The effects of density-dependent polyphenism on circadian biology of the desert locust *Schistocerca gregaria*

Thesis submitted for the degree of

Doctor of Philosophy

at the University of Leicester

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University of Leicester

2016

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Abstract

Locusts demonstrate phenotypic plasticity in behaviour, morphology and physiology, driven by population density changes. At low densities, locusts become 'solitarious', are cryptic in behaviour and appearance, and avoid other locusts. At high densities, locusts become 'gregarious', are conspicuously coloured, primarily day-active, and aggregate with conspecifics. In this thesis I investigate the endogenous circadian clock, its mechanisms and its outputs.

I investigated the timing of both hatching and emergence of eggs from each phenotype. Eggs from gregarious parents hatched earlier than those from solitarious parents but the larvae emerged from the substratum later. I propose that the avoidance response of solitarious animals is expressed in hatchlings and encourages them to escape the egg pod. This represents the first investigation of both hatching and emergence in the desert locust.

Subsequently I investigate electroretinogram (ERG) and behavioural responses to visual stimuli. The behavioural response differed, with solitarious animals less likely to hide than gregarious animals and more likely to startle. The amplitude of hiding response was modulated in a diurnal pattern in solitarious but not gregarious animals. I demonstrate diurnal and circadian rhythmicity in the ERG response, showing that the free-running circadian clock expresses a shorter period in gregarious locusts. I hypothesis that this is due to the continuous social interactions that gregarious individuals experience.

Finally, I used molecular tools to identify key circadian clock genes and their expression patterns under diurnal conditions. I describe differences in patterns of gene expression between gregarious and solitarious animals with significantly greater depth and accuracy than previous work. I indentify diurnally rhythmic patterns of expression in genes that account for 15% of the transcriptome. This work provides the foundation for future molecular work on *S. gregaria*, both in terms of differential and rhythmic expression, but also in identifying genes of interest and enabling structural characterisation of the resulting proteins.

Acknowledgements

I'd first like to thank my supervisors, Dr Tom Matheson and Dr Ezio Rosato, for their help and guidance throughout the long journey. Tom, for his thorough comments on my written work, without which this work would be an incomprehensible shadow of its current form. Ezio, for his encouragement and infectious enthusiasm, without which so would I. I'd like to thank all members of the Leicester Locust Labs, past and present for their help and stimulating conversations over the last four years. You've all been forthcoming with advice and practical help, as well as many hours of (productive?) enjoyment. We are all indebted to those who have helped maintain the Leicester locust colony including, but by no means limited to, Carl and Chanida, without their hard work the experiments described here would have been impossible. I'd also like to thank the members of the genetic department who have been incredibly helpful, sharing their time, knowledge and consumables. Special thanks go to Ozge for helping me find my feet throughout my first two years and Ben and Nathaniel without whose patient help and advice Chapters 4 and 5 would not have been possible. I am also hugely appreciative to Rachel and the rest of the AccessAbilty centre at the University for their help and support in my final year. Without the techniques and support provided writing this work would have been an awful lot harder.

I'd like to thank my family and friends who have provided moral support and encouragement when needed and been understanding of my brief and occasional visits. Here a special thanks go to my parents helping at short notice with everything from the correct, placement, of, commas, to moving house, who'd have thought I'd fit so much 'stuff' in one flat. I am exceptionally thankful to my fiancée Rebecca, putting up with the stress and unusual hours of work over the last four years, despite having her own demanding course to do. Her drive has always encouraged me to knuckle down get the needed work done, but much to her dismay usually not until 10pm. I'd like to thank my friends further afield, Sarah and Tony especially, for many hours of conversation when the rest of the world was asleep.

Finally I'd like to thank the many Content Creators out there in YouTube land, providing a backdrop to soothe the passing of time whilst watching the many hours of footage produced for Chapter 2 and also whilst writing up. Special mentions go to NerdCubed and TotalBiscuit for their marathon videos.

This research used the ALICE High Performance Computing Facility at the University of Leicester and was funded by the Biotechnology and Biological Sciences Research Council (BBSRC).

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The thesis is my original work and has not been submitted, in whole or in part, for a degree at this or any other university. I confirm that all of the work recorded in my thesis is original unless otherwise acknowledged in the text or by references.

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Common abbreviations

AA – Amino Acid(s).

BCTR – BMAL C-Terminus Region: The BCTR is present in mammalian CYC (BMAL), in contrast to the Drosophila CYC which is missing this region. BCTR is involved in binding of CRY1 as well as transcriptional.

bHLH – basic Helix Loop Helix: Common to both CLK and CYC (BMAL) this domain is found in transcription factors. The larger helix binds to a consensus sequence, in this case the E-box, where it acts as a promoter.

BLAST – Basic Local Alignment Search Tool (NCBI).

BRLZ – Basic Region Leucin Zipper: Containing a helix loop helix motif, this transcription factor contains a specific DNA binding domain followed by a leucin zipper that allows for additional dimerisation.

CCGs – Clock Controlled Genes.

DCMD – Descending Contralateral Movement Detector.

DD – Dark Dark: Constant dark conditions.

ERG – ElectroRetinoGram: extracellular electrophysiological recording from the retina.

EST – Expressed Sequence Tag – Short DNA sequence within a coding region of a gene derived from cDNA, often used in microarray assays to bind known genes to establish expression levels.

FAD – Flavin Adenine Dinucleotide : The FAD binding domain is found on CRY and is the location at which Flavin Adenine Dinucleotide binds non-covalently to the protein. The FAD binding domain is involved in regulation of the CRY protein's functions.

FC – Fold Change (log2).

FPKM – Fragments Per Kilobase Mapped.

GO – Gene Orthology: A project that provides consistent descriptions of gene products across databases based on cellular components, molecular function and biological process.

LD – Light Dark: Conditions which alternate between light and dark photic conditions, Often accompanied by thermocycling (hot during light periods, cold during dark periods).

LGMD – Lobula Giant Movement Detector.

LN – Lateral Neuron: Ventral (LNv) and dorsal (LNd) pacemaker neurons in *Drosophila melanogaster*.

ORF – Open Reading Frame: region of a gene that has the potential to code for a protein. Identifiable as a stretch of codons from a start codon (ATG) to a stop codon (TAG, TAA or TGA).

PAS – Period, Arnt, Sim: First discovered in Period, Aryl hydrocarbon Receptor Nuclear Translocator and SIngle-Minded. PAS domains act as signal sensor domains. In Period this location dimerises with TIM or mCRY. CLK and CYC (BMAL) also dimerise via their respective PAS domains.

PDF – Pigment Dispersing Factor.

qPCR – quantitative Polymerase Chain Reaction.

ZT – Zeitgeber Time: time in h from the presentation of a discrete stimulus such as lights on, or temperature change.

Chapter 1:
General Introduction

Locusts are grasshoppers that swarm. The switch from a non-swarming state to a swarming state involves behavioural, physiological and morphological changes driven by population density in a phenomenon called density dependent polyphenism or 'phase change'. At low population densities individuals tend towards a solitarious phenotype. Cryptically camouflaged, these individuals avoid conspecifics and are predominantly active shortly after dusk. At high population densities individuals tend towards a gregarious phenotype. These individuals are actively attracted to conspecifics and form swarms that cause great economic damage (Steedman, 1990).

In this thesis I describe specific behaviours that differ in solitarious and gregarious locusts, molecular components of the endogenous circadian clock and related patterns of gene expression. Because solitarious and gregarious locusts show different diurnal patterns of behaviour, I have focused largely on differences in circadian biology.

Firstly in this introduction I discuss locusts, their ecological importance, the density dependent polyphenism (phase change) they are known for, and the study organism, *Schistocerca gregaria*. Secondly I introduce diurnal and circadian rhythms in organisms and the environment and the mechanisms by which an organism is able to produce their rhythms. Finally I discuss circadian patterns in behaviour, plasticity in these patterns and known circadian differences between the two extreme phenotypes of *S. gregaria*.

1.1 Locust biology, distribution and economic damage

Locusts are well known as pests, with great appetites and the potential to cause massive economic damage, the outbreak in 1949 - 1957 caused approximately £120 million in crop damage alone (Steedman, 1990). *S. gregaria* is the most damaging of the locusts. With an invasion area of 29 million km^2, a desert locust plague has the potential to bring hardship on up to a tenth of the world's population

(Steedman, 1990). *S. gregaria* has been shown to damage food crops such as bulrush, sorghum, maize, wheat, barley, rice and sugarcane, and cash crops such as coffee, cotton and fruit trees (Steedman, 1990). Finally, plague swarms can consume 30 kg/ha of grassland which has potentially devastating consequences for cattle. This damage greatly affects those who rely on crops for their livelihood, but can also bring famine to an area, as well as increasing the risk of disease, although locusts themselves are not known carriers of disease. In 1985 - 89 a large outbreak led to renewed interest in locust neurobiology and behaviour (Skaf, 1990), and since then much work has been done investigating phase change in *S. gregaria* with the aim of reducing the risk of an outbreak; however with a further notable outbreak in 2004 (Bell, 2005; Ceccato *et al.,* 2007) there is still work to be done to understand and combat this pest.

1.1.1 Locust phase change

The common feature of all locusts is their response to increased population density; it is this that results in the outbreaks and damage described. Under low densities individuals tend to a cryptic solitarious phenotype; under high densities individuals tend to the economically damaging swarming gregarious phenotype. In the case of *L. migratoria* these different phenotypes were originally put down to two differing species (*L. migratoria* and *L. danica*), it was not until Uvarov's (1921) Theory of Phase Transformation was put forward that a single genotype was accepted. Plotnikov (1924) observed the change in the lab, however at the time it was put down to related races that could hybridise. Uvarov (1921) termed the two extreme phenotypes 'solitaria' (solitarious) and 'gregaria' (gregarious), while any intermediate phenotypes were known as 'transiens'. These were further divided into 'congregans' (solitarious to gregarious) and 'dissocians' (gregarious to solitarious) by Uvarov and Zolotarevsky (1929). The general criterion for identification has since been a combination of behaviour, pigmentation and morphometric ratios (Nolte, 1974). To avoid confusion with the term 'phase shift' as used in the context of circadian rhythms, these two states will be referred to as 'solitarious' and 'gregarious', or as 'the extreme phenotypes' of *S. gregaria*.

1.1.2 Locust phylogeny

To further understand where *S. gregaria* falls within the evolution of locust-like traits, this section explores the phylogeny of closely related grasshoppers and the distribution of locust-like traits. Seventeen species of locust have evolved in a wide range of geographical regions, for example; *S. gregaria* has invasion regions in North Africa, Asia Minor, and India; *Locusta migratoria* has invasion territories in Malagasy, tropical Africa, south-west France, Middle Asia, Caucasia, Ukraine, central-European Russia, the Mediterranean, south-east Asia and India (Nolte, 1974). These 17 species belong to at least 6 subfamilies within the Acrididae family and are likely to represent multiple evolutionary events (Figure 1).

In the eight true locusts highlighted in Figure 1 it is likely that three independent evolutionary events occurred: *Anacridium melanorhodon*; the *Schistocerca* subfamily; and *Patanga succincta*, *Nomadacris septemfasciata* and *Austracris guttulosa* (Song, 2005). By examining population density dependent phenotypic plasticity as a number of co-occurring traits, for example: colour changes, morphological changes and behavioural changes, it is likely that evolution of all traits would not occur simultaneously with a single mutation. Instead selection pressures result in convergent evolution across disparate species. Indeed it is likely that non-locusts, which are not known to swarm, may have the required machinery to undergo behavioural plasticity (Song, 2005). This is likely the case in *S. interrita* which has only recently shown locust traits.

Figure 1: Cladogram of grasshoppers adapted from Song (2005), generated from the external and internal characteristics including: size of anatomical features, presence or absence of ridges or markings and many others (70 characteristics in total). 65 grasshoppers selected containing 8 true locusts and an additional 8 true locusts are shown above the dashed line. Highlighted species show population density phenotypic plasticity. At each node the Bremer support value is shown.

1.1.3 *Schistocerca gregaria*

This section explains in detail the population dependant phenotypes seen in *S. gregaria*, including behavioural and morphological differences in addition to what is known about the mechanisms underlying these changes. The two extreme phenotypes of *S. gregaria* differ in morphology, physiology and behaviour. Following the life cycle of the locust I will discuss the relevant differences from laying and hatching through to adulthood and sexual maturity. Following that I will discuss what is currently known on the mechanism of this phenotypic plasticity.

Laying females of both phenotypes show a preference for laying adjacent to egg pods derived from gregarious females over laying adjacent to solitarious egg pods or in clear substrate (Bashir *et al.,* 2000). This is likely due to pheromonal signals associated with the egg pods, but also with laying females, both of which promote ovipositing behaviour in other gravid females (Saini *et al.*, 1995; Rai *et al.*, 1997; Torto *et al.*, 1999). In the context of a gregarious swarm this promotes laying over a short period of time in the same site. It is hypothesised that this site preference acts to increase population density for the next generation, helping to maintain gregariousness in gregarious populations (Nolte, 1974; Uvarov, 1977; Bashir *et al.,* 2000). The egg pods laid by the two phenotypes also differ in composition, with gregarious females laying larger eggs in smaller numbers (7.5 mm compared with 6.5 mm length; 48-96 compared with 65-119 eggs per pod; Uvarov, 1966; Nolte, 1974; Maeno *et al.,* 2001). Gregarious hatchlings tend to emerge darker and larger (Uvarov, 1966; Simpson *et al.,* 1999; Miller *et al.,* 2008). They also show increased locomotor activity relative to solitarious (Hunter-Jones, 1958; Harano *et al.,* 2012). Gregarious hatchlings are attracted to conspecifics, moving towards other hatchlings, whilst solitarious hatchlings are not attracted to conspecifics (Uvarov, 1966; Nolte, 1974; McCaffery *et al.*, 1998)

Solitarious animals continue to show conspecific repulsion throughout the juvenile instars. The number of moults differs between the two extreme phenotypes, with solitarious animals often (20%; Cheke, 1978) requiring an extra moult before reaching adulthood compared to the gregarious (Uvarov, 1966; Maeno and Tanaka, 2009; Maeno and Tanaka, 2010). In addition to the extra moult, solitarious adults are larger

than gregarious adults. Colouration of the phenotypes differs, with both taking on a base fawn colouration (from beta carotene), however solitarious animals may also produce mesobiliverdin which combines to give them a distinctive green colouration (Nolte, 1974). The fawn colour of gregarious animals is additionally partially obscured by insectorubin in the hypodermis and melanin in the cuticle, giving rise to a cryptic camouflage pattern (Nolte, 1974). The colouration of gregarious males is a bright yellow colour once the animal reaches sexual maturity; reaching maturity also occurs earlier in gregarious animals (Nolte, 1974; Uvarov, 1966).

There is evidence for increased chiasmata, genetic material exchange during mitosis, in gregarious animals compared to solitarious animals (Nolte, 1974), suggesting an increase in genetic variation in a given population (whole genome, not just phenotype linked genes). An increase in chiasmata results in more varied genetic material exchange; alleles that are proximal to each other have a decreased chance of being exchanged together. The cause of this increase in chiasmata is thought to be the same pheromone that induces melanin production (Nolte, 1974). These developmental differences are not reversible; an adult reared isolated will remain larger than conspecifics even if subsequently crowded and similarly morphometric differences are not reversible. By contrast the behavioural changes discussed below are reversible; crowding a solitarious animal for just 4 h will produce a behaviourally gregarious individual and isolating a gregarious animal for 4 h will begin produce solitarious behaviours in the individual (4 h in final instar, Roessingh and Simpson, 1994; < 48 h in adults, Bouaichi *et al.*, 1995).

Adult individuals show conspecifics attraction and repulsion in gregarious and solitarious individuals respectively. Gregarious animals will tend to congregate together. In addition to the conspecifics attraction/repulsion and general locomotor activity, the flying behaviour also differs between the extreme phenotypes, with solitary individuals tending to fly during the night (Nolte, 1974), compared to gregarious animals which tend to fly during the day. Solitary individuals are also generally better jumpers, possibly due to their increased size (Uvarov, 1977). A significant difference is also seen in lifespan, with gregarious individuals living an

average of 48 days (max 96) and isolated animals living 65 days (max 119). This suggests a biochemical metabolic difference (Uvarov, 1966).

The trigger for gregarisation is density dependent and is composed of visual, tactile and olfactory cues (Nolte, 1974). The main trigger in *S. gregaria* is known to be tactile stimulation of the hind legs, including proprioceptor signals (Rogers *et al.*, 2003), however olfactory and visual cues combined are still sufficient via an alternative pathway (Rogers *et al.*, 2003*;* Anstey *et al.*, 2009). Serotonin, a neurotransmitter, has been shown to be sufficient and necessary for behavioural gregarisation, increasing in concentration in the thoracic ganglia in gregarising animals (Anstey *et al.*, 2009).

This triggering density-increase is often the consequence of climate or available space as well as the more obvious raw population increase. For example, patchy food source distribution tends to promote solitary individuals as they tend to be more separated (Nolte, 1974). In both phenotypes, continued exposure to the triggering factors, or lack thereof, strengthens the phenotype over many generations. This is in part due to the effect of maternal phenotype on hopper development (Nolte, 1974). Hagel *et al.* (2004) showed that this wasn't transmitted directly by ecdysteroid content in the eggs, despite a strong content vs egg size correlation. Interestingly, even given numerous generations of consistent crowding, laboratories populations still tend to be less extreme examples of the crowded phase than are expressed freely in the wild during a swarm (Uvarov, 1966).

1.1.4 Polymorphism, polyphenism and plasticity

This section explains how locust population density dependant polyphenism is related to polymorphism and polyphenism in the wider context. Polymorphism refers to individuals in a population exhibiting different morphological phenotypes. This is due to genetic variation and does not include single mutants within the population (Ford, 1953). The most common polymorphism is sexual dimorphism, whereby male and female individuals display different phenotypes. Polymorphism is also found between populations that live in differing environments. Known as ecotypes, these individuals may express differing phenotypes due to minor genotypic differences or to different environmental pressures. Individuals from different ecotypes may still breed

and are therefore still considered the same species. Whilst polymorphism carries similarities to locust-like traits, the lack of genetic determination and thus the ability of an individual to change from one phenotype to another, e.g. gregarious to solitarious, within one generation means polyphenism is a more appropriate term.

Polyphenism refers to a situation in which multiple phenotypes are produced by the same genotype, distinct from polymorphism where the differences are produced by genotypic differences (Simpson *et al.*, 2011). The most common polyphenism is developmental, seen in holometabolous insects where the juvenile individuals are phenotypically different from the adult. Phenotypic plasticity is defined as 'the ability of individual genotypes to produce different phenotypes when exposed to different environmental conditions' (Pigliucci *et al.*, 2006) which excludes developmental polyphenism. Triggers include diet, season and population density, as in *S. gregaria*. The moth, *Nemoria arizonaria*, shows diet dependent polyphenism in its caterpillar stage; those that feed on oak flowers (catkins) develop physical mimicry, effectively camouflaging them. By contrast those that feed on the leaves of the oak develop to mimic the twigs (Greene, 1988). Honey bees, *Apis mellifera*, represent a model organism for phenotypic plasticity. Individuals fed high levels of royal jelly develop into queens whilst those fed little royal jelly will develop into workers (Shuel and Dixon, 1960). Here the compound has been identified as Royalactin (Kamakura, 2011), which acts to increase body size, ovary development, shorten developmental time and increase juvenile hormone levels.

1.2 Diurnal and circadian rhythmicity

This section introduces cyclic biological rhythms, their importance, how they are generated and their impact on behaviour. Finally this section discusses the current literature surrounding circadian biology in the desert locust including molecular and behavioural studies.

1.2.1 Environmental and biological context

Most environments vary cyclically. There are daily (diurnal) cycles of light and temperature, shorter duration cycles of tidal conditions (lunar), and seasonal changes in temperature and day length. Responding in the optimal fashion to the prevailing

conditions is vital to survival, and being able to predict and anticipate them accurately provides a strong advantage (e.g. cyanobacteria, Ouyang *et al.*, 1998). As a result many organisms have evolved an endogenous clock. In this work I look at the endogenous circadian clock (Latin: circa-diem, "about – a day"), its mechanism and its outputs.

The endogenous circadian clock has three defining features, it must: be entrainable to local rhythmic conditions, free-run under constant conditions, and be temperature compensated (Bell-Pedersen *et al.*, 2005). Under constant conditions the circadian clock will continue to cycle with a period length of between 20-28 h without any environmental inputs. The oscillators must be able to respond to changes in the rhythmic conditions such that the clock can be reset or phase shifted and thus be synchronised with the environment. The period length of the endogenous cannot be susceptible to temperature changes, within the physiological range of the organism, which requires that the system compensates for the higher metabolic rates associated with higher temperature

Entrainment has been shown in response to variables such as light (Bell-Pedersen *et al.*, 2005), temperature (López-Olmeda *et al*., 2006), pressure (Hayden and Lindberg, 1969) and social cues (Marimuthu *et al.*, 1981). These entrainment variables are known as Zeitgebers (German: "time-giver"). The time relative to the Zeitgeber is known as Zeitgeber Time (ZT) and is used to describe how long has passed since a discrete Zeitgeber was given in an laboratory scenario; e.g. dawn is often ZT 0, with light as the Zeitgeber, whilst dusk may be 12 h later at ZT 12. Once entrained to the daily rhythms the clock runs at a period length of approximately 24 h (20 - 28 h), allowing the animal to start to respond in anticipation of upcoming conditions. This may take the form of taking shelter prior to the temperature dropping or light fading, or producing enzymes in anticipation of feeding (Bell-Pedersen *et al.*, 2005; Lorenzo and Lazzari, 1998; Saito *et al.*, 1976). This optimises the production and utilisation of resources within the animal, reducing stresses and cost and thus increasing fitness (Myers, 2003). The accurate cycling of the circadian clock is shown to provide higher fitness in cyanobacteria, less accurate synchronisation between the clock and environment showed a selective disadvantage

(Ouyang *et al.*, 1998). It is also believed that the circadian clock has influences on other rhythms with shorter (ultradian) and longer (circaluna/circatidal and seasonal) period lengths (Ikeno *et al.*, 2010).

1.2.2 The endogenous circadian clock

The previous section has established the circadian clock as a necessary response to a cyclic environment. This section briefly describes the molecular mechanism of the well-studied endogenous circadian clock in *Drosophila.* Further details and comparisons between different models are discussed in Chapter 4. The central endogenous circadian clock consists of a negative feedback transcription-translation loop (Dunlop, 1999). The expression of one set of genes (*clock* and *cycle*) promotes the expression of a second set (*period* and *timeless*) whose protein products in turn feed back negatively on transcription of *clock* and *cycle*. Due to the transcription-translation lag the period length of this cycle is approximately 24 h. The presence of Clock and Cycle proteins promotes clock controlled genes (CCGs) which comprise the output of the clock. One mechanism by which the clock is entrained is via light; Cryptochrome is activated by light and functions to degrade Timeless and Period, allowing *clock* and *cycle* to be expressed and thus resetting the clock. Additional factors that help regulate the cyclic expression of *clock* will be discussed in Chapter 4.

The circadian clock is highly conserved, with homologues present across a wide range of evolutionarily disparate organisms from cyanobacteria to flies to humans (Ouyang *et al.*, 1998; Dunlop, 1999; Czeisler *et al.*, 1999). Differences within these systems do exist, for example Cryptochrome does not have light induced functionality in mammalian systems, and not all invertebrates show the same clock to that of *Drosophila*. The monarch butterfly, *Danaus plexippus*, for example expresses both a *Drosophila*-like Cryptochrome that is light sensitive as well as a mammalian-like Cryptochrome with different functionality. However the presence of a central molecular clock controlling behaviours and metabolic functions with an approximately 24 h period length is almost universal.

1.2.3 Circadian rhythms of behaviour

Although circadian rhythms are generated on a molecular level, they can be studied at a behavioural level. A wide range of behaviours have been shown to be controlled by circadian rhythms. Locomotor activity is a key indicator of circadian patterns, such as the robust rhythms seen in *D. melanogaster (*Allada and Chung, 2010), the house fly, *Musca domestica* (Engelmann *et al.*, 1996) and the blow fly, *Calliphora vicina* (Cymborowski *et al.*, 1994). *Drosophila* shows a diphasic locomotor rhythm, with activity at dawn and dusk. Other behaviours shown to be circadian in *Drosophila* include eclosion, courtship, foraging and sleep (Konopka and Benzer, 1971; Shaw *et al*., 2000; Sokolowski, 2001).

