2.92g | 1.46g | 0.029g |
| Histidine | 0g | 3.10g | 1.55g | 0.031g |
| Phenylalanine | 0g | 3.30g | 1.65g | 0.033g |
| Isoleucine | 0g | 2.62g | 1.31g | 0.026g |
| Threonine | 0g | 2.38g | 1.19g | 0.024g |
| Leucine | 0g | 2.62g | 1.31g | 0.026g |
| Valine | 0g | 2.34g | 1.17g | 0.023g |

### 3.2.4 *Molecular analysis*

*Note: for full methods see chapter 2*

Honey bees from the preliminary study had their brains extracted and sent back from ANU to the UK for further analysis. In parallel to bees used to determine survival for each circadian rhythm, 12 separate cohorts of 12 bees each were reared under the same experimental conditions for harvest of nucleic acid material that would allow the detection of circadian gene expression. At day 7 and day 14 one of these boxes for each treatment was snap-frozen in a -80 ºC freezer at each of 6 evenly spaced intervals or timepoints (TP) **(Figure 3.1)** Frozen bees were transferred to a falcon tube for processing.

Bees were removed from the freezer and the head was separated from the rest of the body, which was returned to storage. For each timepoint, 3 heads were pooled and due to time and financial constraints only TP1, TP3 and TP5 were measured. This meant that for each timepoint, 3 replicates each consisting of 3 individual honey bee heads were used for the analysis **(Figure 3.2)**. Each group of 3 bee heads were crushed in liquid nitrogen using a pestle and mortar and the crushed material was transferred to individual 2 ml microfuge tubes which were stored at -80 ºC for further processing.

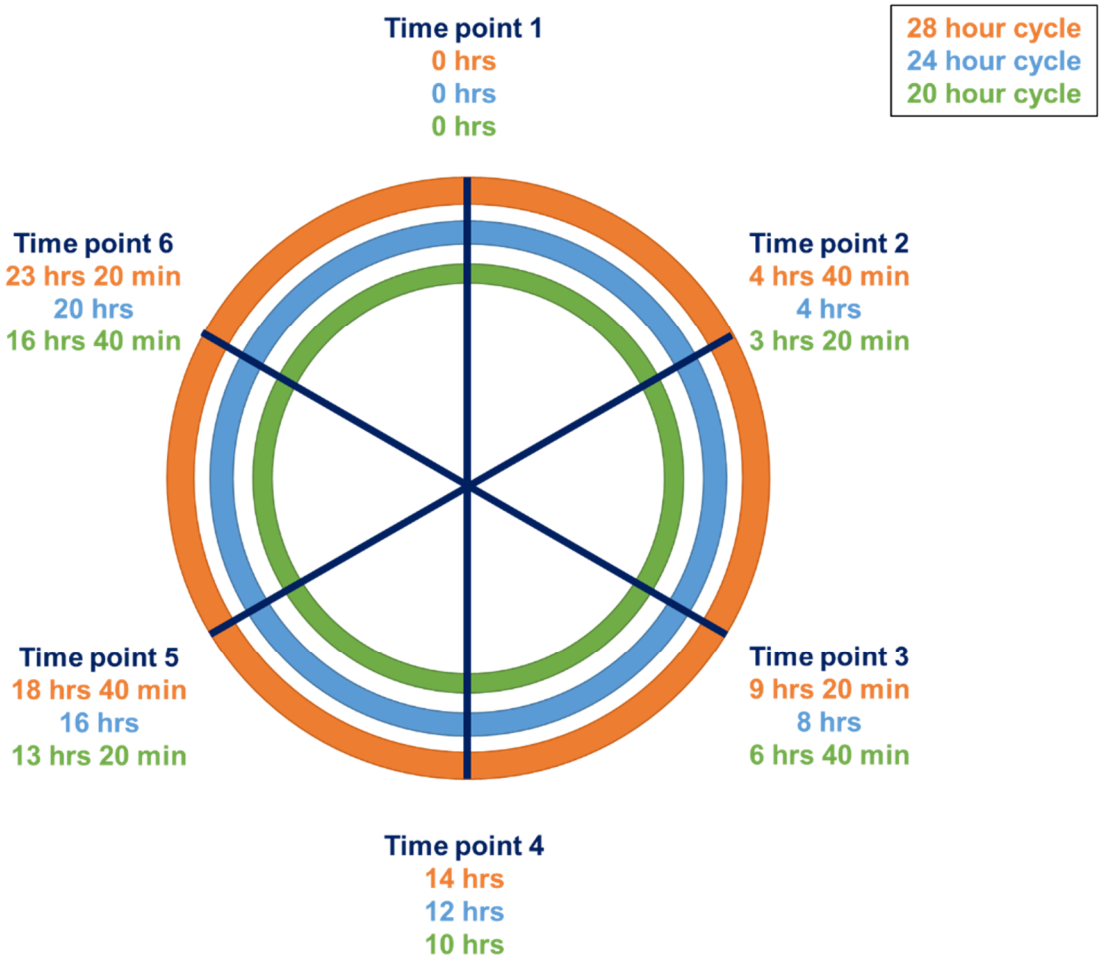


**Figure 3.1** Samples were collected for RNA extraction at 6 'evenly spaced' intervals at both day 7 and day 14. Collections were every 4 hrs for the 24 h control, every 4.66hrs for the 28 h cycle and every 3.33 h for the 20 h cycle. The "light phase" of the cycle occurred between TP1 and TP4, the "dark phase" between TP 4 and TP1.

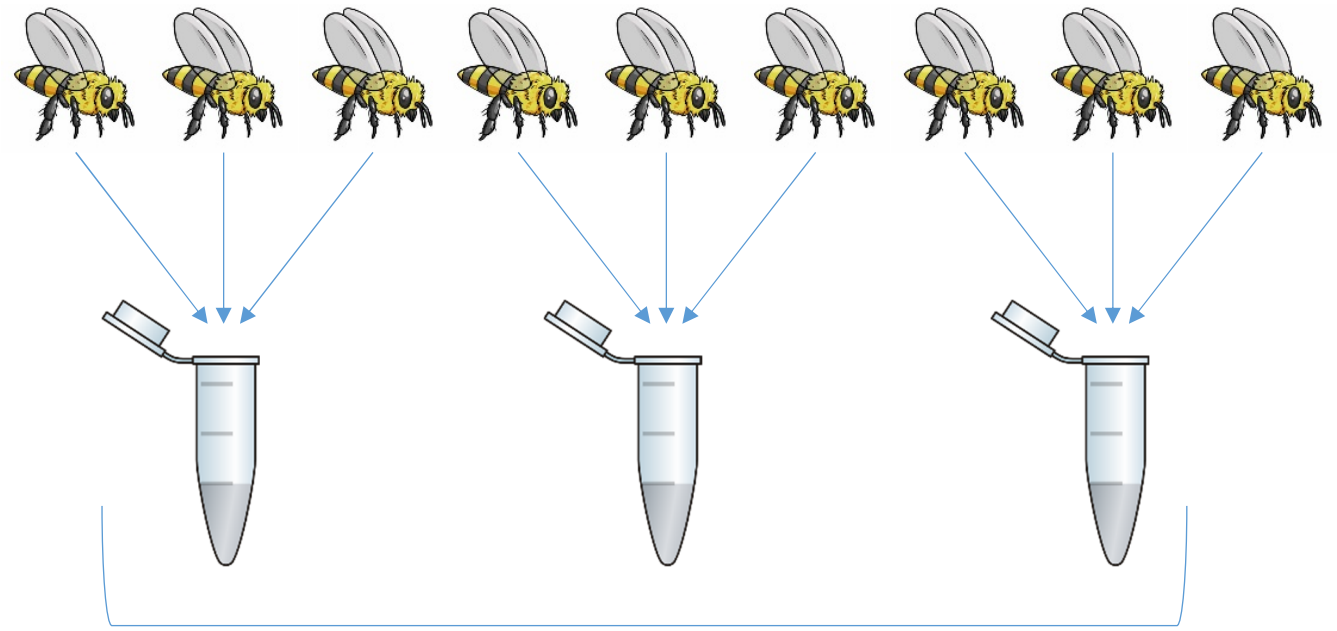


**Figure 3.2** Honey bee heads were pooled in multiples of 3, to make 3 samples per timepoint for RTqPCR analysis. Bees heads were removed and then crushed in liquid nitrogen and stored in 2ml Eppendorf tubes at -80ºC to await RNA extraction.

For RNA extraction samples were stored at -80 ºC. Once defrosted, total RNA was extracted from the heads with Trizol. The next day the pellet was then cleaned with 1 ml of 75% ethanol and centrifuged at 7500 G for 5 minutes. The wash was performed a second time before the wash was removed and the pellet allowed to air dry before resuspension in 10 µl of RNase and DNase free $H_2O$ and stored at -80 ºC. 1 µl of extracted RNA was analysed using a Nanodrop spectrophotometer ND-1000 to quantify total RNA yield for DNase treatment. 4500 ng of RNA in total was treated with RNase-free DNase (Promega) following supplied instructions, 1 ul of sample was then measured again on the Nanodrop using DNase treated water as a blanking solution.

First strand cDNA synthesis from 2 µg of the DNase treated RNA was conducted with iScript™ cDNA Synthesis Kit (*BioRad*) according to the manufacturer's instructions. Negative controls for both template and enzyme were used to ensure no contamination during the reaction. cDNA was then stored at -20 ºC.

Primers were manually designed to span an exon-boundary for *CLK*, *PER* and *Sir2*. *RP49* as in Robertson and Wanner (2006) was used as reference a gene. Confirmation of the primer sequence was obtained firstly by EndPoint PCR using MyTaq HS Mix DNA polymerase (Bioline) and 0.5µl of cDNA and run on a 2% agarose gel against Hyperladder IV (Bioline) to confirm product sizes **(Figure 3.3)**. To further verify the product was the intended sequence the primers were sent for sequence validation (Eurofin genomics).

Real-time quantitative PCR was performed with a BioRad CFX Connect thermal cycler using 10 µl reactions set up in 96-well format. Each gene was measured on a separate plate with duplicates of each triplicate and their water-negatives. A mixture containing 7 µl of $H_2O$ 1 µl iTaq™ Universal SYBR® Green (*BioRad*), 0.5 µM of each forward and reverse primer and 1 µl of cDNA (diluted to 1:2) or a water sample for negative controls was placed into each well. After denaturing for 5 min at 95 °C, 40 cycles were carried out using the following parameters: 95 °C (10 s); 55 °C (10 s) and 72 °C (15 s). Prior to this, standard curves were generated by serial dilution of the cDNA to ensure the efficiency of the primers was approximately 2 to ensure doubling of the product after each cycle. Alongside final quantification, melt curves were plotted to ensure no contamination from reaction reagents or non-specific sequences were present.

### 3.2.5 ***Statistical methods***

The amount of food consumed was analysed with generalized linear models using SPSS (IBM SPSS Statistics 23). The impact of diet and circadian period on survival was analysed using a Cox regression (Coxreg) analysis with circadian period as the covariate to calculate the hazard ratio (HR). The death of a bee was coded as an event and was entered as the dependent variable. Comparisons between groups were evaluated using the 'indicator' contrasts in SPSS. The cycle number ($Ct_{hreshold}$ value) of the mRNA expression measured with RT-qPCR were converted using the Double Delta Ct Value ($\Delta\Delta Ct$) and compared against RP49. Individual timepoints were compared using t-tests (Excel 2016), for analysis of general expression *Sir2*, *Clk* and *Cry2* were measured using univariate ANOVA with LSD *post hoc* in SPSS. Consumption rate was analysed using SPSS with either day or circadian period as a main effect in a univariate ANOVA.

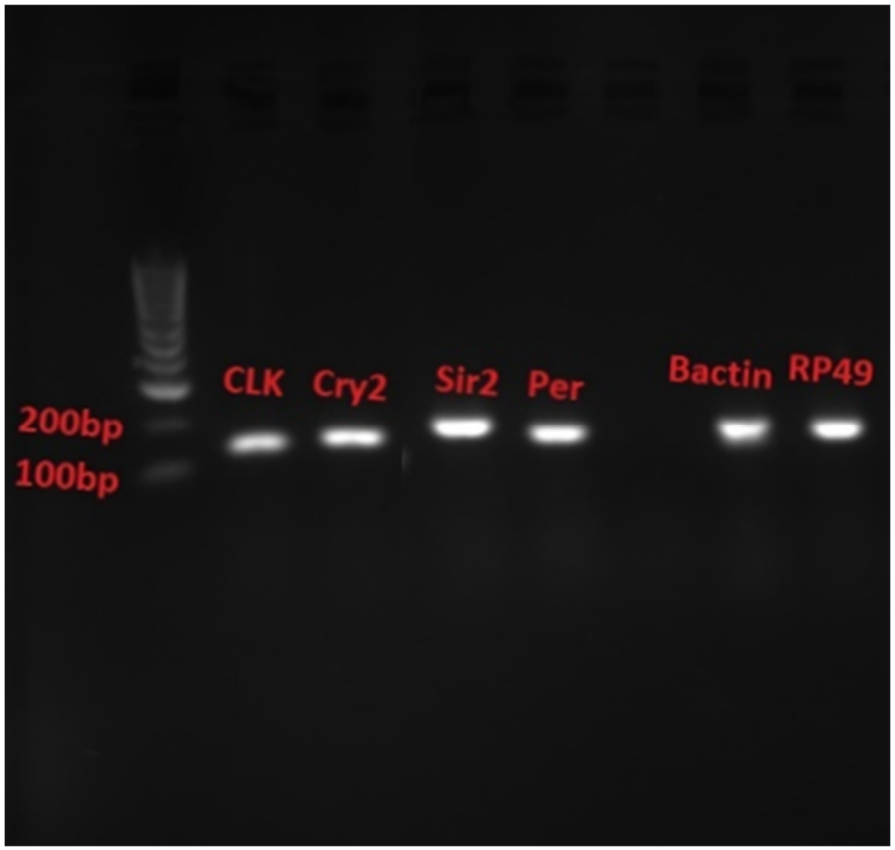


**Figure 3.3** Endpoint PCR products from left to right: *Clock*, *Cry2*, *Sir2*, *Per*, *BActin*, *RP49*.

## 3.3 **Results**

### 3.3.1 ***Rearing honey bees in non-24 h circadian cycles will negatively influence over-all fitness and survival***

When newly emerged honey bees were subjected to a 14:14 LD cycle, they suffered higher mortality than those which were treated with a standard 24 h cycle consisting for 12 hours of light followed by 12 hours of dark used to represent a natural light cycle **(Figure 3.4).** Bees on a 14:14 LD cycle had 1.6-fold greater risk of dying than those on the 12:12 cycle (Coxreg, CircadianPeriod, HR = 1.612, [95 % CI 1.348–1.927], p< 0.005).

Honey bees do not change their total feeding volume based on the length of the circadian period they are exposed to, but do consume more food as they age. The circadian period had no effect on the average amount of diet consumed per 24 h across either test periods, day 6 to day 8 or day 13 to day 15 (Univariate ANOVA, CircadianPeriod: $F_{1, 3} = 4.79$, p= 0.509) **(Figure 3.5)**. While bees ate more per day between days 13 and 15 than they did between days 6-8 (Univariate ANOVA, Day: $F_{1, 3} = 9.885$, p= 0.014) the relationship between these was consistent and did not change, regardless of the circadian period they were subjected to (Univariate ANOVA, CircadianPeriod*Day: $F_{1, 3} = .290$, p= 0.605).

The shorter circadian period of 20 h was less damaging to lifespan compared to the control than the 28 h period, but still negatively impacted longevity. Bees subjected to a circadian period of 20 h, consisting of 10 h of light followed by 10 h of total darkness had a 1.2-fold greater risk of dying than those on the 24 h 12:12 LD control. While not as pronounced as the longer circadian period treatment, this effect was still significant (Coxreg, CircadianPeriod, HR = 1.196, [95 % CI 1.003–1.428], p= 0.047) **(Figure 3.6)**.

As with the previous experiment, the rate of consumption of the 1 M sucrose solution in the modified circadian period (0.190 g/day ±0.006 g) was not significantly different compared to consumption on the control treatments (0.190 g/day ±0.004 g) (Univariate ANOVA, CircadianPeriod: $F_{1, 3} =0.644$, p= 0.445) but older bees did consume the diet at a significantly higher rate than newly emerged bees (Univariate ANOVA, Day: $F_{1, 3} =32.964$, p< 0.005). There was no significant interaction between circadian period or age on total consumption, suggesting that the circadian period did not change the relationship between age and amount of solution consumed (Univariate ANOVA, CircadianPeriod*Day: $F_{1, 3} =0.054$, p< 0.823) **(Figure 3.7)**.

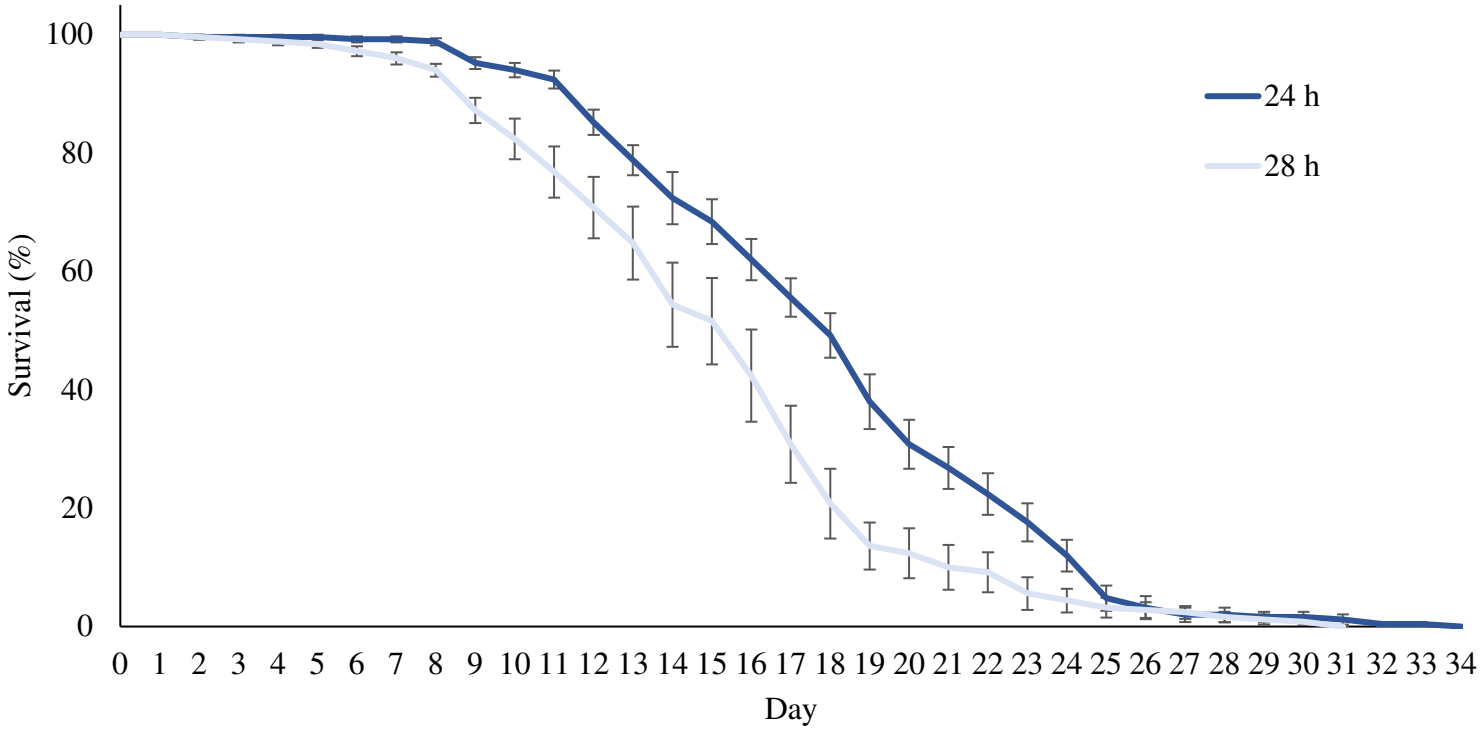


**Figure 3.4** Honey bees exposed to an extended circadian period of evenly distributed light and dark periods totalling 28 h had a 1.6-fold increased chance of death compared to those on a 24 h circadian period (Coxreg, CircadianPeriod, HR = 1.612, [95 % CI 1.348–1.927], p< 0.005) (N = 10 boxes of 25 bees for both treatments.)

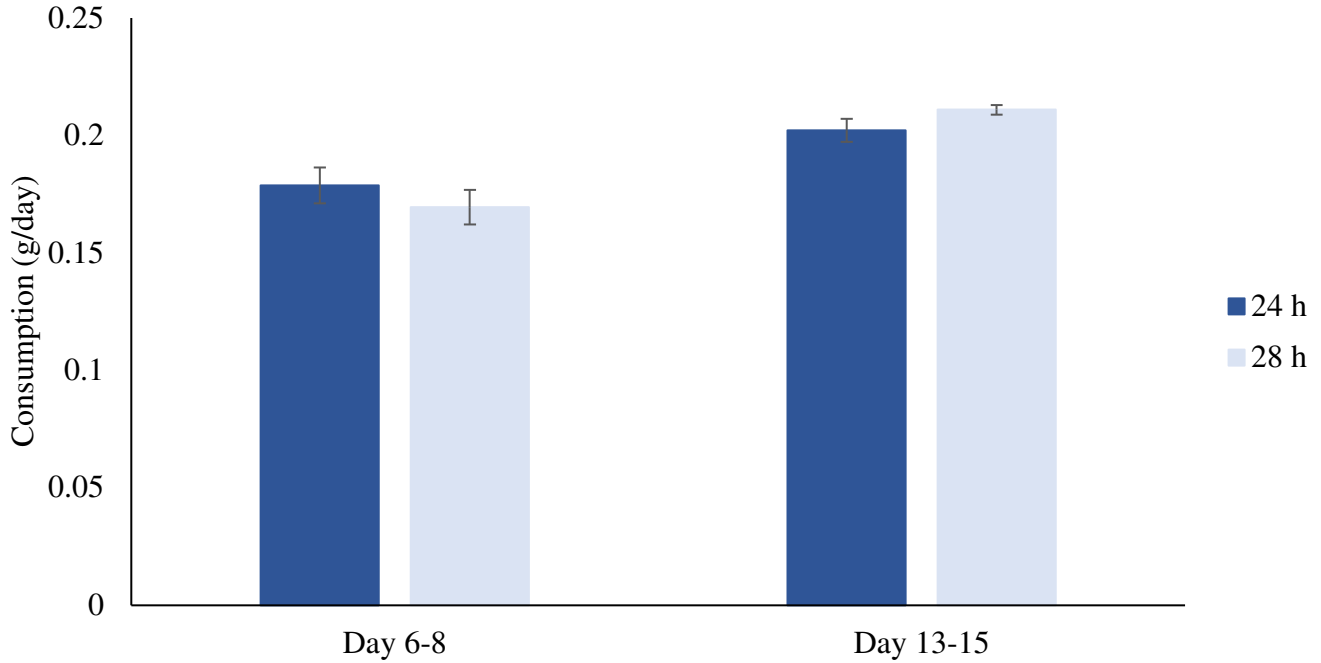


**Figure 3.5** Consumption measured across a 3-day period centred on day 7 and day 14 of life showed that while honey bees consumed more of the 1 M sucrose solution per day between days 13-15 (0.208 g ±0.06) than days 6-8 (0.179 g ±0.06) (Univariate ANOVA, Day: $F_{1,3} = 9.885$, p= 0.014), the circadian period had no effect on the rate of consumption in either of the 2 measuring periods (Univariate ANOVA, CircadianPeriod: F1, 3 = 4.79, p= 0.509) (N=10).

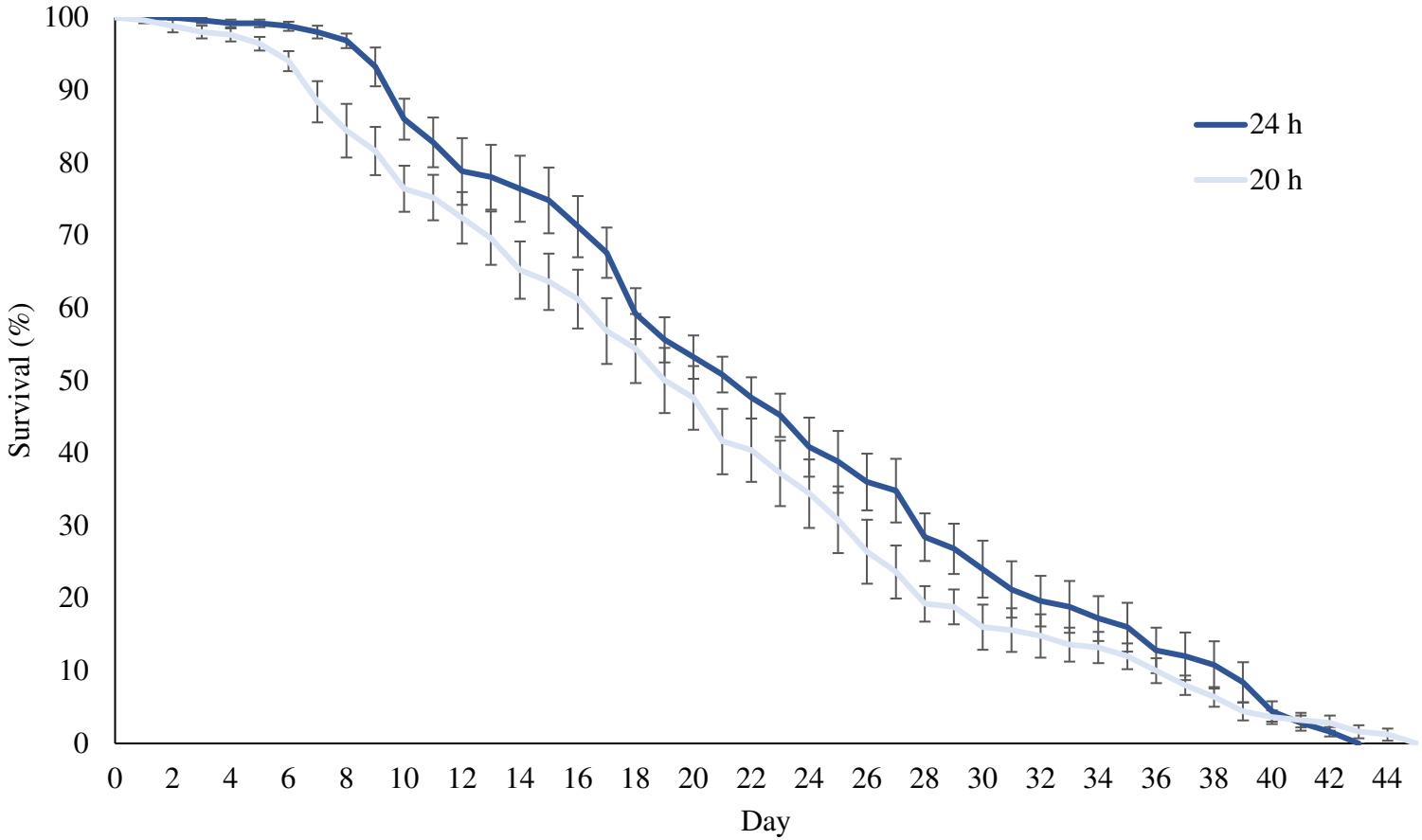


**Figure 3.6** Newly emerged workers entrained to a 20 h cycle were 1.2-fold more likely to die than those entrained to a 24 h cycle from the first day post-emergence. While not as pronounced as the 1.6-fold increase seen in the 28 h treatment, this was still statistically significant (Coxreg, CircadianPeriod, HR = 1.196, [95 % CI 1.003–1.428], p= 0.047). (N = 10 boxes of 25 bees per treatment.).