The freshwater snail, *Bulinus tropicus,* shows rhythms in a similar selection of behaviours, with locomotor activity, feeding, oviposition and hatching all showing circadian patterns (Chaudhry and Morgan, 1983). Copulation is a common circadian behaviour, for example in *Drosophila*, *Hesperia* and *Laupala*, with differences in mating time creating a potential mechanism for speciation (Tauber *et al.*, 2003; Devries *et al.*, 2008; Fergus *et al.*, 2011; Rund *et al.*, 2012).

In addition to daily cycles of behaviour, circadian rhythms are involved in both longer and shorter rhythms, such as circatidal and circanual rhythms. These rhythms frequently affect population scale behaviours such as mating or hibernation. It is thought that the basis of these rhythms is the difference between anticipated dawn and actual dawn as the day length changes with the seasons.

1.2.4 Developmental and phenotypic circadian patterns

Although circadian rhythms are, by definition, stable within the individual, circadian patterns can also vary between individuals of the same genotype, developmentally or through phenotypic plasticity. To illustrate this potential, this section discusses developmentally linked changes in behavioural rhythms in *Gryllus bimaculatus* as well as phenotypic changes due to aging in *A. mellifera*

In the cricket, *Gryllus bimaculatus*, nymphs exhibit a characteristic diurnal activity pattern; activity is higher during the photophase (lights on) and lower during the scotophase (lights off) (Tomioka and Chiba, 1982). Upon moulting to the adult form

the crickets show a brief period of general low activity, seen in other crickets and moulting animals (the grasshopper *Locusta migratoria*: Edney, 1937; the crab *Gecarcinus lateralis*: Bliss, 1962; the cockroach *Leucophaea maderae*: Page and Block 1980), before an immediate reversal of activity timing. Adult crickets show a nocturnal activity pattern, with high activity during the scotophase and low activity during the photophase (Tomioka and Chiba, 1982). This rapid change has been temporally associated with sexual maturation, occurring 3-6 days after imaginable moult (Tomioka and Chiba, 1982) and is associated with an increase in serotonin in the brain (Nishinokubi and Tomioka, 2000). This phase reversal indicates that either the endogenous clock itself or the coupling to the behavioural output changes between the developmental stages. The former theory has been disproved, with no difference in pacemaker activity suggesting changes to the coupling of the clock and the behaviour are responsible (Tomioka and Chiba, 1992).

In addition to the queen-worker phenotypic differences, worker *A. mellifera* show age polyethism, a phenomenon whereby an individual progresses through four distinct sub-castes that focus on particular behaviours: cell cleaning, brood and queen care, food storage and foraging (Seeley, 1982). The majority of these sub-castes are arrhythmic, behaviours occur 24 h a day with individuals working randomly with respect to time. Workers develop diel activity prior to becoming foragers, with activity during the day and inactivity at night (Moore *et al.*, 1998). This is an example of circadian rhythmicity changing within an individual as its circumstances change.

1.2.5 Circadian plasticity in *S. gregaria*

S. gregaria is well studied with regard to density dependant phenotypic plasticity, however little work has been done comparing the circadian biology of the two extreme phenotypes. This section discusses the current knowledge of the endogenous circadian clock in this species, as well as known phenotypic variation in behaviour and neurophysiology with regard to time of day.

The endogenous circadian clock of *S. gregaria* is poorly understood. What is known in the literature is discussed here. Tobback *et al.* (2011) produced partial sequences for *clock*, *period* and *timeless* from an existing database of Expressed Sequence Tags (EST)

utilising Blast (NCBI). Expression levels of these genes were studied using real time qPCR with brain samples collected every 3 h over 24 h. Both gregarious and solitarious locusts, showed circadian expression of the three genes; *clock* is highly expressed at ZT 0 while *period* and *timeless* come to a peak at 12 – 18 h. Tobback *et al.* (2011) states that this expression does not differ between the phenotypes. Tobback *et al.* (2011 & 2012) also attempted knockdown of *period* and *timeless* which resulted in reduced progeny, while knockdown of *clock* produced a lethal effect, confirming the presence of a molecular clock and also the importance of the clock in the animal, both for survivability but also for reproduction. No work gives any indication of the similarities or differences of the *S. gregaria* molecular clock to that of *Drosophila,* nor any indication of how the molecular clock associates with the behaviours discussed below.

In contrast to the limited data available for the molecular machinery of the circadian clock, differences in diurnal and circadian activity patterns exhibited by solitarious and gregarious locusts have been observed both in the laboratory and in the field (Figure 2A; Steedman, 1990; Ely *et al.*, 2011; Gaten, unpublished). Day-time activity is higher in gregarious animals than solitarious animals (Steedman, 1990), with the latter showing peaks in activity 1-2 hours after lights off (Ely *et al.*, 2011). This pattern is similar to the pattern in the firing sensitivity of a loom-sensitive interneuron, the Descending Contralateral Movement Detector (DCMD; Figure 2B; Gaten *et al.*, 2012). While both phenotypes display a cyclic diurnal pattern, the timing of the peaks is significantly different, with solitarious animals peaking in neuronal sensitivity between 19:00 and 20:00 (ZT11 & ZT12) and gregarious animals peaking at 14:00. Tobback *et al.* (2011) state no difference in the endogenous clock between gregarious and solitarious animals and they propose a downstream driver to the differences seen, either visual sensitivity rhythms or downstream genetic control adjusting the interpretation of the clock's cycle. This would be similar to the comparison between nocturnal and diurnal mammals (Challet, 2007) or nymph and adult *G. bimaculatus* (Tomioka and Chiba, 1982), both of which show consistent endogenous clock activity despite reversed activity patterns.

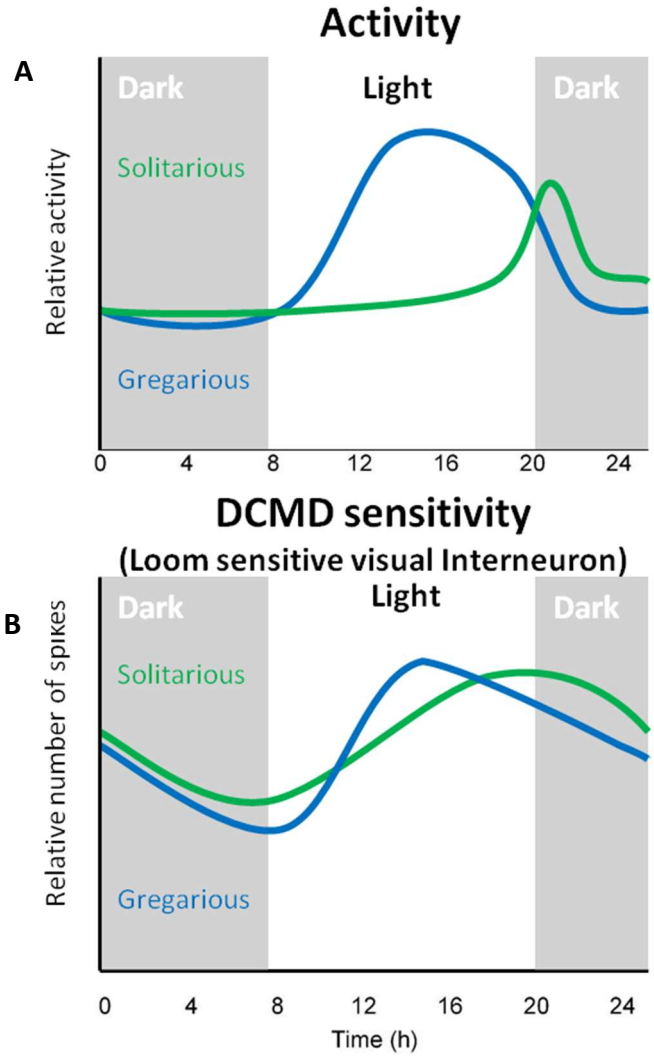


Figure 2: Rhythmic activity and DCMD sensitivity in *S. gregaria* A: Relative activity levels of gregarious and solitarious animals in an arena under 12:12 LD conditions (Gaten, unpublished) B: relative DCMD sensitivity, relative number of spikes produced in response to a looming stimulus, in gregarious and solitarious animals under constant low light (subjective light : dark shown). Adapted from Gaten *et al.* (2012).

In addition to the activity patterns described above, emergence of hatchlings has also been shown to be rhythmic despite arrhythmic laying. In *S. gregaria* eggs may be laid at any time of day (Popov, 1958), however emergence has been shown to be rhythmic in gregarious eggs in the field (Ellis and Ashall, 1957) and under controlled laboratory conditions (Padgham, 1981) with emergence occurring around dawn. Until recently no investigation had been carried out on eggs derived from solitarious females, although hatching has now been shown to be rhythmic in both phenotypes with eggs derived from gregarious females hatching earlier than those from solitarious females (Nishide *et al.*, 2015a). This work is discussed in more detail in Chapter 2. Despite this recent work, emergence is still to be examined in eggs derived from solitarious females.

1.3 Aims and objectives

The main aims of this thesis are, within the context of population density dependent phenotypic plasticity seen in the desert locust, *Schistocerca gregaria:*

1) to investigate behavioural and neurophysiological circadian rhythms,
2) to investigate the molecular basis of changes in circadian biology.

As such, the objectives of this project are:

- To examine hatching and emergence of *S. gregaria*, investigating diurnal patterns in both behaviours as individuals. In addition to investigating the inherent sibling synchronicity as distinct from tactile and chemosensory interactions in eggs derived from females of both extreme phenotypes (Chapter 2).
- To assess circadian influences on the behavioural response to looming stimuli, and on electroretinogram responses of the compound eye to a brief light stimulus in both extreme phenotypes (Chapter 3).
- To investigate the molecular mechanisms of the central circadian clock in *S. gregaria*, using RNAseq to develop a transcriptome with good coverage of poorly expressed genes at all times of day. This transcriptome will then be examined with the purpose of gene discovery of the key circadian genes (Chapter 4).
- To assess the molecular expression differences between the two extreme phenotypes, with greater depth and specificity than work by Badisco *et al.* (2011; Chapter 5). Any diurnal rhythmicity of the central circadian genes identified in Chapter 4, in addition to the remainder of the assembled transcriptome is to be assessed to further establish the functional mechanism of the central clock and the areas affected by diurnal rhythms in *S. gregaria*.

Chapter 2:
Diurnal patterns of hatching and emergence

2.1 Introduction

Throughout this chapter I refer to 'emergence' and 'hatching' in animals which lay their eggs in a substrate. Hatching, or eclosion, is the behavioural event during which an individual leaves its egg. It is a process with many parallels to ecdysis, moulting, during which the animal sheds its outer cuticle allowing growth. Much like hatching, ecdysis is reliant on the correct conditions. Ecydsis may be delayed if conditions are not correct (the desert locust, *Schistocerca gregaria*; Hughes, 1980), for example by removing suitable perches. Delay in egg development and hatching, known as diapause, is seen in many insects including the Australian plague locust, *Chortoicetes terminifera* (Deveson and Woodman, 2014), the migratory locust, *Locusta migratoria* (Ando, 1993) and *S. gregaria* (Shulov and Pener, 1963). In contrast to hatching, emergence is the point at which the individual, having hatched earlier, emerges from the substrate in which the egg is buried. Emergence occurs a period of time after hatching due to the need to move through the substrate and, in some cases, to carry out an initial moult.

2.1.1 Hatching and emergence behaviours

The process of hatching involves the escape of the animal from the enclosing eggshell. In the case of many insects this includes the chorion, the viteline membrane and the serosal cuticle. The first eclosion, moulting to the first larval stage, may occur simultaneously with hatching as in the cockroach, *Periplaneta americana* (Provine, 1976; Provine, 1977), or this may instead occur once the animal has emerged from the surface as in *S. gregaria* (Bernays, 1972). In the locust, hatching involves simultaneous dorsal-ventral contractions of the abdominal segments in an attempt to burst the eggshell that has been weakened by enzymatic action; failure to burst the eggshell results in the animal moving forward within the egg before trying again (Bernays, 1972). Once the eggshell is breached, the animal escapes with similar peristaltic motor patterns to those seen before hatching, but these movements are now rapid and continuous. Once free from the eggshell the newly emerged locust larva

"digs" up through the substrate to the surface, again utilising similar motor patterns (Bernays, 1972); the legs are covered in a membranous cuticle and cannot be used for digging (Uvarov, 1977).

Previous work, as discussed below, tends to focus on either hatching or emergence. Fieldwork has often focused on emergence, while laboratory work has tended to look at hatching. Investigating hatching of individuals, as opposed to the emergence of a whole sibling group, controls for variables such as depth of eggs and any sibling interaction effects, however in limiting investigations to these situations there is little scope to investigate how these variables affect the animal under natural conditions. By investigating both behaviours, hatching and emergence, it is possible to compare the results and gain insight into the strength of these variables.

2.1.2 Circadian entrainment, gating and zeitgebers for hatching and emergence

Hatching and emergence often occur at precisely controlled times of the day, allowing the nymph to avoid the majority of predators, and find a suitable shelter or food prior to the heat of the day (Pittendrigh, 1954). Rhythmic hatching occurs across five orders of insect (Reynolds, 1980). Such rhythms have been shown to be under circadian control in many species, such as the hatching of *Diatraea grandiosella* (Takeda, 1983), *Gryllus bimaculatus* (Itoh and Sumi, 2000), and *Antheraea pernyi* (Sauman *et al.*, 1996). As the behaviour is not rhythmic within an individual (it can only hatch once), this process is referred to as being 'circadian gated'; an animal may only hatch during windows prescribed by the gating factor. A circadian clock is not the only method by which hatching is gated: species such as the bush cricket, *Metrioptera hime*, also hatch in close temporal proximity to dawn (Arai, 1979). Here the hatching is triggered by environmental photic cues, lights-on and lights-off, as opposed to circadian rhythmicity. Behaviours can also be triggered by a change in thermal conditions (known as thermal gating). It has been theorised that emergence tends towards a certain time due to the biomechanical thermal requirements for the muscular contractions utilised in both hatching and surfacing from the substrate (Uvarov, 1977). Alternatively the rise in temperature through a threshold, or a change from falling to rising temperature may act as a trigger for hatching or emergence behaviours. An example for this is the hatching in eastern lubber grasshoppers, *Romalea microptera*,

thought to be dependent on a thermal threshold (Smith *et al.*, 2013). Humidity has also been considered as a potential trigger (Uvarov, 1977), although no experimental evidence exists to support this (Tanaka and Watari, 2009). Light alone has also been shown to be a significant cue when present (*Antheraea pernyi,* Truman, 1971), however it is unlikely to function as such in *S. gregaria* where the egg is not exposed to light due to the surrounding substrate (Uvarov, 1977;Nishide *et al.*, 2015b).

It has been shown that a hybrid mechanism exists, for example in the tsetse fly, *Glossina moristans* (Zdárek and Denlinger, 1995), and the onion fly, *Delia antiqua* (Tanaka and Watari, 2003). In these cases the time of hatching varied based on the amplitude of thermal cycling, with smaller amplitude cycling promoting an advanced hatching time in the diurnal cycle. The interaction of the circadian clock and environmental variations acts to precisely time hatching and emergence to optimal conditions for the hatchlings.

Eclosion and emergence are complex behaviours and often do not show a strict requirement for a single oscillatory entrainment variable. The sunflower moth, *Homoeosoma electellum*, has been shown to entrain to either light or thermal cycles in the absence of the other (Riemann, 1991). Similar patterns have been seen in *Heliothis virescens* (Roush and Schneider, 1985), *Trichogramma evanescens* (Zaslavski *et al.*, 1995) and many others. Due to the ecological importance of precise timing, as well as the multimodal entrainment, eclosion/hatching is a behaviour commonly used as a reliable assay for the internal clock of insects (*Drosophila*, Konopka and Benzer, 1971; Engelmann and Mack, 1978; *Sarcophaga argyrostoma*, Saunders, 1976; *Romalea microptera*, Smith *et al.*, 2013; *Chymomyza costata*, Koštál and Shimada, 2001) and therefore provides a good measure as to the tendencies of the central clock.

2.1.3 Hatching and emergence in *Schistocerca gregaria*

In the field, gregarious *S. gregaria* exhibit synchronised laying, with large numbers of egg pods laid in a relatively small area in the same day. Observed egg pod densities ranged from 0.13 per sq. ft. to in excess of 0.25 per sq. ft. (Ellis and Ashall, 1957), although this may occur at any time during the day or night (Popov, 1958). Egg

development rate is strongly linked to local conditions, with temperature and moisture levels being key predictors of hatch date (Hamilton, 1936), so there is a high probability of multiple egg pods hatching on the same day. In the field, gregarious hatchlings emerge in the 4 h either side of dawn, at the end of the cryoperiod, during which time the air and soil temperatures are low (19-25°C); by contrast the air temperature during the day can reach over 50°C (Ellis and Ashall, 1957). This pattern of emergence has been confirmed in the laboratory for gregarious locusts by Padgham (1981), who showed a circadian pattern of emergence under constant light and temperature conditions. Solitarious locusts, by contrast, do not have the opportunity to lay pods together given their low population density, so there is unlikely to be any significant crowding of hatchlings when they emerge. However, it is likely that emergence is affected by any innate behaviour of the individuals to avoid conspecifics. Emerging at dawn is thought to allow the hatchlings to dry their freshly exposed exoskeleton in the morning, before the increased heat of midday and risk of predators force them to seek cover (Padgham, 1981). Little or nothing is known about the emergence, as opposed to hatching, of solitarious hatchlings in the field or lab.

In *S. gregaria* a decrease of 1°C is sufficient to promote hatching during the expected period of the thermocycle (Nishide *et al.*, 2015a); *S gregaria* hatch during the cryoperiod, the period during which the temperature is low, under laboratory conditions (Figure 1A; Nishide *et al.*, 2015a). Light has been shown to influence hatching when the eggs were exposed to it, but no influence was seen in eggs covered in sand (Nishide *et al.*, 2015b). Despite recent work, the mechanism for the hatching rhythmicity seen in *S. gregaria* is not yet understood.

Any possible inter-sibling influence on hatching, whereby the hatching of one individual tends to promote the hatching of others within the same egg pod either by tactile or chemosensory stimulation, has not been directly investigated in *S. gregaria,* although it has been observed in the field that hatchlings from the same egg pod tend to emerge at a similar time (Ellis and Ashall, 1957). Sibling influence is a driving force in *Nezara viridula* for example, where the physical interaction of siblings in the same egg mass stimulates hatching (Lockwood and Story, 1985; Todd, 1989). It should be noted that sibling influence refers specifically to the influence of an already hatched

individual on one yet to hatch by the above mechanisms; it is not to be confused with the effect of sibling group, which broadly defines the synchronicity of hatching times of a sibling group without assuming a specific mechanism.

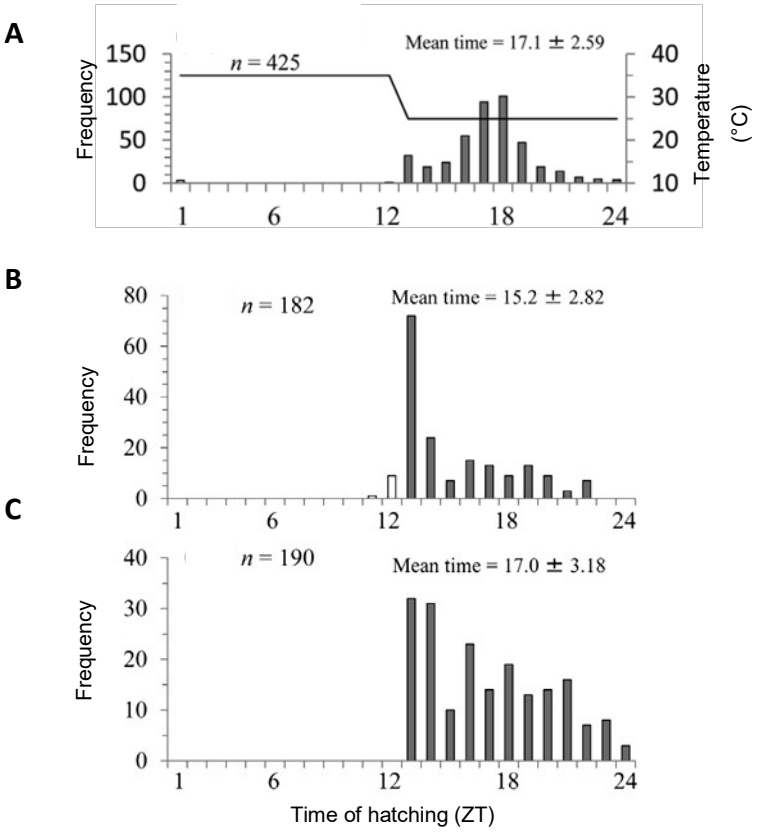


Figure 1: Hatching times of *Schistocerca gregaria* (Nishide *et al.*, 2015a). A: Hatching under 35:25°C thermo cycling, showing thermocycle as a solid black line with temperature (°C) on the right axis. B (Gregarious) & C (Solitarious): comparing phenotypic differences under 35:20°C thermo cycling, gregarious hatchlings eclose earlier than solitarious hatchlings under these conditions with both hatching in the first part of the cryoperiod.

The eggs produced by females of solitarious and gregarious locusts differ in three main ways. First, eggs from gregarious females tend to be larger than those produced by solitarious females (mean ± SD = 7.75 ± 0.06 mm and 6.69 ± 0.06 mm respectively; Maeno *et al.*, 2013). Second, gregarious hatchlings tend to emerge darker and larger than solitarious hatchlings, which is determined prior to ovulation (Simpson *et al.*, 1999; Simpson and Miller, 2007). These larger, darker hatchlings show higher levels of locomotor activity once hatched (Hunter-Jones, 1958; Harano *et al.*, 2012), one of the primary indicators of the gregarious phenotype in adult locusts. By contrast, the smaller, lighter coloured solitarious hatchlings show

reduced locomotor activity, which corresponds with the reduced locomotor activity of adult solitarious animals. Third, gregarious females lay fewer eggs than solitarious females (48 - 96 and 65 - 119 per pod respectively; Uvarov, 1966). It is not known whether any of these factors affect the timing of hatching or emergence but it has been hypothesised by Nishide *et al.* (2015a) that the larger eggs of gregarious females may result in the earlier emergence recorded in that study, due to the hatchlings being larger and therefore able to surface sooner.

2.1.4 Aims and hypotheses

I have carried out a detailed investigation of hatching and emergence patterns in lab-reared gregarious and solitarious locusts under controlled light and temperature regimes (12 : 12 light : dark, 36°C photophase : 25°C scotophase). A key aim was to use these behaviours to gain insight into the central circadian clocks of the two phenotypes. I interpret my results in the context of the possible effects that the timing of hatching may have on local population densities in the field. This information will further our understanding of the differences between the phenotypes and could allow for targeted monitoring and management of gregarious breeding sites.

I hypothesised that gregarious animals would emerge at or around dawn in a circadian entrained fashion (Padgham, 1981), prior to the environmental, particularly thermal, conditions changing. I further hypothesised that solitarious hatchlings would emerge over a longer period of time prior to dawn to permit dispersal and therefore conspecific avoidance in line with the solitary nature of isolated locusts. Work by Nishide *et al.* (2015a) predicts that eggs of both phenotypes should hatch 5 - 7 h prior to dawn, with eggs of gregarious females being expected to hatch prior to those of solitarious females.

2.2 Methods

2.2.1 Husbandry

All animals (*Schistocerca gregaria* Forskål) used in this work were bred at the University of Leicester, U.K. Solitarious animals were isolated from the gregarious colony for 2 generations. Both solitarious and gregarious animals were maintained under a 12 : 12 h light : dark regime, with light levels of 750-1500 lx during the photophase, and 0 lx during the scotophase. Cage temperatures were 36°C during photophase and fell to 25°C during scotophase. Locusts received fresh wheat seedlings and bran flakes *ad libitum*. Solitarious animals were reared individually in 10 x 10 x 20 cm cages isolated from sight and smell of other animals. Gregarious individuals were kept at high density (100-300 per cage) in 50 x 50 x 50 cm cages. These conditions were identical to those used previously in numerous studies of gregarious and solitarious animals (Matheson *et al.*, 2004; Gaten *et al.*, 2012).

Sexually mature gregarious animals were provided with 4 egg tubes per cage which were changed twice a week. The locusts mated and laid spontaneously, with the result that egg tubes frequently contained 8-12 egg pods per tube. Individual pairs of sexually mature solitarious male and female locusts over 1 month past their imaginal moult were placed in 9.5 x 16 x 10.5 cm tanks for a maximum of 6 h or until copulation was complete. The solitarious animals were then immediately re-isolated. The mated females were each provided with a single egg tube, which was changed whenever laying was evident. The dimensions of the egg tubes were 45 mm diameter x 88 mm high for the solitarious cages and 36 mm diameter x 110 or 90 mm high for the gregarious cages. Egg tubes for both phenotypes consisted of sand with 10% water by volume when presented to the animals and were replaced 3-4 days after presentation. Egg tubes containing eggs were placed in an incubator (SANYO, MIR-254) on a 12 : 12 light : dark cycle at constant temperature of 28°C for the first 7 days of development. The eggs normally hatched after approximately 14 days under these conditions, but for these experiments were removed to a controlled temperature and light cycle approximately 5 days prior to hatching as described below.

2.2.2 Hatching

Eggs were separated from the egg pods of gregarious and solitarious parents at least 5 days before their expected hatch date (no more than 7 days after laying). Once separated, excess debris (dried sand or foam) was removed from the individual eggs and the posterior end was identified on the basis of the micropyle. A petri-dish (14 cm diameter) was prepared with a shallow layer of sand (1 cm; 10% water by volume). The eggs were inserted posterior down into pre-formed holes and covered by less than 2 mm of sand (Figure 2). 50 eggs were prepared per egg pod, with two egg pods per petri dish. The eggs from the two pods were demarcated with a line both in the sand and on the lid of the petri dish. The petri-dishes were situated under a camera (Bassler AG021C-2; 6mm/F1.4) for a period of at least 7 days (i.e. until at least 2 days past the expected hatch date). During that time, continuous video recording occurred under constant red light (MARL 084-501-76-99: 610-660 nm, centred at 635 nm; 320 lx), in addition to the 12 : 12 h white light cycle provided by overhead fluorescent tubes. The red light permitted filming during both “light” and “dark” periods. The recording was carried out using VirtualDub software (http://www.virtualdub.org) at 1 frame per second (FPS). In addition, a software filter was used to overlay the current time onto the recording (Alidator; http://voidon.republika.pl/virtualdub). Playback for analysis was initially 30 FPS. Where necessary, analysis was on a frame by frame basis. The time of day at which each hatchling emerged from the sand surface was recorded to the nearest minute. Time of hatching was recorded as the time at which each hatchling emerged from the thin layer of sand. The pod of origin was noted to allow analysis for sibling group effects. The time of hatching was recorded as Zeitgeber time (ZT) relative to the time at which the lights came on and temperature began to increase (ZT = 0).

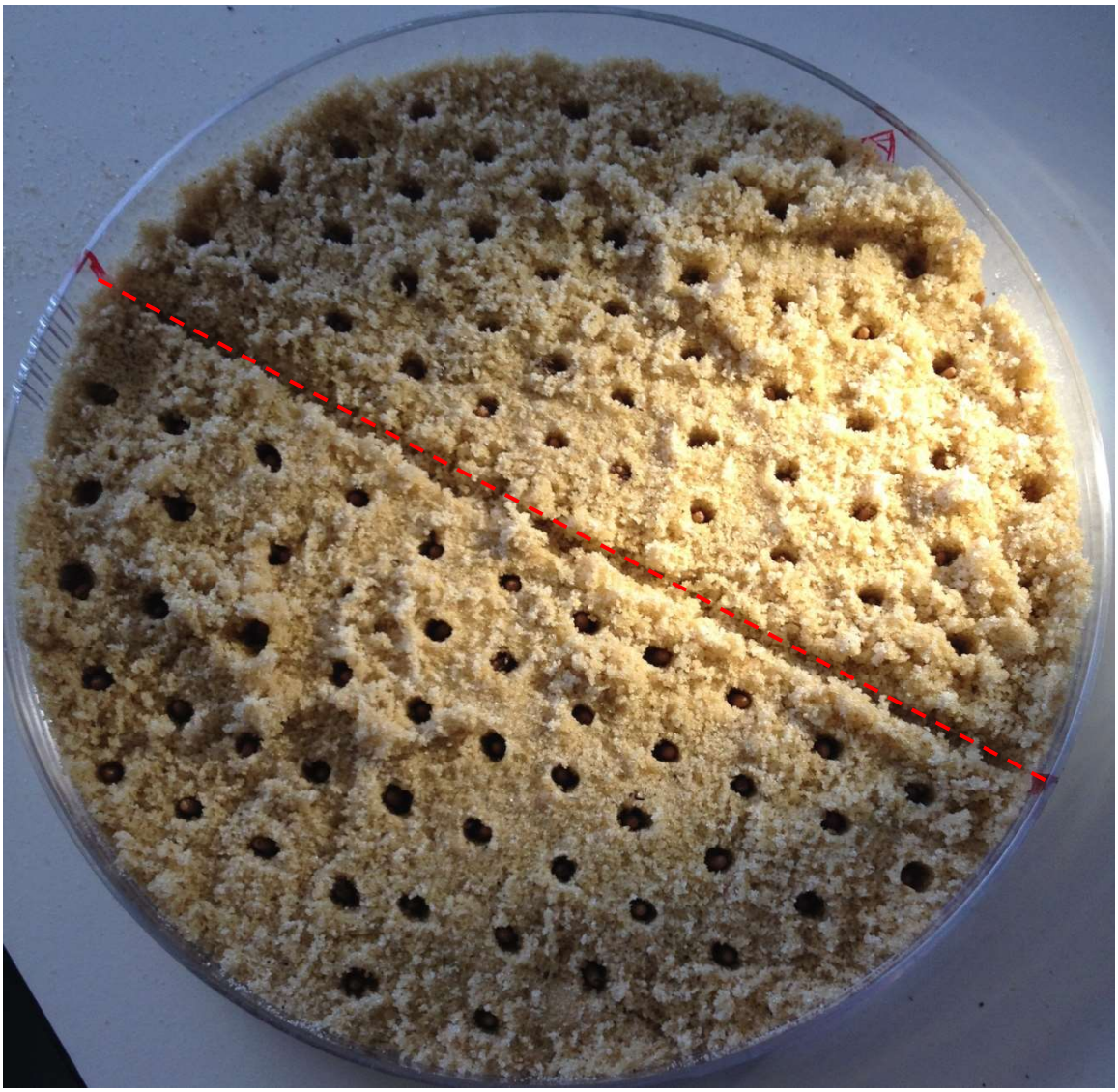


Figure 2: Example hatching experiment prior to covering the eggs with 1 – 2 mm of sand. The dashed red line indicates the divide between two sibling groups, each of which consists of 50 eggs. Petri-dish is 140 mm in diameter.

2.2.3 Emergence

Egg tubes from gregarious and solitarious parents were taken from the constant-temperature incubator and placed under standard lighting and temperature conditions (12 : 12, L : D, 36 : 25°C) at least 5 days before expected hatch date (no more than 7 days after laying). The thermocycle in place for these experiments rose to 36°C over 3 hours and fell to 25°C over the course of the cryoperiod. Egg tubes sourced from solitarious individuals consisted of 1 egg pod, rarely with a second egg pod; whereas egg tubes sourced from gregarious cages consisted of up to 8 individually identifiable egg pods. Identification was based on the location of hatchling emergence.

1 ml of water was added to each egg tube prior to being recorded and analysed as described above (Figure 3). In addition fresh wheat seedlings were available for the hatchlings, and egg-tubes from solitarious animals were maintained in isolated containers. When large numbers hatched simultaneously the number emerging was recorded for each minute. Where possible the originating egg pod was noted. If identification of originating egg pod was not available the data were not utilised in sibling group analyses.

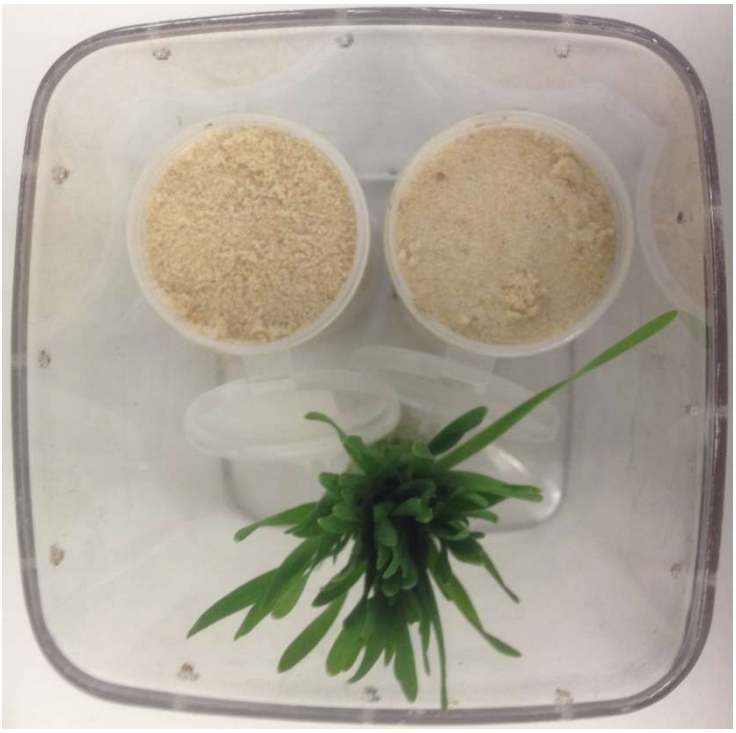


Figure 3: Example layout of emergence experiment (top view). The tubes were continuously recorded, with low level red light in addition to the cycling white light cycle. Container measured 110 x 110 mm at the widest points and was aerated with small holes (approx 1 mm diameter) around the top.

2.2.4 Egg depth

In order to compare the Hatching and Emergence assays (2.2.2 and 2.2.3) the depth of egg laying must be examined. In the Hatching assay the depth was controlled, with each egg isolated and placed at the same depth, in the Emergence assay the depth was not directly controlled, with the naturally occurring egg pod spanning a range of depths from first laid to last laid (Figure 4). As measuring egg depth was destructive, a different set of samples was used. This was carried out for both gregarious egg tubes used (long and short) and the solitarious egg tubes, incubated as described (see Husbandry) prior to measurement. The depths of the highest egg and the lowest egg for each egg pod were recorded relative to the sand surface. From these data the length of the egg pod was calculated.

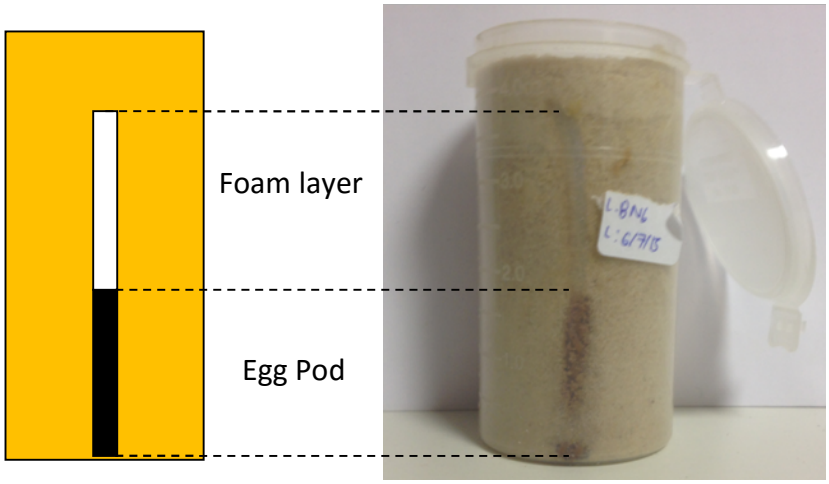


Figure 4: Side view of an egg pod within the egg tube from a solitarious cage. Although the eggs are visible against the side of the tube in this example, not all eggs may be located peripherally. Egg tube was 45 mm diameter x 88 mm height.

2.3 Results

2.3.1 Time of hatching

The time of hatching of eggs positioned just under the sand level was significantly different between gregarious and solitarious populations (N_g = 9 pods, N_s = 9 pods; Mann-Whitney U; P = 0.004; Figure 5A&B, bottom). Gregarious hatchlings emerged 42 min earlier than solitarious hatchlings (median = 21:06 ZT and 21:48 ZT; IQR = 2:45 and 2:20 respectively). The median time of hatching was 2:54 and 2:12 hours prior to dawn respectively and therefore prior to when the lights and temperature began to rise.

The effect of sibling group (Figure 5A&B, top) on the hatching time was significant in both the gregarious and solitarious data (Kruskal-Wallis; both $P < 0.001$). There was no significant difference between interquartile range (IQR) of hatching time in gregarious and solitarious eggs calculated per sibling group (Kruskal-Wallis, $P = 0.895$; median = 1:58, 1:48 respectively). The number of live animals to hatch did not differ significantly between phenotypes (Mann-Whitney U; $P = 0.825$), with an average of 20 and 20.3 hatching per sibling group of gregarious and solitarious respectively.

2.3.2 Time of emergence

Hatchlings from gregarious females emerged 40 min later in the scoto- (dark) /cryophase (median time: 23:43 ZT, IQR = 2:49; N = 832) than those from solitarious females (median time: 23:03 ZT, IQR = 1:18, N = 410; Mann-Whitney, $P < 0.001$) with 54% of gregarious emergences and 89% of solitarious emergences occurring before dawn (Figure 6A&B: Bottom).

The sibling groups (Figure 6A&B: Top) showed a significant effect on emergence time in both solitarious and gregarious populations (Krustal-Wallis; both $P < 0.001$). The IQR of emergence times in each pod differed between the two phenotypes (Figure 7; $t(19.2) = 3.77$, $P = 0.001$). Gregarious sibling groups emerged over a longer period of time (IQR; mean = 1:23, SD = 1:30 h) than the solitarious sibling groups (IQR; mean = 0:07, SD 0:05 h). The number of animals to emerge did not differ significantly between phenotypes (Mann-Whitney U; $P = 0.592$), with an average of 42 and 30 emerging per pod of gregarious and solitarious respectively.

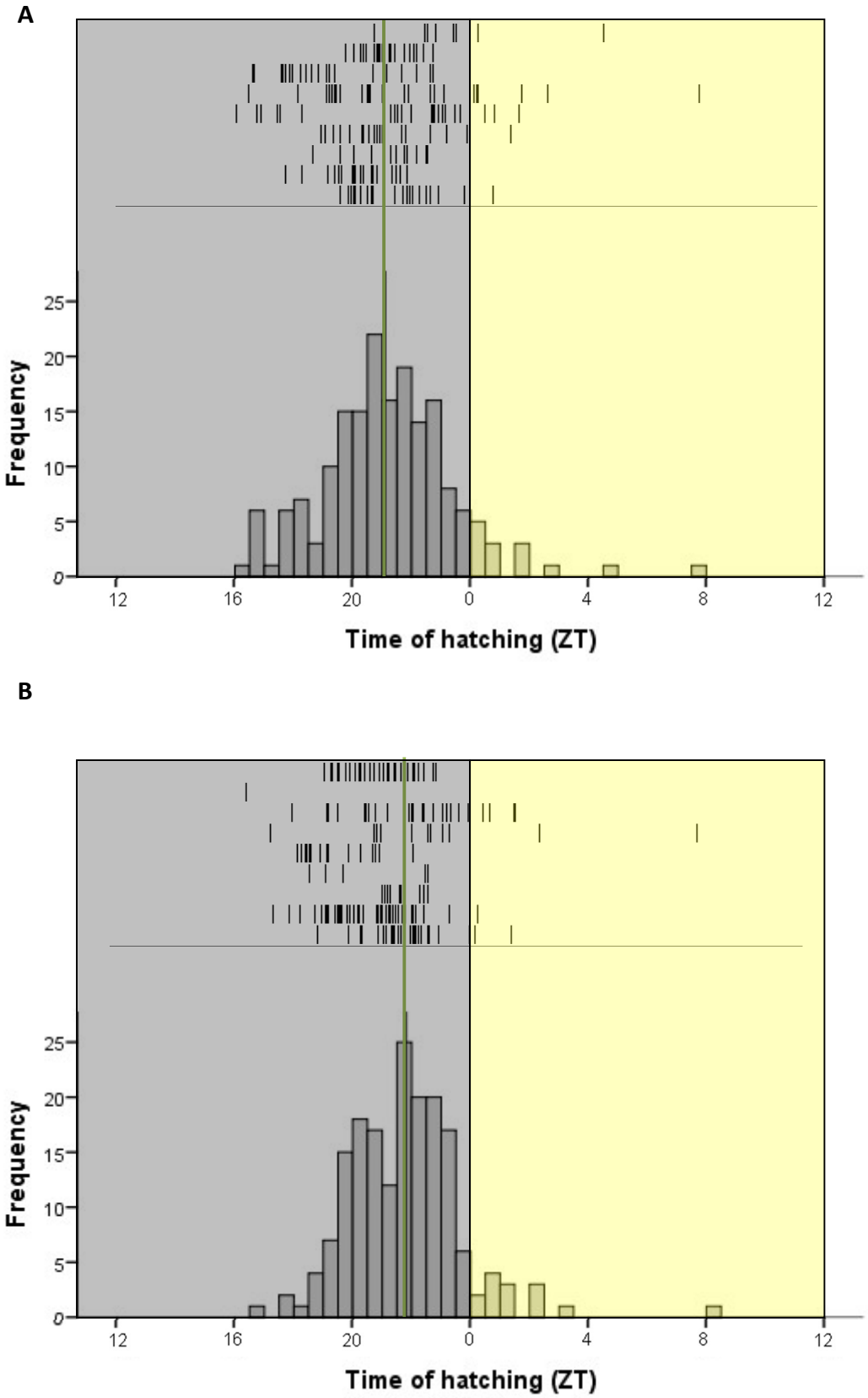


Figure 5: Time of hatching (ZT) of eggs derived from gregarious (A) and solitarious (B) females. Vertical line: median hatching time. A(top) & B(top): Raster plot of hatching time, sibling groups are shown on separate rows. A(bottom) & B(bottom): Histogram of hatching, bins are 30 min. Gregarious eggs hatched significantly earlier then solitarious eggs (21:06 and 21:48; *P* = 0.004)

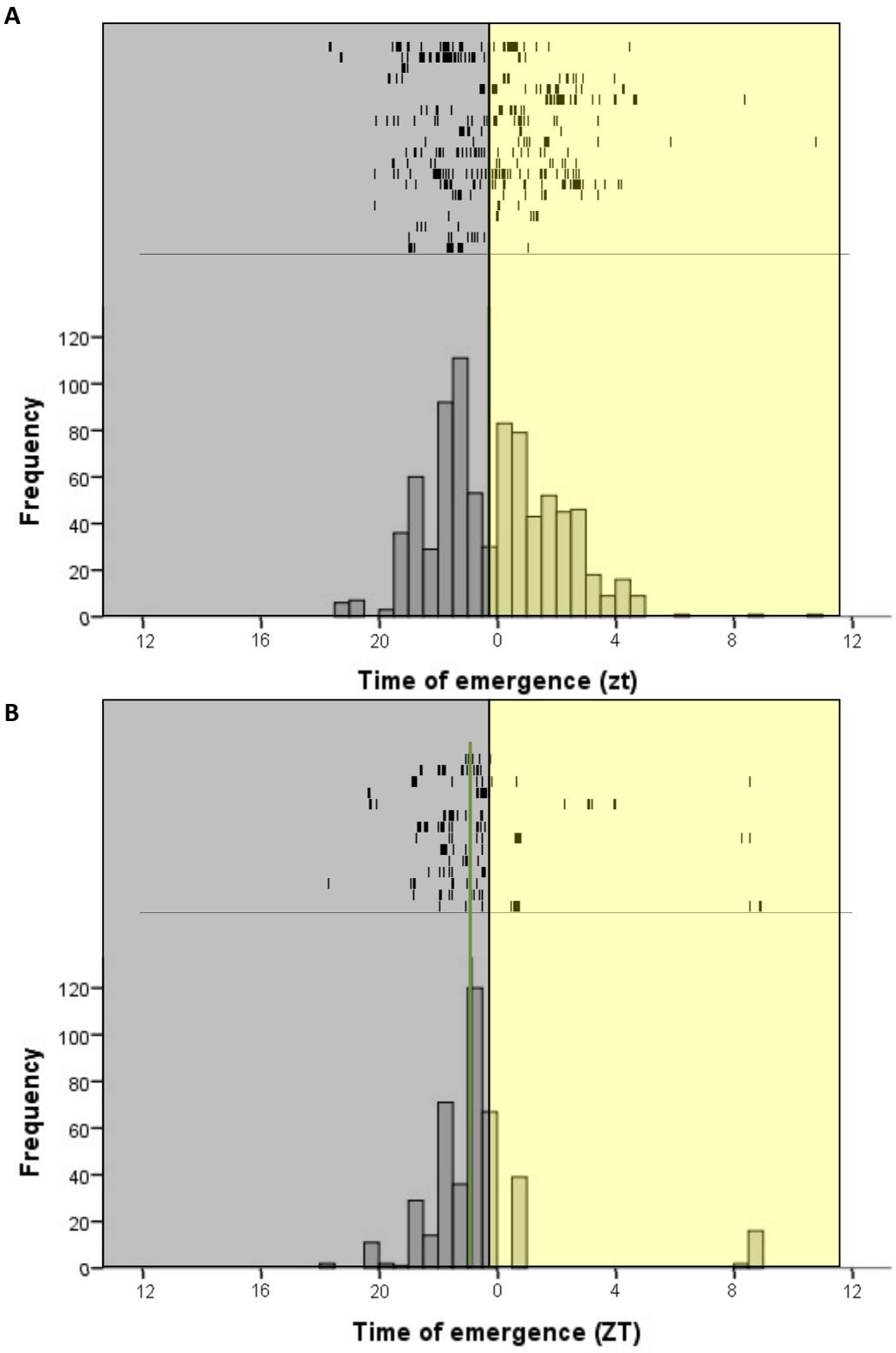


Figure 6: Time of emergence (ZT) of hatchlings derived from gregarious (A) and solitarious (B). Vertical line: median hatching time. A(top) & B(top): Raster plot of emergence time of hatchlings, sibling groups are shown on separate rows. A(bottom) & B(bottom): Histogram of emergence time of hatchlings, bins are 30 min. Solitarious hatchlings emerged significantly earlier then gregarious hatchlings (23:03 and 23:43; $P < 0.001$)

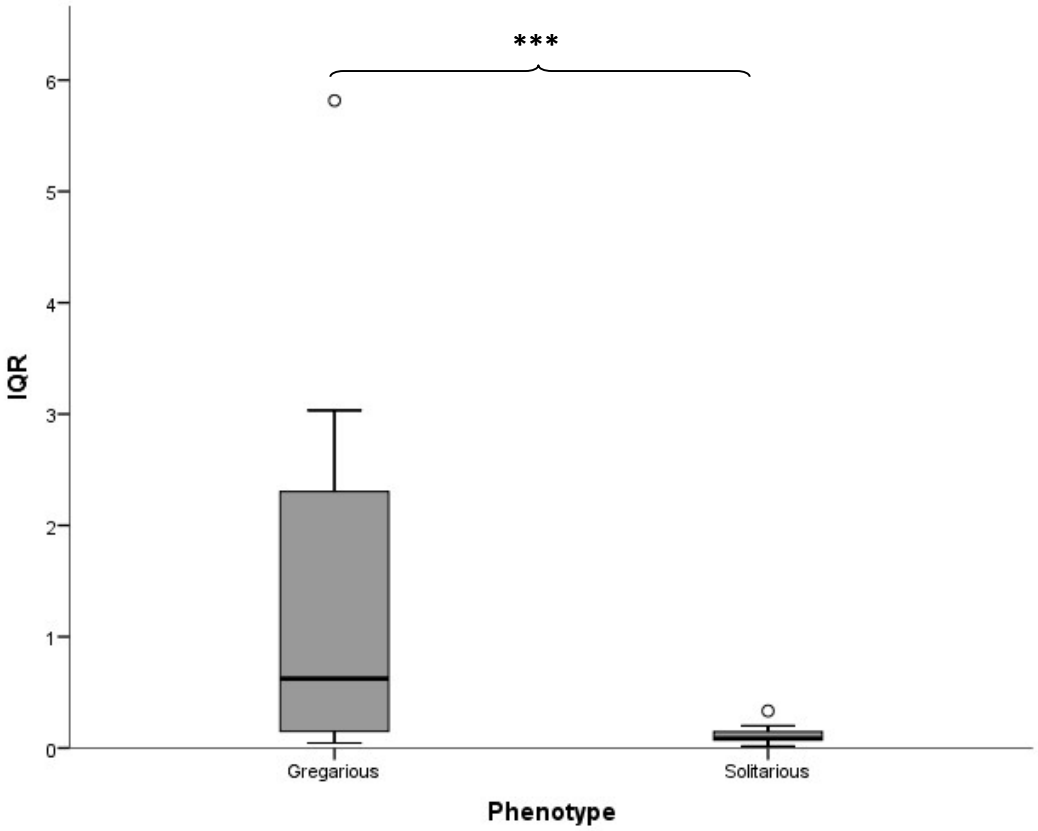


Figure 7: IQR of sibling emergence events of gregarious and solitarious sibling groups. Gregarious sibling groups show a significantly larger distribution than solitarious sibling groups (average IQR: 1:23 and 0:07; *P* = 0.001).