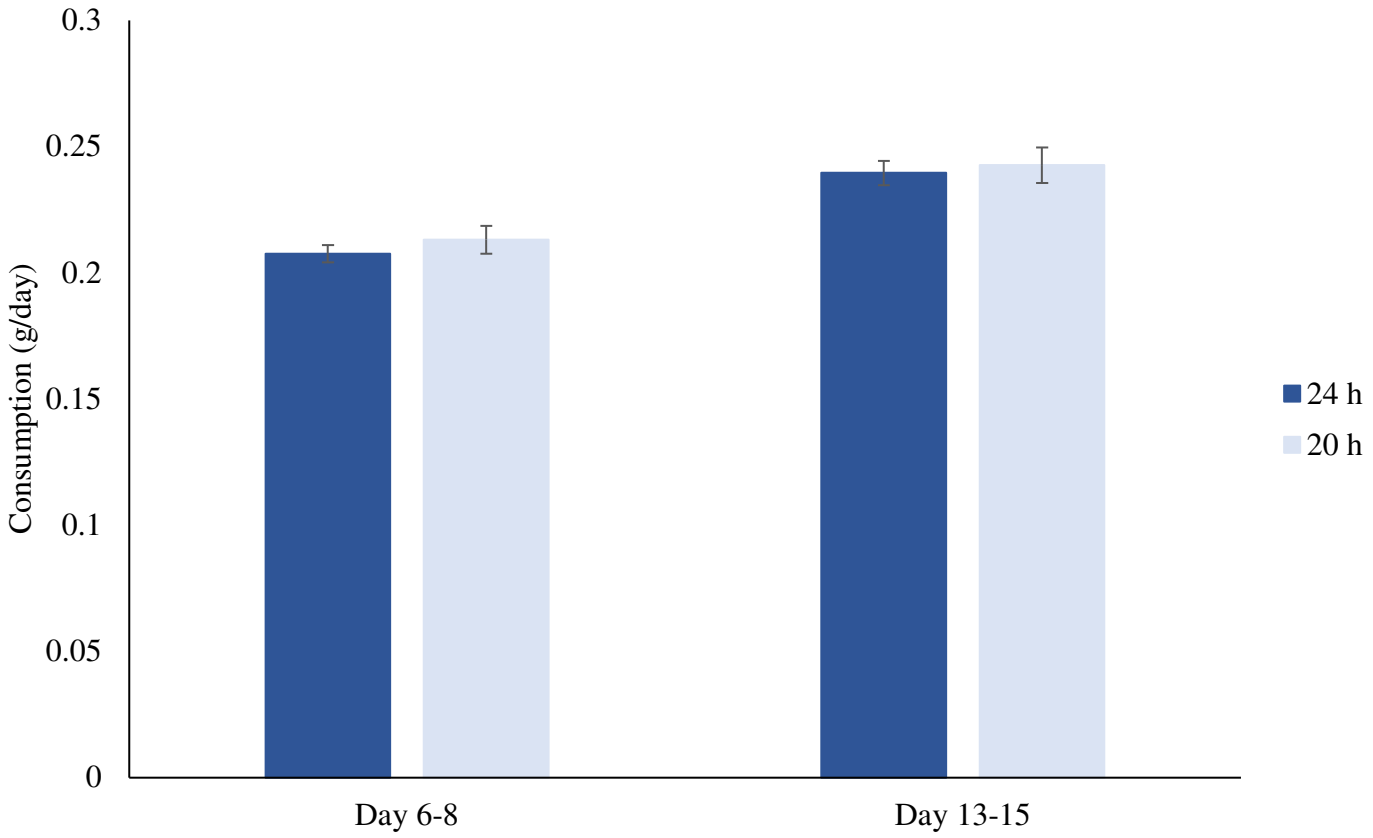


**Figure 3.7** Consumption of 1 M sucrose solution increased as honey bees aged, consuming 0.21 g (±0.04) of solution per day per bee between day 6 and 8, compared to 0.24 g (±0.04) per day per bee between days 13 and 15 (Univariate ANOVA, Day: $F_{1,3}$ =32.964, p< 0.005). Circadian period had no effect on the rate of consumption in either measurement period (Univariate ANOVA, CircadianPeriod*Day: $F_{1,3}$ =0.054, p< 0.823) (N=10)

### 3.3.2 ***Entrainment to a circadian period significantly different from honey bees innate free-running period will cause disruption to key circadian genes that will result in a failure to match expression to environmental cues***

#### *3.3.2.1 Sir2*

*Sir2* expression was measured using a univariate ANOVA with $C_t$ value relative to the reference gene *RP49* as the dependent variable with circadian period (CP), age and time point (TP) set as fixed factors. All results were relative to TP1 of the 24 h treatment on day 7. *Sir2* expression was stable across all ages and regardless of circadian period manipulation. The analysis showed no significant interactions between CP and age on the expression of *Sir2* ($F(2, 54) = 0.623$, $p = 0.540$) **(Figure 3.8A)**. From the results of a simple main effects analysis we can likely reject the hypothesis that overall *Sir2* expression is changed by CP manipulation ($F(2, 54) = 0.470$, $p = 0.628$) and this effect is not influenced by the age of the honey bee. Neither CP and TP ($F(4, 54) = 0.761$, $p = 0.555$) nor a combination of CP, age and TP ($F(4, 54) = 0.904$, $p = 0.468$) showed any statistically significant interaction with regards to *Sir2* expression.

The results of the analysis of *Sir2* expression between the time points was inconclusive. There was also no statistically significant interaction between age and TP ($F(2, 54) = 1.569$, $p = 0.218$) **(Figure 3.8B)**, however both age ($F(2, 54) = 2.225$, $p = 0.142$) and TP ($F(2, 54) = 2.544$, $p = 0.088$) show non-statistically significant differences suggestive of rhythmical cycling which could, with more extensive study potentially lead to significant results. There is also non-significant evidence to suggest that *Sir2* is more highly expressed as honey bees age, with levels at day 14 (1.80x) of the experiment higher than at day 7 (1.21x) (LSD *Post hoc* $p= 0.074$). When results at day 7 are considered independently **(Figure 3.8C)** it is clear that circadian period has no detectable effect of expression of *Sir2* at this age group ($F(2, 27) = 0.652$, $p = 0.529$) and any difference between timepoints is less pronounced ($F(2, 27) = 2.175$, $p = 0.133$) and unlikely to be affected by an interaction with CP ($F(4, 27) = 0.516$, $p = 0.725$). Honey bees at day 14 show no changes in overall *Sir2* expression because of changes to their CP ($F(2, 27) = 0.438$, $p = 0.650$) and also show non-significant differences in *Sir2* expression based on the TP in which the measurement was collected ($F(2, 27) = 1.937$, $p = 0.164$). The interaction between CP and TP is also not statistically significant ($F(4, 27) = 1.155$, $p = 0.353$) **(Figure 3.8D)**. One potential point is the differing behaviour of the 28 h CP for day 7 compared to day 14. While at TP1 both show a decrease in expression relative to

the TP1 of the 24 h treatment on day 7 (0.426x and 0.567x) and show a non-significant increase relative to this by TP2 in both day 7 (1.754-fold, LSD *Post hoc* p=0.107) and day 14 (2.020-fold, LSD *Post hoc* p=0.295), at TP3 on day 7 there is a near significant decrease (0.095x, LSD *Post hoc* p=0.056), while on day 14 by TP3 the 28 h CP has seen a near significant increase of 3.398-fold (LSD *Post hoc* p=0.067). This difference in cycling behaviour could potentially be due to desynchronisation of internal circadian clockwork with external stimulus.

*3.3.2.2 Clk*

*Clk* expression was measured using the same analytical techniques as *Sir2* listed in *3.3.2.1*. All expression was standardised relative to 24 h day 7 TP1 and *RP49* was used as a reference gene. As observed in *Sir2*, *Clk* is more highly expressed in older honey bees than younger bees in this study. Univariate analysis of variance showed a significant interaction between honey bee age, CP and TP for *Clk* expression ($F(4, 54) = 3.000$, $p = 0.026$). While only CP and age had a significant interaction ($F(2, 54) = 3.444$, $p = 0.039$), both CP and TP ($p = 0.089$) and TP and age ($p = 0.113$) both had near statistically significant interactions. A simple factor analysis showed that expression was overall lower on day 7 of the experiment than on day 14, showing only a 1.4-fold increase in *Clk* expression compared to the older bees 6.068-fold increase ($F(1, 54) = 16.577$, $p < 0.005$). However, this increase was only found in the non-standard circadian treatments, whereas the 24 h CP showed stable overall *Clk* expression across the experiment **(Figure 3.9A)**.

Clk showed differential expression based on the period in the CP of the bees in question for both 24 and 20 h treatments, with expression peaking in the later part of the cycle. This was not the case for the 28 h treatment, where no cyclical expression was observed at day 7. Both 7 day old and 14 day old bees saw significant changes in *Clk* expression depending on the TP at which sampling occurred ($F(2, 54) = 4.317$, $p = 0.018$), LSD *post hoc* analysis showed that for both 7 and 14 day old bees *Clk* expression at TP1 was lowest (M=1.430), rising to a peak at TP2 (M= 4.742, p= 0.035) and then remaining above TP1 at TP3 (M=5.709, p= 0.007) **(Figure 3.9B)** This suggests that *Clk* expression is based on external cues rather than locked to an internal rhythm as they both peaked relative to the conditions in the housing chambers, not a preserved internal rhythm. At day 7 both the 20 h (M=1.021, p< 0.005) and 28 h (M=0.248, p< 0.005) treatments have significantly lower levels of *Clk* expression than the 24 h treatment (M=2.994) **(Figure 3.9C)**. However, by day 14 both the 24 h (M= 4.772) and 28

h (M=4.325) show no significant difference between their overall expression (LSD *post hoc* p= 0.876). The 20 h (M=10.403) treatment shows a near significant increase in overall *Clk* expression relative to both the 24 h (p= 0.057) and 28 h (p=0.074) treatments. This is likely due to the increase in expression at TP3 which was significantly higher than either point for 24 h and 28 h (both $p < 0.005$) **(Figure 3.9D)**.

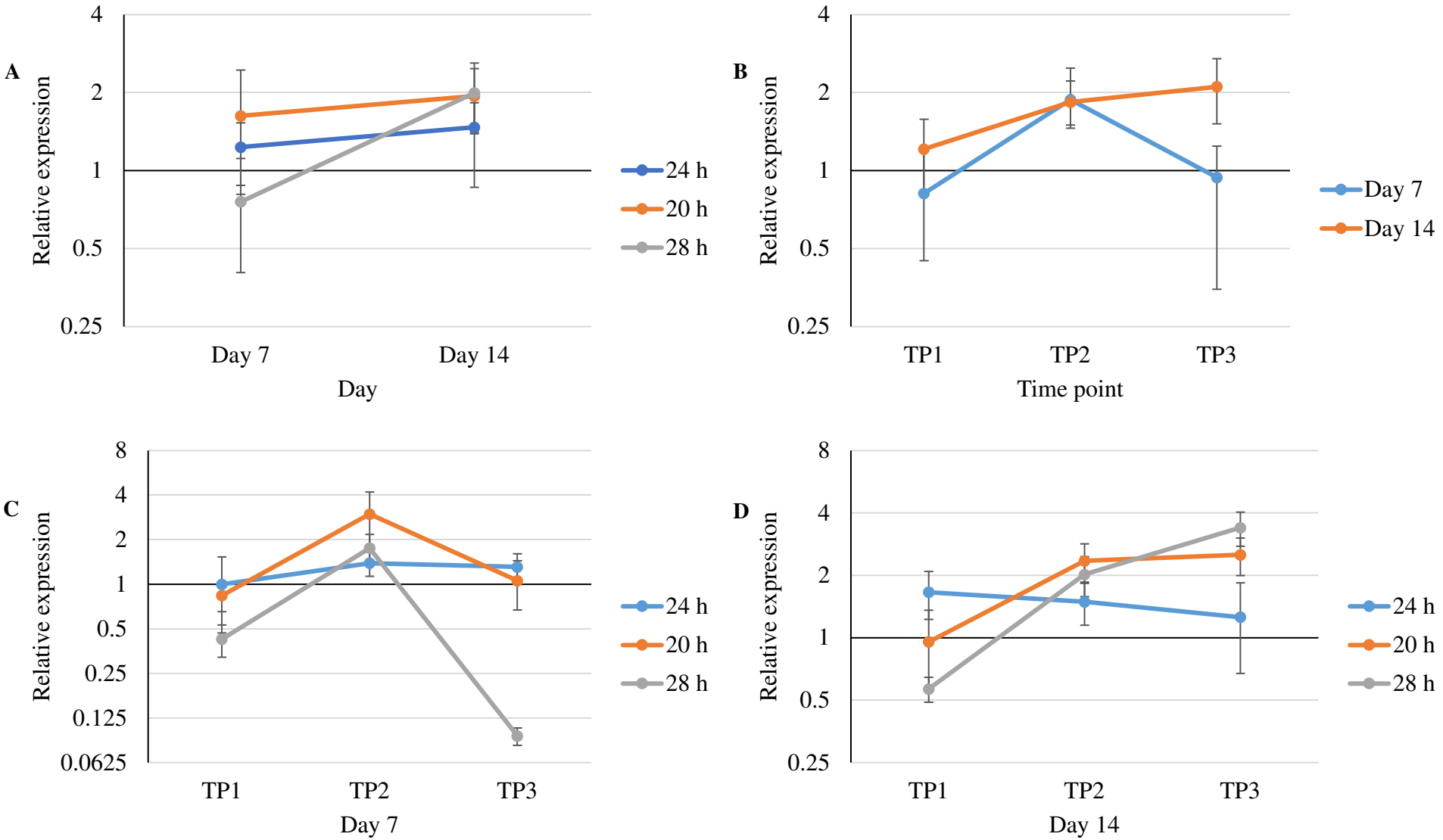


**Figure 3.8** (**A**) Changes in expression of *Sir2* between day 7 and day 14 for all 3 CP tested in the trial (Univariate ANOVA, $F(2, 54) = 0.623$, $p = 0.540$). (**B**) Relative changes in *Sir2* expression across all 3 time points for the combined CPs (Univariate ANOVA, $F(2, 54) = 1.569$, $p = 0.218$). (**C**) Relative changes in *Sir2* expression across all 3 time points for each CP at day 7 of the trial (Univariate ANOVA, $F(4, 27) = 0.516$, $p = 0.725$). (**D**) Relative changes in *Sir2* expression across all 3 time points for each CP at day 14 of the trial (Univariate ANOVA, $F(4, 27) = 1.155$, $p = 0.353$). All expression shown as relative change compared to 24 h TP1 day 7 treatment against reference gene *RP49* ±*SEM*. N = 9

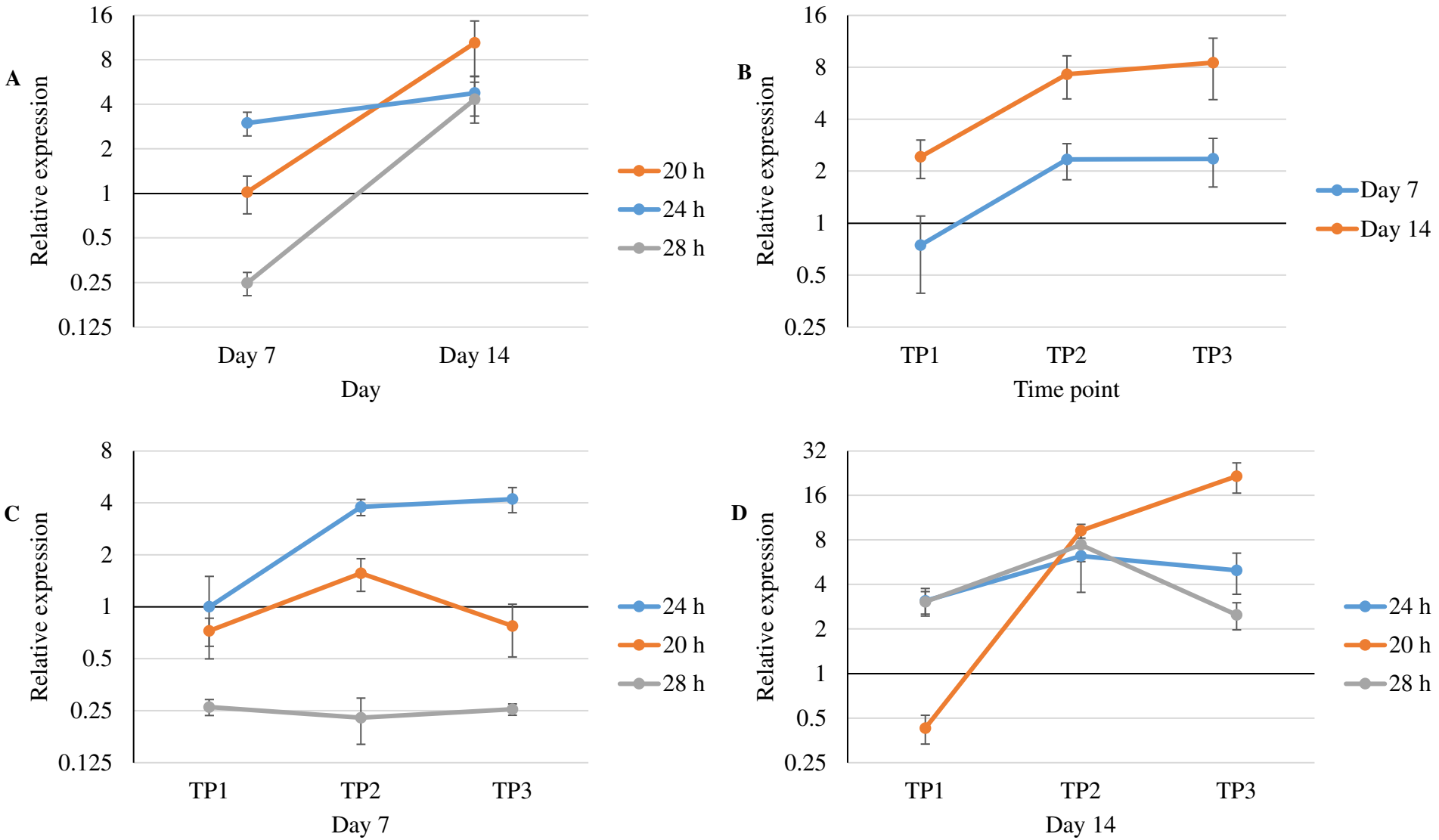


**Figure 3.9** (**A**) Changes in expression of *Clk* between day 7 and day 14 for all 3 CP tested in the trial (Univariate ANOVA, ($F(2, 54) = 3.444$, $p = 0.039$). (**B**) Relative changes in *Clk* expression across all 3 time points for the combined CPs (Univariate ANOVA, $F(2, 54) = 4.317$, $p = 0.018$). (**C**) Relative changes in *Clk* expression across all 3 time points for each CP at day 7 of the trial (Univariate ANOVA, $F(4, 27) = 1.957$, $p= 0.130$). (**D**) Relative changes in *Clk* expression across all 3 time points for each CP at day 14 of the trial (Univariate ANOVA, $F(4, 27) = 2.597$, $p = 0.059$). All expression shown as relative change compared to 24 h TP1 day 7 treatment against reference gene *RP49* ±*SEM*. N = 9

*3.3.2.3 Cry2*

As with the other genes, there was a near significant trend suggesting that increased age leads to greater expression of key circadian genes. Across all treatments *Cry2* was expressed 3.36-fold higher than baseline at 14 days, compared to only 1.39-fold at day 7 ($F(1, 54) = 3.744$, $p = 0.058$) **(Figure 3.10A)**. *Cry2* showed no significant changes in expression across the time points with regards to CP ($F(2, 54) = 0.290$, $p = 0.749$) **(Figure 3.10B)**. There were no consistent patterns in expression between the time points at either day 7 or day 14. While 20 and 28 h treatments appear to show similar trends for both these age groups, the 24 hour treatment does not. This was also found to be the case when only 7 day old honey bees were included in the analysis ($F(4, 27) = 1.334$, $p = 0.283$) however there were near significant differences between TP1 (M=0.46) and TP3 (M=1.78) (LSD *post hoc*, p= 0.085) and between the 24 h (M=2.19) and 28 h (M=0.161) CP treatments (LSD *post hoc*, p= 0.065) **(Figure 3.10C)**. *Cry2* expression for 14 day old honey bees was far more uniform, with no significant interactions observed ($F(4, 27) = 1.430$, $p = 0.251$) and no significant or near significant factors (all P-values > 0.222) **(Figure 3.10D)**. From this we can determine that there is no consistent cyclical behaviour in *Cry2* at 14 days.

*3.3.2.4 Per*

A univariate ANOVA was conducted to determine the impact of age, CP and TP on relative expression of the honey bee gene *per* relative to the reference gene *RP49* as calculated via the ΔΔCt method. It was observed that CP plays a significant role in the expression of *per* in the honey bee ($F(2, 54) = 3.744$, $p = 0.058$). *Per* expression in the 28 h (M=1.925) treatment was significantly lower than both the 24 h (M=5.536, LSD post hoc, p= 0.016) and 20 h (M=5.332, LSD post hoc, p= 0.048) treatments, although like other measure genes there was a near-significant trend showing increased expression of *per* with age ($F(1, 54) = 2.374$, $p = 0.129$) **(Figure 3.11A)**.

Honey bees did not show any measurable cyclical changes in the expression of *Per* regardless of age. No statistically significant interaction was observed between TP and age ($F(2, 54) = 1.428$, $p = 0.249$) **(Figure 3.11B)** and no difference in expression of *per* was observed when using TP alone ($F(2, 54) = 1.055$, $p = 0.355$). It is therefore unlikely that the animals in this study showed observable cyclical changes in their *per* expression based on time point in the circadian cycle in a way consistent across all treatments.

*Per* does not show any measurable or consistent change in its expression across the CP at day 7 of the trial, but by day 14 there is observable decreases in the level of *Per* at the end of the cycle for both the 20 and 28 h treatments. It is possible that *per* is expressed with greater amplitudes in older honey bees throughout the day than in younger honey bees. When analysed separately by age, there was no statistically significant difference between time points for any treatments in 7 day old worker bees (LSD *post hoc*, all P-values >0.341) **(Figure 3.11C)**. However, when only 14 day old bees were included in the analysis TP3 was found to have significantly lower levels of *per* than TP2 (LSD *post hoc*, $p = 0.038$) **(Figure 3.11D)**.

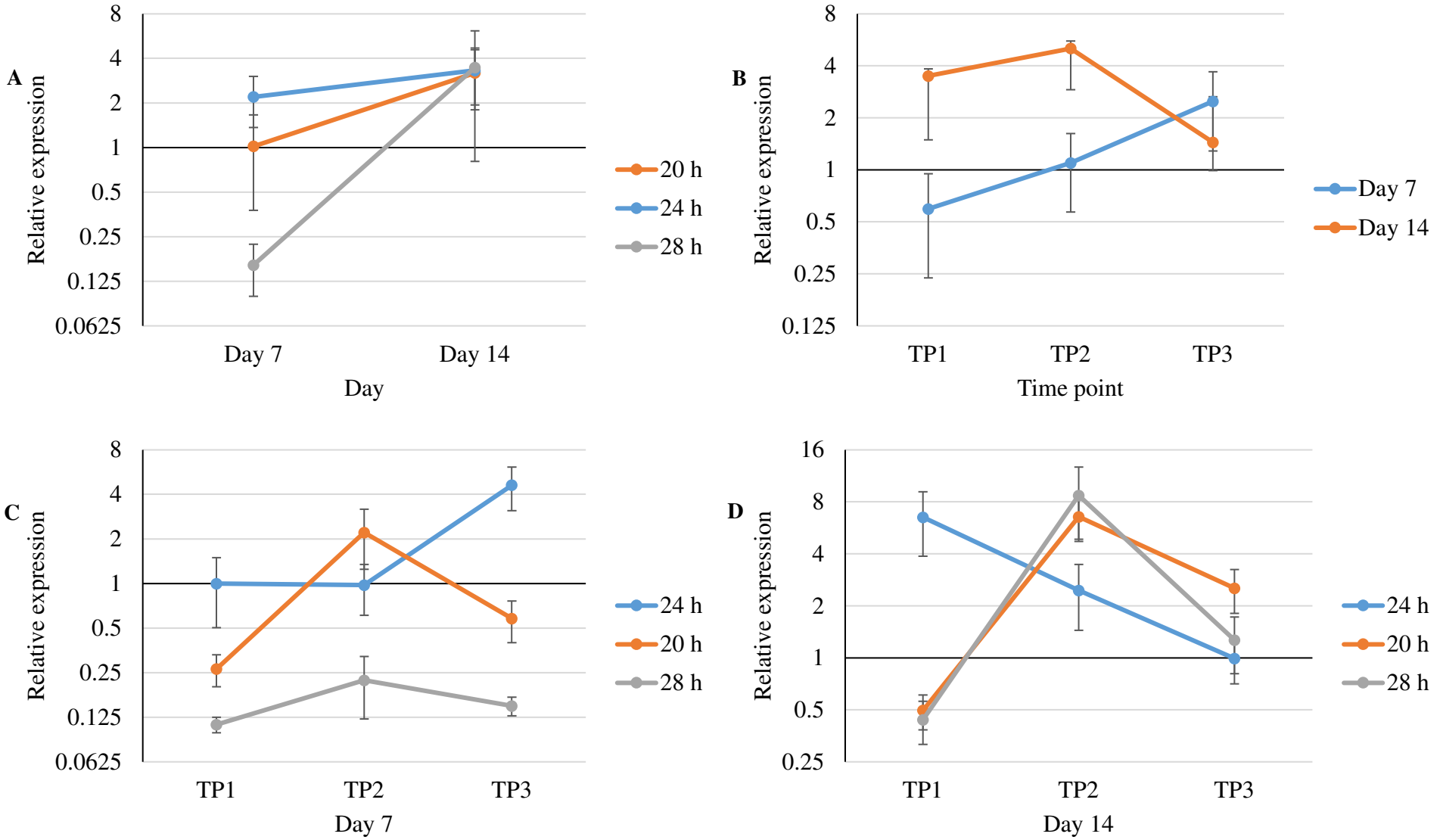


**Figure 3.10** (**A**) Changes in expression of *Cry2* between day 7 and day 14 for all 3 CP tested in the trial (Univariate ANOVA, ($F(2, 54) = 3.744$, $p = 0.058$). (**B**) Relative changes in *Cry2* expression across all 3 time points for the combined CPs (Univariate ANOVA, $F(2, 54) = 0.290$, $p = 0.749$). (**C**) Relative changes in *Cry2* expression across all 3 time points for each CP at day 7 of the trial (Univariate ANOVA, $F(4, 27) = 1.334$, $p = 0.283$). (**D**) Relative changes in *Cry2* expression across all 3 time points for each CP at day 14 of the trial (Univariate ANOVA, $F(4, 27) = 1.430$, $p = 0.251$). All expression shown as relative change compared to 24 h TP1 day 7 treatment against reference gene *RP49* ±*SEM*. N = 9

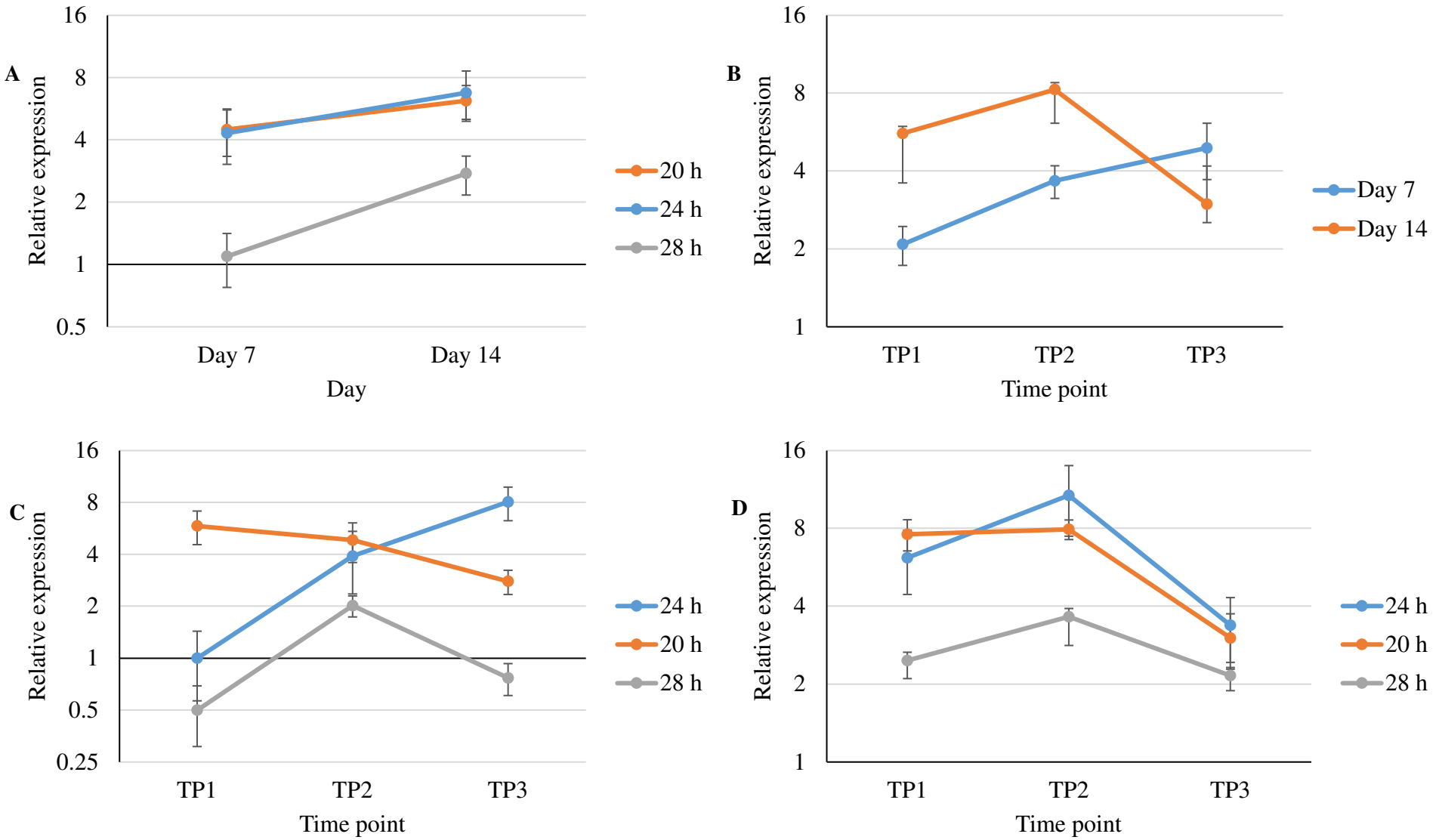


**Figure 3.11** (**A**) Changes in expression of *per* between day 7 and day 14 for all 3 CP tested in the trial (Univariate ANOVA, $F(2, 54) = 3.744$, $p = 0.058$). (**B**) Relative changes in *per* expression across all 3 time points for the combined CPs (Univariate ANOVA, $F(2, 54) = 1.428$, $p = 0.249$). (**C**) Relative changes in *per* expression across all 3 time points for each CP at day 7 of the trial (Univariate ANOVA, $F(4, 54) = 1.896$, $p = 0.140$). (**D**) Relative changes in *per* expression across all 3 time points for each CP at day 14 of the trial (Univariate ANOVA, $F(4, 27) = 0.338$, $p = 0.850$). All expression shown as relative change compared to 24 h TP1 day 7 treatment against reference gene *RP49* ±*SEM*. N = 9

### 3.3.3 ***High amino acid diets have little to no further effect on survival on bees with circadian desynchronization.***

Non-24 h circadian periods dramatically decreased survival for honey bees compared to a standard 24 h period regardless of diet. Bees on the 28 h treatment experienced a 1.5 increased risk of death compared to those on the 24 h control treatment (Coxreg, CircadianPeriod, HR = 1.539, [95 % CI 1.283–1.848], p< 0.005).

As previously observed (see Chapter 2), diets high in EAA proved harmful to lifespan in the honey bee. The risk to bees on a high EEA:C diets was 2 times greater than those on the low EAA:C diets (Coxreg, CircadianPeriod, HR = 1.979, [95 % CI 1.649–2.375], p< 0.005). The interaction between CP and diet on survival was not significant, however given how closely the value approaches significance it is worth further investigation (Coxreg, CircadianPeriod, HR = .802, [95 % CI .623–1.032], p< 0.086) **(Figure 3.12)**.

This effect was also measured in bees on the 20 h treatment. Individuals on the 20 h CP suffered a 1.6 times greater risk of death relative to those on the 24 h control (Coxreg, CircadianPeriod, HR = 1.663, [95 % CI 1.386–1.995], p< 0.005).

In the 20 h study, for honey bees on a low EAA diet, a CP of 20 h significantly reduced survival compared to the bees on a 24 h CP. However, for bees already on high EAA:C diets showed no further risk when placed on the same circadian period that was harmful to bees fed low EAA:C diets. Those bees fed a high EAA:C diet had a 2.4 times greater risk of death relative to the low EAA:C diet (Coxreg, CircadianPeriod, HR = 2.396, [95 % CI 1.988–2.887], p< 0.005). This effect of diet on survival was not consistent between circadian periods however, there was a significant interaction between the effect of day length and diet (Coxreg, CircadianPeriod, HR = .679, [95 % CI .528–.874], p= 0.003) **(Figure 3.13)**.

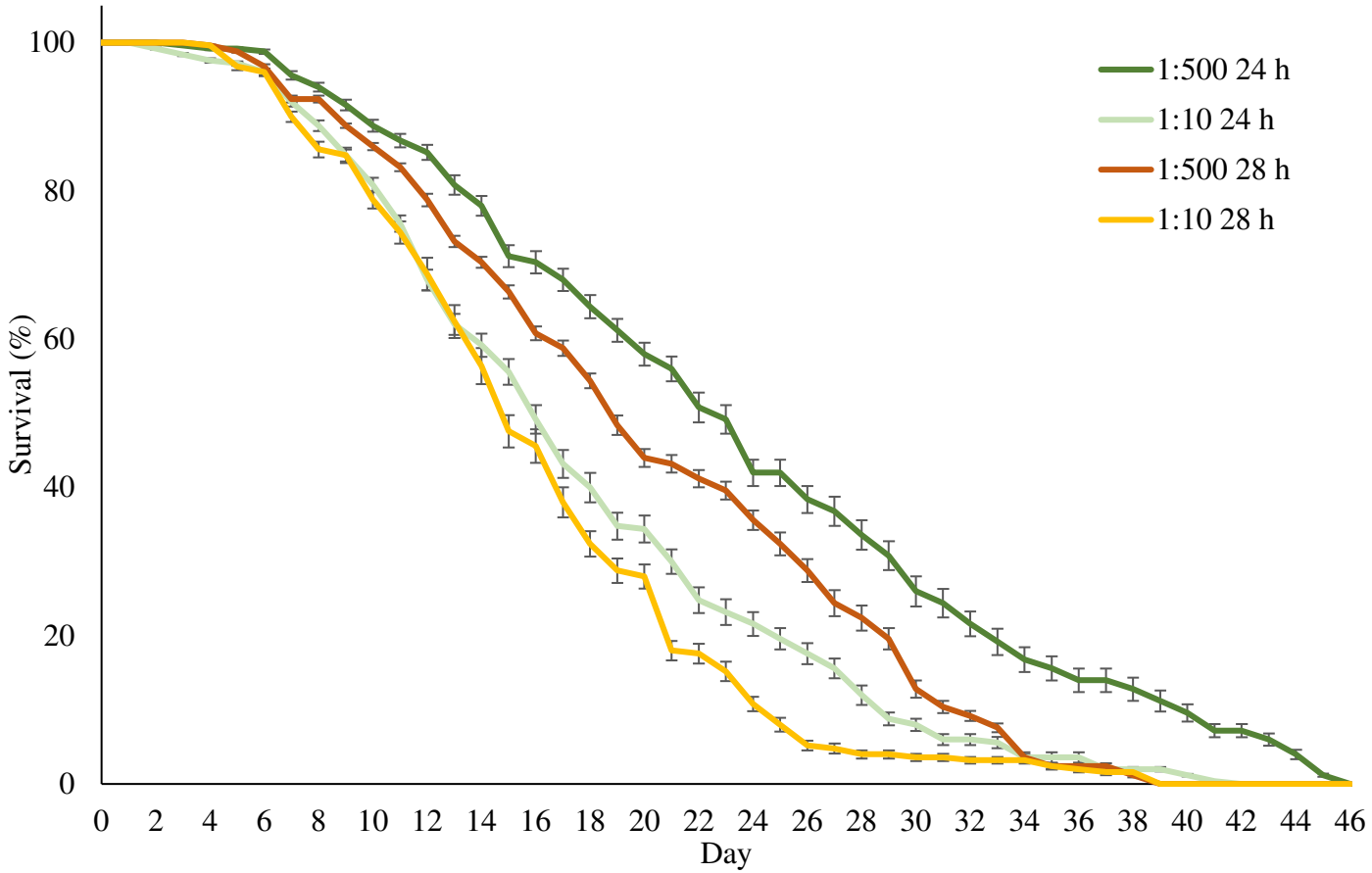


**Figure 3.12** Honey bees fed on low EAA diets had a higher chance of dying throughout the experiment when entrained on the circadian period longer than 24 h compared to the 24 h control (Coxreg, CircadianPeriod, HR = 1.539, [95 % CI 1.283–1.848], p< 0.005). This was also true of individuals fed the high EAA diets (Coxreg, CircadianPeriod, HR = 1.979, [95 % CI 1.649–2.375], p< 0.005). There was no significant interaction between diet and day length on survival at the 5% significance (Coxreg, CircadianPeriod, HR = .802, [95 % CI .623–1.032], p< 0.086). (N = 10 of 25 boxes per treatment)

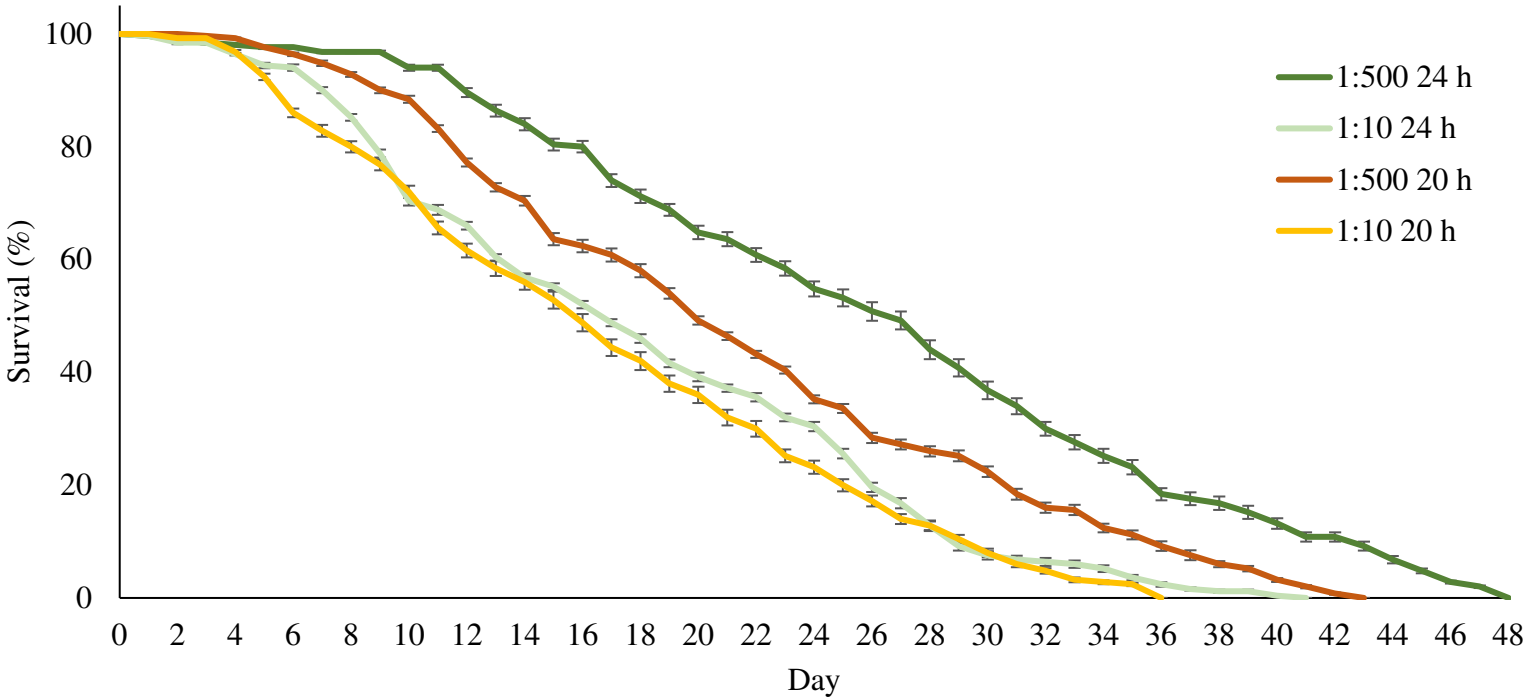


**Figure 3.13** Like the 28 h experiment, the 20 h circadian period and diet seem to negatively influence survival (Coxreg, CircadianPeriod, HR = 1.663, [95 % CI 1.386–1.995], p< 0.005), however unlike the 28 h experiment there is significant interaction between the effects of diet and day length on honey bee survival diet (Coxreg, CircadianPeriod, HR = 2.396, [95 % CI 1.988–2.887], p< 0.005). Unlike bees on the low EAA:C diets those on the high EAA:C diets appear to suffer no further ill-effects of a non-24 h circadian period. This suggests that the underlying mechanisms could share similar pathways. (N = 10 of 25 boxes per treatment)

## 3.4 **Discussion**

### 3.4.1 ***Does rearing honey bees on non-24 h circadian cycles cause them to be subject to constant, daily re-entrainment which will negatively influence over-all fitness and survival?***

Survival of bees in these experiments was clearly affected by the non-24 h circadian light cycles. It was predicted that if these treatments did lead to forced internal desynchronization, this would subject the honey bees to continual re-entrainment over a sustained period. This effect was more pronounced in the longer 28 h circadian period compared to the 24 h control. There could be a physiological basis for this, that drawing out each stage is more harmful than contracting it. However, the answer may be simpler; when manipulating periods of light and dark within 24 hour cycles, Moore and Rankin (1985) found that honey bees tended to have free-running circadian rhythms between 22 and 24 h so it is possible that the free-running rhythm of the honey bees in this study was closer to 20 h than 28 h. Despite the effect being larger in the 28 h treatment, the 20 h cycle displayed a similar trend, these results strongly suggest that both a 20 h circadian cycle and a 28 h circadian cycle are beyond the physiological range of the honey bee to successfully entrain.

This is consistent with the work of Pittendrigh and Minis (1972) who had similar findings in *D. melanogaster*. The subjects in that study survived longer (over 50 days in all treatments) than bees in the present study, but *D. melanogaster* are longer lived generally than honey bees in the height of summer (Lamb, 1968; Seeley, 1978) and the range of cycles in Pittendrigh and Minis (1972) were narrower than in this study (21.5-27 vs 20-28 h respectively). Dalley (1980) showed similar effects in an even more extreme circadian period (16 h) in the shrimp *Crangon crangon*. This adds further credence to the theory that an animal's physiological efficiency is dependent on synchronisation between its internal clock and external cues from the environment. This phenomenon is the first time this has been shown in the honey bee.

### 3.4.2 ***Does entrainment to a circadian period significantly different from their innate free-running period cause disruption to key circadian genes that will result in a failure to match expression to environmental cues?***

As the primary molecular analysis of the study failed to achieve significant results, we cannot definitively state the cause of the increased mortality suffered by bees on abnormal circadian periods, only that the effect itself is present. Circadian disruption has long been linked with cancer, diabetes, heart disease and metabolic diseases in humans which are all leading causes of death in adults. The significant results of the preliminary study show that after 19 days, Clk expression is consistent with the circadian period they are exposed to, further confirming

strong circadian rhythmicity at that stage, which is susceptible to manipulation. One possible reason for the *Clk* being higher during this stage is that collection was timed to coincide with the "night phase" of the 28 h treatment, where Clk is known to peak (Hoeijmakers, 2009). Another reason is that *Clk* is shown to be upregulated during circadian disruption, of which the 28 h cycle could be considered (Olovnikov, 1973).

The cause of entrainment to non-standard circadian periods is almost certainly the result of the photoperiod used, whether light is the direct cause or the driver of a secondary mechanism such as body temperature, metabolic energy expenditure or nutritional feedback is something still not fully understood and cannot be confirmed within the limitations of this study. Worker honey bees have been shown to exhibit strong circadian rhythms between 2- and 3-weeks' post emergence in captivity when housed in groups with or without a queen. In humans, the liver has been shown to keep a clock independent of the suprachiasmatic nucleus (SCN) which relies on nutritional input to regulate its rhythm independent of light. The role of fat bodies in the honey bee has been considered similar to that of the vertebrate liver in terms of their metabolic action. These fat bodies are responsible for the storage of nutrients and the synthesis of proteins, lipids, and carbohydrates. Like the liver, fat bodies are also the method by which honey bees detoxify nitrogenous waste products that build up in haemolymph (Harman, 1956).

Work by von Frisch (1968) showed that honey bees can be entrained to cycles via feeding time even when light is kept constant. As workers, do not consume food during darkness and enter a sleep-like state, it may be that the circadian shift happens in the fat bodies of the honey bees and the localised expression of Clock genes, explaining why we did not find cycling in the analysis of mRNA isolated from the honey bee head. The more likely explanation however, is sample sizes were insufficient to measure genes that which are only expressed at a low level. Snap freezing samples in liquid nitrogen immediately after collection rather than euthanizing the subjects in a -80 $^{o}$C freezer would improve the preservation of RNA but was rejected on safety and logistical grounds. In future, studying measures of the activity of individuals would help to answer these questions but it was not possible in this case due to constraints in time and facilities.

### 3.4.3 ***Is the increased mortality associated with circadian desynchronization due to upregulation of the mTOR pathway? If so do mTOR activators like high amino acid diets further effect survival?***

Statistical analysis indicates that for the 3 genes (*Sir2*, *Cry2*, *per*) analysed, the strongest expression is found during the early hours of darkness for the honey bee, suggesting that whichever circadian oscillations in expression occur in these genes, they occur in tandem with the light manipulations and not the natural, external, environment. These peaks in early night are consistent with findings in mice (Leloup and Goldbeter, 2003) and honey bees (Bloch et al., 2001).

While no other significant conclusions could be drawn from the current findings relating to the clock genes, mRNA analysis of circadian stressed honey bees fed modified diet formulations may help to inform further investigations. As found in Paoli *et al.* (2014) survival was higher in diets low in essential amino acids and with the sucrose only diets; both shorter and longer circadian periods resulted in diminished survival. What is interesting is that the relationship between circadian period and survival was not consistent between the diets on the 20 h treatments. As hypothesised, the effect of a diet high in EAAs is reduced when combined with a circadian period that also reduces survival. While it is possible that this effect was a "bottoming out" of survival stressors it is also worth considering whether both these effects act through a single pathway or mechanism, the most likely of which is mTOR.

The *mTOR* pathway is inhibited by SIRT1 action in SIRT1 deficient mouse embryonic fibroblasts (MEFs). This is also true in non-mammalian cells, e.g. Sir2 inhibits the mTOR pathway in *Saccharomyces cerevisiae* (yeast cells) (Ha and Huh, 2011). BMAL1, a key protein in the cellular clock action also limits mammalian Target of Rapamycin Complex 1 (mTORC1) activity (Ghosh *et al.*, 2010). Work by Paoli *et al.* (2014) showed that Sir2 levels in honey bees fed diets with high enough EAA levels to reduce survival were significantly lower compared to those on diets low in EAAs. Sir2 is also required for successful caloric restriction induced longevity in several organisms (Lin *et al.*, 2000; Rogina and Helfand, 2004). Sir2 is known to not only regulate metabolic processes but interacts with the circadian clockwork in a cyclical manner (Bellet *et al.*, 2011). Interestingly, the crucial interaction for circadian rhythm between BMAL1/CLOCK is dependent on SIRT1/Sir2. In BMAL1 knockdown mice, treatment with the mTOR inhibitor Rapamycin partially restores lifespan (Khapre *et al.*, 2014). Hepatocytes treated with palmitate, a fatty acid shown to promote insulin resistance and trigger cell apoptosis during the development of obesity inhibited and expression of circadian genes like *Per2* are supressed. This is reversed when treated with

SIRT1 activators suggesting that it is SIRT1 inhibition that is responsible for this breakdown (Tong *et al.*, 2015).

While we were unable to confirm this relationship with molecular analyses, there is a compelling case that the effect we observed was due to failure of Sir2 to repress the *mTOR* pathway. As Nakahata *et al.* (2008) showed, Sir2 is a rheostat for metabolic processes and is dependent on circadian feedback to repress *mTOR*. If *Sir2* activity was already repressed by circadian dysregulation, then, as expected, further disruption to the system would have no further effect on lifespan.

One further possible theory for the discrepancy between the 28 h and 20 h results is the timing of the experiments. The 20 h experiment was conducted after the 28 h experiments, at a date later in the summer due to space availability. It has been shown that honey bees that emerge later in the year have larger fat bodies than those born in the height of summer. This increase in fat body mass could well be responsible for increasing their tolerance to the cytotoxic effects of excess dietary amino acids and partially protect them from the combined effects of circadian disruption and diet, which was not the case in the 28 h treatments. This is one potential explanation as to why the interaction was not quite significant, and a high EAA:D diet affected survival even in circadian disrupted individuals.

Honey bees, like most other living creatures, show diurnal rhythms based around our planets rotation as it orbits the sun. These periods of day and night have a profound effect on the conditions faced by organisms living on the earth's surface. As such, it is unsurprising that honey bees are subject to severe physiological pressure when exposed to circadian cycles significantly different from 24 h. Longer cycles appear to apply more stress than shorter cycles, which could provide an important avenue of research in the future. Most interestingly, while diets high in EAAs are shown to negatively impact survival in control treatments, they demonstrated proportionally less harmful effects relative to the severity of the circadian disruption. This could be because the honey bee was already overwhelmed by the circadian disruption, but it is also likely that the stressors caused by a diet high in EAAs also affect the same pathway as those caused by circadian disruption. A likely target for this is the mTOR pathway, due to decreased *Sir2* activity. However, further investigation is needed and could help inform us on the role that diet and circadian rhythm play in the ageing process.

# Chapter 4. The interaction between diet and the mTOR inhibitor rapamycin on survival in the honey bee

## 4.1 **Introduction**

Of the many ways discovered with which we can extend lifespan beyond its natural maximum, simple dietary restriction (DR) was amongst the earliest and broadly effective (Ball *et al.*, 1947). By limiting caloric intake slightly above the point at which malnutrition occurs, without limiting essential nutrient intake, it is possible to decelerate the pace at which cellular ageing occurs (McCay *et al.*, 1935). Through this method, both lifespan and healthspan (Ravussin *et al.*, 2015) can be extended in a wide variety of species including but not limited to yeast (Jiang *et al.*, 2000), nematodes (Schulz *et al.*, 2007), flies (Rogina and Helfand, 2004), crickets (Lyn *et al.*, 2011), mice (Weindruch and Walford, 1982; Sohal *et al.*, 1994b), rats (Yu *et al.*, 1982; Weindruch *et al.*, 1986) and primates (Heilbronn *et al.*, 2006; Colman *et al.*, 2009; Mattison *et al.*, 2012). While longevity studies have not been conducted in humans due to the inherent difficulty of enforcing and recording in such long lived creatures (Racette *et al.*, 2006), markers of longevity, such as reduced inflammation (Johnson *et al.*, 2007; Meydani *et al.*, 2016) without inhibiting immune response and improved insulin sensitivity (Barzilai *et al.*, 1998; Kirk *et al.*, 2009) have been identified, supporting the possibility that DR could also be an effective intervention in humans.

Because DR is such a drastic and all-encompassing intervention there is significant debate about the underlying mechanism or if there even is a single underlying mechanism and rather than several complimentary components (Heilbronn and Ravussin, 2003). Unsurprisingly the mechanism(s) through which this life-extension occurs is a subject of much study (Sohal and Weindruch, 1996; Hursting *et al.*, 2003; Barja, 2004; Ungvari *et al.*, 2008). Theories range from (but are not limited to) a decrease in core body temperature (Lane *et al.*, 1996), DNA damage mitigation (Sohal *et al.*, 1994a; Vermeij *et al.*, 2016) and 'hormesis' (where a low-intensity stressor elicits a response which protects against cellular ageing actors) (Masoro, 2007). One thing that is certain however, is that DR is a major physiological inducer of autophagy ("self-eating") (Yang *et al.*, 2014). Autophagy plays an important role in the removal and recycling of damaged macromolecules and organelles from the cell. One of the hallmarks of ageing is a decrease in autophagic activity, something which is worsened in age-related diseases (Barbosa *et al.*, 2019).

### 4.1.3 *The mechanistic target of rapamycin (mTOR)*

One of the major autophagic regulators is TOR kinase, the mammalian target of rapamycin (*mTOR*). The TOR pathway is an intracellular signal transduction pathway that is highly conserved, being present universally from yeast to mammals. Not only is it a potent inhibitor of autophagy but it is crucial in regulating critical cellular processes such as growth, proliferation, cytoskeletal organization, transcription, protein synthesis and ribosomal biogenesis. TOR integrates metabolic signals from the body (including but not limited to nutrients, hormones, growth factors and stressors) allowing the cell to respond to multiple inputs by acting as a master regulator of nutrient and hormone cues (Sengupta *et al.*, 2010). The *ATG* genes that control autophagy are downstream of TOR kinase which allows mTOR to effectively regulate autophagic processes in coordination with metabolic input.

mTOR is the catalytic subunit of two distinct complexes known as mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) **(Figure 4.1)** (Dowling *et al.*, 2010). These complexes are functionally distinct in both their protein components and the substrates they phosphorylate. mTORC1, formed by mTOR, a protein known as RAPTOR and several others, is acutely inhibited by rapamycin (Dodd and Tee, 2012). Because of this, the compound has been used both therapeutically and as an investigative tool to further understand mTORC1 regulation and function. The physiological function and molecular targets of mTORC2 have been harder to decipher however, although what is known is that it is formed of the mTOR subunit and an associated regulatory protein RICTOR (Frias *et al.*, 2006). Because it is significantly less sensitive to rapamycin, only being effective in certain cell types and with chronic, treatment less is known. The need for more effective tools to further investigate this subunit are evident.

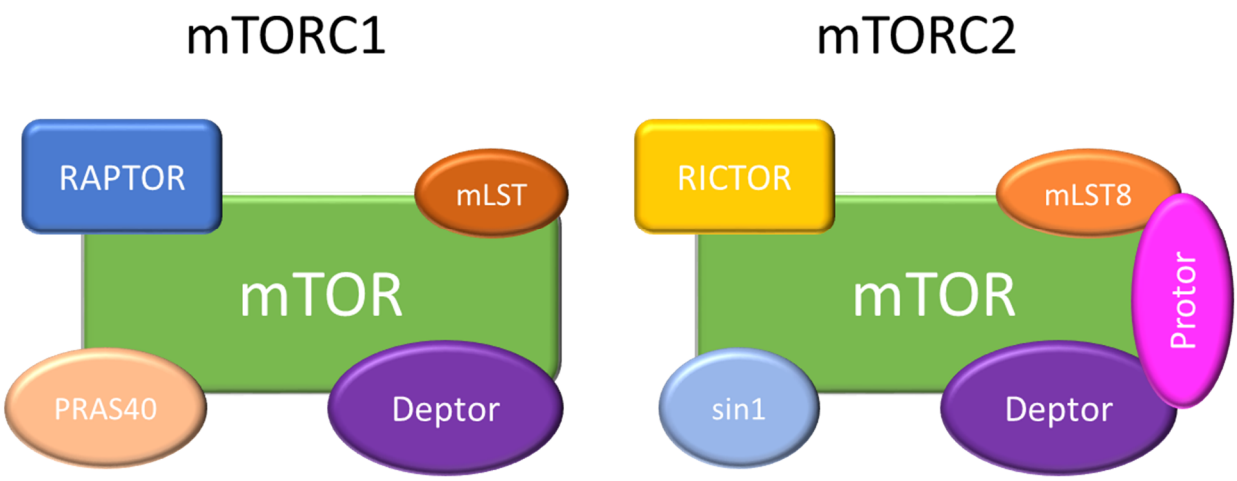


**Figure 4.1** The components comprising the two complexes formed by the mTOR protein in the cell. While rapamycin inhibits the mTORC1 complex directly and immediately, it can also inhibit the formation of the mTORC2 complex with prolonged exposure.

### 4.1.4 *mTOR and metabolism*

mTORC1 integrates four key regulatory inputs, growth factors like insulin, energy status (via AMP-activated protein kinase (AMPK)), oxidative stress and nutrients like free amino acids; it's activity is therefore reflected by the nutritional state of the cell (Wullschleger *et al.*, 2006). Energy in the cell is generated when nutrients are converted to chemical energy stored as ATP by mitochondria. When ATP levels drop, mTORC1 is inhibited (Zoncu *et al.*, 2011). It is this energy sensing by mTORC1 which inhibits autophagy, by phosphorylating Ulk1 Ser 757 preventing activation of the same gene by AMP-activated protein kinase (AMPK) and so inhibiting autophagosomes biogenesis (Kim *et al.*, 2011). Increased mTORC1 activity not only inhibits autophagy but promotes protein and lipid synthesis, as well as mitochondrial biogenesis which all aid in cell growth. The mode of action of mTORC2 remains poorly understood. What is known is that deletion of mTORC2 in mice is lethal, which makes its study difficult and implies an essential role in cell function. The role of mTORC2 in the activation of genes encoding forkhead box proteins, FOXO1 and FOXO3, promote apoptosis and are linked to longevity, is thought to be as an activator of Protein kinase B (PKB) also known as AKT (Laplante and Sabatini, 2009).

Chronic over consumption of nutrients can result in an upregulation of mTOR which in turn leads to a 'metabolic derangement' (Chen *et al.*, 2012). Deregulation of the mTOR pathway has been observed in a number of diseases such as cancer (Sato *et al.*, 2010), diabetes (Fraenkel *et al.*, 2008) and obesity (Wang *et al.*, 2009). As discussed in the previous chapter,

SIRT1 negatively regulates the mammalian target of rapamycin via the mTORC1 complex by interacting with the upstream complex Tuberous Sclerosis Complex 2 (TSC2) (Ghosh *et al.*, 2010). This reduction in mTOR activity is shown more clearly when SIRT1 activators such as resveratrol (a competitor of ATP for the mTOR ATP-binding pocket) are used, and likewise SIRT1 inhibitors such as nicotinamide improve mTOR activity dependent on SIRT1 levels (Wang *et al.*, 2013b). Indeed autophagy in HeLa cells can be induced by resveratrol treatment that promotes SIRT1 activity (Park *et al.*, 2016) but not when mTOR or the autophagic apparatus are disabled (Morselli *et al.*, 2010).

mTOR has long been associated with modifying the ageing process. In yeast, it was discovered that deletion of the gene encoding the ortholog of the downstream target of mTOR S6K would double the duration of time cells were viable during the stationary stage of their cycle. Other methods of downregulating mTOR such as genetic modification (Kapahi *et al.*, 2004; Wu *et al.*, 2013), RNAi knockdown (Fabrizio *et al.*, 2001; Jia *et al.*, 2004) and mTOR specific inhibitors (Powers et al., 2006; Medvedik et al., 2007; Bjedov et al., 2010) have been shown to extend the lifespan of a variety of species. .

### 4.1.5 ***Rapamycin and its role in life extension***

One such specific inhibitor, of mTOR, is rapamycin. This compound, which inhibits eukaryotic cell proliferation, produced by the gram-positive bacterium *Streptomyces hygroscopicus* was first isolated in 1972 by Suren Sehgal and colleagues from samples found on Easter Island (known natively as Rapa Nui, hence the name) (Sehgal, 1998). Licenced as Rapamune or Sirolimus by the US Food and Drug Administration (FDA) in 1999 it was first used as an immunosuppressant to prevent organ rejection in transplant patients and as an anti-fungal agent. However, it was not until the discovery by Powers *et al.* (2006) that rapamycin extended the lifespan of yeast in a dose dependent manner, that focus was given to the compound.

Rapamycin is an inhibitor of both mTORC1 and mTORC2 (Feldman *et al.*, 2009). Intermittent exposure to rapamycin is enough to achieve mTORC1 inhibition in most cases, but only prolonged exposure appears to affect mTORC2 (Sarbassov *et al.*, 2006). Inhibition of mTORC1 signalling by rapamycin is achieved by forming an intracellular complex with the isomerase FKBP12 that then binds to the mTORC1 complex rendering it ineffective (Yip *et al.*, 2010). mTORC2 inhibition is less straightforward however, rapamycin in this case disrupts the formation of the mTORC2 complex, but does not inactivate mTORC2 complexes

that have already formed, through mechanisms that are not yet fully understood (Lamming et al., 2012). Despite this, the inhibition of mTOR1 at least is consistent across multiple taxa (Khor *et al.*, 2016).