2.3.3 Egg depth

Egg pods were laid at depths spanning from 79.4 ± 9.2 mm (average lowest egg value) to 49.5 ± 10.8 mm (average highest egg value). The depth of egg laying did not differ between any of the egg tube types or between phenotypes (Figure 8). This was the case for the depth of the highest egg (ANOVA, $F_{(2,23)} = 0.965$, $P = 0.396$), lowest egg (ANOVA, $F_{(2,23)} = 0.616$, $P = 0.549$) and range of laying within each pod (ANOVA, $F_{(2,23)} = 1.077$, $P = 0.357$).

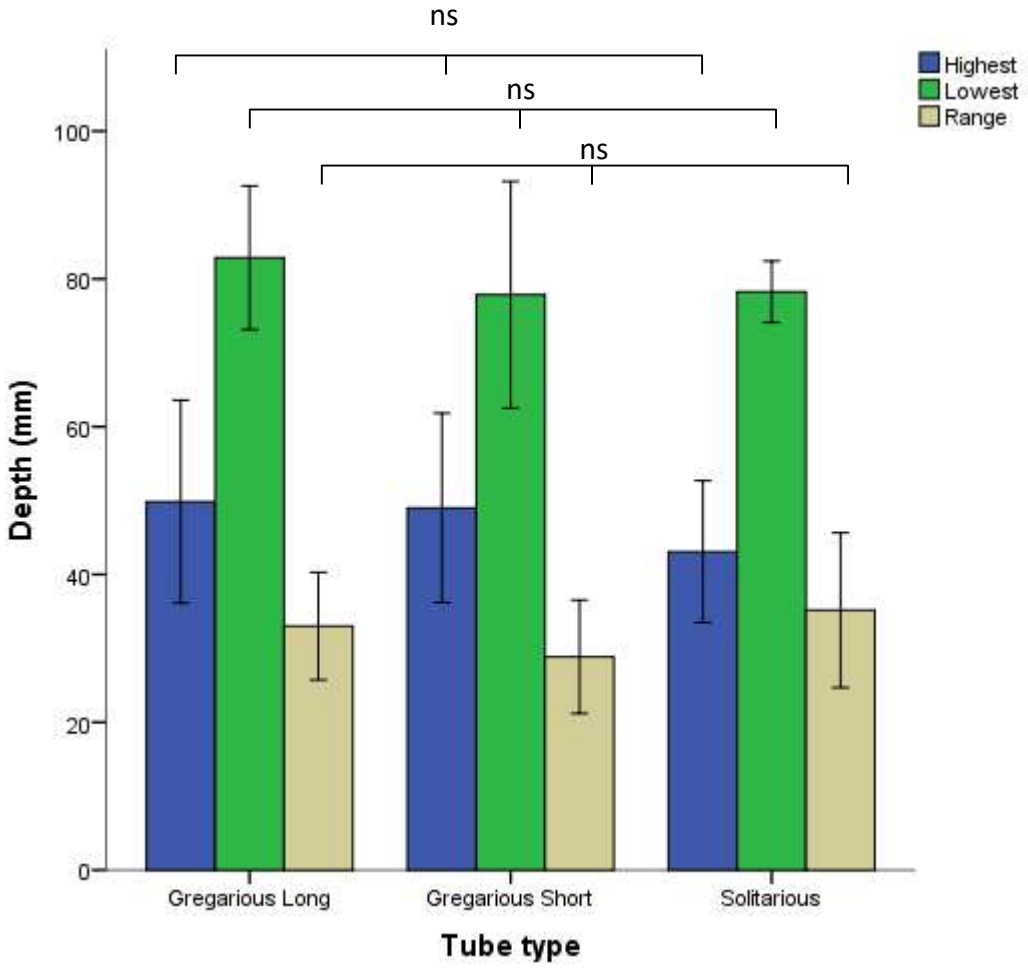


Figure 8: Comparing depths of eggs (mm) in the egg tubes used in emergence experiments. Error bars are 1 SD above and below the average. No significant differences were seen in the depth of the highest or lowest egg (average 49.5 and 79.4 mm) or range of egg depth ($P = 0.396$, $P = 0.549$, $P = 0.357$)

2.4 Discussion

Both the hatching and emergence experiments described here, as well as the preceding husbandry, were carried out under coincident thermoperiod and photoperiod with a period length of 12 : 12 h. At “dawn” the lights turned on and the temperature started to rise. Within 3 h the temperature stabilised at 36°C. At “dusk” the lights turned off and the temperature fell over the course of the scotophase to 25°C. This differs from the thermal conditions examined in Nishide *et al.* (2015a & b), where adults were reared at a constant temperature (31°C) under a 16 : 8 h photoperiod. Experimental conditions also differed, with the thermocycle in the present work showing a more gradual shift from cyro- to thermophase (approximately 3 h) and a gradual cooling over the course of the cryophase (temperature measured in the centre of the widest egg tube) compared to the near instant (within 1 h) condition changes in Nishide *et al.* (2015a & b). No attempt was made in Nishide *et al.* (2015a & b) to track members of the same egg pod, with eggs from many egg pods combined for the assay. Finally the measures in Nishide *et al.* (2015a) were carried out hourly, whilst work in this study has a temporal resolution to the nearest minute.

2.4.1 Hatching

Under the laboratory conditions used here, with coincident thermoperiod and photoperiod, the hatching times of solitarious and gregarious locusts differed significantly. Gregarious eggs hatched 40 min earlier than solitarious eggs. Hatching occurs in both gregarious and solitarious prior to dawn while the temperature is still falling in this study. In contrast the temperature was stable for some time (4 h) before hatching occurred in Nishide *et al.* (2015a). Additionally Nishide *et al.* (2015b) showed rhythmic hatching under constant dark conditions. With the current data and that of Nishide *et al.* (2015a & b) it is highly likely that hatching is entrained to the circadian clock, using temperature as the Zeitgeber, and is not reliant on passing a thermal threshold. Both gregarious and solitarious eggs show a significant difference in hatching times between different sibling groups, suggesting that animals from the same sibling group tended to hatch at a similar time (Figure 5A & B: top). It is worth noting that the layout in this experiment excluded contact between siblings, so any

correlation must be due to either entrainment to the same cues or some residual mutual maternal effect, for example a genetic or epigenetic effect. This differs from mechanical sibling influence, which as previously discussed is driven by tactile or chemosensory stimulation, in that the siblings are not influencing the hatching time of the other individuals.

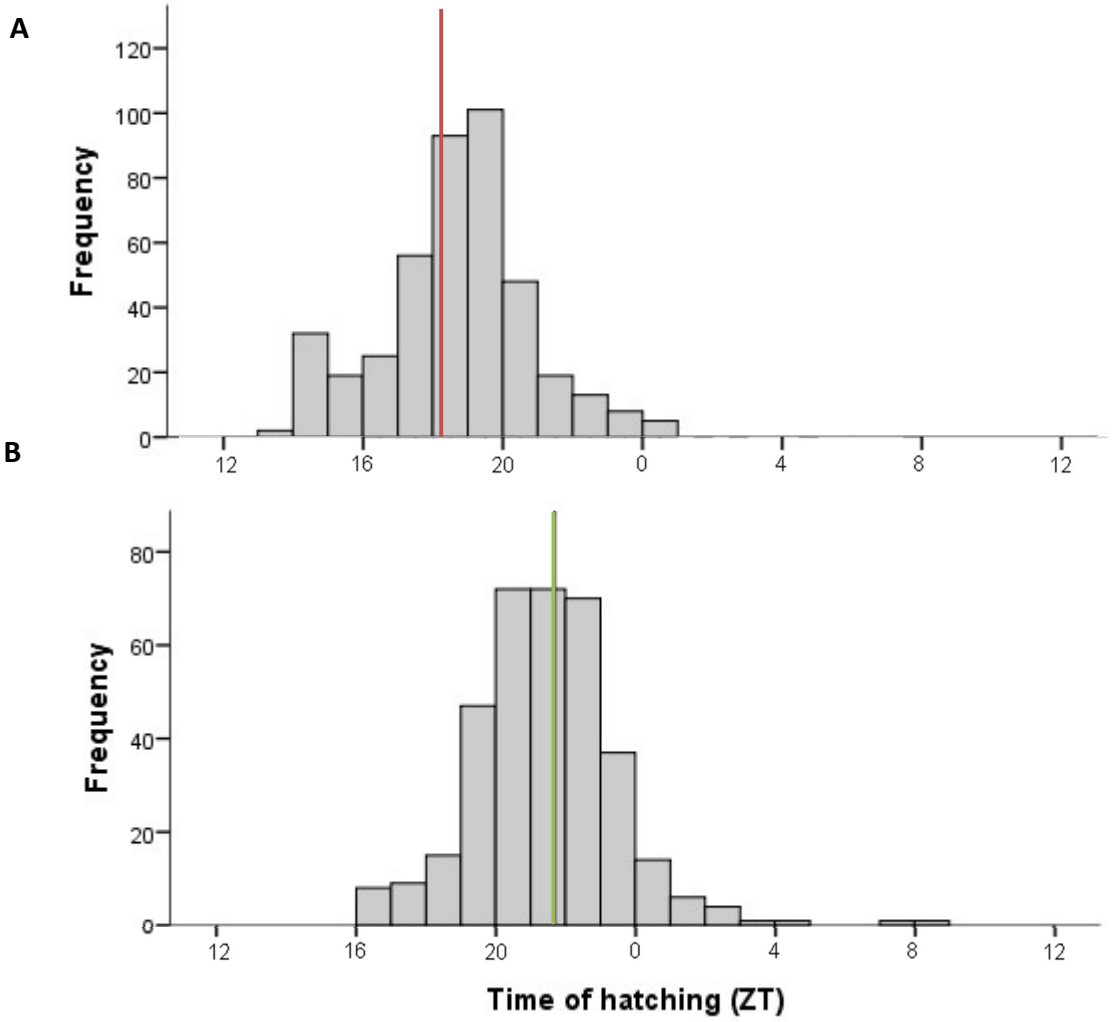


Figure 9: Comparison of gregarious and solitarious data combined from Nishide *et al.* (2015a) and the present study. A) adapted from Nishide *et al.* (2015a), selected to be similar in conditions to current data (35:25°C). n = 425, Mean = 17:06 ± 2:35. B) Whole population hatching from this work. N = 363, Mean = 21:41 ± 2: 48. Data binned into 1 hour periods. Solid vertical lines indicate mean values.

The hatching times of both phenotypes were centred at approximately 21:30 ZT (median: 21:06 & 21:42; gregarious and solitarious respectively), which differs markedly from the data presented in Nishide *et al.* (2015a) which shows a mean time of 17:06 under comparable conditions (Figure 9). The very substantial difference between the two datasets is unexpected. The two dominant factors in explaining these

differences are any possible strain differences and the differences in the thermo cycling. In Nishide *et al.* (2015a) the thermo cycling is more rapid, fully heating or cooling the environment within an hour; in the present study the thermo cycling was more gradual, more closely representing how conditions may vary in the field. The strain differences are not known to be significant, but without testing both strains side-by-side under identical conditions it would not be possible to establish any significance. The work by Nishide *et al.* (2015a) compared an additional strain and found no differences, so it seems unlikely strain differences would result in the difference seen. It is, however, possible a mutation in the Leicester strain could affect the timing of hatching. Previous work on the hamster, *Mesocricetus auratus*, has shown that artificial twilight, with gradual changes in light over the course of dawn and dusk, provides a different stimulus and results in a different behavioural output when compared to an abrupt change in conditions (Tang *et al.*, 1999). It is more likely, therefore, that the different thermocycles are responsible for the majority of the observed differences.

In agreement with Nishide *et al.* (2015a & b) and Padgham (1981) the vast majority of animals hatched during the cryophase of the thermocycle when exposed to the cycling thermal conditions for at least 5 days. This strengthens the argument that thermal entrainment happens in the final 5 days prior to hatching. It is not possible to estimate the period of any circadian rhythm driving the hatching from the current data as in the majority of cases all eggs hatched within one cycle period. This is likely due to the stringent experimental design whereby eggs from the same sibling group were recorded together, discrete from other sibling groups. This allowed the effect of sibling group to be assessed, however this resulted in the vast majority of viable eggs hatching during the same 24 h period. Nishide *et al.* (2015a & b) did not differentiate between sibling groups, combining eggs from various egg pods and thus getting a range of hatching dates for each experimental group. This in turn may have resulted in the less rhythmic distribution of hatching compared to the current study. It can therefore be confidently stated that animals from the same egg pod will tend to hatch within one cryophase, usually occurring within one hatching event per egg pod. Additionally the data collected in this study and that of Nishide *et al.* (2015a & b) combine to provide

strong evidence of an endogeneous circadian clock providing the gating for the hatching behaviour.

2.4.2 Emergence

The data discussed below refer to the animals' emergence from the sand when incubated in the naturally occurring egg pod. In this situation, the recently hatched larvae must make their way to the surface from various depths below the sand. The depth of laying does not differ between solitarious and gregarious animals, and even in the centre of the widest egg tubes the temperature closely follows that of the room (+/-1°C), as such the two phenotypes can be directly compared given the context of the hatching experiment discussed above.

The diurnal pattern of emergence of gregarious hatchlings has been documented previously, with emergence occurring within 2 h either side of dawn under laboratory conditions (Padgham, 1981). This pattern had also been seen in earlier field observations (Ellis and Ashall, 1957). It was hypothesised by Padgham (1981) to be, at least in part, due to a temperature-entrained circadian clock.

I show that gregarious locusts from our laboratory population also emerge around dawn when they have been incubated under a coincident 12 : 12 h thermoperiod and photoperiod. A matutinal emergence pattern was seen (Figure 6) with a median emergence 20 min prior to lights on, with a spread of approximately 3 h (IQR = 2:49). As the temperature rose from dawn onwards in this experimental setup it is more likely that the rhythm seen is circadian, entrained to the thermal cycles, rather than reliant on the thermal variation itself. Photic entrainment is unlikely, given that the eggs were shielded from the light of the room by the surrounding substrate (Nishide *et al.,* 2015b), however if they were uncovered they would be susceptible to light entrainment (Nishide *et al.,* 2015b). Additionally, in the present study the majority of emergence events occurred prior to dawn. The predictive element of the majority of the population's emergence is a strong indicator that this process is driven by the circadian clock, as opposed to any external diurnal stimulus, similar to that seen in *Drosophila* (Qiu and Hardin, 1996) whereby the hatching window started 2 h before lights on with hatching occurring within 6 h. This is confirmed in work carried out by

Nishide *et al.* (2015a & b), where rhythmic *hatching* occurred in populations of mixed sibling groups under constant conditions, following thermocycle entrainment.

Solitarious animals were also shown to emerge just prior to dawn, with an average lead time of 1 h and 6 min. This difference in median time was significant between the phenotypes. The increased lead time of the solitarious animals may enable the individuals to spread out further than their gregarious equivalents before predator avoidance and the need to dry the exoskeleton (Tanaka and Watari, 2003) in the morning sun become of high importance. This could minimise the increased local population density seen as a result of the more cohesive hatching pattern.

Within the two datasets the identifiable sibling groups allowed analysis of emergence cohesion within the same emergence event. The individuals in the same egg pod are genetically related and are exposed to the same environmental experiences, thus they are at a similar developmental stage with the same gating stimulus (circadian or otherwise) which may be driving the observed cohesion. Alternatively it may be due to sibling interaction, whereby one individual, via physical or chemical stimuli, encourages surrounding individuals to hatch and thus emerge at a similar time. These interactions are controlled in the Hatching assay discussed previously (2.4.1).

Solitarious individuals showed a stronger sibling grouping effect, emerging over a much shorter period of time than gregarious emergence events (average IQR: 0:07 and 1:23). Emerging more cohesively would act to increase the local population density immediately following hatching, whereas a less cohesive emergence would result in a lower population density at the emergence site. This behavioural difference is of note as it may be seen to be contrary to one of the fundamental behaviours of the phenotypic change, whereby solitarious animals tend to disperse, reducing the population density, whereas gregarious animals tend to proactively congregate to increase the population density (Uvarov, 1966). An alternative hypothesis is that the individual solitarious hatchlings are in fact actively avoiding each other during emergence through the substrate, thus promoting higher rates of movement towards the surface. This may work to promote an earlier, more synchronous emergence event. Those hatchlings that hatch first would show relatively slow movement through

the substrate, while as the number of hatchlings in the substrate increases the movement speed increases, resulting in all the individuals emerging at a similar time. Conversely gregarious animals may be attracted to each other within the substrate and therefore be less motivated to move towards the surface. This would result in the more dispersed emergence as well as the later central tendency seen.

This work confirms the field observations that siblings tend to emerge together, although the cause of this cohesion is still unknown it. Predator avoidance may well be a factor in the cohesion strategy itself, since the longer an emergence site is active the higher the risk of discovery by a predator. If the site is discovered, emerging en mass may give some solitarious hoppers the chance of escaping while the predator is capturing others. By minimising the duration of the hatching event the individuals are ensured maximal opportunity to emerge and escape before discovery. This is of lesser importance for gregarious sibling groups because each pod is just one of many thousands laid at the same time in a small area (Uvarov, 1966). There is likely limited ecological advantage to a cohesive hatch in this situation since there will likely be others hatching at a similar time, and a broader hatching event may work to prevent crowding within the egg pod, which could lead to individuals getting stuck or being unable to emerge before tiring. Although no study has occurred on this topic, dead hatchlings can often be found partway through the substrate in both phenotypes suggesting they either succumbed to weakness or became stuck before being able to emerge.

2.4.3 Hatching vs. emergence

By comparing the hatching and emergence results it is clear that a lag period exists: the animals in the hatching assay eclose consistently earlier in the scotophase/cryophase relative to the emergence of those in the emergence assay. This is likely due to the depth of sand they must make their way through to reach the surface. The gregarious animals show a relatively similar spread between hatching events (average IQR = 1:56) and emergence events (average IQR = 1:23). This could be the result of 1) precise correction for lay depth, based on the amplitude of thermal cycles resulting in those that are deeper hatching sufficiently earlier to compensate for

the additional distance (Zdárek and Denlinger, 1995; Tanaka and Watari, 2003), or 2) the hatchings occurring in a random order, thus masking the depth effect.

Species such as the onion fly, *D. antiqua*, compensate for depth by varying eclosion time in relation to amplitude of diurnal temperature change; deeper egg positions will experience a smaller cyclical variations and this will promote an earlier hatch (Tanaka and Watari, 2003). This mechanism is unlikely to be at play in my results due to the design of the egg tubes. The minimum distance from the egg to the edge was always less than the distance to the surface. Since the sand was heated from all exposed sides, not just the surface, the rate of temperature change was not systematically related to depth and therefore could not be the driver of depth-related hatching synchronicity. In fact the temperature at the average level of the egg pods was within 1°C of the room temperature, and therefore similar to the temperature of eggs in the hatching assay. As such, it is clear that the depth of laying is not causative of any differences between the hatching and emergence times.

In the solitarious animals by contrast, the distributions of hatching and emergence are significantly different. Hatching occurs with a similar distribution to gregarious animals (average IQR = 1:48), with emergence occurring over a much shorter period of time (average IQR = 0:07). Again, no thermal cues exist to compensate for depth so it must be assumed that hatching occurs as recorded in the hatching assay, however animals emerge more cohesively. Currently the mechanism for this difference is unknown, however a possible explanation could involve the conspecifics avoidance/attraction effect that defines the two phenotypes. As discussed above this could be a factor in the significant difference in emergence given the similar hatching times with solitarious hatchlings driven out of the substrate by repulsion of their conspecifics, whilst gregarious do not experience the repulsion drive and thus make slower process.

2.4.4 Conclusion

I hypothesised that hatching would occur before dawn, as is seen in desert locusts in the field and in a diverse range of species. Additionally I hypothesised that solitarious hatchlings would emerge over a longer period of time in comparison to gregarious hatchlings, allowing them to rapidly disperse.

This work has shown a strong rhythm that is persistent between phenotypes in hatching and emergence behaviour. The emergence pattern had a central tendency just prior to dawn in both phenotypes as expected and confirmed in other recent works (Nishide *et al.*, 2015a & b), however the increased synchronicity of solitarious hatchlings opposed my hypothesis and no mechanism for this behavioural difference is currently known. Taken with the work by Nishide *et al.* (2015a & b) together these show the rhythm to be circadian in nature, which is likely controlled by an endogenous clock.

Chapter 3:
Retinal and behavioural responses to visual stimuli

3.1 Introduction

The previous chapter focused on the timing of a vital developmental process, hatching and emergence. This chapter focuses on behavioural and electrophysiological assays in adult locusts. The visual system has been shown to be adapted to the lifestyle of the species it is found in, in terms of sensitivity to salient stimuli and in terms of adapting to the environments commonly experienced by the animal. Here I discuss vision, how it can be assessed, what is known of circadian influence of the visual system and the role of escape behaviour. I then discuss the specifics of vision in Schistocerca gregaria, with an emphasis on current knowledge of circadian influences, and how it has been shown to differ as part of density dependant plasticity.

3.1.1 Vision

The visual system allows an animal to acquire vital information from its environment that allows it to behave and survive. Neurons within the visual system will therefore be adapted to process the most salient information for the animal's lifestyle, for example collision sensitive neurons in pigeons (Sun and Frost, 1998) and neurons in the toad tectum that preferentially respond to objects that resemble prey (Ewert, 1997). These specialised neurons can then trigger specific behaviours, including escape and prey capture. Visual systems are often specialised for specific roles; for example simple eyes, ocelli, often function to aid in control of flight behaviour (Link, 1909; Simmons, 2002), meanwhile photoreceptors (Laughlin and Weckström, 1993) and interneurons (O'Carroll *et al.*, 1996; Egelhaaf *et al.*, 2002) have both been shown to be spatio-temporally tuned to compensate for visual inputs resulting from the animals' movements.

Mechanistically, invertebrate vision is highly varied (Figure 1, Meyer-Rochow, 1999), however the compound eye of the insect has many conserved features. Large numbers of subunits, known as ommatidia, together form the eye. Each ommatidium is formed of four to eight retinula cells. Each retinula cell contains a rhabdomere which functions

as the photoreceptor, similar to the rods in vertebrate eyes (Wolken and Gupta, 1961). Light entering the rhabdomere is kept within the photoreceptor by means of the high refractive index forming an optic waveguide (Figure 1; Warrant and Nilsson, 2006). This high refractive index is partially due to screening pigmentation that forms a high contrast between the effectively transparent waveguide and the pigmented surrounding cells. As a result a high proportion of the light is kept within the photoreceptor allowing maximal transduction (Warrant and Nilsson, 2006). Each photoreceptor responds best to light entering along its long axis, known as paraxial directional sensitivity; therefore the specific rhabdom in the ommatidium provides information as to the direction the light is travelling. Additional spatial sensitivity comes from a combination of the lens, cone and diaphragm (Warrant and Nilsson, 2006). The lens is formed by the transparent cuticle layers over the facets of the eye; this coarsely focuses the light from a set spatial receptive field into the underlying components. The crystalline cone fine focuses the light coming from the lens onto the photoreceptors (Warrant and Nilsson, 2006). Depending on the type of compound eye the cone takes on different properties.

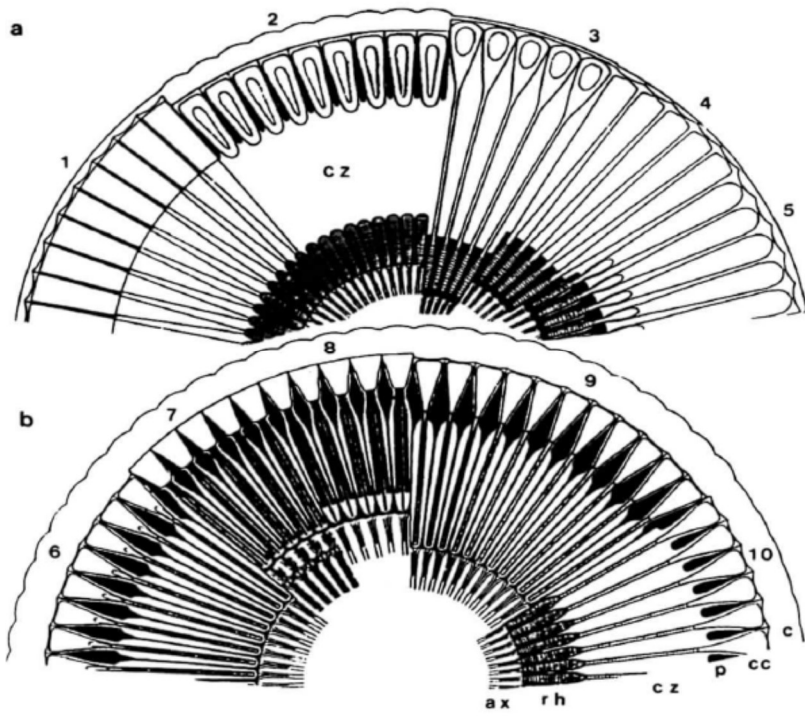


Figure 1: Known compound eyes (Meyer-Rochow, 1999). Including: 1. Reflecting superposition. 2. Refracting superposition. 3. 4. & 5. apposition with light guides. 6. focal apposition. 7. Neural superposition. 8. Open rhabdom apposition 9. Simple apposition. 10. Parabolic superposition. ax. Axons. c. cornea. cc. cone cells. cz. Clear zone. rh. Rhabdom.