There are numerous accounts of rapamycin extending lifespan in organisms ranging from yeast (Powers *et al.*, 2006; Rallis *et al.*, 2013; Lind *et al.*, 2017), long and short lived C. elegans strains (Lind *et al.*, 2017) to rats (Zhang *et al.*, 2013), with trials in primates underway (Ross *et al.*, 2015; Tardif *et al.*, 2015). Not only is it shown to extend lifespan, but delay the onset of age related conditions like cancer (Liu *et al.*, 2005), heart disease (Ramos *et al.*, 2012) and mitochondrial defects (Johnson *et al.*, 2013), and the progress of age related markers such as DNA damage in human fibroblasts (Saha *et al.*, 2014). Extensive work has been conducted in mice, largely due to their well-established lines of cancer and metabolic disorder models and their inherent short lifespans making them useful systems for investigating human ageing. The literature on the subject is conclusive and unanimous that rapamycin successfully extends both mean and maximal lifespan in various mouse models (Swindell, 2016). There is significant debate about the effect of gender on rapamycin's potency (Harrison *et al.*, 2009), as well as whether the life-extending effect is due to inhibiting the occurrence and growth of cancer (Jin *et al.*, 2010) or other, more general factors. It appears however that there is significant evidence that rapamycin has effects other than that of cancer prevention (Anisimov *et al.*, 2010). It is also shown to alleviate a variety of age related diseases in mouse models (Shioi *et al.*, 2003; Spilman *et al.*, 2010) and mimics the effects of DR on organ development in females (Cheng *et al.*, 2015) and improves epigenetic age markers (Liao *et al.*, 2016; Wang *et al.*, 2017).

#### 4.1.6 ***Insect models and inconsistencies; a new problem?***

The use of rapamycin in insect models has largely focused on *D. melanogaster* because, like mice, it is a widely-established model organism with a well-documented genome and many disease mimicking strains. The status of rapamycin as a tool for life extension in this species is however not so clear. While some studies found positive results (Chen *et al.*, 2011), others have in fact found that rapamycin has a detrimental effect on survival in generic strains but beneficial on disease models such as ALS-TDP, Friedreich's ataxia and mitochondrial disease (Calap-Quintana *et al.*, 2015; Cheng *et al.*, 2015; Wang *et al.*, 2016) or even sex differences whereby males are unaffected but females see reduced survival (Harrison *et al.*, 2010) in contrast to findings in mouse studies. Bjedov et al. (2010) found that in *D. melanogaster* rapamycin extended life span, increased stress resistance, reduced fecundity, and increased lipid levels when fed a diet containing 200 μM of rapamycin; this effect was larger in females

than males like the mouse models. They also determined that their treatment did not affect mTORC2, instead it was inhibition of mTORC1 that resulted in an increase in lifespan. This increase in lifespan was also associated with an increase in lysosomes in the cell, which indicates autophagy induction. This life-extension was greater than any they could achieve with DR.

It is clear therefore, that if insect models are to be used to study mTOR and autophagy then either there needs to be a diversification of species used to help clarify the findings in *D. melanogaster* and ensure they are consistent across all *Insecta*, or a more suitable model is found. The honey bee may prove to be a useful model in this research given its closeness to mammals in respect to circadian clockwork (Rubin *et al.*, 2006) and methylome (Wang *et al.*, 2006).

### 4.1.7 ***The honey bee; a sweet solution?***

The honey bee (*A. mellifera*) is a successful model for dietary restriction (Paoli *et al.*, 2014). Thus, it is reasonable that, given the previously stated similarities to humans, they may prove a more interesting and useful model for human ageing than *D. melanogaster*, even with inherent limitations of working with a eusocial animal with a painful sting.

One potential reason of interest for the honey bee is the action of p10-Hydroxy-2-decenoic acid (10-HDA), also known as "Queen bee acid" the major lipid component of royal jelly. This lipid has proliferative qualities when fed to *C. elegans* which is believed to be mediated by TOR signalling and provides protection from thermal and oxidative stresses (Honda *et al.*, 2015). While 10-HDA is not the only potentially proliferative compound in RJ (Honda *et al.*, 2011; Detienne *et al.*, 2014), and it is not known if it plays a part in caste differentiation, the TOR pathway certainly does (Patel *et al.*, 2007; Mutti *et al.*, 2011; Wheeler *et al.*, 2014). Given the vastly different lifespans of the castes it is an interesting observation.

Resveratrol is considered a mimetic of DR, known to extend lifespan in a significant number of species including mice (Baur *et al.*, 2006), fish (Valenzano *et al.*, 2006), *C. elegans* (Bass *et al.*, 2007), metazoans (Wood *et al.*, 2004), but not all (Miller *et al.*, 2011). Its effects are also dependent on gender and dietary composition in some species (Wang *et al.*, 2013a). Resveratrol tests in *A. mellifera* are limited to one study, however the results are worthy of further investigation. Resveratrol treatments of 130 μM not only increased median and maximum lifespan but also reduced sugar responsiveness; overall bees ate less food containing the compound compared to control-fed bees during the trial (Rascon *et al.*, 2012). Given the success of this experiment and that these beneficial effects are dependent on SIRT1

(Price *et al.*, 2012), even though it is not a direct activator (Ghosh *et al.*, 2013) and that SIRT1 negatively regulates mTOR (Ghosh *et al.*, 2010) this suggest the potential of other mTOR inhibitors in increasing lifespan in *A. mellifera*. There is a clear need for further investigation of both the role of mTOR and the effects of rapamycin in the honey bee model.

### 3.1.8 ***Aims and hypothesis***

In this study, we investigate the effects of the mTOR inhibitor rapamycin fed as part of a prolonged liquid diet on the lifespan and appetite of newly-emerged honey bees (*A. mellifera)*. We suggest that given pervious findings, rapamycin should extend lifespan relative to untreated groups by downregulation of mTORC1.

## 4.2 **Materials and methods**

### 4.2.1 ***Sample collection***

(*For more detailed descriptions of sample collection see Chapter 2*)

In this study, brood frames from hives located on Newcastle University campus, Newcastle-upon-Tyne (during either May 2014 or July 2015) were removed and stored in in ventilated mesh brood boxes (275 mm X 440 mm X 140 mm) in incubators at 34ºC, 70% RH with total darkness. Newly emerged honey bees (*Apis mellifera* Buckfast*)* were collected from brood frames in waste sacks and then collected in vials and moved to boxes on a daily basis. This method ensured that all cohorts were within 24 hours of age, and removed the need for chilling. In the 2014 trial, newly emerged bees were removed from the frame and stored in 10 groups of 25 without a queen in modified Polypropylene boxes (195mm X 135mm X 110mm; Really Useful Products Ltd) and stored at 34ºC and 70% RH in climate chambers. Each treatment consisted of 250 newly emerged workers and a total of 1000 for the whole trial.
In the 2015, trial newly emerged bees were stored in groups of 30 without a queen in purpose-built *Perspex* boxes (110mm X 200mm X 60mm) with sliding side panels to allow for removal of dead individuals and stored at 34ºC and 70% RH. Each treatment consisted of 300 newly emerged workers and a total of 1200 for the whole trial. The initial trial took place in total darkness whereas due to scheduling conflicts the 2015 trial took place in a chamber set to a 12:12 L:D cycle.

### 4.2.2 ***Diet preparation and feeding measurements***

This experiment was conducted as a 2 x 2 between-subjects factorial design. Bees were allowed to feed *ad libitum* on a liquid diet of 1 M sucrose with 1:10 EAA:C or 1:500 EAA:C in modified 2ml *Eppendorf* tubes in combination with either rapamycin dissolved in 100% ethanol, at a concentration of 200 μM or an equal volume of 100% ethanol as a control. Rapamycin was purchased in powered form *(LC Laboratories, Woburn, MA, USA)* and dissolved to a stock concentration of 50 g/L in 100% ethanol, then added to diets to reach the final required concentration. 1 L of each stock solution was aliquoted into 50ml falcon tubes and frozen at -20 ºC then defrosted at room temperature daily as needed. Each box also contained a single water tube to allow bees to drink freely without consuming the diet. Bees were allowed to feed *ad libitum* throughout the trial and feeding tubes were removed and replaced with fresh tubes containing the diets each day when the survival count was taken, minimising disruption to the colony. Consumption was measured on two separate occasions by weighing the tubes prior to administration and then at removal 24 hours later. Each period

lasted 3 days between day 6 and day 8, then day 13 and 15 and the mean consumption across each period calculated. Evaporation was accounted for by measuring the change in volume at the same time as the consumption was measured in 10 empty, but otherwise identical housing cages. The mean weight change of the evaporation boxes for each diet was subtracted from the overall weight change for each respective 3-day period to give the final consumption value.

#### 4.2.3 ***Survival analysis***

Survival was measured daily by conducting a visual count of the number of dead bees and removing them from the housing chambers with forceps and frozen at -80$^{o}$C. Any liquid waste on the base of the housing box was removed with a dry paper towel where possible. The survival count was taken by subtracting the number of dead from the total at the start of the trial (either 25 or 30 depending on trial). This process was repeated until all individuals had died. Empty boxes were cleaned of any liquid waste and then replaced back in the climate chambers until the end of the study to prevent any changes in microclimate for the remaining boxes.

#### 4.2.4 ***Statistical methods***

All statistical analysis was performed using SPSS v 23.0 (IBM SPSS Statistics 23). The amount of food consumed was analysed with generalized linear models with *post-hoc* LSD pair-wise comparisons. Water consumption was not measured and therefore not included in the statistical analysis. The impact of diet and rapamycin on survival was analysed using a Cox regression (Coxreg) analysis with rapamycin or diet as the covariate to calculate the hazard ratio (HR). The death of a bee was coded as a timed event and was entered as the dependent variable. Comparisons between groups were evaluated using the 'indicator' contrasts in SPSS with all diets compared to the 1:500 "low" EAA diet without rapamycin. Comparisons between treatments were conducted using a Kaplan–Meier log-rank pair-wise comparison with 'Treatment' as a factor.

## 4.3 **Results**

### 4.3.1 ***Survival of the 2014 cohort of newly emerged bees was negatively affected by rapamycin in a diet dependent manner.***

As in previous chapters, the consistent theme of high EAA diets causing reduced longevity was observed. Bees fed a diet high in EAAs experienced a 2.7 times increased risk of death compared to those on the low EAA diets **(Figure 4.2)** (Coxreg, HR = 2.727, [95 % CI 2.223–3.345], P < 0.001). There was a small, nonsignificant difference in the way that rapamycin affected longevity in the honey bee depending on diet. While there was no effect of rapamycin on survival overall (Coxreg, HR = 0.905, [95 % CI 0.738–1.109], P = 0.335), there was a significant interaction between the effect of diet and rapamycin on survival (Coxreg, HR = 1.367, [95 % CI 1.030–1.814], P = 0.031). This interaction is due to a non-significant negative effect of rapamycin on survival in honey bees fed diets low in EAAs (Kaplan–Meier, logrank pairwise comparison, L-R vs LP+R, df=1, $\chi 2$=1.211, P=0.271), whereas there was a non-significant *positive* effect of rapamycin on survival in honey bees fed on high EAA diets (Kaplan–Meier, logrank pairwise comparison, HP-R vs HP+R, df=1, $\chi 2$=3.028, P=0.082). Rapamycin appears to have a small positive effect on survival for high EAA diets early in life but a negative impact on survival long term for both. Survival from Day 7 shows that while rapamycin in low EAA diets continue to have a non-significant negative effect on survival time (Kaplan–Meier, logrank pairwise comparison, LP-R vs LP+R, df=1, $\chi 2$=1.648, P=0.199), rapamycin in high EAA diets has a significant negative effect on survival time (Kaplan–Meier, logrank pairwise comparison, HP-R vs HP+R, df=1, $\chi 2$=17.718, P< 0.001) in contrast to the overall nonsignificant reverse trend. Thus, the effects of rapamycin are influenced by the EAA status of the diet.

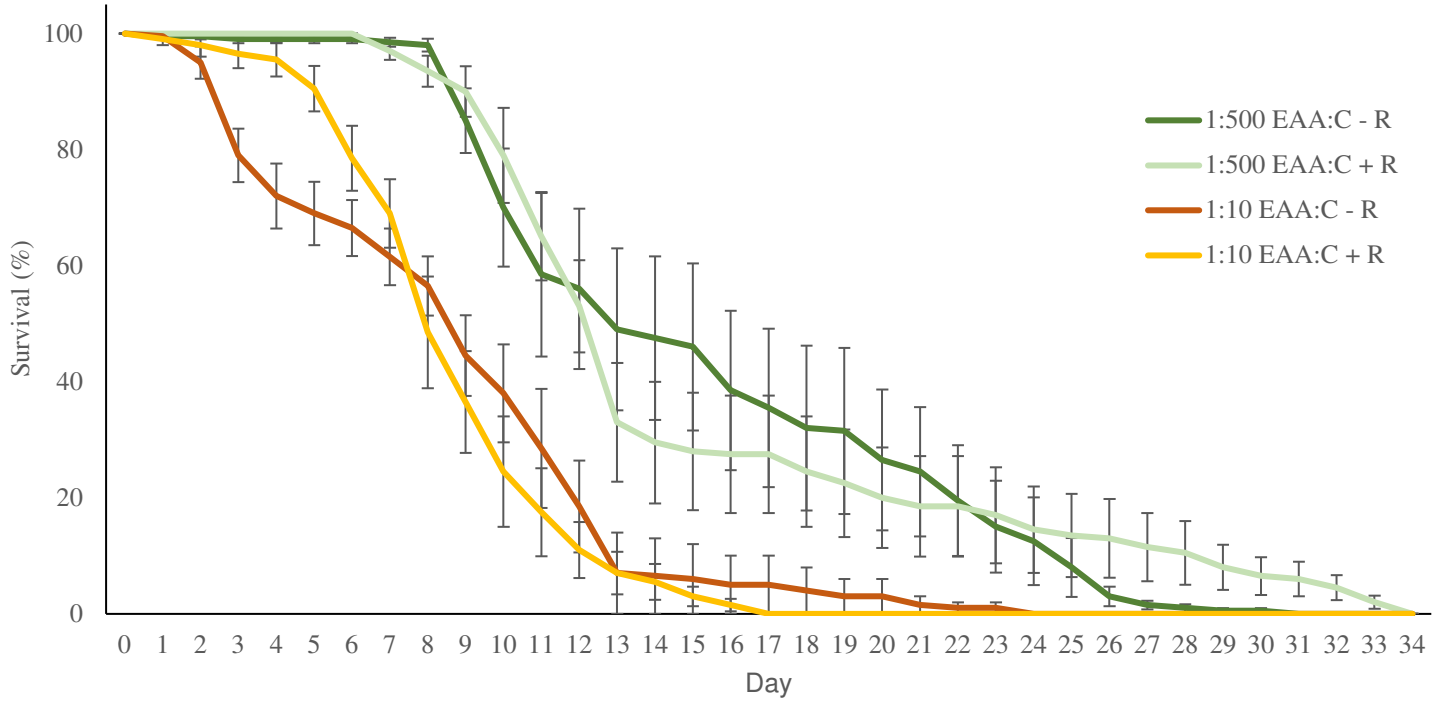


**Figure 4.2** Survival of young honey bees fed a diet high or low in EAAs with or without rapamycin. Diets high in EAAs proved to be a consistent source of mortality, increasing the likelihood of death by 2.7 times throughout the experimental period (n = 10 cohorts of 25 bees each per rail). Rapamycin resulted in 1.4 times increased chance of mortality regardless of EAA concentration after 14 days of the trial, but not after 7 days. (N=10 boxes per treatment, 25 bees per box)

### 4.3.2 *Rapamycin supresses increase in appetite with age when combined with a low EAA diet, but not with a high EAA diet.*

Increasing age appears to have been a factor in increased consumption of all diets. Mean daily consumption increased significantly between day 7 and day 14 for all treatments (ANOVA $F(3, 3.005) = 39.118$, $P = 0.008$, LSD post hoc) low EAA ($p<0.005$), low EAA + R ($p<0.005$), high EAA ($p<0.017$), high EAA + R ($p<0.005$).

At day 7 of the trial there was no significant difference in mean daily consumption between + R or – R for any of the treatments (ANOVA $F(3, 36) = 1.052$, $P=0.382$). At day 14 this was also true (ANOVA $F(3, 36) = 1.099$, $P=0.362$) (**Figure 4.3**). Post-hoc LSD pairwise comparisons revealed that there was a non-significant ($P=0.086$) decrease in mean daily consumption for honey bees fed a high EAA diet in combination with rapamycin compared to rapamycin combined with low EAAs. Due to low numbers of surviving bees in the high EAA treatments at day 14 this result needs further investigation.

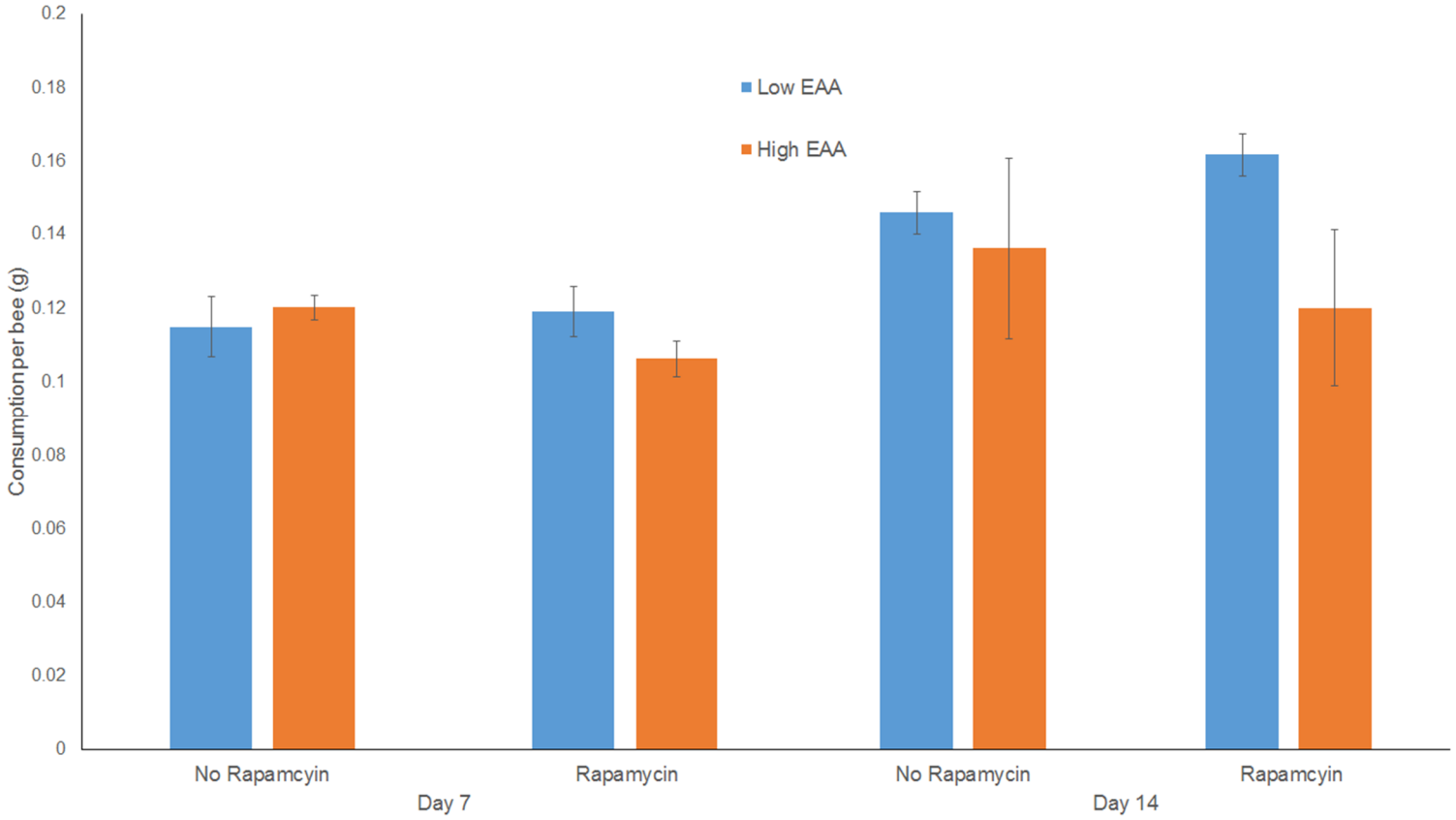


**Figure 4.3** Mean daily consumption for a three-day period around day 7 and day 14 for each treatment. Neither rapamycin nor amino acid content have any effect on the daily consumption of a sucrose diet after 7 days. By 14 days the nutritional requirements of all honey bees on all diets have increased (ANOVA $F(3, 3.005) = 39.118$, $P = 0.008$). Rapamycin appears to have no significant effect on consumption rates at either timepoint or diet. (N=10 boxes of 25 bees per treatment)

4.3.3 ***In the 2015 cohort Rapamycin had a negative effect on honey bee survival across the experimental period when combined with a low EAA diet, but no effect when combined with a high EAA diet.***

Two broadly observable trends occurred in this study. Bees fed a diet high in EAAs had a 2.7 times greater risk of death during the course of the experimental period compared to those fed a low EAA diet **(Figure 4.4)** (Coxreg, Diet, HR = 2.730, [95 % CI 2.300–3.241], P < 0.001). Likewise, being fed rapamycin resulted in a 1.5 times increased risk of death during the trial regardless of EAA content in the diet (Coxreg, rapamycin, HR = 1.460, [95 % CI 1.236–1.724], P < 0.001).

This effect was not consistent between both diets however (Coxreg, Diet*rapamcyin HR = 0.712, [95 % CI 0.566–0.897], P=0.004). While rapamycin combined with low EAA diets significantly reduced survival compared to an ethanol control (Kaplan–Meier, logrank pairwise comparison, LP-R vs LP+R, df=1, $\chi 2$=22.670, P < 0.001), high EAA diets combined with rapamycin did not adversely affect survival compared to controls (Kaplan–Meier, logrank pairwise comparison, HP-R vs HP+R, df=1, $\chi 2$=0.222, P=0.638). Rapamycin appears to have had a negative effect on survival for bees fed a low EAA diet, but had no effect on survival in bees on a high EAA diet.

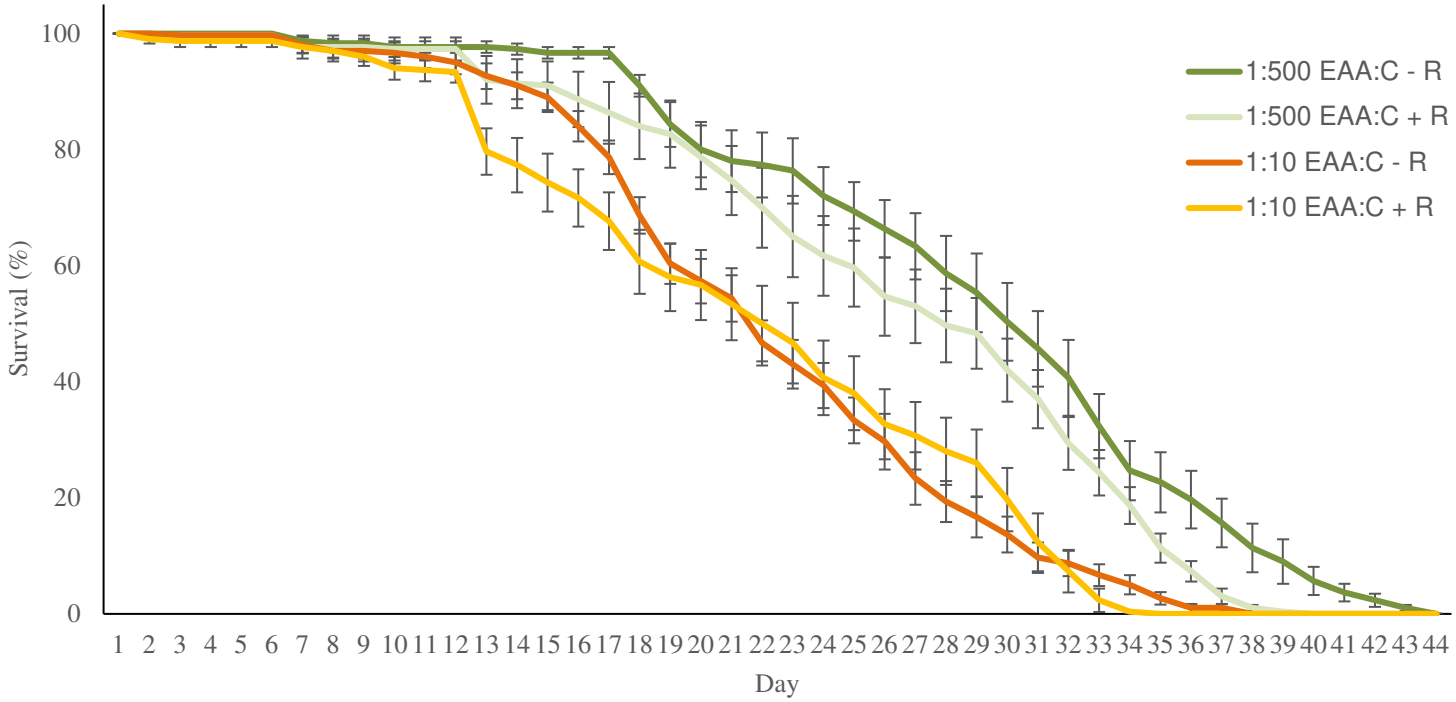


**Figure 4.4** Survival of second cohort of newly emerged bees fed on high and low EAA diets with or without the presence of rapamycin. Survival was curtailed by diet regardless of the presence of rapamycin. By the end of the trial period rapamycin was detrimental to survival when honey bees were fed the low EAA diet, but had no effect when combined with a diet high in EAAs. (N=10 boxes per treatment, 30 bees per box)

### 4.3.4 *Rapamycin increases consumption of low EAA diets but not high EAA diets at day 14, but this effect is not present at day 7*

Younger honey bees did not change their overall consumption of the diet based on the presence of rapamycin or the EAA content. Mean daily consumption measured across 3 days centred on day 7 was not significantly different between treatments (ANOVA Diet ($F(3, 36) = 1.018$, $P=0.396$).

By day 14 however this had changed (ANOVA Diet ($F(3, 36) = 9.480$, $P < 0.001$) **(Figure 4.5)**. Post-hoc analysis revealed that there was significantly higher consumption for the low EAA + R ($P < 0.001$) compared to the low EAA - R, while there was no difference between high EAA treatments regardless of rapamycin presence ($P > 0.593$).

As previously observed, total consumption of diet increases in all treatments with age. There was a significant increase in the mean consumption between day 7 and day 14 (ANOVA Diet ($F(3, 1) = 14.882$, $P=0.031$) which was more pronounced in the low EAA plus rapamycin treatment than the others (ANOVA Diet ($F(3, 72) = 3.724$, $P=0.015$).

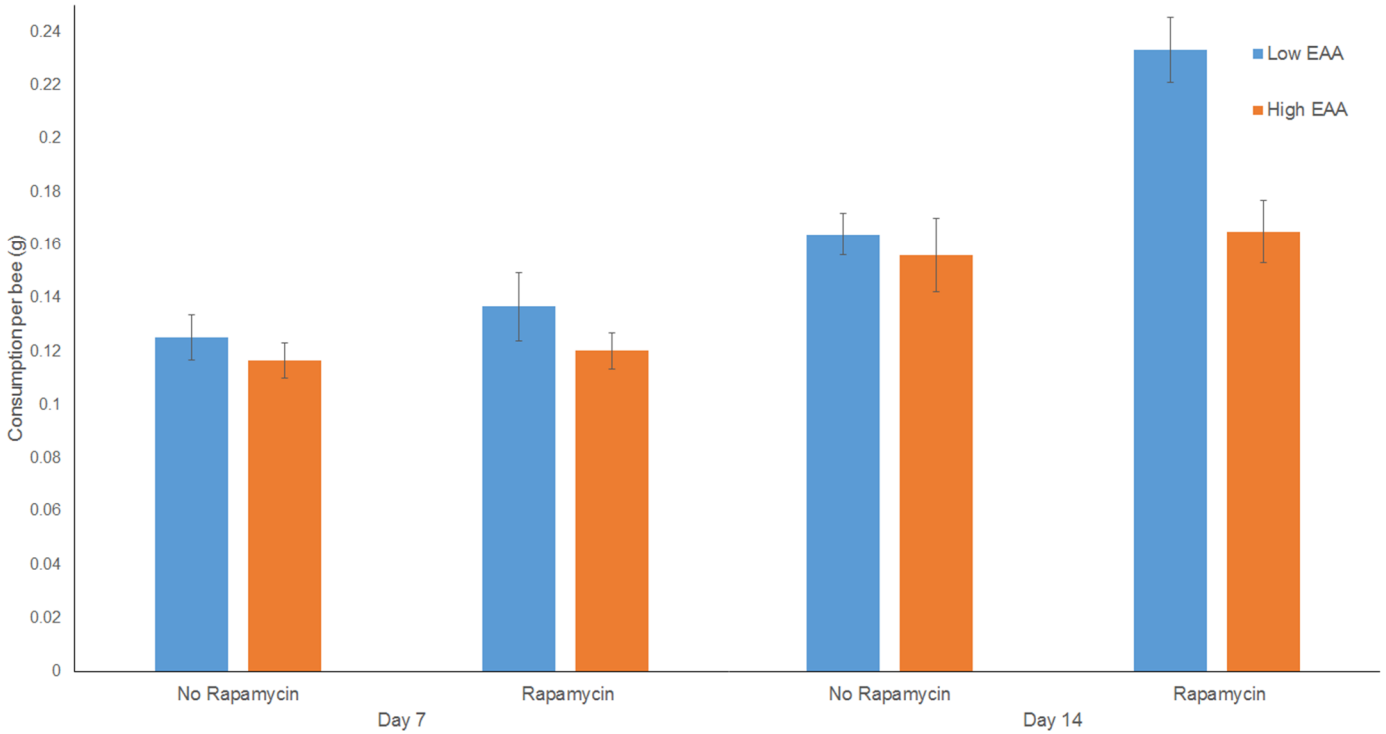


**Figure 4.5** Mean daily consumption for all diets with and without rapamycin across a 3-day period centred on day 7 or day 14. Honey bees ate the same quantity of all diets at day 7 and more of all diets at day 14 relative to day 7 due to higher metabolic demands. At day 14 rapamycin in addition to a low EAA diet resulted in higher daily consumption compared to the control, but not when combined with a diet high in EAAs. (N=10 boxes per treatment, 30 bees per box)

## 4.4 **Discussion**

Rapamycin has been widely lauded for its proliferative properties having extended the lifespan of several model organisms as well as its anti-cancer and immunosuppression capabilities (Sehgal, 1998; Harrison *et al.*, 2009; Miller *et al.*, 2011). However, not all treatments have been successful and its ability to extend lifespan in insects is currently disputed (Harrison *et al.*, 2010; Cheng *et al.*, 2015). Rapamycin is known to inhibit the action of mTORC1, an important component in mTOR action which plays a crucial role in cell proliferation and growth. Retarding mTORC1 action is thought to be one of the key components of dietary restriction (Powers *et al.*, 2006), an intervention known to extend lifespan in virtually every model organism to date (Weindruch and Walford, 1982; Lane *et al.*, 1996; Barzilai *et al.*, 1998; Min and Tatar, 2006; Schulz *et al.*, 2007).

### 4.4.1 ***Rapamycin and longevity***

Unlike several previous studies, neither trial found a proliferative effect of rapamycin and indeed in several treatments it proved to have the opposite effect. It seems that prolonged, continuous feeding of rapamycin in a liquid diet has a negative impact on the survival of newly emerged honey bees which does not remain in combination with a second stressor like a diet containing a high ratio of EAA to carbohydrate. We also found significant polyphagia in one of the rapamycin treatments.

This study reached similar conclusions as those conducted in *D. melanogaster* by Harrison *et al.* (2010) and Cheng *et al.* (2015). Both discovered a negative effect of sustained rapamycin treatment on long-term survival on non-mutant strains. In Harrison's study, *D. melanogaster*'s dose dependent decline in survival started at approximately day 18 of the study period. While this study only tested one dose of rapamycin (200 μM) in contrast to those of Harrison *et al*, which investigated a range of different concentrations, we too found there was no immediate effect and the negative effects of rapamycin took approximately 18 days to manifest in both trials. Despite our experimental design being most similar to Bjedov et al. (2010) we did not find similar life-extending properties. Polyphagia, defined as excessive or persistent appetite is one of the key signs of human diabetes. Sustained rapamycin treatment in mice by Wang *et al.* (2015) lead to increased polyphagia, insulin resistance and hyperglycaemia. Our observations in the second trial showed honey bees also exhibit increased polyphagia, albeit only at 14 days into the study. While the honey bees' metabolic demands increase as it ages and matures into a role outside the hive where flying is necessary, honey bees on low EAA diets plus rapamycin had a disproportionate increase in consumption relative to the controls.

### 4.4.2 *Seasonal effects on honey bee longevity*

Our first trial has several results that contradict the findings of the second trial. Firstly, mortality in high EAA diets appear to be masked by the addition of rapamycin during the initial stages of the experiment which is not the case in the second trial. Similarly, consumption did not differ significantly, regardless of rapamycin or EAA:C treatment used at day 14, which was not the case in the second trial. There are two reasons I believe go some way to explaining this contradiction and validate the assumption that the latter experiment is more robust in its findings. Firstly, the first experiment was conducted using a housing box that was top opening and required the entire lid to be removed if access was required meaning it was impossible to remove dead bees from the bottom of the box on a daily basis. Honey bees are known to be avid cleaners of the hive and remove dead individuals regularly (a process known as necrophoresis) (Visscher, 1983).

The first trial was distinctive because of its very early initial mortality on the low EAA:C w/o rapamycin treatment, which was not observed in any other treatment in either study. Not only is it understandable that inability to remove carcasses in organisms that are programmed to perform the task immediately could cause stress to individuals, but hygienic behaviours are one of the key tools used by the honey bee to combat the spread of pathogens. This is especially important as the hive (like our experimental chambers) are an ideal combination of high temperature and humidity for microbial growth and the genetic homogeneity of eusocial insects makes them especially vulnerable (Naug and Camazine, 2002), particularly as rapamycin is a known immunosuppressant (Dumont and Su, 1996). Furthermore, I observed on multiple occasions that individuals became entangled with carcasses in an attempt to move them, which could be a source of physical damage to explain the early mortality.

Lastly the first trial was carried out in the month of May (2014) and thus took place in the spring when the colonies were less well established; the second trial (July, 2015) took place during the summer months. As discussed in previous chapters the lifespan of honey bees in experimental conditions is heavily influenced by the season (Ament *et al.*, 2008). The early-season brood combined with constant exposure to decomposing carcasses is the likely reason for both the immediate high mortality of the low EAA:C without rapamycin treatment as well as the very truncated lifespan compared to the second trial. It was on this basis that the second trial was conducted in July with boxes that allowed for the removal of dead individuals daily.

### 4.4.3 ***Mechanisms through which rapamycin could curtail lifespan***

The main finding from the amended (second) trial is that the mTOR inhibitor rapamycin decreases the longevity of newly emerged honey bees under experimental conditions when paired with a low EAA:C diet. This diet has been found to be the best at extending lifespan compared to high EAA:C diets and sucrose alone controls so this finding is unexpected. Harris et al. (2010) used a nutrient rich 10% sugar/10% yeast which is known to reduce lifespan during their observations about the detrimental effects of rapamycin in *D. melanogaster*, but honey bees fed our analogous high EAA diet did not experience any curtailment of lifespan in combination with rapamycin. This might well be because the doses used by Harris where this effect was most strongly measured was 5 times higher than the dose we used and it may well be that a higher dosage is necessary for a similar impact on lifespan.

While rapamycin is a specific inhibitor of mTORC1, when provided continuously as in this study it also inhibits the formation of mTORC2 (Sarbassov *et al.*, 2006). mTORC2 is not inhibited by dietary restriction like mTORC1 and inhibition of mTORC2 by rapamycin across multiple tissues is known to increase glucose intolerance and decrease insulin sensitivity in mice and rats (Lamming et al., 2012). Its inhibition reduces male survival but not female survival in *D. melanogaster* (Lamming *et al.*, 2014). The reasons for this difference between sexes is not known, but as worker honey bees are sterile it is possible that their survival is being damaged in a similar way as reported for male *D. melanogaster*. It is possible that the reason Resveratrol worked in previous studies in *D. melanogaster* (Bass *et al.*, 2007) and honey bees *(Rascón et al., 2012)* whereas rapamycin has not is that Resveratrol regulates mTOR indirectly through sirtuin activation. Not only does Sirt1 not inhibit mTORC2 in mice models, it is crucial in its function and Sirt1 deficient mice have impaired mTORC2 signalling which results in diabetes-like symptoms (Wang *et al.*, 2011).

Ament *et al.* (2008) observed that rapamycin delayed onset of foraging in a seasonal manner, only delaying onset in honey bees during June-July while observing an adverse effect during August. The onset of foraging is associated with decreased lipid stores and an acceleration of ageing (Toth and Robinson, 2005). It is possible that as the summer in Newcastle is significantly shorter than that of the Urbana, Illinois rapamycin administer in July was having the adverse effect of promoting early-onset foraging and speeding up the ageing process.

Nurse honey bees have significant lipid stores in their head and abdomen in the form of the glycolipoprotein Vitellogenin (VG) (Omholt and Amdam, 2004). At emergence these stores dictate several factors such as longevity, foraging preference and the time at which an

individual will progress to foraging (Amdam *et al.*, 2004). The nutrition signals that activate juvenile hormone biosynthesis and vitellogenin production are mediated by the TOR pathway (Maestro *et al.*, 2009). Rapamycin prevents lipid accumulation and mTORC2 is necessary for lipogenesis/adipogenesis (Laplante and Sabatini, 2009). These stores are lost at onset of foraging and is indeed thought to be an important trigger (Antonio *et al.*, 2008), one of the triggers for the onset of foraging is the nutrient deprivation. As mTORC1 inhibition is thought to play a crucial role in the life extending properties of dietary restriction, it may be that rapamycin is initiating a response to nutrient deprivation and promoting foraging behaviour earlier and so reduces survival (Woyciechowski and Moroń, 2009). This behaviour is unique to eusocial insects and would make any attempt to use honey bees as models for ageing difficult, especially if the effect is seasonally variable.

I propose that long term inhibition of mTORC2 by rapamycin is the reason we see reduced survival in individuals on a low EAA diet. This is for two reasons: the onset of reduced survival was delayed, which rules out any immediate toxic effect, and our persistent administration was similar to that which inhibited mTORC2 signalling in other studies in a delayed manner. Secondly the presence of polyphagia in the rapamycin treatment is typical of metabolic syndrome which is a known side-effect of mTORC2 signalling disruption (Cho *et al.*, 2001). There are several potential reasons for why this effect was not present in honey bees on high EAA diets. The diet alone is extremely toxic and it may be that it already overwhelms the honey bee's physiology and causes mortality before mTORC1 inhibition can take effect. Amino acids are known to activate mTORC2 signalling (Tato *et al.*, 2011), and our high EAA diets are high in amino acids which could be upregulating mTORC2 and offering partial protection. Methionine, a nutrient that inhibits autophagy by promoting mTOR signalling (Zhou *et al.*, 2016), is one of the ten EAAs used in the diet and may be responsible for the observed effects. Furthermore, limiting methionine is known to mimic the effects of DR (Lee *et al.*, 2016) and methionine levels in the 1:10 diet were higher than previously observed nutritional targets for the honey bee (Paoli *et al.*, 2014).

Our results add further confusion to the role of rapamycin as a tool to study the mTOR pathway and ageing. Even in mice, where most of the success in the area has come, it is not universal. Meta-analysis by Swindell (2016) "demonstrated significant heterogeneity across studies, with hazard ratio (HR) estimates ranging from 0.22 to 0.92" with both sex and genotype proving significant factors in successful life extension. There is no clear consensus on this issue and further research is required.

Rapamycin is a proven mTOR inhibitor and has been shown to extend lifespan in a variety of species, but most commonly the mouse. There is also evidence that it can negatively affect survival, with a possible mechanism being mTORC2 inhibition. Our tests found no positive effect of rapamycin on survival and under certain circumstances a significant negative effect. The evidence is consistent with mTOR2 inhibition but further analysis would be required to confirm this. Future tests should either use intermittent exposure (which does not inhibit mTORC2), directly measure mTORC1 and mTORC2 activity or downstream targets of longevity such as lysosome formation. However, any such research must take into account the unique physiology of the honey bees in its experimental design, confounds such as seasonal effects, which may influence potential findings.

# Chapter 5. Exploration of methods used to quantify methylation changes in the genome of eusocial insects

## 5.1 **Introduction**

### 5.1.1 ***Epigenetics and ageing***

While there is a strong basis for a genetic component in lifespan, it is clear that it is not the only determining factor of longevity. The ageing process is influenced by the environment in a way which cannot be accounted for by genotype or accidental death alone. Analysis of the longevity of some 2872 pairs of monozygotic twins found that only 25% of longevity was heritable (Herskind *et al.*, 1996), the rest was determined by external factors. One such factor is DNA methylation, the addition of a methyl group ($-CH_3$) to the position 5 carbon in the base of a DNA molecule, most commonly at 'CpG' (cytosine-phosphate-guanine) sites.

DNA methylation, specifically in vertebrates, is extremely common. Conservative estimates range from 60-80% of CpG sites in vertebrate somatic cells being methylated (Ehrlich *et al.*, 1982). DNA methylation is an essential process in development. Changes to the methylome have a significant stochastic component, as well as being vulnerable to environmental modification (Feinberg and Irizarry, 2010). These influences are felt most strongly during development and early life, where conditions can have a large impact on future health and lifespan (Sharp *et al.*, 2015).

The ageing process itself results in distinct changes to the methylome over time, known as epigenetic drift (Heyn *et al.*, 2012). Studies suggest that globally, there is large scale hypomethylation of the genome (Wilson and Jones, 1983). Conversely, there is also prominent hypermethylation at promoter sites for specific age-related genes (Fraga *et al.*, 2007). Several age-related diseases such as cancer (Ahuja *et al.*, 1998), auto-immune disorders (Pinney and Simmons, 2010) and dementia (Delgado-Morales and Esteller, 2017) also have associated methylome changes as part of their pathology. Since, by definition, epigenetic changes like DNA methylation are not permanent, they are potential targets for therapeutic interventions to lengthen lifespan.

It has been proposed that the life-extending effects of DR are at least in part due to the Sirt1-mediated maintenance of DNA methylation patterns (Wakeling *et al.*, 2009). DR has shown to reduce the instances of age associated diseases in organisms in which it extends lifespan (Fontana and Klein, 2007), but also extends lifespan in mice which are disease-free at the time of death (Shimokawa *et al.*, 1993). This is in keeping with the disease specific, and general

age-related changes in DNA methylation patterns. As discussed in previous chapters, Sirtuins are crucial in this life extending effect (Cohen *et al.*, 2004). Sirtuins are responsible for the deacetylation of histones around which genomic DNA is coiled. Methylation is crucial to sirtuin-mediated histone deacetylation action. It is methyl-CpG-binding proteins that recruit the deacetylases to histones and histone deacetylation occurs largely around CpG islands (Irvine *et al.*, 2002). Not only this, but sirtuins are important in the activities of DNMT1, a key enzyme in the methylation of genomic DNA (Peng *et al.*, 2011).

### 5.1.2 ***Methylation and circadian rhythmicity***

Circadian behaviours vary based on fixed factors such as age (Russo *et al.*, 2007), gender (Campbell *et al.*, 1989), ethnicity (Kim *et al.*, 2002) and environmental inputs such as stress (Caplan *et al.*, 1979), light exposure (Pauley, 2004) and diet (Goel *et al.*, 2009). As discussed in previous chapters, circadian disruption is a key symptom of age-related diseases like prostate cancer (Jung-Hynes *et al.*, 2010), metabolic syndrome (Maury *et al.*, 2010) and Alzheimer's (Wu and Swaab, 2007). An important factor in this disruption is alterations to the methylome. Promoter region methylation is known to down-regulate important circadian genes (Yang *et al.*, 2006). Circadian genes *PER1*, *PER2* and *CRY1* show substantial promoter methylation in cancerous endometrial tissues but significantly less so in non-cancerous tissue (Shih *et al.*, 2006), a condition characterised by circadian disturbances (Shih *et al.*, 2005). Additionally, a high fat diet produces significant promoter-region methylation in *CLOCK* (*CLK*) and *PER2* genes in obese women with metabolic syndrome (Milagro *et al.*, 2012).

Circadian disruption, while also being a symptom of age-related changes to the methylome, is also a driver of it. Extensive research has indicated that shift-work (working non-standard and non-sociable hours) causes extensive changes to promoter region methylation in tumour suppressor genes and glucocorticoid receptors (Bollati *et al.*, 2010). This is important because a move towards a 24 h society is one of the defining features of our ever more urbanised lifestyle and has pressed upon the population a powerful stressor, which potentially has serious health and economic costs (Åkerstedt, 2003). Exposure to light during hours of darkness, known as light-at-night (LAN), results in a suppression of melatonin production by the pineal gland. Melatonin has been shown to protect against cancer associated hypomethylation in mice models for breast cancer (Schwimmer *et al.*, 2014). One of the main risk factors in breast cancer is exposure to LAN (Davis *et al.*, 2001) and hypermethylation of core circadian clock genes *PER1*, *PER2*, *CRY1* and *BMAL1* is not only present in breast cancer pathologies, but also correlates with strength of prognosis (Kuo *et al.*, 2009). The hypermethylation is not limited to promoter regions alone however, breast cancer is also

associated with hypermethylation in gene-bodies (Flanagan *et al.*, 2009). Along with other circadian genes, *Clk* has been hypothesised as a key regulator of tumorigenesis, and hypermethylation of its promoter region increases the risk of breast cancer (Hoffman *et al.*, 2010), something known to happen in shift workers (Zhu *et al.*, 2011).

### 5.1.3 ***Methylation in the honey bee***

One particularly striking example of the power of DNA methylation is the honey bee. Workers in the hive are relatively small, infertile, show strong circadian oscillations in behaviour and are short lived; in contrast, honey bee queens are large, can produce thousands of eggs in a single day, exhibit non-cyclic circadian behaviour and live for several years (Dietz and Lambremont, 1970). While such pronounced differences in morphology and physiology suggest at least some genetic component, caste determination occurs after the egg has been deposited (Weaver, 1966). The difference in physiology comes from differential exposure to nutrients during the larval development stage. During the first 7 days of development all individuals are fed Royal Jelly (RJ), a secretion high in protein and carbohydrates, produced by the hypopharyngeal gland of nurses. Around day 7 of larval development those destined to be workers are swapped from a diet of RJ to a combination of honey and 'bee bread' (processed pollen). Those destined to be queens remain on a pure RJ diet, which they are fed in high quantities. This change in diet has the effect of extending the average developmental period from 16 days to approximately 21 days (Winston, 1991).

This divergence in diet results in profound epigenetic changes. At the 2$^{nd}$ instar of larval development there is significantly higher expression of insulin receptors (Wheeler *et al.*, 2006) and upregulation of larval ribosomal and metabolic gene expression (Cristino *et al.*, 2006). Kucharski *et al.* (2008) showed that silencing the expression of DNA methyltransferase (Dnmt3) resulted in queenlike characteristics; including fully functioning ovaries and extended longevity, which could be derived from larvae reared in the absence of royal jelly. This finding provided strong evidence to suggest that differential methylation states were at least in part responsible for the marked difference in queen and worker morphology. It has been proposed that insects may be an innovative model for studying DNA methylation (Glastad *et al.*, 2011). It is unlikely that they play a significant role in repression of transcription as is common on vertebrate cells (Wolffe and Matzke, 1999) but many have potential roles in transcript stability and alternative splicing which occurs extensively in the vertebrate genome (Schwartz *et al.*, 2009; Laurent *et al.*, 2010). While there is not a fully functioning methylome in all insects, including the common model organism *D. melanogaster,* there is in the honey bee (Wang *et al.*, 2006). Lyko *et al.* (2010) found over

560 genes which were differentially methylated between workers and queens. This was even more pronounced in larval DNA, with 2399 differentially methylated genes (DMGs) (Foret *et al.*, 2012). The authors propose that in the case of the honey bee, methylation is focused around highly conserved and ubiquitously expressed genes which, while needed for development, are not consistently expressed throughout the honey bee lifespan. Their findings suggest that methylated CpG clusters in honey bee exons are analogous to those found around intron/exon boundaries in the human genome and are responsible for splice variants in key conserved genes. One such example of this is two classes of histone genes in *A. mellifera*. Only those with introns are methylated whereas intronless genes are completely unmethylated (Wedd and Maleszka, 2016).

Work investigating the effects of light pulses on differentially expressed genes (Becker *et al.*, 2016) show that not only do disruptions to circadian rhythm result in differentially expressed genes in the honey bee brain, but the bubblegum gene (*bgm*), which is responsible for brain long-chain fatty acids metabolism (Min and Benzer, 1999) and epigenetic control of transcription by suppling acetyl-CoA for histone acetylation (Takahashi *et al.*, 2006) shows significant increases in methylation when the organism is exposed to light. This provides direct evidence that circadian disruption alters DNA methylation of metabolic genes in the honey bee and that honey bees might be a promising model for further investigation.

### 5.1.4 ***Measuring changes to methylation in non-human model organisms***

There appears to be significant potential for the honey bee model to play a role in understanding how methylation changes affect metabolism, ageing and circadian disruption, but there are several key issues that must be addressed first. Methylation levels in human embryonic stem cells (hESCs) is approximately 80% at CpG sites (Lister *et al.*, 2009). Plants by contrast range from 24% in Arabidopsis (Cokus *et al.*, 2008) to 86% in maize (Cokus *et al.*, 2008). However, what was found in the genome wide analysis of the honey bee methylome was a general scarcity of methylated CpG sites. Of the 600 million cytosine's in the genome, only 70,000 were methylated and a large majority of these were in exons and virtually all were found amongst the 10~ million CpG sites (Lyko *et al.*, 2010). This poses its own problems since at 0.7% CpG methylation this is approaching the sensitivity level for ease-of-use kits designed for mammalian cell lines. Despite claims of universality for any species and detection thresholds of 50pg, the suitability of such kits to measure global methylation changes has not previously been tested in honey bees (Yong *et al.*, 2016).

One of the more common methods of methylation analysis that has had success in the honey bee is bisulfite conversion-based sequencing, where genomic DNA is treated with sodium bisulfite. The sodium bisulfite deaminates unmethylated cytosine to uracil, while leaving methylated cytosine unaffected. This method provides single-base resolution so can be used to investigate specific sequences such as CpG islands (Mor *et al.*, 2015) or whole-genome bisulfite sequencing (WGBS) (Legendre *et al.*, 2015). WGBS provides greater resolving power and sensitivity than anti-body based kits but also requires more starting material, a bisulfite conversion step which damages genomic DNA and is more expensive and time consuming (Hsieh *et al.*, 2009).

### 5.1.5 ***Aims and hypothesis***

In this study dietary and circadian interventions were tested to produce global changes in methylation levels and to test the ability of conventional ELISA kits to detect these changes. Furthermore, we investigated whether honey bee circadian and metabolic genes contain CpG sites with measurable methylated changes.

## 5.2 **Materials and methods**

### 5.2.1 ***Sample collection***

Honey bee samples used for 5-mC quantification were collected during the summer of 2012. Brood frames were collected from hives on the roof of the Ridley Building (Newcastle University campus) and allowed to emerge in total darkness in incubators set to 34 $^{o}$C and 70% RH, overnight. Newly emerged bees were then removed into a yellow recycling bag where they were divided out into groups of 20 and caged in Perspex boxes (for detailed description see Chapter 2). Bees were fed on either 1:5 EAA:C (high EAA) or 1:500 (low EAA) diet (**Table 3.1**). A further treatment group fed only on 1:500 was treated with one of two types of SiRNA used to knock down *Sir2* expression or a control siRNA. Measurement of *Sir2* by RT-qPCR showed that one of the two siRNAs (siRNA2) was successful in reducing *Sir2* expression. Samples were collected for measurement at day 7 and day 14 of the trials.

Ants used in this study were supplied by colleagues from the University of Leeds from studies conducted between January and March 2014. Pharaoh ant colonies (*Monomorium pharaonis*) were fed on diets with high or low protein content and with or without Rapamycin in a 2x2 experimental design. Colonies were harvested and stored on dry ice during transport. Upon arrival workers were sorted from brood and queens and moved to a-20 $^{o}$C freezer for storage.

Honey bees for MiSeq bisulfite sequencing (*A. mellifera ligustica)* were collected from a managed, queenless microhive in Canberra's Australian National University. A single cohort of honey bees were allowed to emerge together over the course of 3 days. Each day a different colour marking was applied to the thorax with enamel paint and was used to identify day of emergence. As the hive was of uniform age and contained no queen, some individuals were forced into either precocious foraging to supply the hive with nectar or delayed nursing and hive maintenance behaviour. To maintain the need for nurses a small amount of brood was added to the colony every 7 days. On days 7 and 14 of the experiment we collected 9 individuals exhibiting foraging behaviour (identified returning to the hive with a pollen load) as well as 9 exhibiting nursing behaviour (collected from the brood frames) from the marked cohorts. These individuals were placed in perforated Eppendorf tubes and immediately frozen in liquid nitrogen.

### 5.2.2 ***Nucleic acid preparation***

Honey bees were euthanized by confinement at -20 $^{o}$C for 10-30 min. Material was then immediately frozen in liquid nitrogen and ground gently into powder with a pestle and mortar.

Both pestle and mortar were cleaned with 70% ethanol and then cooled with liquid nitrogen before use. Crushed material was then stored at -80 ºC in 2 ml Eppendorf tubes until extraction. To extract DNA, ground bees were treated with 200 µl of chromosomal breakage buffer (See general introduction for further details) and left to incubate at room temperature with brief vortexing for 2-3 min. 0.2 ml of phenol-chloroform buffered to pH8 was added and the mixture was vigorously shaken by hand for 15 s. The mixture was then allowed to incubate for 5 min, before being centrifuging at 10000 G for 5 min. The aqueous layer was then extracted to a new tube and 0.2 ml of phenol-chloroform was added and centrifuged again for a further 5 min at 10000 G. The aqueous layer was extracted to a new tube along with 20 µl 3M NaClAc, 400 ml of ice-cold ethanol and then stored at -20 ºC for 24 h. The following day the mixture was then centrifuged for 30 min at 10000 G and the supernatant removed. The pellet was left to air dry for 5-10 min before being resuspended in 10 µl of twice autoclaved $H_2O$ and stored at -20 ºC until testing.

For ant samples 200 µl of chromosomal breakage buffer was added to each tube of workers and the material was crushed using a tissue grinder. After the tissue was sufficiently broken apart the phenol-chlorophorm extraction took place, as described for honey bee.

Samples prepared for bisulfite sequencing were moved to dry ice in preparation for full-brain extraction. Heads were separated from the thorax with forceps and placed in 50 mm NaCl, 25 mm Tris, 5 mm EDTA, pH 8 (0.5× NTE buffer) buffer solution. The upper chitin of the head was removed and the hypopharyngeal glands were examined to ensure all samples were correctly identified according to their role within the hive. All foragers showed atrophy of the gland, which is considered an accurate way to identify foragers (Ohashi *et al.*, 2000). The glands were then discarded and the remaining brain tissue was pooled and homogenised using a motorised homogeniser. DNA extraction was conducted as described in chapter 2.

### 5.2.3 ***Optimisation of the ELISA 5-mC assay***

Extracted DNA was measured using a nanodrop and then diluted with twice autoclaved $H_2O$ to a concentration of 50 ng/µl. Three experimental replicates containing 100 ng of DNA of each treatment were measured, in duplicate, to ensure validity of the signal generated. Initial measurements of global DNA methylation were measured using the fluorometric MethylFlash™ Methylated DNA Quantification Kit (Fluorometric) (Epigentek). The kit measures levels of 5-methylcytosine (5-mC) through the use of antibodies. Samples were incubated with 5-methylcytosine antibodies affixed to the bottom of strip wells. A binding step followed (90 min, 37 °C) and after multiple wash samples were incubated with another

antibody alongside a fluorogenic compound, which was used to measure binding. Substrate and fluorescence was measured. Reagents were added with a multi-channel pipette and aerosol-barrier pipette tips as specified in the accompanying documentation. We rejected a single point positive control (PC) in favour of the more robust standard curve approach which allowed us to perform absolute quantification measurements instead of simple relative measurements by using a positive control of pre-prepared DNA of known methylation profile supplied with the kit. A negative control (NC) was followed by a series of increasing concentrations of PC from 0.5 ng/µl to 10 ng/µl **(Table 5.1)**.

**Table 5.1.** Plate layout for initial ELISA runs. Standards were run in duplicate descending from lowest to highest concentration across the first two columns as recommended in the protocol (Epigentek). All treatments comprised of 3 experimental replicates ran in duplicate. All diets were 1:500 EAA:C unless stated as 1:5 EAA:C.

| Strip | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| A | MF3 | MF3 | 1:5 (1) | 1:5 (1) | siRNA C (1) | siRNA C (1) |
| B | MF4 0.5ng | MF4 0.5ng | 1:5 (2) | 1:5 (2) | siRNA C (2) | siRNA C (2) |
| C | MF4 1 ng | MF4 1 ng | 1:5 (3) | 1:5 (3) | siRNA C (3) | siRNA C (3) |
| D | MF4 2 ng | MF4 2 ng | 1:500 (1) | 1:500 (1) | siRNA1 (1) | siRNA1 (1) |
| E | MF4 5 ng | MF4 5 ng | 1:500 (2) | 1:500 (2) | siRNA1 (2) | siRNA1 (2) |
| F | MF4 10 ng | MF4 10 ng | 1:500 (3) | 1:500 (3) | siRNA1 (3) | siRNA1 (3) |
| G | | | | siRNA2 (1) | siRNA2 (2) | siRNA2 (3) |
| H | | | | siRNA2 (1) | siRNA2 (2) | siRNA2 (3) |

The plate was read using a 'Infinite 200pro' (Tecan) microplate reader (excitation 530 nm, emission 590 nm) after 2 mins post administration of the fluro-development solution. The reflective fluorescence unit (RFU) values were then used to calculate the absolute quantification of 5-methylcytosine using the method provided in the documentation. Linear regression to determine the slope of the standard curve was performed using Microsoft Excel (2013) inbuilt functions and the absolute quantification was calculated by the following equation:

$$\text{5-mC (ng)} = \frac{\text{Sample RFU - NC RFU}}{\text{Slope x 2*}}$$

The automatic 'gain' recording showed that recordings were 53% more intense than recommended levels for the machine recording during the first recording. This was likely due to the exceptionally intense readings from final row of wells, but to ensure readings were closer to the ideal intensity for all subsequent tests, plates were measured a second time, 2 mins after the initial reading. This produced gain measurements of 126% and 111%, far closer to the ideal 100%, suggesting that the exceptionally high gains from the first reading were likely due to contamination in the final row of wells. An alternative plate layout was also designed which used a 2 ng positive control in several positions across the plate **(Table 5.2)**. This design was used to address concerns about the time between reading the first row of wells and the final row of wells by the plate reader, thought to be a confounding factor in the analysis.