3.1.2 Electroretinogram

The sensitivity of the retina to light can be measured using an electroretinogram (ERG), made by inserting an electrode through the cornea into the space surrounding the photoreceptive cells (Stark, 1975), with a reference electrode either in the extracellular space of the head (Schmachtenberg and Bicker, 1999; Hussain *et al.*, 1984), thorax (Chang and Lee, 2001) or in blanketing agar (Chen *et al.*, 1992). As the recording electrode is extracellular, the measured electrical activity represents the net activity of the surrounding cells. Recordings are made during a brief flash of light, for example in the range of 8 to 1000 ms (Hussain *et al.*, 1984). The type of signal recorded depends on the layer of the compound eye in which the electrode is situated (Figure 2; Naka and Kuwabara, 1959). Recordings from the crystalline cone layer consist of a rapid hyperpolarising on-transient, followed by a sustained on-plateau (Figure 2A; Stark, 1975). Repolarisation occurs once the light stimulus is removed. Recordings from the inner receptor layer feature a strong positive on-transient (Figure 2C). In the outer receptor layer a negative sustained off depolarisation is seen in addition to the on-transient (Figure 2B). This is effectively a hybrid of the crystalline cone and inner receptor layer recordings. Recordings proximal to the basement membrane show a gradual positive response, a sustained on-positive plateau, followed by a gradual repolarisation (Figure 2D). Due to the changing potential shape it is important to be consistent in electrode depth to minimise variation. Dark adapted ERGs show a intensity response curve best fit by the naka-rushton model (see Methods; Evans *et al.*, 1993). This models the sigmoid relationship whereby response increases logarithmically with respect to intensity, peaking at a saturated level.

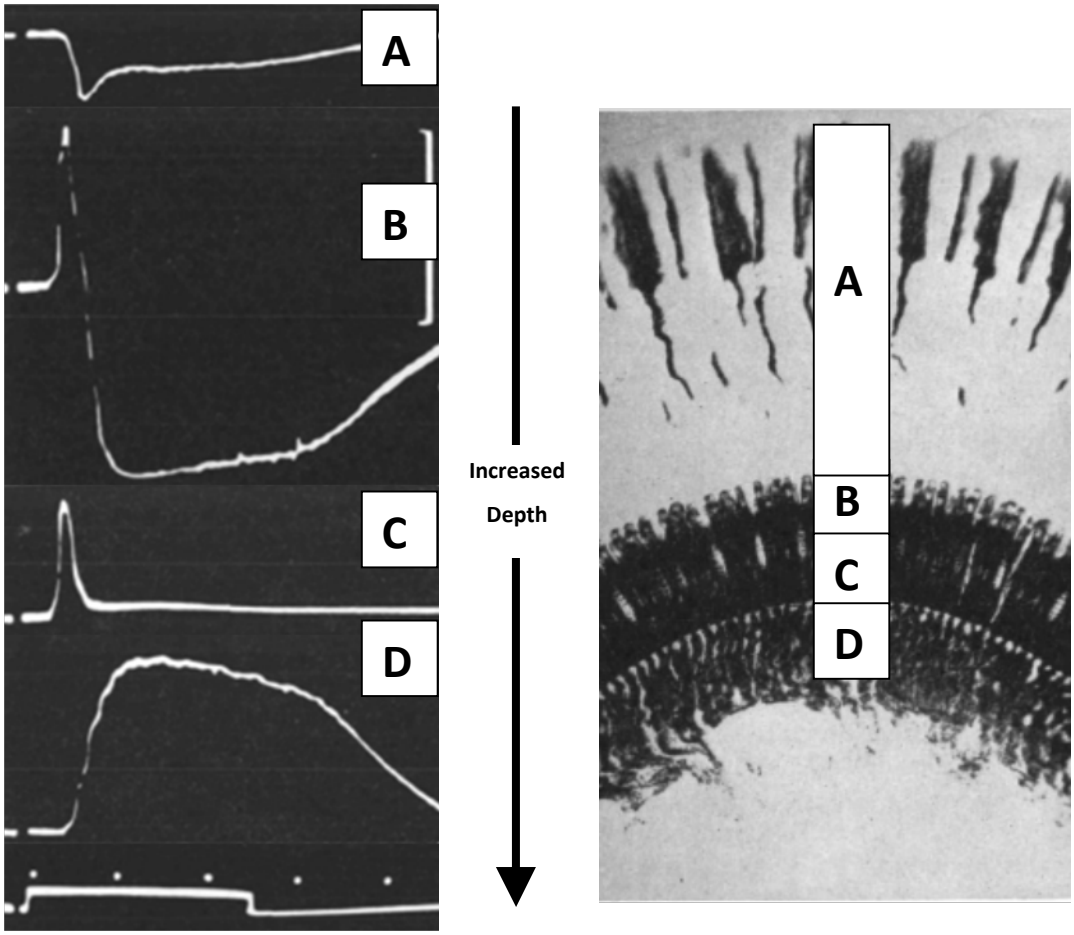


Figure 2: ERGs recordings from various depths in a Crayfish eye (adapted from Naka and Kuwabara, 1959). Top to bottom: A. Crystalline cone layer. B. Outer receptor layer. C. Inner receptor layer. D. Proximal to basement membrane.

3.1.3 Vision and circadian rhythms

In a similar way to the need for the visual system to be tuned to the lifestyle of the animal it must also be able to respond to changes in the environment. One of the key variables that fluctuates predictably throughout the life of the animal is light intensity, varying by as much as a million fold in a 24 h period (Bunning and Moser, 1969). As such the visual system is adapted to respond and predict the changes, allowing the animal to have efficient and effective sight over a large range of visual conditions (Figure 3; Warrant and Nilsson, 2006; Autrum, 1981; Meyer-Rochow, 1999). There are two main mechanisms by which the visual system adapts (Warrant and Nilsson, 2006). Experiments based on light sensitivity must try to isolate one form of adaptation from the other. The first mechanism involves light dependent changes: a responsive change to the current prominent conditions. Brought on by variations in intensity of the ambient light, these allow the system to adapt precisely to non-diurnal fluctuations (Warrant and Nilsson, 2006). The structural changes are visible in Figure 3, comparing Day Light Adapted (DLA) to Day Dark Adapted (DDA) and Night Light Adapted (NLA) to Night Dark Adapted (NDA). While an awareness that these changes exist is useful, they are not the primary focus of the present work so will not be explained further.

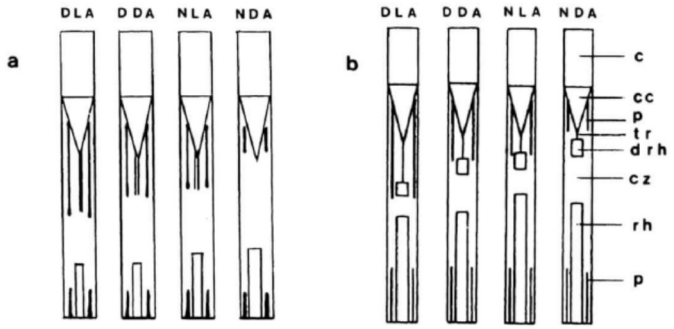


Figure 3: Dark/Light adaptation changes under DLA (day light adapted), DDA (day dark adapted), NLA (Night light adapted), NDA (Night dark adapted) conditions (Meyer-Rochow 1999): a. Scarab beetle. b. Carabids. c. cornea. cc. crystalline cone. cz. Clear zone. drh. Distal rhabdom. p. Pigment. rh. rhabdom.

The second control mechanism is via the central circadian clock, which is common to both vertebrates and invertebrates (Bell-Pedersen *et al.*, 2005; Damulewicz and Pyza, 2011). Figure 3 shows examples of these changes in the scarab beetle and carabids, comparing DLA to NLA and DDA to NDA. These rhythms are light-independent, free-running with an approximate 24 h period under constant conditions. A common example is the rhythmic eye-glow or eye-shine of moths and

beetles; here the mirror-like tapetum reflects light during the night and this acts to double the sensitivity of the eye. During the day, however, the tapetum is obscured by pigments. This cycle is maintained even in constant light or dark (Warrant and Nilsson, 2006; Pyza and Meinertzhagen, 1997). Similar rhythms are present at a neuronal level in the sea slug, *Aplysia*. In this case the central clock is located in the basal retinal neurons, which set the membrane potential for the photoreceptors, thereby adjusting their sensitivity (Geusz and Page, 1990; Pyza and Meinertzhagen, 1995). Similarly, in *Drosophila*, there are axonal diameter variations in two large monopolar neurons in the lamina (L1 & L2) as well as synaptic changes of the same neurons (Pyza and Meinertzhagen, 1995; Warrant and Nilsson, 2006). Turnover of visual membrane in mammalian photo transducers shows a circadian pattern, with rods shedding discs at dusk (LaVail, 1980; Young, 1978). Invertebrates show a similar pattern in rhabdom maintenance, with the horseshoe crab *Limulus* showing dawn degradation, and crabs internalising the membranes at dawn (*Limulus*, Chamberlain and Barlow, 1979; Barlow *et al.*, 1987; crab, Stowe, 1980). In *Drosophila*, a similar reduction in visual pigmentation was seen in the hours following dawn (Stark and Sapp, 1988; Zinkl *et al.*, 1990), however no assessment as to the mechanism of this change was made.

Circadian rhythms are present in all organisms and allow them to prepare in advance of predictable environmental conditions or events (Bell-Pedersen *et al.*, 2005; Lorenzo and Lazzari, 1998; Saito *et al.*, 1976). It is this need to prepare that drives the circadian changes in the visual system. The light differences between day and night can be as high as a million fold (Bunning and Moser, 1969). Consequently the light dependent mechanisms alone would not be able to change the sensitivity sufficiently rapidly to prevent blinding (Warrant and Nilsson, 2006), requiring predictive adaptation to reduce the sensitivity prior to the potentially dangerous increase in stimulation. To illustrate the importance of this cycle to animals that are active at different times, crepuscular, nocturnal and facultative diurnal ants do have an endogenous cycle, while strictly diurnal ants do not (Menzi, 1987). The ant species with an endogenous cycle in sensitivity were found to be those able to function in more than one environment, whilst the species with no cycle were only in a single

environment (Menzi, 1987). More generally, day active invertebrates show only transduction changes, and less frequently structural changes, while nocturnal individuals show both.

The visual system, and light itself, is also intrinsically linked to the endogenous circadian clock by the use of light as an entrainment condition (Zeitgeber; Time-giver). Pulses of light or a phase shift in lighting conditions can rapidly re-entrain the central clock of an animal and thus result in shifts in the patterns of behaviours and physiological processes. This is consistently seen wherever light entrains the circadian clock. In *Drosophila* for example, the clock is shifted as a result of the seasonally changing day length (Stoleru *et al.*, 2007; Dubruille and Emery, 2008), thus allowing behaviour to remain appropriate.

Photoreceptor cells are often circadian oscillators, they carry out molecular cycling that makes up the endogenous circadian clock; additionally glial cells are thought to be oscillators in the lamina. These oscillators are linked to changes in the number of synapses as well as in morphology of interneurons and glial cells (Dunlap, 1996). Possible candidates for transmission factors for circadian oscillators in *Drosophila* are pigment dispersing factor (PDF) neurons, FMRamide-like neurons, and serotonin neurons (Pyza and Meinertzhagen, 2003; Warrant and Nilsson, 2006; Nassel, 1988). The first two produce peptides that have been shown to be instrumental in the circadian axonal diameter variations seen in L1 and L2 (Pyza and Meinertzhagen, 2003; Warrant and Nilsson, 2006), monopolar neurons in the lamina that carry out motion detection.

3.1.4 Escape behaviour

One of the key uses of visual information is in predator-prey interactions. Due to the high information density of visual information, predators and prey often use visual cues to detect each other (Curio, 1993; Hemmi, 2005a & b). The importance of this detection role can be seen in the visual adaptations to enhance performance in this role; e.g. prey specific neurons in toads (Ewert, 1997). Information derived from visual cues drives decisions in escape and capture behaviours, however the information is limited by constraints placed on the eye and the visual system

(Bouskila and Blumstein, 1992; Koops and Abrahams, 1998; Luttbeg and Schmitz, 2000; Luttbeg, 2002).

Escape or avoidance behaviours are seen in response to a looming stimulus in a variety of species (Locust: Gabbiani *et al.*, 1999; Rind and Simmons, 1992; Simmons and Rind, 1992; Goldfish: Preuss *et al.*, 2006; Cat: Sherk and Fowler, 2001; Pigeon: Sun and Frost, 1998; Frog: Yamamoto *et al.*, 2003). The widespread nature of these behaviours indicates their evolutionary importance, and within these species there are adaptations in photoreceptors and interneurons to tune the system to relevant information (Egelhaaf *et al.*, 2002; Laughlin and Weckström, 1993; O'Carroll *et al.*, 1996). For any animal, there are various possible behavioural responses to an approaching predator or other threat. Consequently there is a decision point as to which behaviour to carry out. In fiddler crabs, the first response is often to return to the burrow entrance, while a more severe or persisting threat will result in the animal going beyond the entrance into the burrow (Nalbach, 1990; Land and Layne, 1995; Jennions *et al.*, 2003). These responses are known to be triggered by visual information (Nalbach, 1990; Land and Layne, 1995). Responses and decisions may occur close to the resolution of the visual system while the precise nature of the stimulus is still unknown, and are therefore likely to be context dependent (Land and Layne, 1995), with arousal state and priming, previous stimuli affecting the next response, being key considerations. *Larinioides cornutus*, an orb-spinning spider, shows a circadian pattern of 'huddle' response, a form of startle death feigning (Jones *et al.*, 2011). Here the response is strongest during the evening, in the build up to the night when the animal is at peak activity. Other behavioural responses to looming stimuli have also been studied in, amongst others, flies (Jablonski and Strausfeld, 2000; Tammero and Dickinson, 2002), locusts (Rind and Simmons, 1992; Gabbiani *et al.*, 2002; Gray, 2005), crabs (Hemmi, 2005a; Hemmi, 2005b), and humans (Regan and Hamstra, 1993).

3.1.5 Vision in *S. gregaria*

Schistocerca gregaria have compound eyes of the apposition type with no significant structural differences between solitarious and gregarious beyond the amount of pigmentation (Roonwal, 1946). Each compound eye consists of 2470 ommatidia in a 1st instar juvenile and 9400 in an adult male. As the animal develops, the size of all

aspects of the eyes increases proportionally (Uvarov, 1966). In different areas of the eye the acceptance angle, the angle from which light is accepted into the ommatidium, varies (Stavenga, 1979) allowing greater resolution of more relevant visual information. The cornea forms a bi-convex lens above each ommatidium focusing light through the crystalline cone and onto the rhabdoms and enveloping pigment cells (Uvarov, 1966). These cells form the retinula, the axons from which enter the optic lobe. As the eyes are close together, distances are established by lateral scanning movements of the head and body, which allow estimation of distance based on the extent of the movement over the retina (Sobel, 1990). In addition, locusts have ocelli, which are used primarily for flight stabilisation (Taylor and Thomas, 2003); there are two lateral and one median ocelli.

In the desert locust there is a well characterised visual pathway that is integral to the detection of looming stimuli. This pathway includes two giant interneurons, the Lobula Giant Movement Detector (LGMD) and the Descending Contralateral Movement Detector (DCMD) that are both driven by inputs from the compound eyes. In addition, L-type ocellar neurons detect a sharp fall in light intensity, boosting the LGMD/DCMD pathway, increasing their sensitivity to the loom specific inputs (Simmons, 1981). The general schematic of this inherently complex system consists of four distinct layers (Figure 4). Light is detected in layer 1 by the photoreceptor; within this layer postsynaptic neurons detect changes in illumination such as light-dark or dark-light transitions (O'Shea and Rowell, 1976; Rowell *et al.*, 1977). This results in a brief excitation which is passed on to layer 2 (Figure 4), here both excitatory and inhibitory synapses to layer 3 are made. The inhibitory synapses are involved in lateral inhibition whereby the excitatory signal sent to surrounding visual units is countered. It is the inhibitory synapses which tune the LGMD to moving edges such as looming stimuli (Rind and Bramwell, 1996). The excitatory synapses pass the signal to the next layer where, when the excitation is sufficient to overcome the lateral inhibition and pass threshold, an excitatory signal is passed to the LGMD (Figure 4: layer 4).

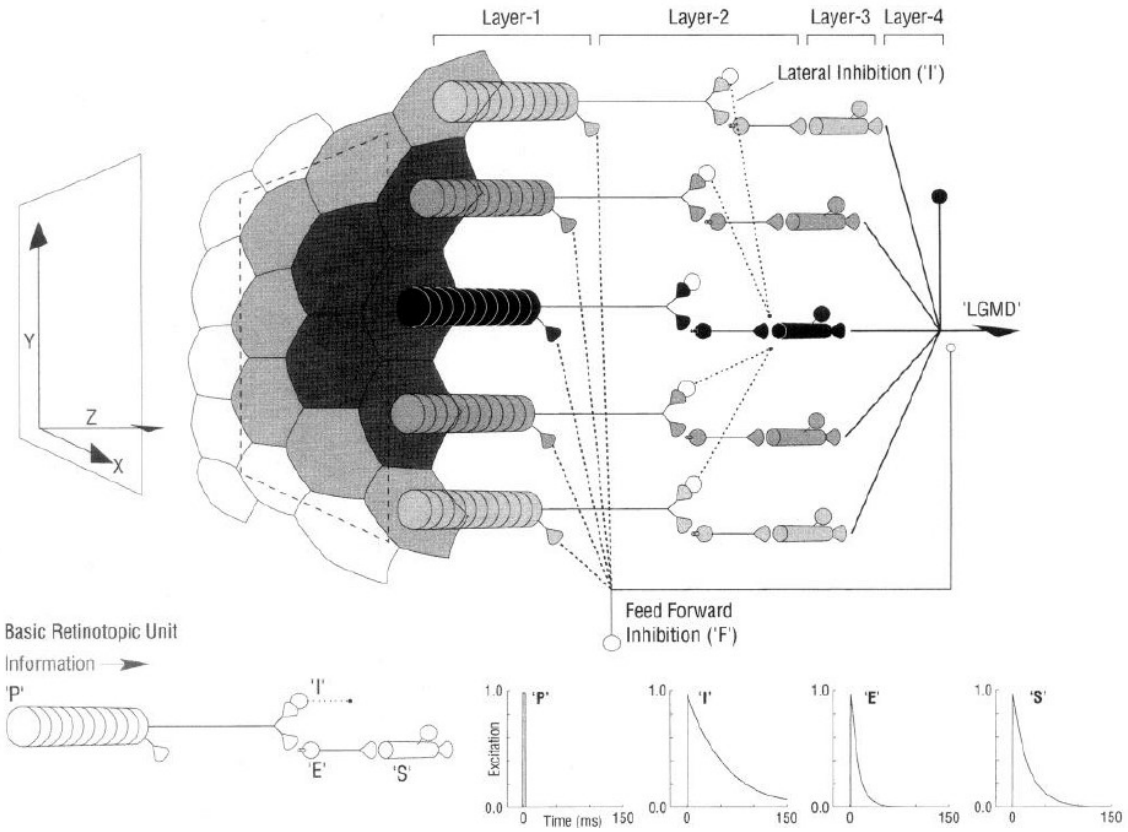


Figure 4: Schematic representation of the neural network involved in LGMD signal generation (Rind and Bramwell, 1996). Layer 1: Photoreceptor units (P) respond to change in illumination with an excitatory burst which was passed to layer 2. Layer 2: consists of three units, excitatory (E): which pass the signal on to layer 3, inhibitory (I): which carry out lateral inhibition with inhibitory synapses to the surrounding layer 3 sum (S) units, and finally feed-forward inhibitors (F): which bypass layer 3 directly inhibiting the LGMD. Layer 3: sum units (S) for each photoreceptor unit summed the E and I signals, exciting the LGMD (Layer 4) when above threshold. Upon activation the S units enter a refractory period.

The LGMD responds less strongly to non-looming stimuli, suggesting specific adaptations to expanding stimuli (Schlotterer, 1977; Rind and Simmons, 1992; Simmons and Rind, 1992; Hatsopoulos *et al.*, 1995; Gabbiani *et al.*, 1999), as opposed to simple translational stimuli (Krapp and Gabbiani, 2005; Simmons and Rind, 1992) or indeed non-collision looming stimuli (Gray *et al.*, 2001; Judge and Rind, 1997). The LGMD forms a 1 : 1 synapse with the ipsilateral DCMD, transmitting action potentials reliably (O'Shea and Williams, 1974; Rind and Bramwell, 1996), and is believed to be the sole input to the DCMD. The DCMD then carries these loom triggered potentials down the length of the central nervous system, making excitatory synapses in the thoracic ganglia to leg and flight motor neurons where it is believed to trigger avoidance behaviours (Simmons, 1980; Santer *et al.*, 2006; Fotowat and Gabbiani, 2007; Rogers *et al.*, 2010).

The DCMD is easily recorded from, since it is the largest axon in the longitudinal connectives of the ventral nerve cord (Simmons, 1981; Gaten *et al.*, 2012). Extracellular recordings from the neck connectives are dominated by large spikes from DCMD with spikes from other neurons less prominent. Circadian changes have been seen in the spiking activity of DCMD in response to a simple repeated looming stimulus (Figure 6; Gaten *et al.*, 2012). It is possible that the DCMD rhythmicity originates from changes either in the LGMD or DCMD itself (Gaten *et al.*, 2012). Alternatively it is possible the changes are evident in the rentinal sensitivity or the neurons and synapses prior to LGMD synaptic input (Figure 4, layers 1-3; Gaten *et al.*, 2012). As discussed previously, circadian oscillators have been detected in the lamina (represented by: Figure 4, Layers 2-3) and retinal region (Figure 4, Layer 1) of many species. Retinal sensitivity can be directly queried using the ERG technique. Work by Horridge *et al.* (1981) in gregarious *Valanga irregularis* has shown a diurnal difference between night and day adapted ERG intensity response curves (Figure 5b; Horridge *et al.*, 1980). These differences were seen despite dark adapting all animals for 15 min prior to recording. However Horridge *et al.* compare only two timepoints, and are therefore unable to make any claims of true diurnal rhythmicity and certainly not of circadian influence. This current work will aim to gather ERGs from a range of time-points in order to establish if the differences seen are present in *S. gregaria* and are diurnal or indeed circadian; in addition this work will compare solitarious and gregarious individuals. Further to the visual rhythms discussed, behavioural changes have been seen in activity level where general activity level was monitored over the course of 24 h (Figure 5; E. Gaten, unpublished) with gregarious animals showing a peak earlier in the photoperiod when compared to solitarious animals.

In the locust, FMRFamide neurons are present in the accessory medulla (Würden and Homberg, 1995; Pyza and Meinertzhagen, 2003), while serotoninergic fibres are thought to fire rhythmically during the night. These serotoninergic fibres originate in the proximal rim of the medulla and run to the lamina and into the eye (Nassel, 1988). Input from these fibres increases photoreceptor sensitivity (Cuttle *et al.*, 1995), so a lack of efferent input reduces sensitivity (Warrant and Nilsson, 2006). Whilst rhythmic firing of the serotonergic fibres occurs

during the night, they are silent during the day. Thus far this rhythm has not been investigated under constant light or constant dark conditions in the locust, and is therefore best described as diurnal (Cuttle *et al.*, 1995), however serotonin has been shown as a potential circadian driver in the visual system of the blow fly, *Calliphora vicina* (Cymborowski, 1998). The circadian rhythms in sensitivity seen in the DCMD activity (Figure 6; Gaten *et al.*, 2012), could be explained by such sensitivity changes at the retinal level. Alternatively serotonin could be modulating the sensitivity of LGMD/DCMD in addition to sensitivity of phototransduction. In addition to circadian rhythms, DCMD activity has been shown to be significantly affected by arousal (Rind *et al.*, 2008). It has been suggested that this could be due to raised octopamine levels given that responses reduce upon addition of epinastine (Rind *et al.*, 2008).