**Table 5.2** Modified plate layout using a spread of 2 ng standards to measure variance in fluorescence across the plate. All other standards kept as closely to their original positions as possible. All diets were 1:500 EAA:C unless stated as 1:5 EAA:C.

| Strip | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| A | MF4 2 ng | MF3 | MF3 | siRNA2 (1) | siRNA2 (3) | siRNA2 (3) |
| B | MF4 0.5ng | MF4 2 ng | MF4 0.5ng | siRNA2 (1) | siRNA1 (1) | siRNA1 (1) |
| C | MF4 1ng | MF4 1ng | MF4 2 ng | siRNA2 (2) | siRNA1 (2) | siRNA1 (2) |
| D | MF4 5ng | MF4 5ng | MF4 2 ng | siRNA2 (2) | siRNA1 (3) | siRNA1 (3) |
| E | MF4 10ng | MF4 10ng | 1:5 (1) | MF4 2 ng | 1:5 (1) | 1:5 (2) |
| F | 1:500 (1) | 1:500 (1) | siRNA C (1) | siRNA C (1) | MF4 2 ng | 1:5 (2) |
| G | 1:500 (2) | 1:500 (2) | siRNA C (2) | siRNA C (2) | 1:5 (3) | MF4 2 ng |
| H | 1:500 (3) | 1:500 (3) | siRNA C (3) | siRNA C (3) | 1:5 (3) | MF4 2 ng |

A second round of measurements were taken using the MethylFlash™ Methylated DNA Quantification Kit (Colorimetric) (Epigentek). The procedure was followed as documented in the manual, to the same standards and measurements as the fluorometric tests except for the quantity of DNA which was increased to 200 ng to aid detection in the less sensitive kit. When the final stop solution was added the plate was allowed to incubate in darkness at room temperature for 2 min and then absorbance was measured on a plate reader at 450 nm. The optical density (OD) values were then used to calculate the absolute 5-mC content using the following equation:

$$5\text{-mC (ng)} = \frac{\text{Sample OD - NC OD}}{\text{Slope x 2*}}$$

Once absolute quantification has been calculated, percentage DNA methylation was obtained by using the following equation:

$$5\text{-mC (ng)} = \frac{\text{5-mC amount (ng)}}{\text{Sample DNA (ng)}} \text{X 100\%}$$

### 5.2.4 ***Optimisation of the bisulphite DNA methylation quantification assay***

#### *5.2.4.1 CpG site quantity and suitability*

CpG sites were identified using HiSeq methylation data obtained and collated from multiple studies into a database (http://dna.anu.edu.au/) by the Australian National University in Canberra. CpG sites were considered to be methylated if methylation levels were present in at least 5% of all runs. First promoter regions where analysed, of which no suitable sites were found in any region, then introns and exons were examined. No CpG sites in *Cry2*, *Clk* or *Per* were found to be methylated at the required threshold so were not used. The honey bee *Bmal* homologue *CYC* had one CpG site which was significantly methylated but was also discarded as the number was deemed insufficient. *Sir2* was found to have 19 methylated CpG sites within 450 base pairs, making it a suitable candidate for further study.

*5.2.4.2 Primer design*

A primary and 'nested' primer pair were designed around known methylated CpG sites in the *Sir2* gene **(Table 5.3)**. A PCR reaction was conducted using the primary primer and then a secondary PCR with was used on the product of the first using the nested primer, increasing the specificity.

**Table 5.3** Primary and nested PCR primers for bisulfite sequencing. Sequences listed in the 5'-3' direction. Subscript numerals refer to positions within the sequences. Their Forward (5') then reverse (3') primer sequences are stated in each case.

| **Gene** | **Primer** | **Sequence (position in sequence) 5'-3'** | **Product length** |
|---|---|---|---|
| Sir2 histone deacetylase, transcript variant 1 (*Sir2*) | Primary | $_{12482859}$ggagaatcaagcatagaaagtagc$_{12482884}$ | 568 |
| | | $_{12483452}$gaataatgataaatgtagaaataa$_{12483476}$ | |
| | Nested | $_{12482892}$gaatgaactttggcagcaattgtg$_{12482916}$ | 436 |
| | | $_{12483352}$gtcttgctgaaagattaccaggta$_{12483373}$ | |

*5.2.4.3 Bisulphite conversion*

2 µg of genomic DNA was bisulfite converted (bsDNA) over-night using the QIAGEN Epitect® Bisulfite Kit, following the manufacturer's protocol. The step was repeated the following day to ensure complete conversion and the final product eluted into TE buffer. bsDNA was amplified using a two-step 'nested' PCR process using GoTaq® Flexi DNA Polymerase (Promega) as described in Chapter 2. In the primary reaction 2 µl of bsDNA was amplified in a final reaction volume of 20 µl containing 5 µl 5X reaction buffer, 2.5 µl 25Mm Mg, 0.5 µl of primer, 0.25 µl of GoTaq and the remaining being water. The secondary reaction contained the same mixture, with the exception of 2 µl of the unpurified "primary" reaction being used as the template instead of the original bsDNA.

*5.2.4.4 Amplicon purification*

Amplicons were purified using a gel extraction technique. The entire reaction from the secondary "nested" PCR was run on a 1.5% agarose gel with ethidium bromide. Bands containing the product were excised and purified using QIAquick® Gel Extraction Kit (Qiagen). 1 volume of gel was incubated with 3 volumes of QG buffer for 10 min at 50 $^{o}$C with intermittent vortexing. After complete dissolution 1 volume of isopropanol was added per volume of initial band. The sample was hand mixed and then added to QUIquick spin columns. The columns were centrifuged at 10,000 G for 60 s and flow-through was collected and discarded. 500 µl of QG buffer was then added to the column and centrifuged a further 60 s, the flow-through discarded. This process was then repeated once more. 750 µl of PE buffer was added and the column allowed to incubate for 2 min; the sample was centrifuged again for 60 s and the flow-through discarded. The product was eluted in 10 µl of EB buffer applied directly to the membrane of the column and incubated for 60 s at RT. The sample was then centrifuged at 12,000 rpm for 1 minute to a fresh micro-centrifuge tube. A further 10 µl of EB buffer was added to the membrane and the step repeated to ensure maximum extraction.

*5.2.4.5 Library preparation*

Libraries were prepared using the amplicons described in the previous step with NEBNext® DNA Library Prep Master Mix for Illumina® and NEBNext® Multiplex Oligos for Illumina®NEBNext. Firstly, each amplicon underwent end-repair. 10 µl of NEBNext® End-Repair Reaction Buffer (10X) was added to 500 ng of each amplicon. 5 µl of NEBNext® End-Repair Enzyme Mix was added and the final volume brought to 100 µl with water and incubated at 20 $^{o}$C for 30 min. To ensure that all samples were pure for library preparation each sample was cleaned using Agencourt AMPure® XP beads (Beckman Coulter) using a

standard protocol. Each sample was cleaned with a bead:DNA ratio of 1.6X, then eluted into a final volume of 45 µl.

The end-repaired sample then underwent the addition of dAMP to the blunt 3' end of the amplicons. This process is known as dA-tailing and is required to enable the subsequent ligation of adapters. 42 µl of DNA was added to 5 µl NEBNext® dA-Tailing Reaction Buffer (10X) and 3 µl Klenow Fragment (3´→ 5´ exo–). The mixture was incubated at 37 $^{o}$C for 30 min according to the standard protocol and then cleaned with 1.8X AMPure® XP beads in the same way as previously described. dA-tailed DNA then underwent adaptor ligation. 33 µl of DNA, 10 µl of Quick Ligation Reaction Buffer (5X), 2 µl of NEBNext® adaptor and 5 µl of Quick T4 DNA ligase were incubated on a thermal cycler for 15 min at 20 $^{o}$C. A further 3 µl of USER$^{TM}$ enzyme mix was added and the mixture was briefly vortexed and incubated at 37 $^{o}$C for 15 min.

Prior to PCR enrichment, samples underwent a 3-step size-selection process with AMPure® beads. The first step being a clean-up by addition of 1.8X beads, eluted into 100 µl $dH_2O$. After this, large fragments were removed by 70 µl (0.7X) beads and the supernatant collected. Final cleaning of the adaptor ligated sample was achieved by 20 µl of AMPure beads (0.2X) and eluted into a final volume of 50 µl of $dH_2O$.

Finally, adaptor ligated DNA was enriched by PCR prior to library construction. 15 µl of purified product was added to 5 µl of Universal PCR primer, 5 µl of a unique index primer to identify the sample during sequencing and 25 µl of NEBNext Q5 Hot Start HiFi PCR Master Mix. The cycling steps were as follows: an initial 30 s denaturation step (98 $^{o}$C), then 6 repetitions of denaturation (10 s, 98 $^{o}$C), annealing (30 s, 65 $^{o}$C), extension (30 s, 72 $^{o}$C). The reaction concluded with a final extension step of 5 min (72 $^{o}$C). The final library was cleaned with 1X AMPure beads and eluted in 30 µl of 0.1x TE solution.

### 5.2.5 ***Statistical analysis***

For global 5-mC measurements all results are shown as the mean ± SEM (standard error of the mean) of experimental triplicates, run in duplicate. Statistical significance was calculated using a one-way ANOVA with Bonferroni post hoc tests (IBM SPSS 23).

Library sequencing was conducted off-site at the AGRF Biomolecular Resource Facility in The Medical Research Centre of The Australian National University. Raw data was processed in 'R' by Dr Robert Kucharski.

## 5.3 **Results**

### 5.3.1 ***Examination of global methylation measures in the honey bee***

#### *5.3.1.1 Fluorescence-based ELISA*

Due to significant over-fluorescence in the final row of the plate in the first run of the ELISA kit **(Table 5.4)**, results for siRNA control, siRNA 1 and siRNA 2 were discarded. As a result the only direct comparison made was between the two untreated diets, 1:5 EAA:C and 1:500 EAA:C. There was no significant difference found in the global 5-mC (5-methylcytosine) content between the 1:5 (1.48% ± 0.73%) and 1:500 (0.95% ± 0.168%) diets (ANOVA $F(1,4) = 0.545$, $P=0.501$). The standard curve calculated for this reaction had an $R^2$ value of 0.883 suggesting a good fit with the data **(Figure 5.1)**.

Our results showed that when a 2 ng/µl sample was plotted in multiple positions across the plate, there was a high degree of variance **(Table 5.5)**. The mean of these measurements was 25639.6 with a standard deviation (σ) of 13858. Standard curve calculations **(Figure 5.2)** showed an $R^2$ value of 0.668 and 0.626 for the first and second reads, respectively. This suggests only a moderate fit with the data. We found that there was a significant effect of diet on the 5-mC content (ANOVA $F(1, 20) = 6.944$, $P=0.001$), but there was no significant difference between the first and second reads (ANOVA $F(1, 20) = 0.20$, $P=0.889$), nor any significant interaction between the reads and diet (ANOVA $F(4, 20) = 0.029$, $P=0.998$). Because of this, data was pooled for analysis. Post-hoc LSD pairwise comparisons revealed that honey bees fed the 1:5 EAA:C diet had significantly higher global methylation (0.851% ± 0.269%) than the 1:500 diet ($P < 0.001$) and all 3 diets containing siRNA (all P-values < 0.009). There were no further significant results from the pairwise comparisons, but near-significant trends ($P=0.077$ and 0.067 respectively) that indicated higher global 5-mC content in the siRNA control and siRNA 2 diets compared to the 1:500 diet alone **(Figure 5.3)**.

**Table 5.4** Heat map of RFU values from first measurements alongside their corresponding map. RFU values for column 6 were significantly higher than their duplicate in column 5. These values had to be discarded and a second plate setup was designed to measure any variance in RFU across the plate. The most likely source of this variance was contamination.

| Strip | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| A | MF3 | MF3 | 1:5 (1) | 1:5 (1) | siRNA C (1) | siRNA C (1) |
| B | MF4 0.5ng | MF4 0.5ng | 1:5 (2) | 1:5 (2) | siRNA C (2) | siRNA C (2) |
| C | MF4 1 ng | MF4 1 ng | 1:5 (3) | 1:5 (3) | siRNA C (3) | siRNA C (3) |
| D | MF4 2 ng | MF4 2 ng | 1:500 (1) | 1:500 (1) | siRNA1 (1) | siRNA1 (1) |
| E | MF4 5 ng | MF4 5 ng | 1:500 (2) | 1:500 (2) | siRNA1 (2) | siRNA1 (2) |
| F | MF4 10 ng | MF4 10 ng | 1:500 (3) | 1:500 (3) | siRNA1 (3) | siRNA1 (3) |
| G | | | | siRNA2 (1) | siRNA2 (2) | siRNA2 (3) |
| H | | | | siRNA2 (1) | siRNA2 (2) | siRNA2 (3) |

| Strip | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| A | 0.285866 | -0.28587 | 2.79322437 | 3.044649 | -0.01065 | 11.5026 |
| B | 1.069572 | 0.271777 | 0.69102636 | 1.294383 | -0.24422 | 8.668044 |
| C | 2.059616 | 1.378609 | -0.0995679 | 1.153172 | 0.357255 | 8.154236 |
| D | 1.15505 | 0.625587 | -0.0475922 | 1.594026 | 0.45557 | 7.882773 |
| E | 2.008266 | 1.450623 | 0.35443672 | 1.126871 | 0.372597 | 8.490826 |
| F | 8.449496 | 2.992047 | 0.05886405 | 2.463523 | 0.942138 | 6.461269 |
| G | | | | 4.265452 | 1.806938 | 13.27729 |
| H | | | | 2.899994 | 4.168076 | 8.385309 |

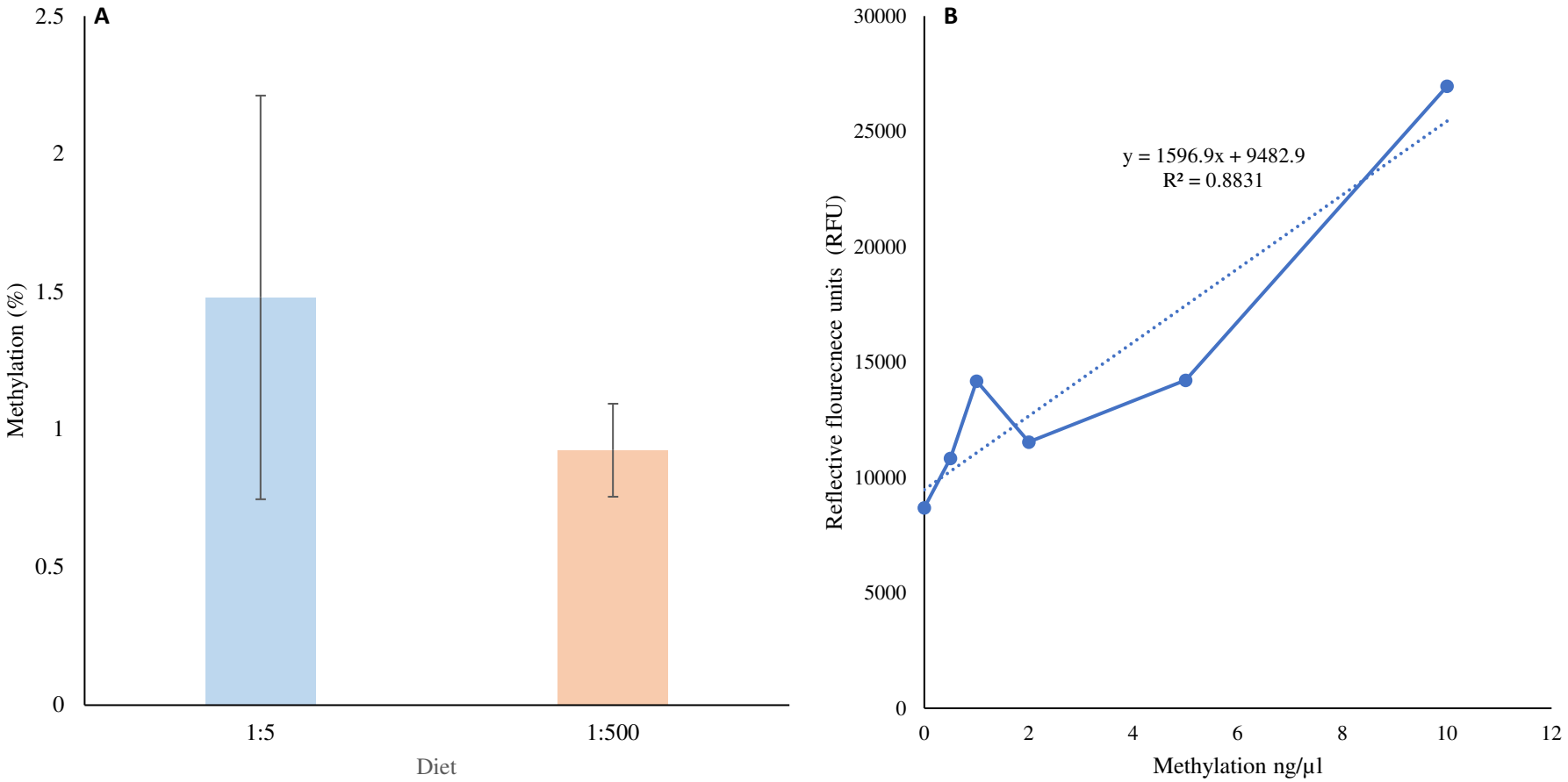


**Figure 5.1** (A) Honey bees fed a diet high in EAAs (1:5 EAA:C) (1.48% ± 0.73%) showed no significant difference in their global methylation levels compared to honey bees fed a diet low in EAAs (1:500 EAA:C) (0.95% ± 0.168%). (B) Standard curve fit was not good ($R^2 = 0.883$) N = 3 bees per sample.

**Table 5.5** RFU values from second read with heat map for 2 ng/µl standards alongside their plate layout. The mean RFU was 25639.6 (± 4899.5) but there was significant variation between replicates.

| Strip | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| A | MF4 2 ng | MF3 | MF3 | siRNA2 (1) | siRNA2 (3) | siRNA2 (3) |
| B | MF4 0.5ng | MF4 2 ng | MF4 0.5ng | siRNA2 (1) | siRNA1 (1) | siRNA1 (1) |
| C | MF4 1ng | MF4 1ng | MF4 2 ng | siRNA2 (2) | siRNA1 (2) | siRNA1 (2) |
| D | MF4 5ng | MF4 5ng | MF4 2 ng | siRNA2 (2) | siRNA1 (3) | siRNA1 (3) |
| E | MF4 10ng | MF4 10ng | 1:5 (1) | MF4 2 ng | 1:5 (1) | 1:5 (2) |
| F | 1:500 (1) | 1:500 (1) | siRNA C (1) | siRNA C (1) | MF4 2 ng | 1:5 (2) |
| G | 1:500 (2) | 1:500 (2) | siRNA C (2) | siRNA C (2) | 1:5 (3) | MF4 2 ng |
| H | 1:500 (3) | 1:500 (3) | siRNA C (3) | siRNA C (3) | 1:5 (3) | MF4 2 ng |

| Strip | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| A | 29990.5 | 1251.5 | 1337.5 | 2882 | 5095 | 2460.5 |
| B | 16334.5 | 8558 | 39725.5 | 2772.5 | 2554.5 | 2355 |
| C | 11213.5 | 19835 | 43205.5 | 5417 | 4237.5 | 2125 |
| D | 33916.5 | 22865.5 | 57965.2 | 6175.5 | 3855.5 | 1942 |
| E | 54627 | 37301 | 11057.5 | 20084 | 9460.5 | 4354 |
| F | 2526 | 1404 | 4263 | 2507.5 | 35858 | 6554.5 |
| G | 1650.5 | 2816 | 5755.5 | 4780.5 | 3914 | 17432 |
| H | 2776 | 3023 | 4846.5 | 2403.5 | 5525 | 24837 |

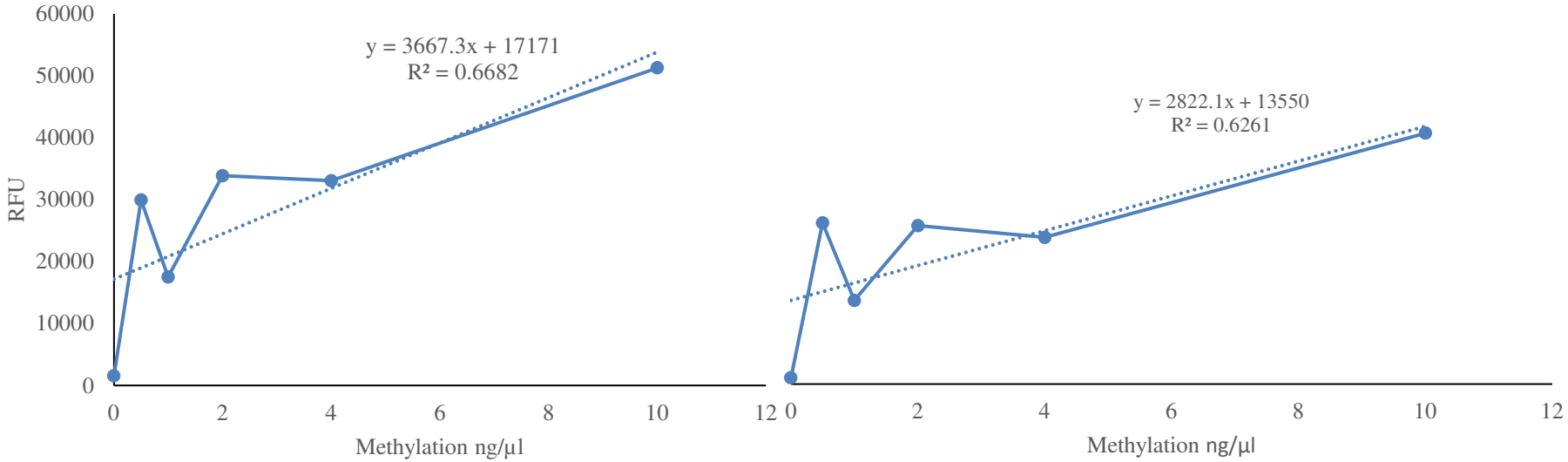


**Figure 5.2** Plots of standard curves and slope calculations derived from the RFU of the supplied methylation standards for the first and second reads of the modified plate layout. Neither $R^2$ value showed a good fit to the data.

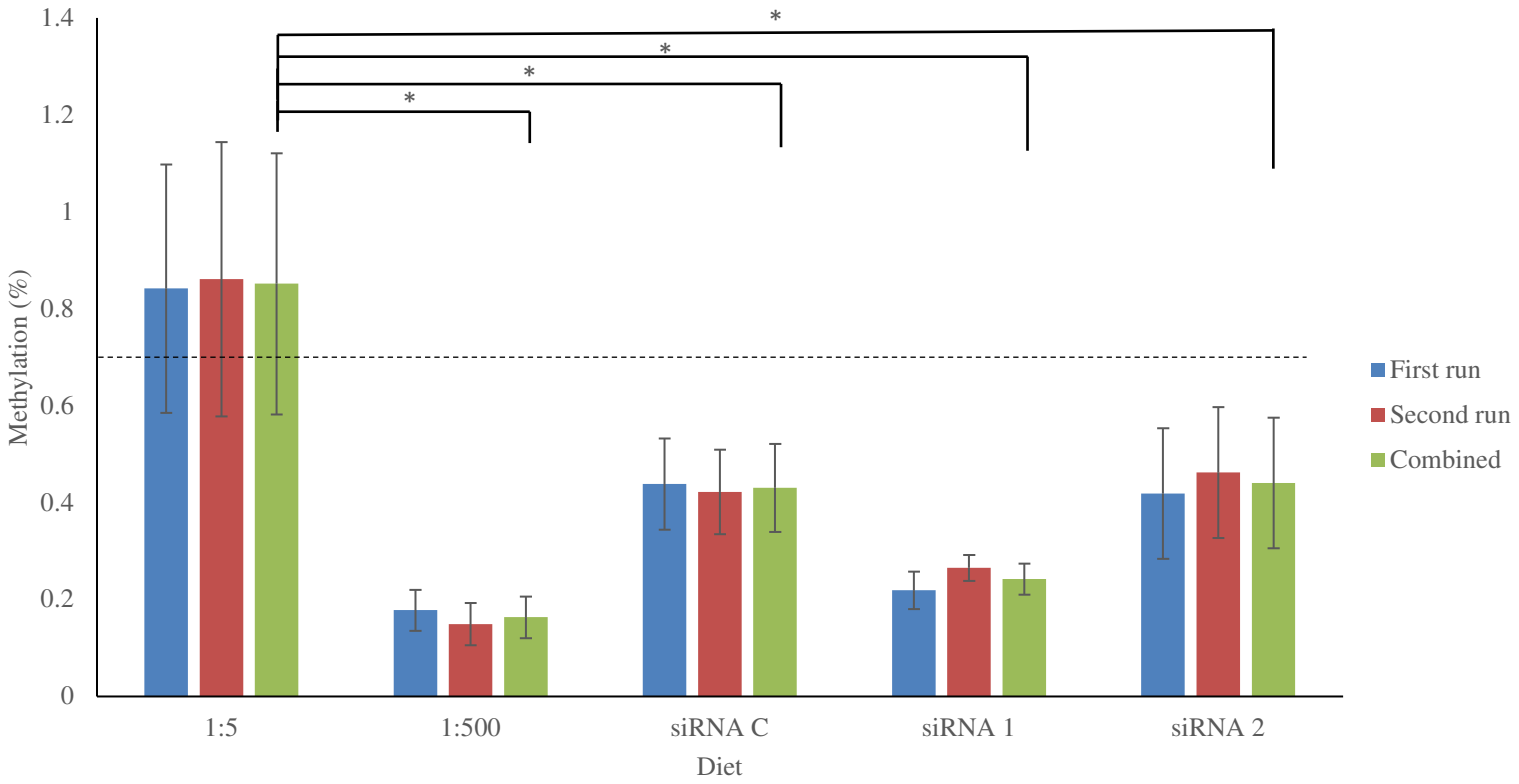


**Figure 5.3** There was no significant difference between methylation measurements in the first and second read. When the data was combined and averaged, the high EAA diet (1:5 EAA:C) had a higher percentage of CpG sites methylated (0.851% ± 0.269%) globally than any other treatment (Next highest siRNA 2 0.440% ± 0.135%). Dashed line indicates 0.7% methylation observed by Lyko (2010).

*5.3.1.2 Colourimetry-based ELISA*

Honey bees

Standard curve calculations showed $R^2$ values of 0.976, suggesting a good fit to the data. Comparison of the first and second reads showed no significant difference (ANOVA $F(1, 20) = 0.019$, $P=0.892$) so data was pooled. There was no significant interaction between treatment and read number (ANOVA $F(4,20) = 0.005$, $P=1.000$). One observable effect was the increase in global methylation in the 1:5 EAA treatment group compared to all other diets with the exception of those with SiRNA2 present. There was a significant effect of treatment (ANOVA $F(4,20) = 2.933$, $P=0.046$) which when post-hoc analysis was performed showed significantly higher 5-mC content in the 1:5 EAA:C (high EAA) diet than all but the siRNA 2 diet ($P<0.02$) **(Figure 5.4)**. While performance of the standard curve was better in the colourimetric kit than the fluorescence-based kit, there was still high variance in the results which made any significant findings unlikely.

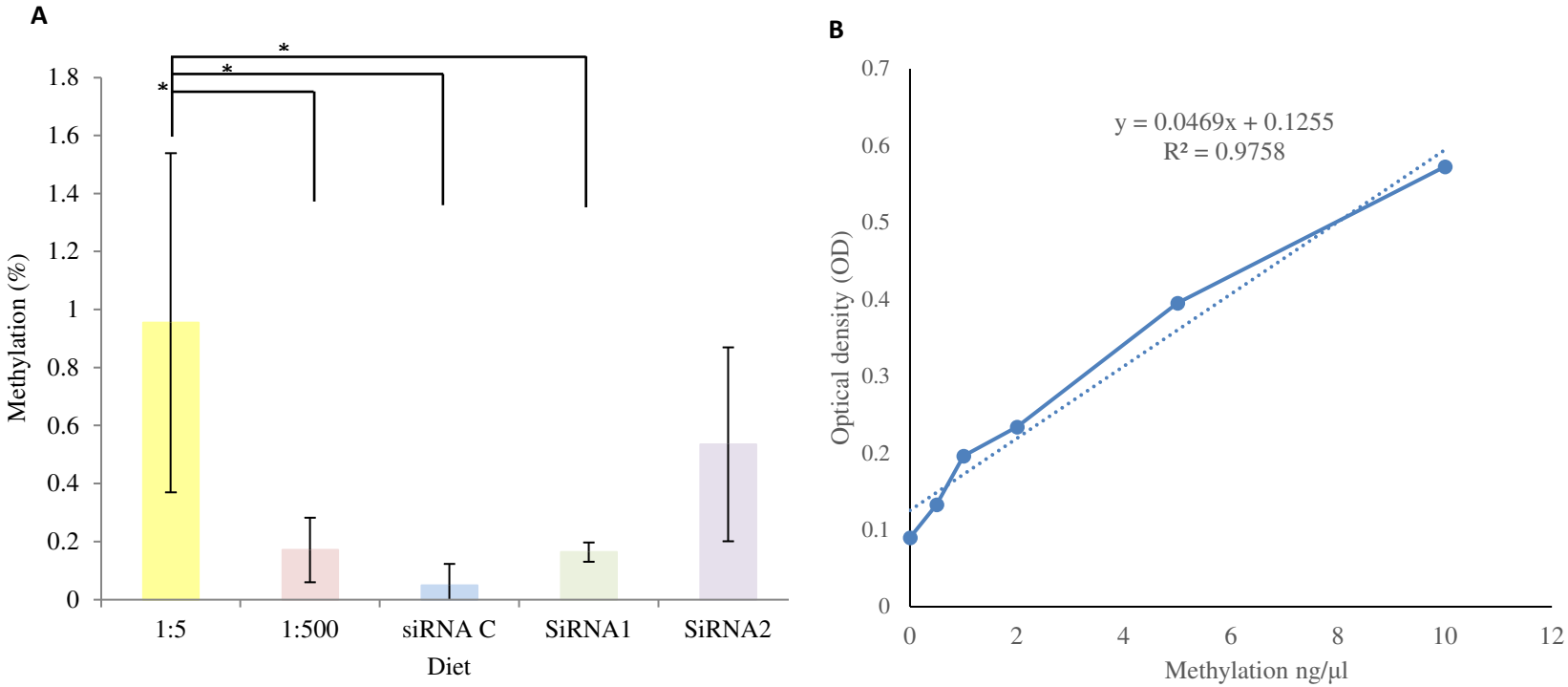


**Figure 5.4** (A) Global CpG methylation was higher in the 1:5 EAA:C diet (high EAA diet) than all other treatments except for siRNA 2. The overall methylation levels were low in all treatments. (B) Standard curve calculated from the OD readings of the colourimetric methylflash ELISA showed good fit ($R^2 = 0.976$) (N=3 bees per treatment).

Ants

Global methylation was also determined in the Pharaoh ant *M. Pharaonic* when fed one of the following five diets: Sucrose control, low protein, low protein + rapamycin, high protein, high protein + rapamycin. As with the honey bee, across both experiments, *M. pharaonic* 5-mC content was low compared to vertebrates (0.112% ± 0.027%). The ELISA kit failed to detect any statistically significant differences between treatments (ANOVA $F(4,20) = 0.672$, $P=0.645$) or experiment (ANOVA $F(1,20) = 2.430$, $P=0.191$), nor was there any overall interaction between the two (ANOVA $F(4,20) = 1.009$, $P=0.426$). As with previous colourimetric assays, the overall fit of the standard curve was high ($R^2 = 0.990$) **(Figure 5.5)**.

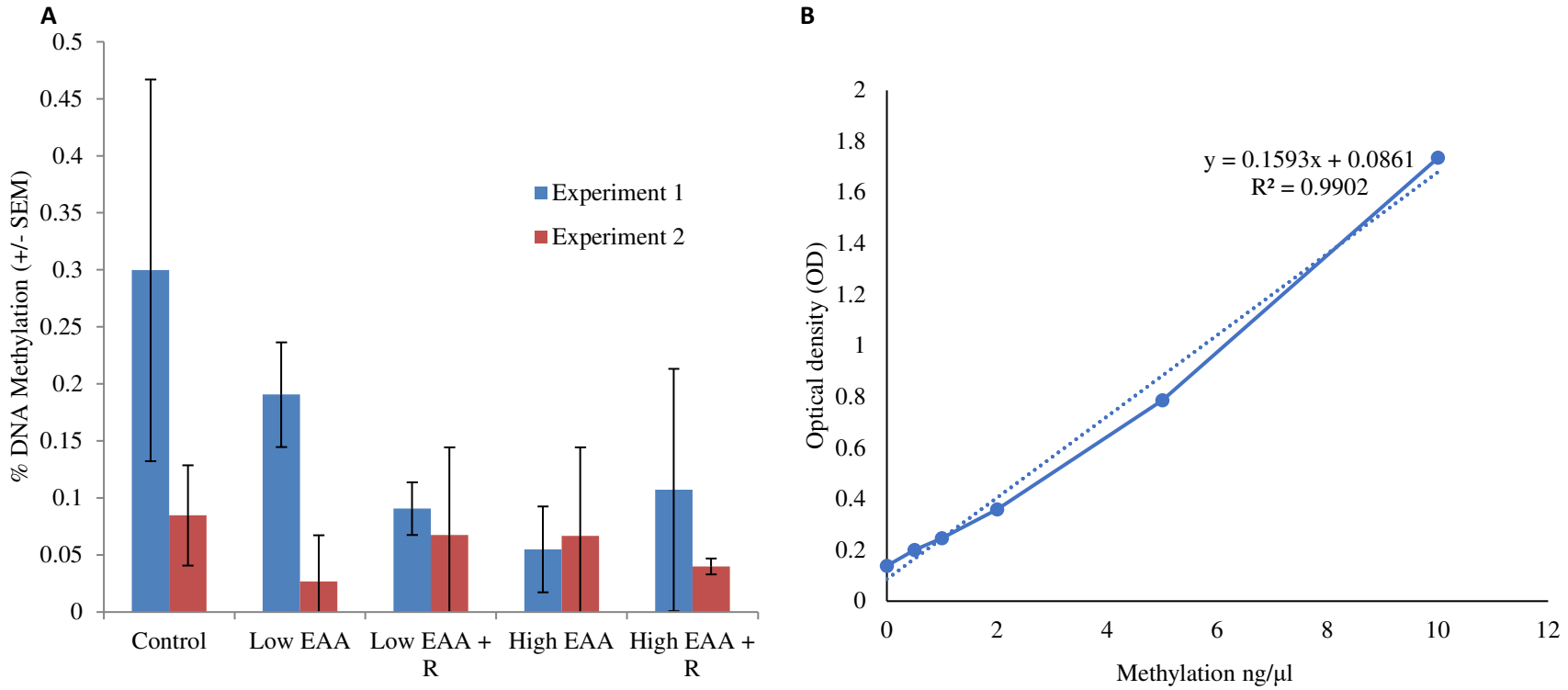


**Figure 5.5** Diet has no effect on global CpG methylation in the Pharaoh ant M. *pharaonic*. (A) There was no significant change in the proportion of methylated CpG sites regardless of the diet used. (B) The standard curve derived from measurements of a control with known CpG content showed good fit ($R^2 = 0.990$).

### *5.3.2 CpG site methylation*

Of the 4 treatment groups, day 7 foragers, day 21 foragers and day 21 nurses resulted in successful reads; however, analysis of the nurse day 7 group revealed that the library was unsuccessful. As displayed in **Figure 5.6**, there was no significant methylation found in CpG sites 1-4 in any of the tests, nor in 9-12 and 15-17. In contrast, high levels of methylation were observed in sites 5-8, 5-7 and 5-8 for day 7 foragers, day 21 foragers and day 21 nurses, respectively; all three groups also showed high methylation in sites18-19. Interestingly, Sir2 CpG methylation shows some variation with age and role within the hive, in particular differential methylation is found in site 14 for both foraging groups but not within the nurse group, which showed methylation at site 13. The day 7 foragers and day 21 nurses showed similar patterns in CpG site 8 which were absent in day 21 foragers. CpG sites 18 and 19 were also highest in the nurse group.

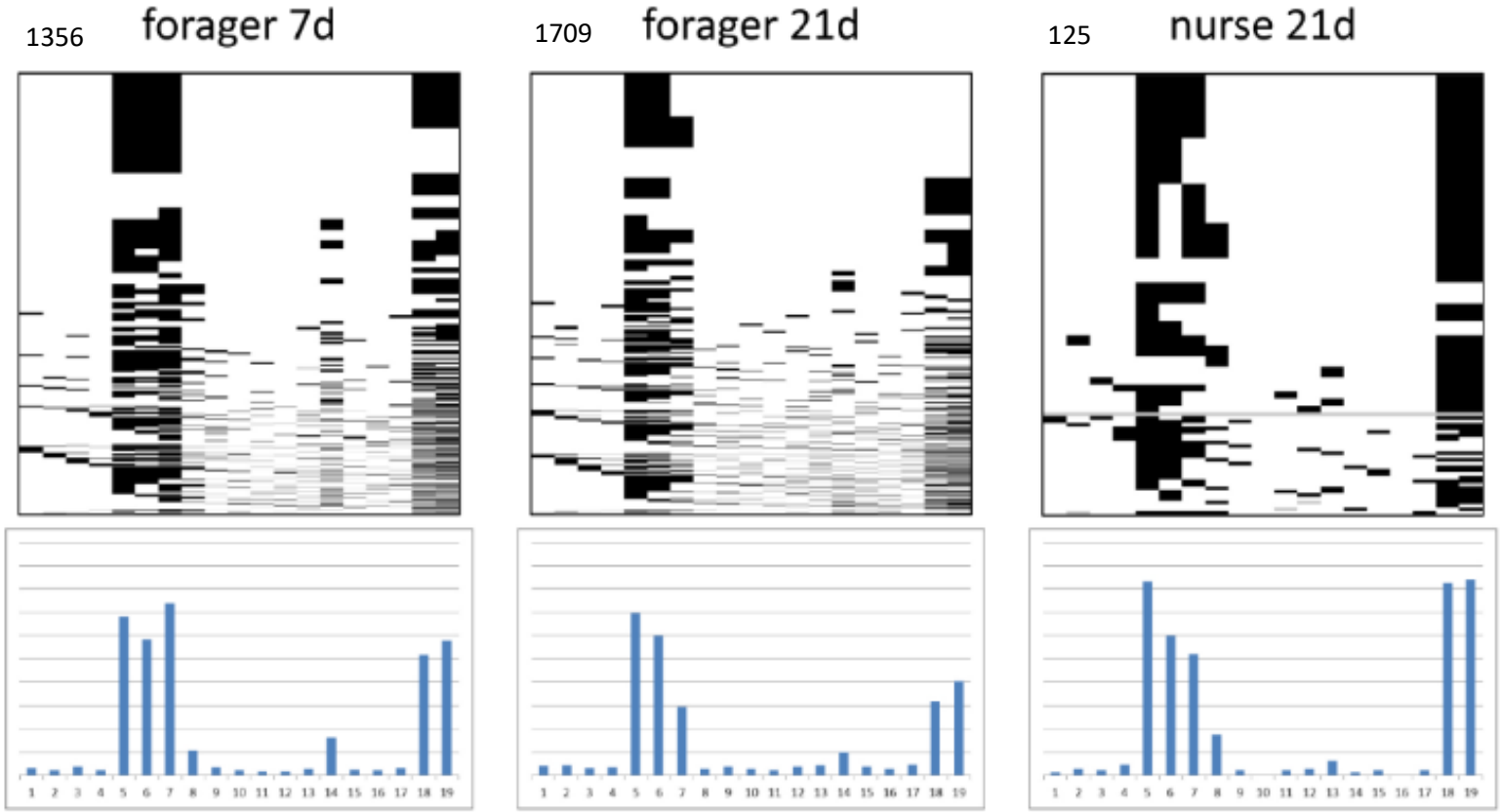


**Figure 5.6** Effect of age and role on the methylation pattern of Sir2. DNA samples were taken from three groups of insects, as shown above panels (a).
Panels (a); Methylation patterns in Sir2 revealed by MiSeq sequencing. Each row represents the methylation pattern of an individual read at CpG positions 1-19 (black: methylated CpGs, white: unmethylated CpGs). The patterns in panel (a) are sorted from the most abundant at the top to the least abundant at the bottom; each column represents an individual CpG site. The number of sequenced reads for each group is shown above each panel (a).
Panel (b); The height of each column in panel (b) represents the average methylation level for each of the 19 CpG sites in the amplicon, corresponding to the columns in panel (a).

## 5.4 **Discussion**

The honey bee model provides a potentially useful but also challenging model to study methylation changes associated with age and diet. The methylation changes which are responsible for the vastly different life outcomes of queen and worker bees provide a tantalising target for life extending therapeutics, however the model is not without flaws. Low global DNA methylation and a lack of promoter site methylation make it a poor fit for some lines of investigation and the lack of suitability of "user friendly" kits makes the study of the honey bee methylome expensive and time consuming relative to more heavily methylated organisms.

### 5.4.1 ***Global methylation measures in the honey bee***

Results from this study add further evidence to the theory that global CpG methylation is generally low in eusocial insects. Whilst the human genome is extensively methylated (Suzuki and Bird, 2008), the genomes of invertebrates are relatively sparsely methylated. The majority of this methylation is contained within gene bodies (Zemach et al., 2010). This was consistent with the findings in this study. Regardless of measurement method, global CpG methylation rates were less than 1% which was a consistent finding throughout. Fluorescence based ELISAs appeared to exhibit greater levels of sensitivity but at the expense of significant error in the standard controls. In contrast, colourimetric ELISA's lacked the sensitivity of the fluorescence kit but showed highly reliable measurements of the standards. Neither were capable of discerning all but the most drastic changes in global methylation, making them unsuitable for use in eusocial insects. *M. pharaonic* did not appear to show any changes in global methylation based on dietary changes nor the presence of rapamycin, so it is unlikely that this is a specific issue with the honey bee, but a fundamental flaw in the kit with regards to these insects. Both the honey bee *A. mellifera* and the pharaoh ant *M. pharaonic* showed low global levels of DNA methylation at CpG sites. The levels of methylation in *M. pharaonic* are comparable to that of carpenter ants (*Camponotus sp.*) and *Harpegnathos sp.* (Bonasio et al., 2012). Bisulphite sequencing by Foret *et al.* (2012) of the honey bee genome found a similar proportion of methylated CpG sites as found in the present study.

One novel finding from this study was the increase in methylated DNA associated with high EAA diets in the honey bee compared to those on low EAA diets. It is difficult to make direct comparisons to honey bees allowed to feed freely due to the impossibility of ensuring age

matching with honey bees in the hive, and the confounding effect of pheromones from the queen. However, all methylation levels observed in our study where within the bounds of other studies (Foret et al., 2012, Kucharski et al., 2008). One possible explanation for this increase is the abundance of methionine in the high EAA diet. Methionine is a methyl donor (Niculescu and Zeisel, 2002) and in some cases, can increase DNA methylation (Cooney *et al.*, 2002; Parrish *et al.*, 2015). It cannot be ruled out that other effects were not missed due to the limitations of the ELISA kits used. The stated accuracy of the kits (0.05%) is too large to effectively detect more subtle changes given the low basal level of methylation, even assuming it reaches the specified accuracy. One of the kits, the fluorometric ELISA, has since been removed from the market. The reasons for this are unknown despite my attempts to contact the supplier. It is possible this is due to the ineffective standard curve supplied with the kit. In future studies involving eusocial insects, alternatives to using global 5-mC detection kits should be found as currently the accuracy cannot be assured at such low methylation levels.

### 5.4.2 ***Site specific methylation measures in the honey bee***

Our findings show that CpG methylation is present in gene bodies within the honey bee. Bisulfite sequencing has previously been shown to be effective at determining differentially methylated genes in the honey bee (Foret *et al.*, 2012). In the present study the test was successful, albeit with low run numbers for each group allowing us to derive limited conclusions for our specific test. Our failure to find suitable CpG sites in promoter regions is in line with previous findings by Elango *et al.* (2009) which suggests promoter region suppression is less common in the reduced genome of the honey bee. Our failure to find suitable CpG sites in all but one of our circadian targets was surprising. One possible reason for this is described by Lyko *et al.* (2010) who proposes that these methylated CpG sites mark critical genes which are conserved and ubiquitously expressed and whose activities cannot be switched off in most tissues. However, they are not required consistently throughout development or during times of environmental stress. He proposes that methylation could be the mechanism by which these genes are "managed" in the form of alternative splicing. Due to the highly specific nature of our circadian target proteins it is possible that the absence of methylation at CpG sites within the genes is due to the fact that they are not alternatively spliced and rather turned 'on' or 'off'. The control of such genes is usually through methylation of upstream products (Azzi *et al.*, 2014). This form of regulation

has also been observed in human cells (Shukla *et al.*, 2011) so it is possible that observations in *A. mellifera* might well also have implications for vertebrates.

This study also confirmed that CpG sites within the *Sir2* gene are methylated. There appears to be differences within the methylation patterns based on age and role, but a more compete and detailed study is required to confirm these findings. These is findings are consistent with findings in ant and honey bee studies, where several genes exhibit caste-specific and developmental changes in DNA methylation (Bonasio *et al.*, 2012). *Sir2* is also important in far more processes than circadian genes as described previously, so its alternative splicing is far more likely.

The honey bee, unlike *D. melanogaster*, has a functional, but simplified methylation system and could prove important as an *in vivo* system in which to understand the impact of environmental and developmental changes on longevity and health. It's low basal levels of CpG methylation and its absence of promoter methylation make it a highly specific, simplified model for gene body methylation. However, it does come with limitations, more general, easy to use measures such as ELISA assays are unlikely to find modest changes in the methylome and techniques like bisulphite sequencing require significantly more time and resources, even more so when attempting to measure global methylation. Future studies aiming to determine the role of DNA methylation in regulating metabolic and circadian genes in eusocial insects should focus on identifying the upstream factors responsible for changes in expression. Once targets have been identified the scope of analysis required should be determined and a decision made on the choice of measurement made.

# Chapter 6. Methionine and its effects on *Sir2* and *FOXO* expression and survival in the honey bee

## 6.1 Introduction

### 6.1.1 *Methionine restriction and lifespan*

It has long been established that methionine restriction extends lifespan in a similar fashion to DR and it has been proposed that these effects share the same underlying mechanisms to that of caloric restriction, and are in fact one and the same (Orentreich *et al.*, 1993; Grandison *et al.*, 2009; Hine and Mitchell, 2015). However, there has been significant evidence to suggest that these life-extending effects are separate and either work in tandem or completely individually (Sun *et al.*, 2009). In mice, it has been shown that restricting methionine intake extends lifespan even when caloric deficit is absent (Majtan *et al.*, 2019) and has been implicated in obesity and insulin dysfunction resistance (Ables *et al.*, 2012) as well as reduced risk of mammary tumours (Hens *et al.*, 2016).

### 6.1.2 *Methionine and regulation of the mTOR pathway*

As discussed in previous chapters, Paoli et al. (2014) showed that Sir2 expression was greatly increased in honey bees fed high EAA:C diets and that Sir2 knockdown via siRNAs reduced lifespan, although not to the same level as high EAA diets. They suggested that the life extending effects of Sir2 in the honey bee were at least partially the result of its downregulation of mTOR to combat high EAA status.

mTOR is a central metabolic regulator whose downregulation has been shown to play a critical role in the life extending effects of DR. mTORC1 activity is regulated by the availability of nutrients, growth factors and ATP. Amino acids, and methionine in particular, have been shown to be a necessary part of mTORC1 activation in normal cells (Arriola Apelo *et al.*, 2014) and methionine exposure has been shown to promote protein synthesis, which is halted when treated with rapamycin (Zhou *et al.*, 2016). Exposure to high levels of amino acids have been shown to have negative health consequences to many model organisms including *D. melanogaster* and *A. mellifera* (Grandison et al., 2009; Paoli et al., 2014).

In mouse cells, when Taste receptor type 1 (T1R), a G protein-coupled receptor on the cell membrane, detects free extracellular methionine it stimulates an increase in $Ca^{2+}$ concentrations in the intracellular space which in turn promotes the mTORC1 activators ribosomal protein S6 kinase (RSK) and extracellular signal–regulated kinases 1 and 2

(ERK1/2) (Sengupta *et al.*, 2010). As methionine has been shown to be crucial in the function of mTORC1, it has been suggested that the life extending effects of methionine restriction are due to the suppression of mTORC1. Further to this, as a negative regulator of mTOR, it has been proposed that sirtuins could be a mediator in the upregulation of mTOR under high methionine states (Sanchez-Roman *et al.*, 2011).

### 6.1.3 ***Methionine oxidation and stress resistance***

Methionine is an essential amino acid which is highly susceptible to oxidation by free radical oxygen species (ROS), this reaction produces methionine sulfoxide (Met-O) (Stadtman *et al.*, 2002). Organisms can reverse this reaction via the methionine sulfoxide reductase system (Msrs), which is now considered an important component of a defence mechanism against oxidative damage. However, the effectiveness of this process declines with age and this decline is implicated in the ageing process (Stadtman, 2006).

Effective or upregulated function of the Msrs has been shown to increase longevity and resistance to oxidative stress across multiple species including yeast, *D. melanogaster* and *Arabidopsis sp.* (Koc *et al.*, 2004; Chung *et al.*, 2010; Lim *et al.*, 2012; Châtelain *et al.*, 2013; Lee *et al.*, 2018), while downregulation has been shown to reduce longevity and accelerate age related decline (Oien and Moskovitz, 2007; Wassef *et al.*, 2007; Salmon *et al.*, 2009). One of the key Msrs genes, *msra-1* has been shown to play a crucial role in protein repair and ROS scavenging via methionine sulfoxide reduction. *C. elegans* carrying a deletion in the *msra-1* gene have been shown to be more sensitive to oxidative stress, present with locomotive difficulties including dysregulated chemotaxis and a reduction in median survival time (Minniti *et al.*, 2009).

The *msra-1* homologue *AccMsrA*, which was identified in the Eastern honey bee, has been shown to serve a similar function to its mammalian counterpart. Not only is it highly expressed in the heads of adult workers, but is upregulated by external stressors like ultra violet (UV) light, heat and $H_2O_2$, indicating a critical role in response to environmental stressors (Gong *et al.*, 2012). *MsrA* is also one of the genes shown to be upregulated in the much longer-lived queen bee (Corona *et al.*, 2005). It has recently been observed that MsrA expression in *C. elegans* is moderated by the *DAF-16* pathway, an ortholog of the human forkhead box protein FOXO3a (Lee *et al.*, 2001). Upregulation of the DAF-16/FOXO3a pathway has been shown to extend longevity in *C. elegans* and other species through a range of mechanisms including regulation of stress resistance, metabolism and apoptosis (Ogg *et*

*al.*, 1997). FOXO also plays an important role in the removal of the primary methionine metabolite S-adenosyl-methionine (SAM) via the promotion of SAM catabolism enhancer glycine N-methyltransferase (Gnmt). Methylation levels are detected indirectly through SAM levels via the mTOR pathway. The SAMTOR protein binds to the GATOR1 protein which forms the SAMTOR-GATOR1 complex, an important step in the activation of *mTORC1*. When methionine levels are high, SAM levels increase and bind with SAMTOR, disrupting the mTOR pathway (Gu *et al.*, 2017). Since *FOXO* expression is downregulated by mTOR signalling (Robida-Stubbs *et al.*, 2012) it is possible that life extending effects of methionine restriction are a result of the downregulation of the mTOR pathway and thus preventing the inhibition of *FOXO*.

### 6.1.4 ***Aims and hypothesis***

In this study, dietary manipulation and RTqPCR was used to investigate the effects of variable methionine content on longevity and healthspan in the newly emerged honey bee and to determine whether methionine restriction acted on the *Sir2* or *FOXO* pathway.

## 6.2 Materials and methods

### 6.2.1 ***Insect husbandry***

Honey bees (*A. mellifera buckfast*) were collected from an isolated brood frame, removed from hives on the Ridley Building roof, Newcastle University. These were stored at 34 °C and 70% RH and allowed to eclose. Newly emerged workers were collected daily and placed in Perspex housing chambers (11x5x5 cm) in cohorts of 20 bees (n = 10 boxes). Bees were kept in incubators in constant darkness at 34 °C and 70% RH for 14 days. Diets were provided in 2 x 2 ml Eppendorf tubes with four 3.0 mm holes drilled along on surface. Newly emerged bees were allowed to feed *ad libitum*. Diets were made using a base diet of 1:500 (EAA:C) ratio using 1M sucrose as the fixed carbohydrate component, as described in chapter 2, with the exception of methionine which used the default amount in the 1:10 EAA:C diets. Five alternative diets were then prepared: A base 1M sucrose only solution with only methionine (Sucrose + Meth); a 1:500 EAA:C diet with 10x the base level of methionine (1:500 + 10x Meth); a 1:500 EAA:C diet with $1/10^{th}$ of the base level of methionine (1:500 + $1/10^{th}$ Meth); a 1:500 EAA:C diet with no methionine (1:500 + No Meth); and a 1:10 EAA:C diet with standard methionine levels (1:10). Where an amino acid was removed from the diet, either partially or in full, the remaining 9 were adjusted to maintain the same minimum 1:500 ratio of EAA: C (Table 6.1).

**Table 6.1** Components of each experimental diet used in experimental study

| Diet | Sucrose | Methionine | Other EAAs |
|---|---|---|---|
| Sucrose + Meth | 342.3g | 1.49g | 0g |
| 1:500 + 10x Meth | 342.3g | 14.9g | 0.268g |
| 1:500 + 1/10$^{th}$ Meth | 342.3g | 0.149g | 0.268g |
| 1:500 + No Meth | 342.3g | 0g | 0.298g |
| 1:10 EAA | 342.3g | 1.49g | 13.42g |

### 6.2.2 ***Daily consumption***

Feeding tubes were weighed after filling and then weighed again after 24 h to measure total consumption, per box, per day. Diets were replaced daily from a pre-frozen stock. A control box containing no bees for each diet was used to record daily evaporation rates for each diet; the final data for the consumption of each diets was adjusted for the evaporation for each specific diet. The number of dead bees were also recorded for each cohort every 24 h and dead bees were removed from the housing enclosure during this process.

### 6.2.3 ***RNA isolation and qRT-PCR***

Honeybees were collected at 14 days, at the end of the experimental trial period and euthanised in a -20 °C freezer for 10 min. Bees were then snap-frozen in liquid nitrogen and stored at -80 °C until processing. Whole bees were ground in liquid nitrogen and RNA was prepared using Trizol reagent (Invitrogen), following the manufacturer's instructions. First-strand cDNA synthesis was carried out on RNA using SuperScript II transcriptase (Invitrogen), following the manufacturer's instructions. Quantitative real-time PCR was performed in a Roche LightCycler 480 with 20 µl reactions set up in 96-well format containing LightCycler SYBR Green I Master (Roche), 0.5 µM of each primer and 1 µl of cDNA (diluted to 1:4). The reference gene used was *Apis mellifera* ribosomal protein 49 (*RP49*). After denaturing for 5 min at 95 °C, 50 cycles were carried out using the following parameters: 95 °C, 10 s; 55°C 10 s; 72 °C, 15 s. Levels of specific RNAs relative to control, and corrected according to levels of reference gene RNAs, were calculated using the ΔΔCt method.

### 6.2.4 ***Statistical analysis***

Consumption data was measured for each individual box on a daily basis and then normalised against number of surviving bees on that day. Total consumption per bee was then calculated for each diet and compared using a One-way ANOVA with LSD post-hoc. Relative expression levels were analysed using the same statistical test. Survival was calculated using a Cox Regression, comparing all treatments to the sucrose only control. All statistical analysis was conducted using SPSS v.21 software and graphs were compiled using Microsoft Excel.

## 6.3 **Results:**

### 6.3.1 ***High levels of methionine reduce overall consumption in worker honey bees***

There did not appear to be a difference in preference between the 1:10 EAA diets and the sucrose only control. Total consumption of 1:10 EAA:C diets (M = 0.000186 ± .000018) in worker honey bees was similar to those fed on a solely sucrose diet (M = 0.000235 ± .000024) (Univariate ANOVA, Diet: $F_{4, 70} = 4.998$, $p = 0.001$, Post hoc LSD $p = 0.112$).

High methionine diets such as 10x methionine (M = 0.000166 ± .000014) however, inhibited total consumption compared to all other diets ($p < 0.29$) with the exception of the 1:10 EAA:C ($p = 0.51$). Diet containing the lower levels of methione like $1/10^{th}$ methionine (M = 0.000277 ± .000024) and no methionine (M = 0.000266 ± .000026), were found to be the most appetising to the honey bees, showing significantly higher consumption than either the 1:10 or 10x methionine diets ($p < 0.012$ and $<0.002$), but not compared to sucrose alone ($p>0.168$) **(Figure 6.1)**.

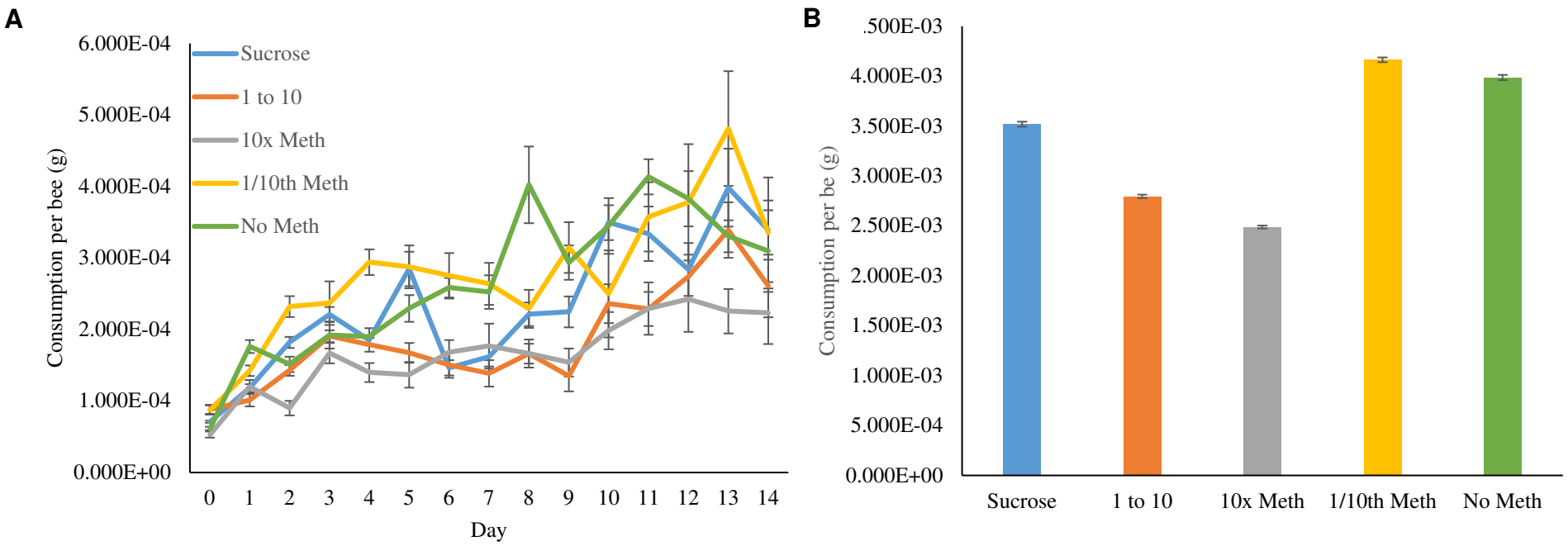


**Figure 6.1** High methionine content significantly decreased total consumption of diet, whereas reduced or no methionine increased consumption relative to a 1:10 EAA:C diet. **a.** Daily consumption/bee consumption for each diet. **b**. The cumulative consumption per bee for each diet across the whole 14-day measurement period. Only the 10x methionine diet caused significantly lower consumption than the sucrose only control, while both $1/10^{th}$ methionine and no methionine diets increased consumption compared to the 1:10 EAA diet and 10x methionine. $n$ = 100, data displayed as mean ± SEM.