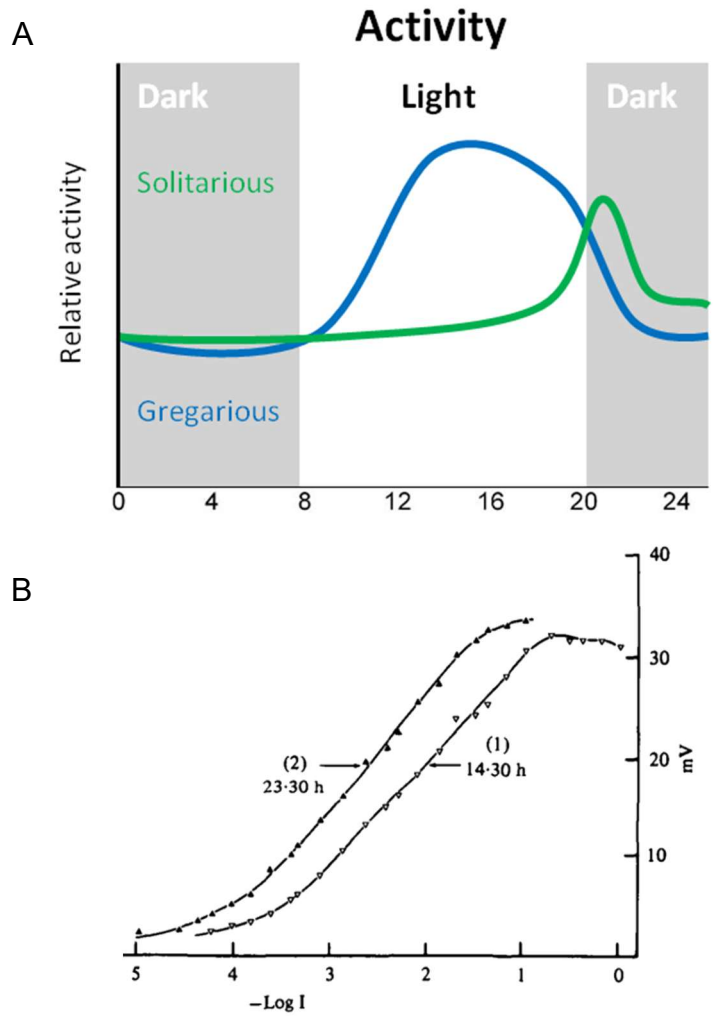


Figure 5: A: Relative activity levels of gregarious and solitarious animals in an arena recorded over 24-72 h periods. (E. Gaten, unpublished). B: Intensity response curve during the day (1) and night (2) in *Valanga irregulari* (Horridge *et al.*, 1980).

As in many species the desert locust's visual system plays a vital role in avoidance of predators and other threats. Loom sensitive visual pathways are tuned to stimuli looming on a direct collision course (Schlotterer, 1977; Hatsopoulos *et al.*, 1995; Rind and Simmons, 1992; Simmons and Rind, 1992; Santer *et al.*, 2006; Gabbiani *et al.*, 1999), these pathways are thought to be involved in both escape behaviour and collision avoidance in flight.

Behavioural responses of locusts to looming stimuli can consist of startle, withdrawal or a jumping response that acts to remove the animal from danger. The startle behaviour of resting locusts is characterised by a brief movement of the legs and body of the animal which results in no overall positional change (Friedel, 1999). Experiments involving behavioural response to looming stimuli in *S. gregaria* have either taken place with the animal fixed in a wind tunnel (e.g. Gray *et al.*, 2001), simulating flight, or resting on a pole free to move around the pole (e.g. Hassenstein and Hustert, 1999). The avoidance responses are defined as follows: in flight based assays the animal will move to change their direction of flight and avoid the stimulus in manoeuvres known as collision avoidance or alternatively rapidly lose height in a glide-dive (Gray *et al.*, 2001); in basking situations the animal rotates around the object it is resting on to create a barrier between itself and the approaching threat in a movement known as hiding (Gabbiani *et al.*, 1999; Hassenstein and Hustert, 1999). In jumping behaviour the animal kicks its hind legs, propelling the animal away from its current location, this allows the animal to become airborne and escape from a potential threat. The expansion velocity of the stimulus has been shown to affect the type of behaviour carried out; both extreme fast and slow stimuli result in startling behaviour, while moderate speeds favour a large angular hiding motion (Hassenstein and Hustert, 1999).

3.1.6 Population dependent phenotypic plasticity

The Desert Locust, *S. gregaria*, exhibits phenotypic plasticity in response to changes in population density (Uvarov, 1966; Roffey *et al.*, 1970; Simpson *et al.*, 1999; Rogers *et al.*, 2014). The phenotypes differ on morphological, neurophysiological and behavioural levels, with touch or a combination of smell and sight being key factors in

triggering the change (Simpson *et al.*, 1999; Rogers *et al.*, 2014). The solitarious phenotype is less active (Bouaïchi *et al.*, 1995), relying more heavily on cryptic appearance to avoid predators, as in the related species *Schistocerca emarginata* (Sword, 1999). Gregarious animals are more active generally, have a more general diet, and brighter colouration. A key behavioural difference between the phenotypes is the conspecific attraction exhibited by gregarious individuals (Rogers *et al.*, 2014; Uvarov, 1977), not present in solitarious animals that show active repulsion (Rogers *et al.*, 2010; Sword *et al.*, 2010; Rogers *et al.*, 2014). This conspecific attraction tends to reinforce the already increased population density, leading to the formation of large swarms.

Of particular relevance to this chapter are the population density dependent phenotype specific differences seen in the responses of DCMD. Generally gregarious animals show stronger DCMD responses compared to solitarious animals. In addition these responses show greater resistance to habituation (Matheson *et al.*, 2004; Rogers *et al.*, 2010). This is frequently explained in the context of the social environment in which the phenotypes occur, the gregarious animals must be able to continue to avoid repeated collision approaches from conspecifics (Matheson *et al.*, 2004; Rogers *et al.*, 2004, 2007). However, the mechanistic basis of the differences is unknown. The receptive field of LGMD (and thus DCMD) differs between the phenotypes (Rogers *et al.*, 2010); however this is limited to specific small regions, with large areas of the receptive field showing near equal responsiveness despite phenotypic differences in eye size, position and binocular overlap (Rogers *et al.*, 2010). Within this central region, peak DCMD firing rates and numbers of spikes evoked by looming stimuli were similar in both phenotypes (Rogers *et al.*, 2010).

The two phenotypes display different patterns of daily activity (Roffey and Popov, 1968; Uvarov, 1977; Steedman, 1990; Ely *et al.*, 2011; E. Gaten, unpublished) with gregarious animals being more active during the day and solitarious animals more active in the hours just after dusk (Steedman, 1990; Ely *et al.*, 2011). Steedman (1990) theorised that this was likely to be due to the diurnal activity of their avian predators. DCMD activity in response to looming stimuli also shows circadian

differences between the phenotypes (Gaten *et al.*, 2012). Gregarious animals exhibit an earlier peak in DCMD responsiveness compared to the solitarious animals. This phase shift between phenotypes may be driven in part by social interactions present in the crowded conditions used to rear the gregarious phenotype (Gaten *et al.*, 2012). This is in line with work on *Drosophila* and honey bees (Toma *et al.*, 2000; Levine *et al.*, 2002), which both show an effect of social interaction on the circadian clock. *Drosophila* shows a forward phase shift, while honey bees show an increase in *period* (a circadian gene) expression. In order to assess the hypothesis that social interaction drives a forward shift in the DCMD peak activity it is important to gain further insight into the circadian rhythmicity in the two extreme phenotypes, establishing if this is a consistent phase shift, including other circadian behaviours, indicative of an effect of crowding.

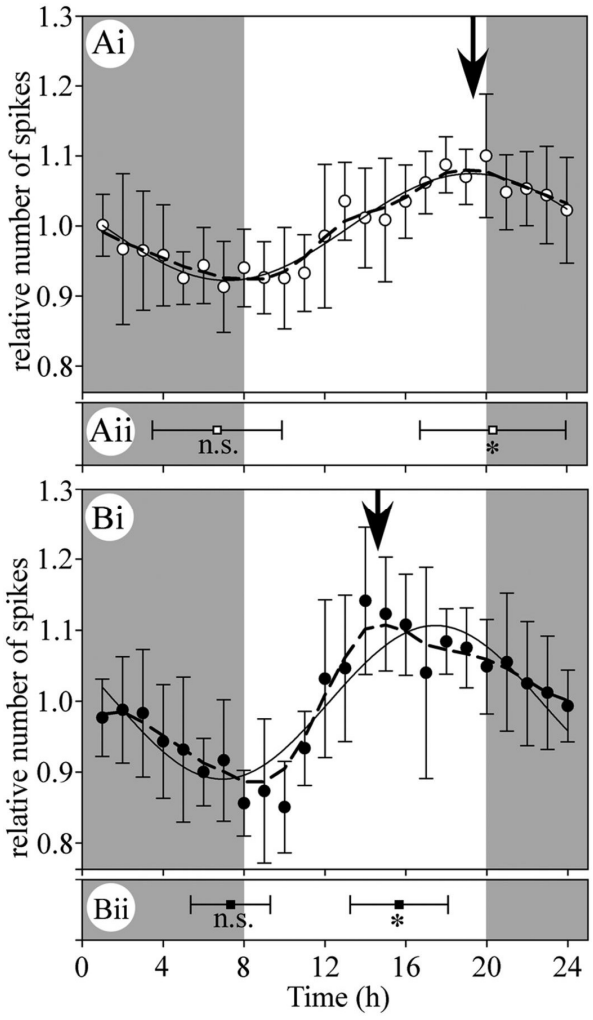


Figure 6: Relative number of spikes ± SD in DCMD elicited by a set looming stimulus (Gaten *et al.*, 2012). Solid lines represent a sinusoidal fit of the data, dashed lines represent a curve of best fit. Solid arrows show peak in number of spikes. Ai: Solitarious animals. Bi: Gregarious animals. Aii and Bii: mean minimum and peak response times ± SD. Solitarious and gregarious animals show different peak response times indicated by the black arrows.

3.1.7 Hypothesis

Here I use ERGs to investigate how the retinal response to standard light stimuli varies within diurnal and circadian frameworks and if any variations seen in the retina account for the DCMD rhythmicity reported in previous studies. The behavioural response to visual stimuli is also compared to the electrophysiological results in order to assess the impact DCMD activity may have on the behavioural output.

Electroretinograms are expected to follow the same pattern as DCMD spike count; with larger amplitude ERG responses correlating with higher spike counts. This outcome would suggest that circadian changes in retinal sensitivity are primarily responsible for the DCMD rhythmicity previously seen.

The type of behaviour expressed in response to a looming stimulus is expected to match general activity levels of the animal, with relatively passive startle responses occurring at times when the animals' activity levels are low and more active hiding occurring when the activity is high. If DCMD is directly involved in hiding, then hiding behaviour amplitude should be correlated with the number of DCMD spikes, regardless of stimulus properties.

By virtue of the expected links between ERG sensitivity and DCMD activity, as well as DCMD and hiding behaviour amplitude, it is expected that there will be a correlation between ERG sensitivity and behavioural amplitude. Larger ERG responses are expected to occur at the same time as larger behavioural responses.

3.2 Methods

3.2.1 General husbandry

Both solitarious and gregarious *Schistocerca gregaria* Forskål were maintained under 12:12 h light-dark, 36°-25 °C conditions. Light levels were between 750-1500 lx during the day and animals were fed *ad libitum* (fresh wheat seedlings and bran flakes). Solitarious animals, 2nd or 3rd generation isolated from the gregarious colony, were reared in 10 x 10 x 20 cm containers isolated from sight and smell of other animals. Gregarious individuals were kept at high density (100-300 per cage) in 50 x 50 x 50 cm containers. These conditions are identical to those used previously in numerous studies of gregarious and solitarious animals (Roessingh *et al.*, 1993; Matheson *et al.*, 2004; Gaten *et al.*, 2012).

3.2.2 Looming stimulus avoidance behaviour

Adult animals between the age of 1 and 3 weeks post imaginal moult were selected to maintain an even gender distribution. Experiments were carried out at key time points that were selected based on previous work on DCMD sensitivity to a looming stimulus (Figure 6; Gaten *et al.*, 2012). The times selected were 12:00, 15:00, 19:00 and 21:00, with the 15:00 and 19:00 time points expected to align with peaks in gregarious and solitarious response levels respectively. All experiments were carried out under ambient lighting of 200 lx to maintain the contrast conditions of the stimulus. Lights were extinguished at 20:00 (as per normal rearing conditions). All animals were allowed to acclimatise at the 200 lx light level at 25 °C for 15 min prior to the start of the experiment to prevent light adaptation affecting responses (Figure 7; Cosens, 1966). Acclimatisation was limited to 15 min to prevent solitarisation of gregarious animals and to permit the animals to be processed within 30 min of the required time-point.

Animals were placed head up on a vertical 8 mm diameter pole 18 cm from a cathode ray tube (CRT) monitor displaying a looming stimulus (Figure 8A), the pole was rotated where possible to avoid handling the animal. The looming stimulus (Gaten *et al.*, 2012) was generated by custom software (S. Huston) triggered by an external pulse. It consisted of a black square on a green background, sequentially enlarging to simulate the approach of an object with an L/V ratio (half-length/velocity) of 20 s^{-1}. The CRT

monitor had a refresh rate of 160 Hz and had an angular resolution of < 0.22° measured at the eye. These values ensured the animal would not perceive any stepwise increments in stimulus size and would perceive the stimulus as a continuously expanding shape (Howard, 1981; Horridge, 1978). The animal was allowed 5 min before being rotated such that the animal was perpendicular to the stimulus with the left eye facing the stimulus (defined as 0°).

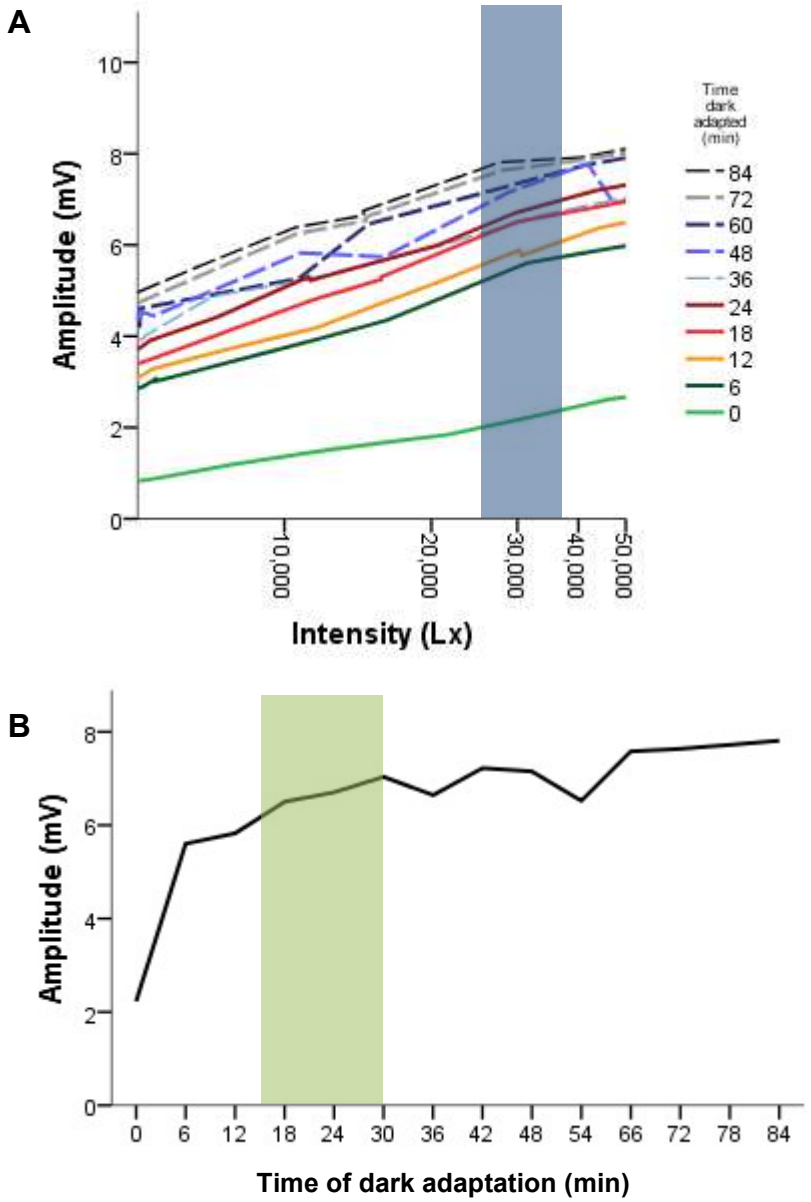


Figure 7: Effect of dark adaption on the ERG of an individual animal. A: amplitude of ERG response plotted against intensity of light pulse. ERG recordings were taken every 6 minutes after lights off. Recordings more than 30 min after lights off are shown as dashed lines. The blue box indicates data shown in B. B: ERG response amplitude in response to 25000-35000 lx stimuli plotted against length of dark adaption (minutes). The green box indicates the period during which recordings were carried out both in 3.2.2 and 3.2.3 (15-30 min).

For the duration of the experiment the animal was recorded from above (Bassler AG601FC; 16 mm/f 1.4), allowing the angle of the animal relative to the stimulus to be measured. A red LED in the field of view of the camera flashed at the start of a stimulus, enabling synchronisation between the video recording and the stimulus (Figure 8B). The video recording and stimulus were synchronised by a data acquisition computer (Micro1401 Mk II; Cambridge Electronic Design (CED)) running Spike2 (10.4 Hz; CED), which allowed each stimulus to be easily identified by the stimulus trigger. The computer providing the looming stimulus also fed-back a sustained pulse to the recording computer indicating the stimulus was active. Should the animal jump off the pole at any stage it was replaced on the pole and allowed to rest for 2 min. The starting angle was recorded to account for misaligned animals. After the first stimulus and response, the pole was rotated to align the animal to the 180° position, such that its right eye was facing the stimulus. The animal was allowed 5 min before the second stimulus was provided. Thereafter the animal was rotated to either the 0° or 180° position between each stimulus. The inter-stimulus interval was at least 120 s after the initial two stimuli. As many stimuli were presented to the animal as possible within a 30 min period, giving a maximum of the initial two stimuli (10 min) and a further 10 stimuli. The behavioural response to a looming stimulus was analysed by defining each event as one of three behaviour types: startle, hide or jump. In a startle response the animal stopped movement (such as grooming or swaying) upon initiation of the stimulus and carried out no overall locomotion during the stimulus, although they may jerk or twitch. Hiding responses were defined by the animal rotating around the pole whilst remaining in contact with it. These responses were measured in terms of angular rotation during the stimulus. A response was defined as a jump if the animal left the pole during the course of the stimulus. The end angular data in this case was defined as the angle at which the animal left the camera's field of view while in the jump.

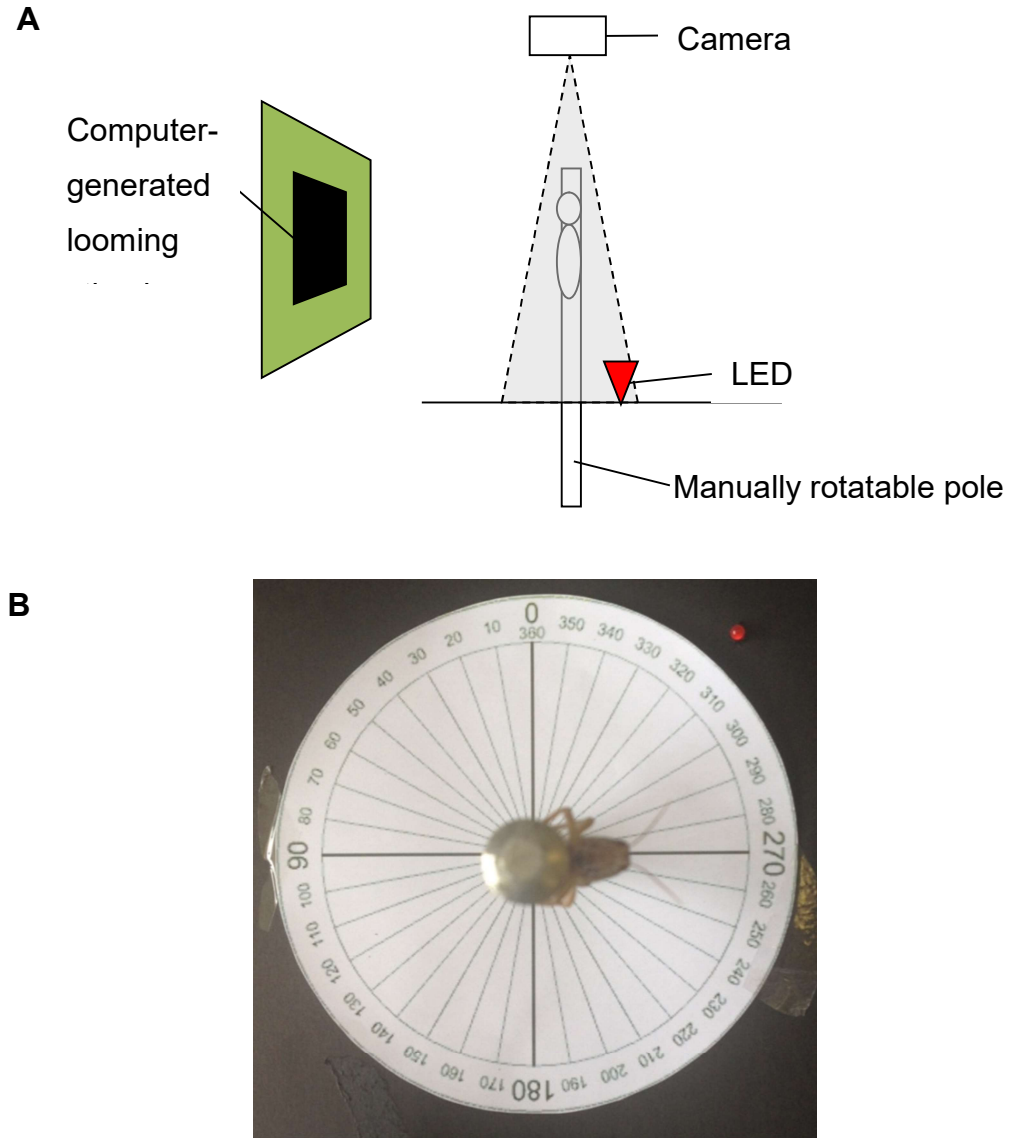


Figure 8: A) Experimental schematic for loom response assay. Animal situated on manually rotatable pole, perpendicular to the CRT monitor providing the stimulus. The stimulus was synchronised to the recording via a red LED. B) The stimulus was presented to the bottom of the field of view of the camera, and the red LED at the top right. The pictured animal is recorded as at 5° from perpendicular to the approach of the stimulus.

3.2.3 Electroretinogram

Adult animals were used, between the age of 1 and 3 weeks post-imaginal moult. Previous experiments on the visual system revealed no difference between sexes (Matheson *et al.*, 2004; Gaten *et al.*, 2012) so animals were utilised as available, although a balance of males and females animals was maintained where possible. Experiments were carried out using both solitary and gregarious animals at 8 time points per condition: first in normal lighting (LD, 12:12 h, light : dark cycle), and then in two subsequent 24 h periods of constant dark. Each animal was used for one time point only. 3 animals were used per time-point per condition.

For recordings, an animal was secured in a 1.5 cm diameter plastic tube and the head immobilised with beeswax. A silver reference electrode was inserted into the haemolymph of the head, centrally above the brain, and secured with beeswax. A 10 MΩ tungsten electrode (insulated until 1 mm from the tip) was inserted 15 µm beyond the surface of the cuticle of the animal's left eye through the use of a precision manipulator; the same electrode was used throughout all experiments. Recording depth was consistent between experiments because response amplitude varies with depth (Naka and Kuwabara, 1959). Experiments carried out at night were set up under red light.

Before proceeding, the animal's eye was exposed to a single white light pulse, and the recording was compared to a typical crystalline cone layer ERG response (Figure 2; Figure 9B), all recordings satisfied this criterion. All lights were then switched off and the animal left to acclimatise to the conditions at 25 °C for 15 min. Each recording set consisted of 30 light pulses of 200 ms duration from a 100 W xenon arc lamp at a distance of 100 cm focused on the eye subtending 3° at the animals eye; the shutter was automatically activated by the recording computer. The intensity of these pulses was controlled by a combination of manually adjusted static neutral-density filters and an automated rotating neutral-density filter housed in a custom-built apparatus (Figure 9A). Stimuli spanned a 4 log unit range from 8 to 160000 lux (cd·sr/m^2) covering a full range of responses, from just above threshold to saturation (Figure 9C). Stimuli were presented in three groups of 10 at high, medium and low light intensities pseudo-randomly selected. The light-intensity of the pulse was recorded using a light-sensitive

diode (TSLG257; Texas Advanced Optoelectronic Solutions) shielded by a static neutral-density filter to bring the pulse within the sensitive range of the diode. Prior to the experiment, light intensity at the location of the animal's eye was calibrated with respect to the signal received by the diode under each combination of filters utilising a PhotoMeterPMLX (Quantum Instruments Inc.). Data were recorded at 10.4 kHz using a Cambridge Electronic Design data acquisition system (Micro1401 Mk II; CED) and Spike 2 software (CED). Analysis was carried out using a custom written Spike2 script which calculated the maximum amplitude of the ERG in response to the light pulse (the maximum difference between baseline and the response - this value was therefore positive), the maximal slope of the response, the time taken to depolarise to 50% and the latency between the light pulse and the start of the response (Figure 9B).

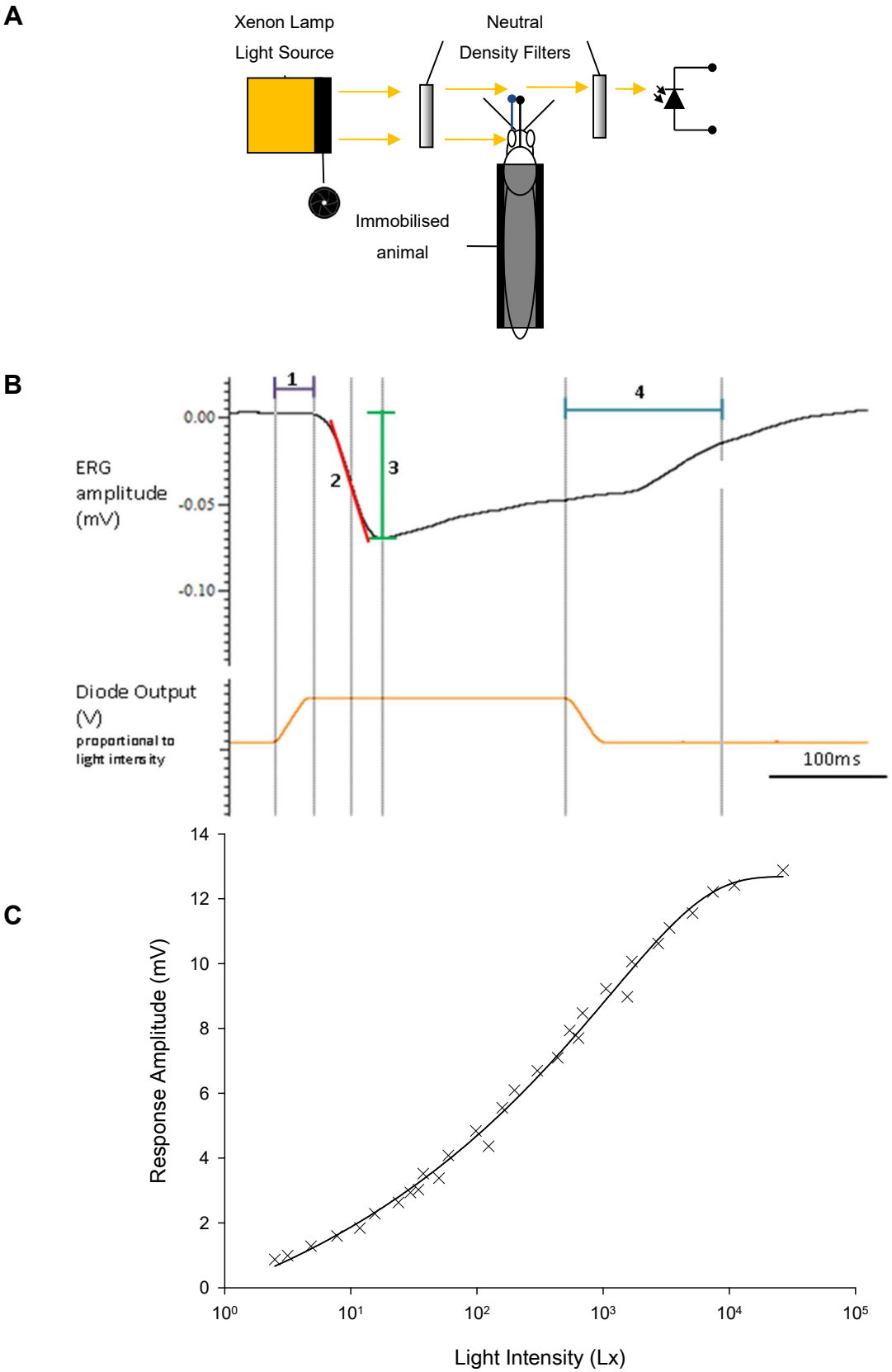


Figure 9: A) Schematic diagram of electroretinogram experiment. The photosensitive diode was connected to the recording computer. B) Example of typical electroretinogram trace with measures shown. Purple (1): latency; Red (2): maximal slope; Green (3): maximal response amplitude; Blue (4): 50% repolarisation time. C) Example intensity response plot with Naka-Rushton model plotted. Increasing intensity of light beyond the threshold level led to an increase in response amplitude. In this example between 10^3 and 10^4 lx the relationship is logarithmic. Above 10^4 lx the relationship begins to saturate, with the highest intensity stimuli generating approximately 8 mV in extracellular response.

3.2.4 Naka-Rushton

The ERGs were analysed not only by extracting the key features as described above, but also by characterising the intensity-response (IR) curve by fitting it with a Naka-Rushton model as is appropriate for retinal sensitivity (eq. 1; Evans *et al.*, 1993). The maximum response indicates the saturated response amplitude. The half saturation intensity is the intensity of light that is required for the model to reach 50% saturation. The exponent controls the shape of the model, the rate of change of gradient in the log regions.

$$R = R_{max}\frac{i^n}{(i^n + i_{50}^n)} \qquad (1)$$

Where: R = response (mV) Rmax = maximum response (mV) i = intensity of stimulus (lx) i50 = half saturation intensity (lx) n = exponent

3.2.5 Cosinor analysis

Analysis of the diurnal rhythmicity of the data was carried out using Cosinor analysis that is appropriate for short or sparse datasets (Cornelissen, 2014). Cosinor analysis is a process by which a sinusoidal relationship (eq. 2) can be fit by linear regression by considering the time as two derivative functions (eq. 3). The significance of these two functions within the regression gives the significance of a 24 h cycle on the data. The null hypothesis is that there is no rhythmicity. By rejecting the null hypothesis it can be stated that the sinusoidal fit has a significant influence on the tested variable. The resulting model fit may not fully fit the variable for many reasons, as other factors may be affecting the result; however a significant Cosinor fit confirms that a cyclic rhythm is one factor. This fitting process usually assumes a 24 h cycle, and therefore should be carried out separately for each 24 h period. If multiple-cycle datasets are tested, any phase shift due to a shorter or longer cycle will reduce any significance of the test. By changing the assumption of the period length it is possible to test for a specific cycle period.

With that in mind the ERG data were first analysed by carrying out a linear least squares regression fitting the Cosinor functions (eq. 3) to the data for each 24 h period. This gives significant results where the data show diurnal (LD) or circadian (DD)

patterns. Understanding that the 24 h period length assumption may not hold, particularly under DD conditions, each dataset (gregarious and solitarious) was tested with a non-linear least squares regression of the sinusoidal (eq. 2). On the basis of the results of these fits it was decided to use both the 24 h period length and a 20 h period length; the best fitting of these two fits was used to visually display the rhythm. Significance of the model was determined by ANOVA. Where the period length assumption is anything other than 24 h in Cosinor analysis this is explicitly stated.

$$Y_t = M + A\cos\left(\frac{2\pi t}{\tau} + \phi\right) \quad (2)$$

$$Y_t = M + A.\sin(2\pi \times \frac{t}{\tau}) + B.\cos(2\pi \times \frac{t}{\tau}) \quad (3)$$

Where: **t = time,** τ **= assumed period length** **M = Meso (average response)**

A = Amplitude of cycle ϕ **= acrophase (time of peaks)** **Y_t= Amplitude at time t**

3.3 Results

3.3.1 Looming stimulus avoidance behaviour

Locusts were exposed to looming stimuli in the visual field of the eye at four time-points spanning noon to dusk. In response, locusts carried out one of three characteristic behaviours: startle; hiding; or jumping. The type and amplitude (angle of movement) of response was recorded.

The type of behaviour triggered by the stimulus was calculated as a percentage of responses per animal (N_g = 25; N_s = 31) per time point. Each animal was tested at each time point. Cosinor analysis of frequency of behavioural response types showed that no significant circadian pattern was present (Table 1). The frequency data for jumping behaviour showed high kurtosis (18.12) preventing Cosinor analysis. As a result of the lack of rhythmicity, data were pooled across time points to compare the two phenotypes. The frequencies of startle (Mann-Whitney, P < 0.001), hide (Mann-Whitney, P < 0.001) and jump (Mann-Whitney, P = 0.005) behaviours were significantly different by phenotype (Figure 10). Gregarious animals startled on 0% of trials (median; IQR = 15.1) while solitarious animals startled in response to 37.5% of stimuli (median; IQR = 29.8). Gregarious animals hid in response to 87.5% (SD = 15.1) of stimuli while solitarious animals hid in response to 60% (SD = 29.7) of trials. Jumping accounted for 0% (median; IQR = 8.6) of responses in gregarious animals and 0% (median; IQR = 0.2) of responses in solitarious animals.

The angular amplitudes of hiding responses were compared in a second analysis. The two phenotypes differed significantly in terms of angular amplitude (Mann-Whitney U, P < 0.001), with gregarious animals rotating an average 16° (median; IQR = 32°) in response to the stimuli, the solitarious animals rotating an average of 9° (median; IQR = 18°) (Figure 11A). The effect of starting angle (median = 0, IQR = 12) in these experiments did have a significant effect on turn angle (Kruskal Wallis, df = 84, *P* = 0.007; Figure 11A) although start angles were not significantly different between solitarious and gregarious (Mann-Whitney U, *P* = 0.863).

Cosinor analysis of the angular amplitude of hiding responses (Figure 11b) revealed a significant fit of the Cosinor functions in solitarious animals (ANOVA, F(2,577) = 5.541,

P = 0.004). Similar analysis of gregarious data was not significant (ANOVA, F(2,641) = 1.914, P = 0.148). The significance of the solitarious pattern is probably driven in large part by the low response amplitudes at 21:00. The coefficients of the Cosinor analysis indicate a peak response at 15:20. The difference between the response amplitudes at 19:00 and 21:00 in solitarious animals is the only significant difference between concurrent time-points in either phenotype.

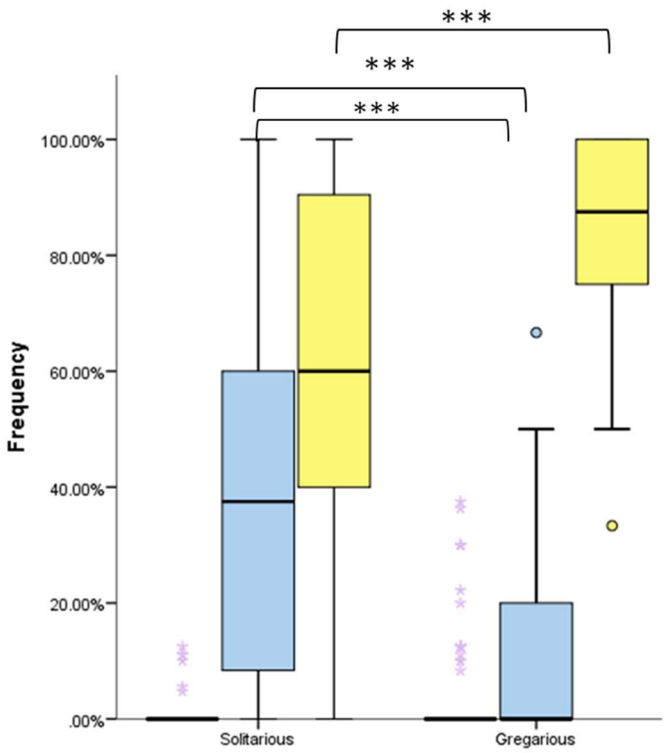


Figure 10: Percentage of responses categorised as startle, hide or jump. Significant differences were seen between phenotypes for all behaviours, with gregarious animals tending to hide and jump more often than solitarious animals which tended to startle more frequently. Jump: purple; Startle: blue; Hide: yellow.

Table 1: Cosinor analysis, assuming 24 h period, of behaviour type in response to looming stimuli across the 4 time points. Each behavioural type was reported as percent of response for each animal at each time point.

ANOVA				
Phenotype	Behavioural type	df	F	Sig.
Gregarious	Startle	(2,84)	2.044	0.136
	Hide	(2,84)	1.379	0.257
	Jump	No	Test	-
Solitarious	Startle	(2,100)	1.543	0.219
	Hide	(2,100)	1.281	0.282
	Jump	No	Test	-

By including angular data for all behavioural types (startle and jump as well as hiding) the significance of the solitarious rhythmicity increases (ANOVA, $F(2,960) = 7.253$, $P = 0.001$) with a similar indicated peak (15:22) and the gregarious data produce a significant (ANOVA, $F(2,764) = 3.348$, $P = 0.034$) regression with an indicated peak at 16:23.

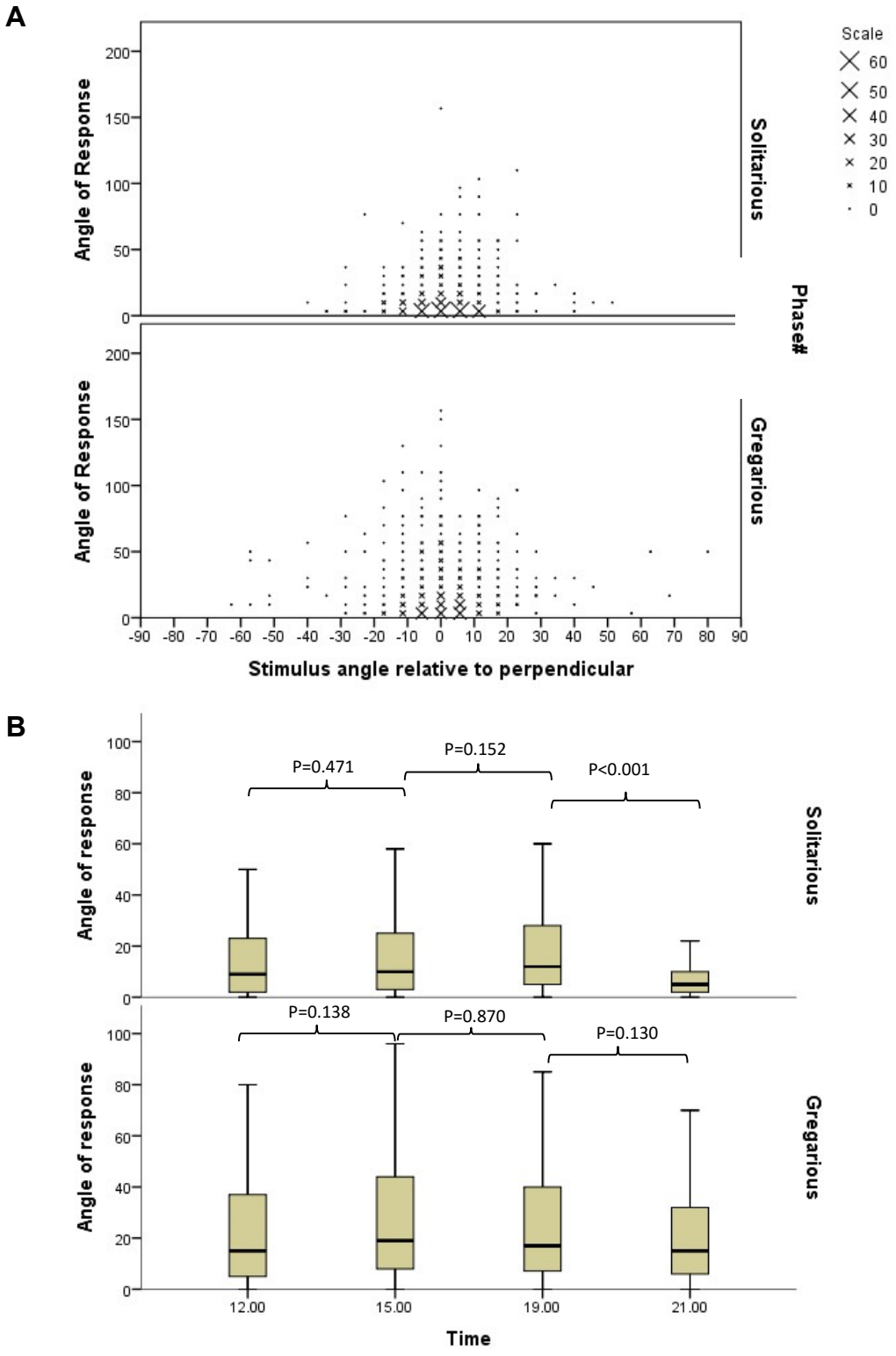


Figure 11: A) Distribution of response angles against starting angle (Stimulus angle). Positive start angles represent the animal rotated away from the stimulus, negative start angles represent the animal rotated toward the stimulus. Data are binned per point with the size of the data point indicating the number of responses. The starting angle is normally distributed. B) The angular amplitude of the hiding behaviour plotted for each time point. Times are in GMT, with lights off at 8pm (20:00). Solitarious animals show a maximal response at approximately 19:00 with a marked reduction after dusk, gregarious animals showed an insignificant difference with a maximal response between 15:00 and 19:00. Significance determined by Mann-Whitney.

3.3.2 Electroretinogram

To determine whether the electroretinogram response was modulated in a circadian or diurnal pattern, the ERG amplitude, slope and recovery time were extracted for each individual response. The amplitude of response was plotted against the intensity of the stimulus for each animal, and most datasets were successfully fit by the Naka-Rushton model (eq 1.; average R^2 = 0.974, Standard deviation = 0.038; N = 127, 60 gregarious of 69, 67 solitarious of 75). Datasets that were not successfully fit were excluded from the downstream analysis as the associated parameters were not representative of the intensity response curve. Figure 12 shows these intensity-response functions separated by phenotype and time-point. Multiple animals are shown in each plot (N = 2-4 per time point) to show the consistent sigmoidal shape of the data. The shape of the gregarious plots at ZT49 and ZT52 are unexpected and this should be considered when examining the data.

The three parameters of the Naka-Rushton fits to the intensity-response plots (see Methods) were subsequently fit with Cosinor functions to seek circadian patterns of change in the ERG responses (Figure 13). The maximal response and exponent variables were not well fit by 24 h Cosinor functions in either phenotype, using all time-points or restricting the fits to each 24 h period (Figure 13, Table 2; Table 3). The log of the half saturation intensity was also not significantly fit by 24 h Cosinor functions using all time points in gregarious or solitarious groups (linear regression ANOVA; gregarious F(2,57) = 1.608, *P* = 0.209; solitarious F(2,64) = 0.618, P = 0.542). A significant fit between the Cosinor functions and the log of half saturation intensity was seen in the first 24 h period (Figure 13, linear regression ANOVA; gregarious F(2,17) = 12.779, *P* < 0.001; Solitarious F(2,18) = 7.775, *P* = 0.04) (Figure 13). Peak sensitivity, lowest value, was estimated at 16:09 in gregarious animals and 15:45 in solitarious animals. This significant relationship was not maintained for the 24-48 h or 48-72 h periods). In all three cases 20 h Cosinor function fits were also unsuccessful.

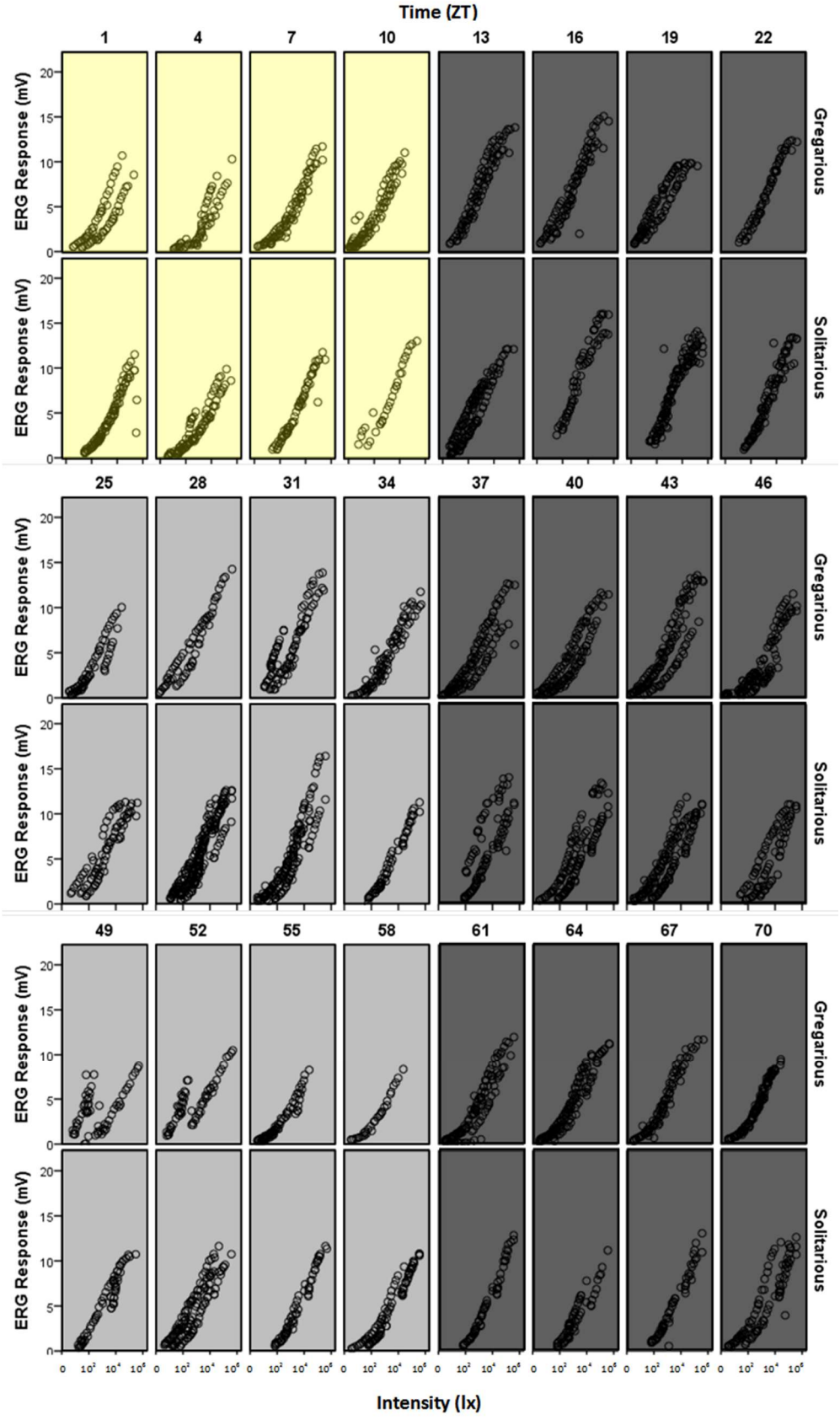


Figure 12: Intensity-response graphs for all collected data, multiple animals are shown per time point. Yellow background indicates where the animal was under light conditions prior to the experiment being set up, both light and dark grey indicate dark conditions, with light grey indicating subjective day and dark grey subjective night.