### 6.3.2 *High levels of dietary methionine reduce survival of worker honey bees*

High methionine diets heavily curtails survival in newly emerged honey bees compared to other treatments in this study. Worker honeybees died at a faster rate when fed diets containing 10x methionine (Coxreg, sucrose x 10x M, HR = 1.449 [95% CI 1.082-1.940], p = 0.013) or the 1:10 EAA:C diets (Coxreg, sucrose x 1:10, HR = 1.595 [95% CI 1.169-2.178], p = 0.003) compared to sucrose **(Figure 6.2)**. In contrast, bees fed on the low methionine diet ($1/10^{th}$ methionine) (Coxreg, sucrose x $1/10^{th}$ M, HR = 0.326 [95% CI 0.231-0.462], p < 0.001) and no methionine diet (Coxreg, sucrose x No M, HR = 0.689 [95% CI 0.515-0.922], p = 0.012) showed increased survival relative to the sucrose control diet **(Table 6.2)**.

Compared to the honey bees fed the 1:10 EAA:C diet, only the 10x methionine diet did not improve survival (Coxreg, 1:10 x 10x M, HR = 0.908 [95% CI 0.727-1.134], p = 0.395), confirming previously observed harmful effects of high EAA diets on worker honey bees. Sucrose (Coxreg, 1:10 x suc, HR = 0.627 [95% CI 0.459-0.856], p = 0.003), $1/10^{th}$ methionine (Coxreg, 1:10 x $1/10^{th}$ M HR = 0.205 [95% CI 0.151-0.277], p < 0.001) and no methionine diets (Coxreg, 1:10 x No M, HR = 0.432 [95% CI 0.344-0.543], p < 0.001) all showed improved survival in worker honey bees relative to the 1:10 EAA diet **(Table 6.3)**.

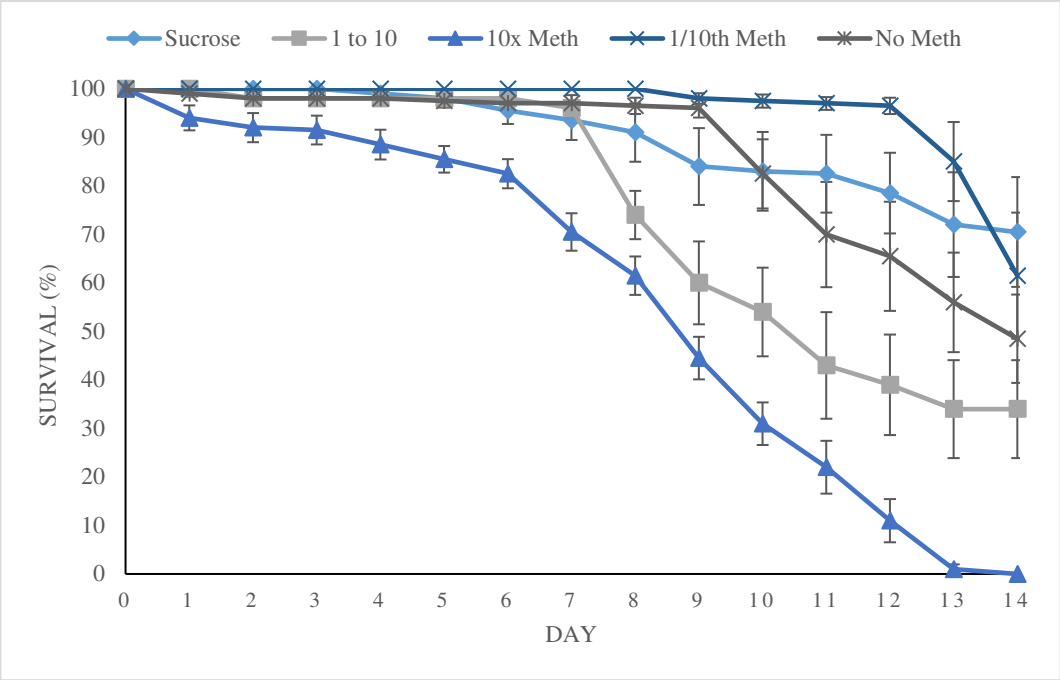


**Figure 6.2** Honey bee survival curves for all 5 treatment diets, sucrose, 1:10 EAA:C, 10x methionine, 1/10$^{th}$ methionine and no methionine. Both 1:10 EAA and 10x methionine diets showed reduced survival relative to the sucrose only control, while 1/10$^{th}$ methionine and no methionine diets improved survival across the study. $n$ = 100, data displayed as mean surviving % ± SEM.

**Table 6.2** Survival statistics from Cox regression comparing survival of worker honey bees on sucrose only diets to the four other diets containing methionine

| | B | SE | Wald | df | Sig. | Exp(B) | 95.0% CI for Exp(B) | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | Lower | Upper |
| Sucrose | | | 148.715 | 4 | 0.000 | | | |
| 1:10 | 0.467 | 0.159 | 8.650 | 1 | 0.003 | 1.595 | 1.169 | 2.178 |
| 10x M | 0.371 | 0.149 | 6.197 | 1 | 0.013 | 1.449 | 1.082 | 1.940 |
| $1/10^{th}$ M | -1.120 | 0.177 | 39.948 | 1 | 0.000 | 0.326 | 0.231 | 0.462 |
| No Meth | -0.372 | 0.149 | 6.274 | 1 | 0.012 | 0.689 | 0.515 | 0.922 |

**Table 6.3** Survival statistics for Cox regression analysis comparing standard 1:10 EAA:C diet relative to diets which varied in their methionine content or contained no EAAs.

| | B | SE | Wald | df | Sig. | Exp(B) | 95.0% CI for Exp(B) | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | Lower | Upper |
| 1:10 | | | 148.715 | 4 | 0.000 | | | |
| Sucrose | -0.467 | 0.159 | 8.650 | 1 | 0.003 | 0.627 | 0.459 | 0.856 |
| 10x M | -0.096 | 0.113 | 0.724 | 1 | 0.395 | 0.908 | 0.727 | 1.134 |
| $1/10^{th}$ M | -1.587 | 0.155 | 104.742 | 1 | 0.000 | 0.205 | 0.151 | 0.277 |
| No Meth | -0.839 | 0.117 | 51.733 | 1 | 0.000 | 0.432 | 0.344 | 0.543 |

### 6.3.3 *Dietary methionine content has no effect on Sir2 expression in the worker honey bee*

The potential effects of methionine on expression of *Sir2* was investigated in worker honey bees fed the five different diets (see above) from eclosure to death. The results show that there were no significant differences between the different diets, and hence methionine content, on *Sir2* expression (one-way ANOVA, $F_{4,25} = 1.094$, $P = 0.381$) **(Figure 6.3A)**. However, bees fed two of the diets showed almost statistically significant differences in expression (Post-hoc LSD, $p = 0.072$) when fed either the 1:10 EAA diet ($M = 4.28 \pm 1.20$) or the no methionine diet ($M = 10.15 \pm 5.01$), but larger sample sizes would be required to determine if this effect, was indeed, significant.

### 6.3.4 *Increasing the dietary methionine content available to worker honey bees significantly increases the expression of FOXO*

The analysis of *FOXO* expression in worker bees fed the five different diets demonstrated a distinct increase in FOXO expression for honey bees fed the diet containing 10x methionine (one-way ANOVA, $F_{4,25} = 9.833$, $P < 0.001$). No other diets lead to such a detectable increase in *FOXO* expression. While there was no differences found between the sucrose only, 1:10 EAA, $1/10^{th}$ methionine or the no methionine diets (post-hoc LSD, $P > 0.552$), the diet containing high levels of methionine i.e. 10x the standard quantity of methionine, resulted in a 10-fold increase ($M = 19.96 \pm 5.35$) in *FOXO* expression relative to any other treatment (post-hoc LSD, all p-values $< 0.001$) **(Figure 6.3B)**.

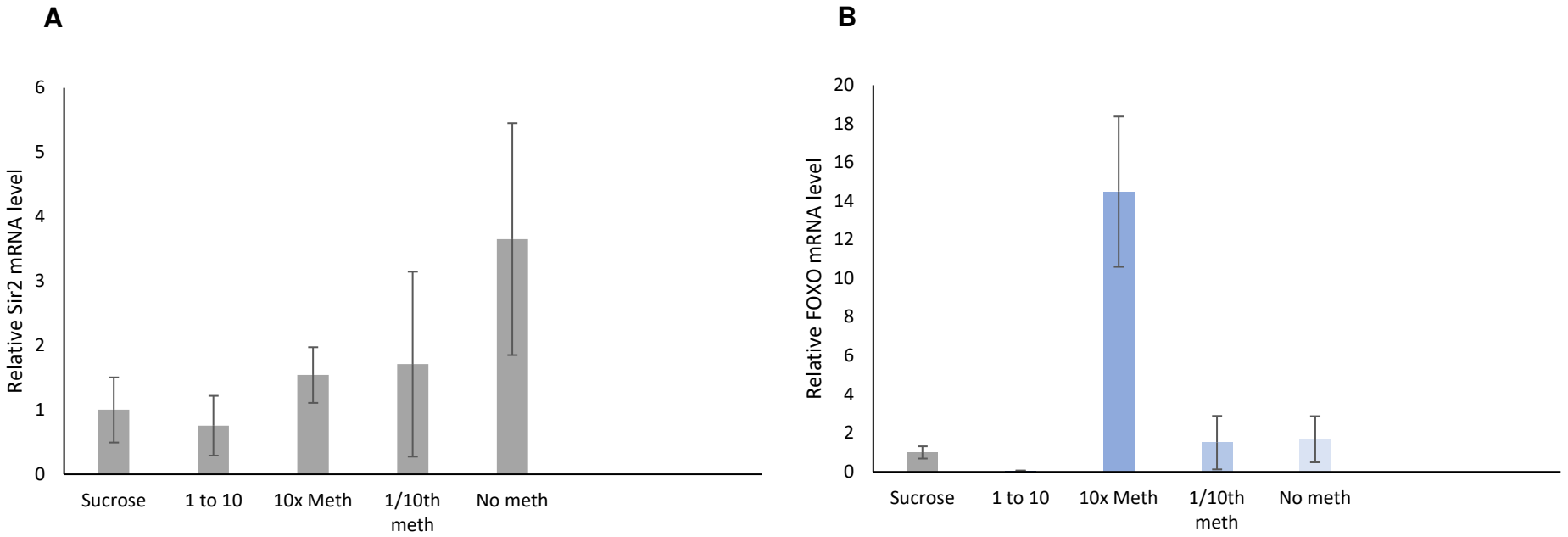


**Figure 6.3** Dietary methionine affects expression of *FOXO* but not *Sir2* in worker honey bees. **a.** Honey bees fed diets with varying quantities of methionine showed no change in expression of the metabolic gene *Sir2*. **b.** Honey bees fed a diet high in methionine showed a significant increase in *FOXO* expression relative to lower or no methionine diets. $n = 6$ per treatment, data displayed as mean ± SEM.

## 6.4 **Discussion**

Restriction of methionine has been shown to extend lifespan in a range of species, even when calories are maintained. The mechanisms which control this life extension are currently not well understood. This study demonstrates that high methionine diets curtail lifespan relative to diets containing low levels of methionine. Two contesting theories of life extension through methionine restriction are (i) suppression of the mTOR pathway through upregulation of *Sir2* and (ii) a reduction in oxidative damage caused by methionine. We found that the latter explanation is the more probable based on our findings, but more work is required to confirm this.

### 6.4.1 ***Methionine and consumption***

This study establishes three important findings. Firstly, diets high in methionine reduce the total energy intake of honey bees across the first 14 days of development by reducing overall consumption. This finding is consistent with results in mice, which showed that food intake relative to body weight was higher in the methionine restricted group than the control group (Sun *et al.*, 2009). This effect is also present in the standard 1:10 EAA diet. Lowering the methionine content in the diet while keeping the overall EAA:C ratio the same increases overall consumption, but not to higher levels than a 1 M sucrose only diet.

The interaction between amino acids and carbohydrates in energy intake is a complex one. This data suggests that the presence of methionine in sufficient quantities suppresses the intake of food through the same mechanisms as it does in mice, although these mechanisms are not well understood. Unfortunately, we were not able to conduct body weight analysis to see if the increased food intake per bodyweight was matched by a decrease in bodyweight due to the difficulty in accurately measuring honey bee mass in the experiment. One possible explanation for this behaviour is the role of methionine supplementation as an appetite suppressant. This has been shown in chicken, especially in the early stages of life (Moskalev and Shaposhnikov, 2010), so it is possible that a similar effect is present here. Work by Howitz *et al.* (2003) indicate that honey bees can taste methionine in the concentrations presented in this study and that the presence of methionine in sucrose solutions reduced the honey bee's willingness to perform memory tasks for a food reward. Another alternative is that honey bees have very low methionine requirements relative to other insects like *Drosophila* (Cota *et al.*, 2006), this could lead to either negative post-ingestive feedback or an inherent taste aversion for the high methionine diet. These results are also consistent with

the consumption data from chapter 4, where no significant difference in overall consumption was found between high and low EAA:C diets.

### 6.4.2 ***Methionine and survival***

The second principle finding of the study was that diets with high methionine content reduces survival, relative to a sucrose control. Lower levels or a complete absence of methionine, alongside a standard amount of the other EAAs improve longevity relative to a sucrose only diet. Diets high in EAA but with proportional levels of methionine, reduced survival, but not to the same extent as the high methionine diet. Both of these findings are consistent with the available literature on insects (Lee *et al.*, 2014) and mammals (Lees *et al.*, 2017) which found that the life extending effects of methionine restriction required low EAA status, unlike our 1:10 EAA diet. The role of methionine in longevity has been widely explored (Sauve *et al.*, 2006; Smith *et al.*, 2007; Kanfi *et al.*, 2012). Methionine restriction has been shown to replicate the effects of dietary restriction, even when no other nutrients are controlled (Fargnoli *et al.*, 1990). Their work suggests that the decrease in mitochondrial ROS (mitROS) generation and oxidative damage to mitochondrial DNA (mtDNA) that occurs during dietary restriction is due to restriction of methionine. Powers *et al.* (2009) found that when everything but methionine is restricted, this effect is not present. There are some cases when methionine is shown to improve longevity (Harrison *et al.*, 2009), but the role is in preventing muscle degradation due to environmental stressors. A factor that our worker honey bees were not exposed to. Diets containing minimal amounts of methionine improved survival beyond those containing only sucrose, but this was also true of the diet absent of methionine. It is likely that this was due to the beneficial effect of the other EAAs in the diet which were absent in the sucrose only diet and that these diets did not have enough methionine to be harmful. The presence of small quantities of amino acids has been shown to improve longevity in the honey bee more than sucrose alone, in part because newly emerged honey bees have higher requirements for EAAs than their older counterparts due to role differentiation (Stenesen *et al.*, 2013).

### 6.4.3 ***Sir2 and FOXO gene expression***

Honey bee *sir2* expression is not changed by the quantity of methionine in the diet, or indeed the quantity of any essential amino acid used in the study. This suggests that it is not the presence of methionine that increases the expression of *Sir2* in honey bees exposed to high EAAs, in contrast to the investigation by Paoli *et al.* (2014). One possible reason for this is

that the concentrations of other EAAs used in that study (1:5 EAA:C) were of a significantly higher concentration than the diets used in this study. However, while overall EAA concentrations were higher, methionine specific levels were lower than the highest treatments in this study, suggesting that any effect is driven by either overall EAA levels or an individual or combination of the remaining 9 EAAs in higher quantities which caused the reduction seen in the prior study. This would be consistent with findings from Paoli *et al.* (2014) which indicated that a 1:5 C:EAA diet curtailed survival significantly more than a 1:10 diet in newly emerged honey bees.

In contrast to the effects observed on *Sir2* expression, our study found that the highest methionine diet significantly increases *FOXO* expression relative to all other treatments. One possible explanation for this is that the decreased consumption caused by the high methionine diet mimicked the effect of DR. Shavlakadze *et al.* (2018) found a high, sugar-only diet similar to those in our study inhibited *FOXO* expression in a persistent manner, even when diet improves. It is possible that by decreasing total consumption, bees on the high methionine treatment were able to avoid the long-term suppression of *FOXO*. As previously explained, DR leads to an upregulation in *Sir2*, which in turn supresses the mTOR pathway, extending lifespan (Ma *et al.*, 2015). The result of this is the upregulation of *FOXO* (Mori *et al.*, 2014), as observed in this study. However, upregulation of *FOXO* has been shown to extend lifespan and our results showed no change in the levels of *Sir2* expression, contrary to the findings of Martins *et al.* (2016). Another potential cause of this upregulation of *FOXO* is the effect of methionine restriction on the prevalence of ROS. In a range of species, low methionine diets induce low ROS levels, which in turn leads to reduced oxidative damage (Sanchez-Roman *et al.*, 2012; Wu *et al.*, 2013; Ruckenstuhl *et al.*, 2014). When cells suffer increased oxidative stress the small GTPase *Ral* is activated. Activation of *Ral* results in the phosphorylation and activation of *JNK* and *JNK*-mediated phosphorylation of FOXO, which in turn leads to an increase in the transcriptional activity of *FOXO* (Essers *et al.*, 2004). A study using high fat diets in mice found that *FOXO* expression increased without any associated rise in *Sir2* expression (Relling *et al.*, 2006), which validates our findings. While our diets were not variable in their fat content, honey bee fat bodies are analogous to mammalian liver and adipose tissue and are significantly altered by changes to their proteome (Chan *et al.*, 2011).

In conclusion, this study has demonstrated that increasing methionine levels in a honey bee diet will reduce total consumption, whether this is due to decreased palatability or a post-

ingestive effect is a potential area for further study. Increased methionine also reduces lifespan significantly, even relative to similarly high EAA diets. While this is represented by an increase in the expression of *FOXO*, it does not lead to significant changes in *Sir2* expression.

# Chapter 7. General Discussion

The aim of this thesis was to explore the effects of sirtuin mediated circadian rhythmicity and diet on longevity in the honey bee. The initial experiments aimed to identify possible effects of circadian disruption on longevity and expression of key circadian and metabolic genes. This study provides the first evidence to support the theory that disruption to circadian rhythm curtails lifespan in the honey bee, it also provides evidence that these effects are not worsened by a diet high in essential amino acids (EAAs). Later experiments aimed at targeting the mTOR pathway with specific interventions, such as the drug rapamycin, to determine the mechanisms behind these findings, as well as attempting to evaluate the feasibility of the honey bee as a model for studying circadian rhythms.

As medical and cultural advancements increase longevity in humans, the burden of age-related diseases like Alzheimer's and cancer have increased significantly. Interventions which can prevent or alleviate these diseases are of great social and economic value. One symptom of many age-related illnesses is deregulation of circadian rhythms. One proposed mechanism for this dysregulation is activation of *mTOR*; not only does increased *mTOR* expression lead to circadian disruption (Cao, 2018), but *mTOR* suppression has been shown to strengthen circadian rhythms and that circadian genes even play a role in supressing mTORC1 activity (Greenhill, 2019). *mTOR* is also an important nutrient sensor and has been shown to be heavily implicated in the life extending effects of dietary restriction (Papadopoli *et al.*, 2019). Honey bees provide an intriguing model for study of both of these areas. Not only are honey bees important pollinators of both wild plants and agricultural crops, so worthy of study in their own right, but they are also capable of vastly different morphological characteristics and lifespans with no change in genome (Remolina and Hughes, 2008); furthermore, they possess circadian genes that are far closer to mammalian circadian genes than that of *Drosophila* (Rubin et al., 2006). Honey bees are, however, not as well established as biological models as *Drosophila* and so this study aimed to identify methods which would be suitable to investigate these systems more effectively. In chapter two we showed that honey bees were unsuitable models to use when attempting to study individuals over an extended period of time. We theorised that, being eusocial insects, being kept alone prevented them from performing critical inter-individual behaviours to maintain their wellbeing, causing increased mortality.

### 7.1 The effect of altered circadian period on survival in the honey bee

In Chapter 3 we used external light cues to alter the circadian period of newly emerged honey bees from 24 h to either 20 h or 28 h periods and measured survival rates and expression of the key circadian genes *Per*, *Cry2* and *Clk*, as well as the key metabolic regulator *Sirt1* and homologue *Sir2*. Previous literature has shown that newly emerged workers are acyclic and do not develop a robust free-running circadian period until they commence foraging behaviour at approximately two weeks (Bloch *et al.*, 2002). Light manipulations have been used before to entrain insects to altered circadian periods (Sailaja and Sivaprasad, 2011) and Pittendrigh and Minis (1964) propose that it is the key driver behind circadian dependent behaviours such as diapause in insects, something further supported by the work of Saunders and Sutton (1969). This study observed a 1.6-fold and 1.2-fold increases in mortality of newly emerged honey bees when placed on a 28 h and 20 h circadian periods, diverging from the control group at around day 10. This is consistent with the findings in studies across species, from *Drosophila* (Pittendrigh and Minis, 1972) to mice (Wyse *et al.*, 2010) and adds further validity to the contestant re-entrainment theory of Withrow (1959).

The results of the analysis of the effect of circadian manipulation on *Sir2*, *Cry2*, *Clk* and *Per* were inconclusive. While it is likely that *Sir2* expression does not change with circadian period, *Sirt1* is known to play a key role in the negative reciprocal regulation of *Per2* in mammals and *Sirt1* knockdown leads to dysregulation of the circadian period due to *Per2* over expression (Wang *et al.*, 2016). Our study found no circadian oscillation in *Sir2* expression which is in keeping with the findings of Nakahata *et al.* (2008) who showed that although expressed at a constant level, its activity as a histone deacetylase was cyclical in nature, peaking at a time of minimal transcription for circadian genes like *BMAL1*. For key circadian genes we found significant increases in expression for two out of three genes and a near significant increase in *Per*, which is explained by the observable increase in circadian activity as the honey bees age. Both *Per* and *Clk* showed circadian osmications based in their expression which were out of phase between circadian treatments, giving further credence to the theory that the decreased longevity is due to desynchronisation of circadian genes with outside cues.

Sirtuins have long been shown to control metabolic signalling through the modulation of enzymatic activity and transduction of nutritional cues into the circadian clock of both central and peripheral circadian clocks (Orozco-Solis *et al.*, 2015). Our study shows that a diet

containing high enough concentrations of EAAs to reduce longevity in the honey bee does not further reduce survival when combined with a circadian period which was also shown to decrease survival moderate levels (20 h), but shows a near-significant trend when subjected to more severe circadian periods (28 h). While it has been observed in *Drosophila* that strong circadian rhythmicity is required for dietary restriction to be effective in extending lifespan, this is the first study to show that harmful diets curtail lifespan in at least a partially shared mechanism with circadian disruption in insects.

### 7.2 The effect of prolonged rapamycin exposure on honey bee survival

Rapamycin is an mTOR inhibitor and has been used as an effective mimic of the life extending effects of DR through *mTOR* repression in multiple species, both by increasing longevity and reducing incidents of age-related illness. The effect of rapamycin on *Drosophila* have been mixed at best, and the effect on the honey bee health and longevity had never previously been observed. This study is the first to illustrate the negative effect of sustained rapamycin exposure in bees, from emergence to death. This further adds to the discord surrounding the effectiveness of rapamycin as a life-extending intervention in insects. While Bjedov *et al.* (2010) showed a positive effect, our results were more in line with those of Harrison *et al.* (2010) and Cheng *et al.* (2015) who observed negative effects on *Drosophila*. We also observed polyphagia, a known side-effect of extended rapamycin exposure (Wang *et al.*, 2015). As this effect was present in the low EAA diets but not in high EAA diets, we propose that polyphagic effect of rapamycin is offset by the honey bees' aversion to high EAA diets (Inouye and Waller, 1984).

One of the potential mechanisms through which rapamycin could be curtailing lifespan is through inhibition of the mTOR subunit mTORC2. While suppression of mTORC1 is a necessary part of the life-extending effects of dietary restriction (DR), restricting access to some nutrients below levels which would be reached *ad libitum*, inhibition of mTORC2 can lead to metabolic syndrome and is a common feature of age related illnesses (Luo *et al.*, 2018). mTORC2 is required for both amino acid and glucose metabolism in a *FOXO* dependent manner and inhibition of the mTORC2 complex can reduce survival in insects (Lamming *et al.*, 2014). Rapamycin acts directly as an allosteric inhibitor of mTORC1 (Li *et al.*, 2014) but extended exposure can lead to inhibition of mTORC2 indirectly, by binding to subunits of mTORC2 and blocking assembly (Sarbassov *et al.*, 2006). The composition of the diets used in this study gives credence to this theory.

### 7.3 Problems with the honey bee model

Chapter four highlights the limitations with using the honey bee as a model to study biological systems beyond having a potentially nasty sting. As explored in chapter two, honey bees are eusocial insects and keeping them housed individually for extended periods of time has significant consequences for their mortality compared to those housed together. Even the biology, which makes them potentially useful, can be problematic. For example, this study conducted two very similar experiments measuring the effect of rapamycin on survival, but the results obtained were markedly different; this was attributed, at least in part, to the dramatic changes in morphology between bees which emerged early in the summer and those which emerged later. Honey bees which emerge later in the year have significantly higher vitellogenin stores and live up to 2 months longer than worker honey bees in the peak of the summer, to allow them to help the hive survive over-winter (Amdam and Omholt, 2002). This would account for honey bees in the trial starting in July living relatively longer than those on similar treatments in May.

Chapter 5 explores the evaluation and development of methods for the measurement of DNA methylation changes in the honey bee. Changes to the methylome of circadian genes is a key symptom of age (Field *et al.*, 2018) and metabolic related diseases (Ramos-Lopez *et al.*, 2018), both of which are associated with shift work (Karlsson *et al.*, 2001; Bokenberger *et al.*, 2018), a behaviour known to cause methylation changes in circadian genes (Zhu *et al.*, 2011). Honey bees are a potentially viable model for the study of DNA methylation changes because methylome changes are capable of producing genetically identical caste members with dramatically different lifespans. These differences are induced by variation in larval diet but are also replicated by genomic interventions such as silencing the expression of DNA methyltransferase (Dnmt3) (Kucharski *et al.*, 2008).

Modern kits have allowed the measurement of global changes in DNA methylation to be performed accurately, cheaply and quickly in mammalian test samples (Kurdyukov and Bullock, 2016). In contrast insects present a potential problem as global methylation levels can be as low as 0.7%, approaching the limits of the reported detection limits of such kits. We measured the effectiveness of two methylation ELISA kits, a colourimetic and fluorescence-based kit. We found that for both ants and honey bees the methylation levels were below the sensitivity of the kits to reliably detect DNA methylation, making them unsuitable for use in analysing global methylation changes in the honey bee or ant. We also attempted to use a

bisulfite sequencing technique known as "MiSeq" to identify site specific changes in DNA methylation of key circadian clock genes in the honey bee. While we successfully identified CpG sites in *Sir,2* we were unable to identify such sites in the key circadian clock genes *Clk*, *Cry2* and *Per*. Although we identified potential differences between the methylation patterns of CpG sites in the *Sir2* gene based on caste differences, definitive analysis was outside the scope of the project, both financially and temporally. We also did not find any potentially differentiated CpG sites in the promoter region of any of the genes investigated, in keeping with the findings of Lyko and Maleszka (2011), suggesting that they are of limited use as a model for cancer where methylation of the promoter region of circadian genes is a key component (Shih *et al.*, 2006).

### 7.4 Dietary methionine content

Methionine is an essential amino acid which plays a crucial role in the formation of cystine and taurine as well as various other metabolic compounds such as S-adenosyl-methionine (SAM). However, restriction of methionine has been shown to increase survival in fruit flies (Grandison *et al.*, 2009) and mice (Lee *et al.*, 2016). Chapter 6 aimed to discover the effects of dietary methionine on the honey bee. Methionine acts as an *mTOR* promoter and as such has been shown to suppress macroauthophagy (Lieberman *et al.*, 2020), a necessary process for the beneficial effects of DR to occur. *FOXO* is a key metabolic transcription factor and not only mediates the beneficial effects of DR, but also controls autophagy through histone modification (Zhou *et al.*, 2012) and controls glucose metabolism in a circadian dependent manner (Chaves *et al.*, 2014). This study found that high quantities of dietary methionine reduced survival and lead to increased *FOXO* expression but not any changes in *Sir2*. We propose that this effect is due to an increase in the production of ROS caused by a high methionine diet (Sanz *et al.*, 2006), which stimulates FOXO through the Ral-mediated, JNK-dependent phosphorylation of FOXO (Essers *et al.*, 2004).

### 7.5 Conclusions

This thesis has shown the potential of the honey bee as a model for investigating the effects of dietary and circadian manipulation on longevity and health. We have determined that honey bee survival is negatively regulated by both circadian disruption and dietary exposure to high levels of essential amino acids, including a specific negative effect of methionine. In this work we observed that continued exposure to dietary supplementation of rapamycin reduced lifespan and promoted polyphagic behaviours similar to those found in metabolic

disorders and showed the effect of seasonality on the survival characteristics of the honey bee. Further to this, we showed that conventional kits designed to measure mammalian DNA methylation levels are not currently sensitive enough to reliably measure insect methylome changes. Lastly, using RTq-PCR we showed that increased methionine, an amino acid known to stimulate mTOR, when added to the diet of newly emerged honey bees increased *FOXO* expression but not *Sir2* and negatively impacted on the survival.

### 7.6 Future work

There are 3 immediate areas of study which could answer direct questions raised by this work:

1. Conduct further RTq-PCR analysis of key circadian genes using liquid nitrogen to snap freeze samples immediately upon collection. These circadian manipulations could be modified to better represent the circadian disruption faced by shift workers.

2. Use immunoblotting to measure honey bee mTORC2 levels during prolonged exposure to rapamycin to determine if the negative effects are due to the disruption to mTORC2 formation.

4. Use electron spin resonance spectroscopy to determine the difference in ROS generation between honey bees fed diets of variable methionine content.

More broadly, following on from this work the direct mechanisms through which mTOR curtails survival in both circadian and metabolically disrupted honey bees should be determined. I propose that this would allow for targeted interventions for this system, which could potentially improve longevity for subjects exposed to either stressor simultaneously and could be a powerful tool in the fight to combat the effects of ageing and age-related diseases.

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