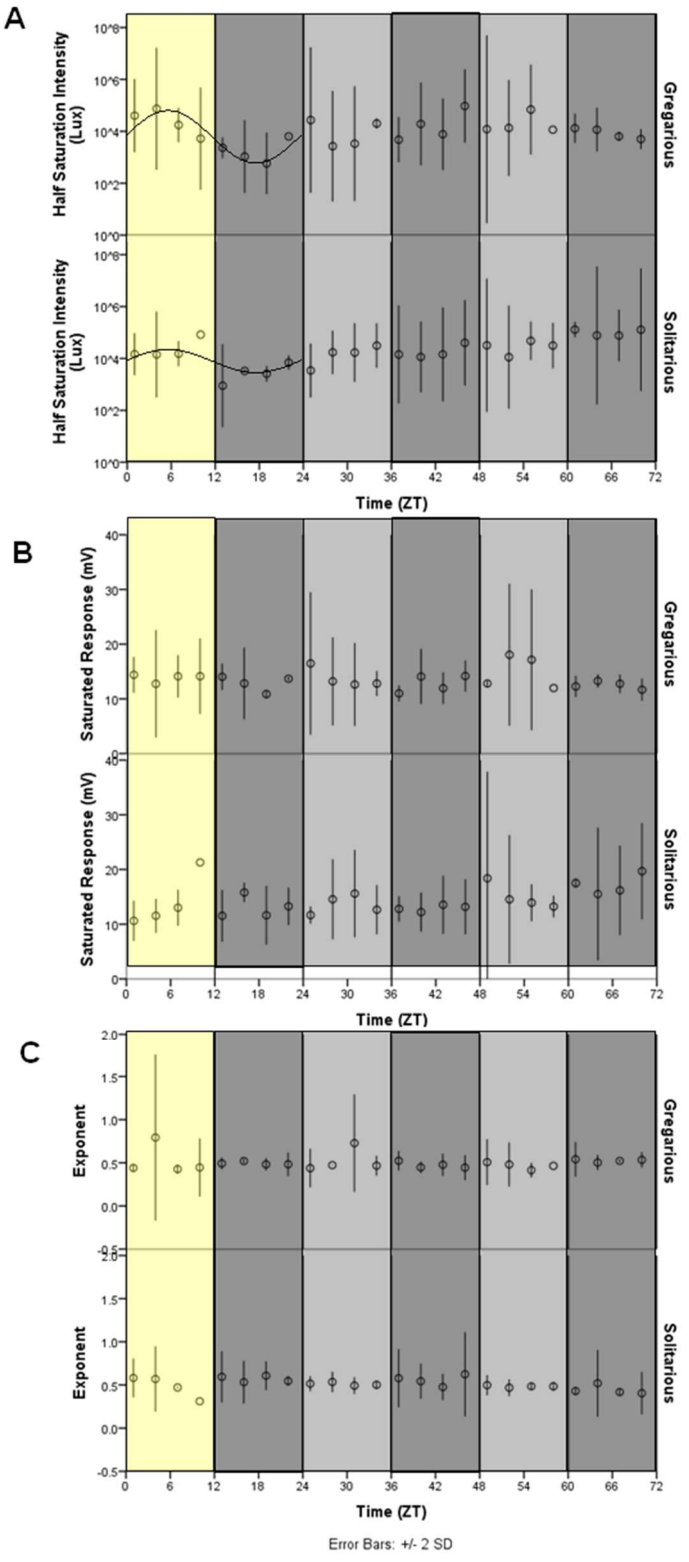


Figure 13 – Values of Naka-Rushton fit parameters: half saturation intensity (A), saturated response (B) and the exponent (C). Yellow background indicates the animal was under light conditions prior to the experiment, both light and dark grey indicate dark conditions with light grey indicating subjective day and dark grey subjective night.

Table 2: 24 h period length Cosinor analysis of Naka-Rushton model exponent parameter. Red cells are non-significant (p > 0.05)

Time	ANOVA			
	Phenotype	df	F	p
0-24	Gregarious	2	0.280	0.759
	Solitarious	2	0.947	0.405
24-48	Gregarious	2	2.450	0.108
	Solitarious	2	0.087	0.917
48-72	Gregarious	2	1.457	0.258
	Solitarious	2	0.550	0.587
All time points	Gregarious	2	0.497	0.611
	Solitarious	2	0.487	0.616

Table 3: 24 h period length Cosinor analysis of Naka-Rushton model maximal response parameter. Red cells are non-significant (p > 0.05)

Time	ANOVA			
	Phenotype	df	F	P
0-24	Gregarious	2	1.048	0.371
	Solitarious	2	0.974	0.395
24-48	Gregarious	2	1.604	0.223
	Solitarious	2	1.215	0.311
48-72	Gregarious	2	3.158	0.065
	Solitarious	2	1.578	0.235
All time points	Gregarious	2	2.907	0.062
	Solitarious	2	0.077	0.926

Having compared the parameters of the Naka-Rushton fits response amplitude, response slope, and response recovery time of the individual ERGs (see Methods) recorded during exposure to stimuli of 250-750 lx were compared pooled across all time points to establish differences between phenotypes in these variables. Following that they were fit with Cosinor functions (Figure 14). This range was selected prior to analysis on the basis of preliminary work (e.g. Figure 9C) which showed this range to be where the intensity response curve showed a logarithmic relationship.

Response amplitude (ANOVA, $F(1,670) = 14.103$, $P < 0.001$) and the maximal slope of the response (ANOVA, $F(1,670) = 11.045$, $P = 0.001$) both showed significant differences between the phenotypes. The maximum ERG onset slope was steeper (more negative) in gregarious animals (mean = -141 mVs^{-1}, SD = 63.0 mVs^{-1}) than in the solitarious animals (mean = -124 mVs^{-1} SD = 68.4 mVs^{-1}). Gregarious animals showed higher response amplitude (mean = 4.07 mV, SD = 1.58 mV) compared to the solitarious animals (mean = 3.26 mV, SD = 1.51). The 50% repolarisation time did not differ significantly (ANOVA, $F(1,577)$, $P = 0.264$), with an average of 0.09 s (SD = 0.038 and 0.023) in both phenotypes.

Cosinor analysis was carried out with both a 20 h and 24 h period length (see Methods). Where both tests showed significance only the strongly significant fit (adj $p < 0.001$) was plotted (Table 4; coloured fits Figure 14).

Response amplitude was fit significantly with the Cosinor functions when using the whole dataset (coloured fit Figure 14; Table 4). To establish any diurnal (in LD) or circadian (in DD) effects each 24 h period was analysed individually. Significance was found in the first 24 h period (linear regression ANOVA; gregarious, $F(2,85) = 86.939$, $P < 0.001$; Solitarious, $F(2,109) = 72.558$, $P < 0.001$, black fits in Figure 14A) and in the third 24-hour period (Linear regression ANOVA; gregarious, $F(2,92) = 7.002$, $P = 0.001$; solitarious, $F(2,98) = 17.657$, $P < 0.001$, black fits in Figure 14A). No significant pattern was found in the second 24 h period. Response amplitude difference in the first 24 h (gregarious: 6 mV; Solitarious: 4 mV) is larger than the third 24 h difference (1 mV; 1 mV).

The response gradient (Figure 14b) was strongly correlated with the peak response amplitude across the dataset (ANOVA, $F(1,670) = 1958$, $P < 0.001$). Strongly significant fit were seen by Cosinor functions (Table 4) across the whole 72 h (coloured fit in Figure 14B), the first 24 h and the last 24 h (black fits in Figure 14B). A significant rhythm was also present in gregarious animals in the second 24 h period (black fit in Figure 14B).

The 50% repolarisation time (Figure 14c) was not fit significantly by 24 h Cosinor functions across all the time points (Table 6). Significance of the fit of 50% repolarisation time by 24 h Cosinor functions was seen in the first 24 h period in both phenotypes as well as the second day (black fits in Figure 14C). No pattern was seen on the third day.

Table 4: Cosinor analysis with 20 h compared to 24 h period length. p values have been adjusted to take into account repeated tests. Green cells are highly significant (adj p <0.001), orange cells are significant (adj p<0.05), red cells are non-significant (adj p > 0.05).

Variable		20 h			24 h		
	Phenotype	df	F	Adj p	df	F	Adj p
Response Amplitude	Gregarious	(2,234)	24.083	<0.001	(2,234)	7.070	0.002
	Solitarious	(2,339)	5.184	0.012	(2,339)	10.541	<0.001
Peak gradient	Gregarious	(2,236)	15.858	<0.001	(2,334)	2.879	0.116
	Solitarious	(2,341)	6.344	0.004	(2,339)	10.144	<0.001

Table 5: Cosinor analysis with 24 h period length of ERG response gradient for three 24 h periods and all 72 h combined. Green cells are highly significant (p <0.001), orange cells are significant (p<0.05), red cells are non-significant (p > 0.05).

Time	ANOVA			
	Phenotype	df	F	p
0-24	Gregarious	(2,85)	53.774	<0.000
	Solitarious	(2,109)	50.775	<0.000
24-48	Gregarious	(2,110)	5.496	0.005
	Solitarious	(2,160)	1.046	0.354
48-72	Gregarious	(2,92)	3.573	0.032
	Solitarious	(2,98)	4.869	0.010
All time points	Gregarious	(2,334)	2.879	0.058
	Solitarious	(2,339)	10.144	<0.001

Table 6: Cosinor analysis with 24 h period length of ERG 50% repolarisation time, for three 24 h periods and all 72 h combined. Green cells are highly significant (p <0.001), orange cells are significant (p<0.05), red cells are non-significant (p > 0.05).

Time	ANOVA			
	Phenotype	df	F	p
0-24	Gregarious	(2,61)	5.713	0.005
	Solitarious	(2,81)	18.260	<0.001
24-48	Gregarious	(2,86)	12.622	<0.001
	Solitarious	(2,160)	3.241	0.042
48-72	Gregarious	(2,81)	0.754	0.474
	Solitarious	(2,92)	0.396	0.674
all time points	Gregarious	(2,234)	0.301	0.740
	Solitarious	(2,339)	1.558	0.212

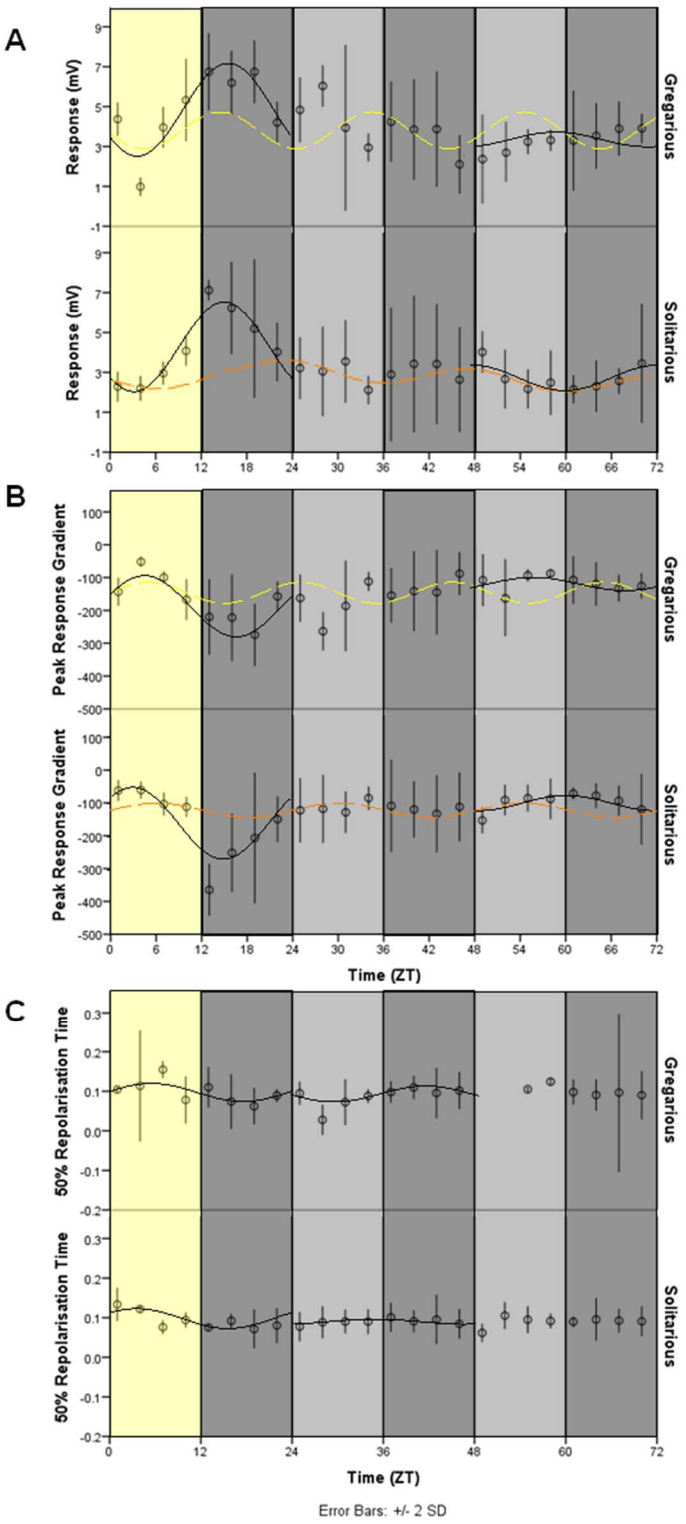


Figure 14 – ERG responses from light intensities 250-750 lx. Response amplitude (A), response gradient (B) and 50% repolarisation time (C). Yellow background indicates the animal was under lit conditions prior to the experiment, both light and dark grey indicate dark conditions with light grey indicating subjective day and dark grey subjective night. Black solid lines show Cosinor fit for 24 h periods. Dashed lines show Cosinor fit for 24 h (yellow) and 20 h (orange) across all time points.

3.4 Discussion

This work has investigated two main variables in each experiment, the phenotype of the animal and the time of day. This discussion will first focus on differences between solitarious and gregarious animals before turning to the effect of the time of day on the two phenotypes.

3.4.1 Population density dependent differences

In this section I discuss the differences between the two extreme phenotypes pooled across all time points as well as the possible causes and implications.

The two extreme phenotypes differ in their behavioural response to a looming stimulus. Solitarious animals are 35.7% more likely to startle than gregarious animals. Conversely, gregarious animals are 27.5% more likely to actively move around the pole in a hiding manoeuvre. The two phenotypes thus utilise two distinct behavioural response preferences despite being in identical environments, specifically both were tested in the absence of conspecifics.

The type of behavioural response shown to a looming stimulus in *Anolis cristatellus*, the Puerto Rican crested anole, and *Anolis stratulus*, the barred anole, has been shown to depend on the effectiveness of the camouflage. Lizards better camouflaged remained stationary for a longer duration of the approach, whilst those less well camouflaged moved to escape sooner (Heatwole, 1968). It is therefore likely that the different behavioural preferences are due to the differing camouflage exhibited by the two phenotypes. Solitarious animals have a more uniform light straw colouration whereas gregarious animals are brighter yellow with black patches that provide them with more broken edges (Uvarov, 1966). The startle response in solitarious animals is likely to act to maximise the efficiency of their cryptic appearance; conversely gregarious animals move, which may act to confuse or divert the predator onto a nearby conspecifics making best use of their high contrast colouring and high population density. The gregarious results are similar to the results of Hassenstein and Hustert (1990) in *L. migratoria* where the gregarious animals responded to looming stimuli from angles 70-120° provoking only active hiding, with no startle responses seen. In the current data some startle occurred. It is likely that this is due to the two

stimuli not being identical; stimuli used by Hassenstein and Hustert (1990) had constant radial expansion whilst this current work used a more complex stimuli that models more closely an approaching object (see Methods). A further difference is that the experiments carried out by Hassenstein and Hustert (1990) utilised 5th instar animals, whilst this current work utilised adult animals. By contrast the behaviour of solitarious locusts seen in this current work is most similar to that seen in extreme dorsal stimuli in gregarious animals by Hassenstein and Hustert (1990). This represents a significant shift in behavioural preferences between the two extreme phenotypes.

The angular amplitude of the active hiding responses is 60% higher in gregarious animals. In the context of Hassenstein and Hustert (1990) the majority of solitarious movements would not have been classed as hiding (< 20°), by contrast the majority of gregarious responses would have been classed as hiding (> 20°). The smaller movement in solitarious animals may act to reduce detection, with larger movements more likely to draw the attention of predators that may not yet have detected the animal. The movement itself would still require a correction by a predator targeting the locust and as such represents a balance between avoidance and detection. This preference to remain hidden is discussed in more detail previously in the context of hiding behaviour compared to startle behaviour. It is possible that gregarious animals turn further because their eyes are more sensitive, as seen in the ERG results as discussed below and DCMD responsiveness (Rogers *et al*., 2010). This may increase the perceived significance of an identical stimulus to the gregarious animals, prompting a larger behavioural response amplitude.

Hassenstein and Hustert (1990) saw hiding achieved most reliably between -40 and +30° relative to the perpendicular. The vast majority of stimuli provided in the current study were within this range. Hassenstein and Hustert (1990) showed that the majority of animals rotate such that they are able to track the target with the ipsilateral eye, stopping short of being opposite the stimulus direction. This suggests a compensation for stimulus approach angle, turning further if the animal starts turned towards the stimulus and less far if turned away. While this is seen in the current study, with a significant relationship between start angle and turning response amplitude, the range

of start angles was intentionally minimised and centred on 0 and as such there is a bias towards these values.

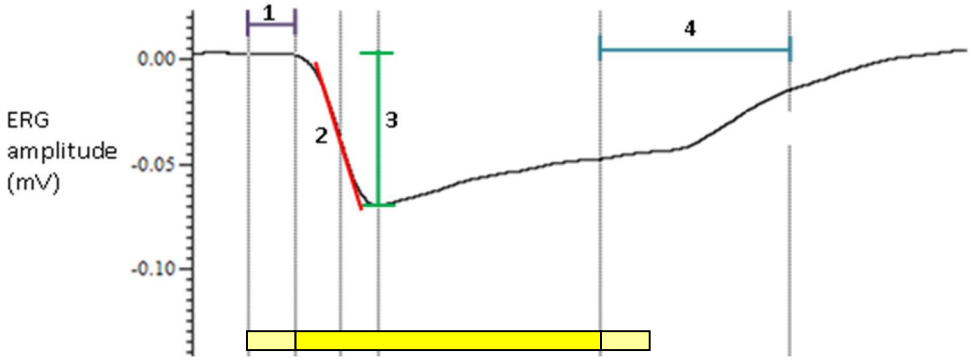


Figure 15: (as in Figure 8B) Example of typical electroretinogram trace with measures shown. Purple (1): latency; Red (2): maximal slope; Green (3): maximal response amplitude; Blue (4): 50% repolarisation time. Yellow bar indicates light pulse (faded yellow indicates transition)

Gregarious locusts show 30% higher ERG amplitude and a 15% steeper onset gradient in response to similar visual stimuli compared to solitarious locusts (Figure 15, 2 & 3). The ERG in this study was recorded from the crystalline cone region of the eye and therefore this increased peak is indicative of an increased on-transient, generated by neurons sensitive to the light-on stimulus (Naka and Kuwabara, 1956), this will contribute to the layer 1 P units of Rind and Bramwell (1996; Figure 4). This increased contribution may be expected lead to increased LGMD and therefore DCMD sensitivity in gregarious animals, however Matheson *et al.* (2004) saw differences in DCMD firing (number of spikes) only in habituated animals, with gregarious animals habituating less strongly than solitarious animals. The larger ERG response seen in the current study represents an increase in sensitivity, with identical stimuli eliciting a greater response in gregarious animals; this may be responsible for the resistance to habituation seen. Habituation occurs at individual presynaptic terminals within the LGMD dentritic tree (O'Shea and Rowell, 1976). It is possible that the increased P unit activity in this region acts to reduce habituation by sensitisation. Sensitisation refers to the ability of a strong, or noxious, stimulus to enhance subsequent responses (Rankin and Carew, 1988). The 50% repolarisation time does not differ between gregarious and solitarious animals, suggesting the shape of the off-transient does not differ despite the increased on-transient. The data shown here provide evidence that

gregarious animals are more sensitive to changes in illumination, and therefore likely to be better able to detect and respond to small moving threats.

3.4.2 Diurnal and circadian rhythms

In this section I discuss the diurnal and circadian patterns of behavioural responses to looming stimuli and of ERG responses, and how these differ between gregarious and solitarious animals. I discuss the possible causes and implications of these differences.

The type of behaviour exhibited in response to a looming stimulus did not differ with time of day in either extreme phenotype. This is likely due to the behavioural decision between active and passive camouflage that differs between the phenotypes (see Section 3.4.1). As shown in Heatwole (1968), lizards with more cryptic camouflage are less likely to move. As the camouflage of locusts does not change over the course of the day it follows that the response to stimuli will not differ. Having developed cryptic camouflage, solitarious locust survival is likely to be maximised by utilising this characteristic at all times of day. In a similar way, because gregarious individuals lack cryptic camouflage, and instead rely on avoidance, maximal survival is likely to be achieved by always preferring this strategy. This is in contrast to DCMD spike firing which differed over the course of the day in both phenotypes (Gaten *et al.*, 2012). If DCMD spike firing was involved in the type of behaviour in response to a stimulus a rhythm in behavioural type would be expected to be seen. This is consistent with analysis of hiding behaviour by Hassenstein and Hustert (1990), which states the DCMD cannot be the sole driver of this behaviour, although it may serve to prime the motor control systems. Additionally *S. gregaria* has been described as showing a diurnal rhythm in behavioural response type to distant moving stimuli in the field (H. Wilps, personal communication in Hassenstein and Hustert, 1990) with jumping occurring during the day and hiding occurring in the evening. This rhythm was not seen in this current study, possibly due to the very low number of jump behaviours elicited. It is possible that jumping vs. hiding frequency show diurnal rhythmicity, which would not be seen in this study, whilst hiding (or jumping) vs. startle frequency does not. As such active and passive behavioural responses represent two distinct paradigms. Repeating the experiments with stimuli more likely to elicit jumping behaviour may enable the rhythm described by H. Wilps to be seen in controlled conditions.

Solitarious animals turned less far in response to a looming stimulus at 21:00 compared to the 19:00 time point. This dip aligns with the peak in DCMD spike count for solitarious animals (Figure 6; Gaten *et al.*, 2012). Although not significant in gregarious animals (P = 0.138 & 0.130) the Cosinor fit suggests that there was a peak in hiding response angle at 15:00. This peak aligns with peak DCMD spike count in gregarious animals but not solitarious animals. This is confirmed with the full solitarious dataset (peak at 15:22). The gregarious dataset does show significant fit using the full dataset, peaking an hour later (16:23). Neither of these peaks coincide with the DCMD activity peaks (15:00 in gregarious; 19:00 in solitarious), and as such increased DCMD activity is unlikely to be responsible for the changes in behavioural amplitude. This is consistent with the view of Hassenstein and Hustert (1990) that DCMD firing is not involved in the amplitude of hiding behaviours. Instead Hassenstein suggests the optic neuropiles to be the location of the primary analysis with regards to turn angle. The optic lobe is known to be the location of the central circadian clock in crickets, cockroaches and beetles (Nishiitsutsuji-Uwo and Pittendrigh, 1968; Tomioka and Chiba, 1984; Abe *et al.*, 1997; Fleissner, 1982), and as such it is likely that the rhythmicity described here is circadian in nature. Repeating the experiment with animals under constant conditions (DD) would therefore be expected to show the same rhythmicity, albeit with reduced amplitude changes. The stronger significance of the solitarious Cosinor fits suggests the behaviour in this phenotype is controlled more strongly by cyclic outputs, such as diurnal conditions or a circadian central clock.

Both phenotypes showed strong diurnal changes in ERG properties during the first 24 h when the animals were under normal lighting conditions (12 : 12 h, L : D). These rhythms confirmed the diurnal pattern as suggested by Horridge *et al.* (1981) with the sensitivity shift described in their work matching that seen in the current study. The additional data provided in the current work shows the adaptation is gradual over 12 h prior to each extreme. Both phenotypes lose most rhythmicity in the second day (which is the first day under D : D conditions). This non-rhythmic period is seen in other ERG analyses (*Calliphora*; Chen *et al.*, 1999). The parameters produced by the Naka-Rushton fit do not show rhythmicity beyond the first day. The accuracy of the Naka-Rushton fit may be decreased as the saturated response may not have been achieved

with the tested light intensities. The rhythmicity returns in the third day (second day under D : D) for response amplitude and peak slope of the response

The diurnal, as opposed to circadian, influence on the ERG in the first 24 h is a major component; with ERG amplitude peak to trough differences in the LD condition 4-6 times that of the second day in DD. This suggests that while sensitivity of the retina is under circadian control, there is an additional and stronger influence of diurnally changing light intensity despite the 15 min acclimatisation undergone in this experiment. The effect ambient light conditions can have on sensitivity is large, accounting for a 3 log unit range (Warrant and Nilsson, 2006). Of particular interest is the amplitude of ERG response on the second day of DD where the amplitude has fallen to sensitivity levels similar to that of day adapted responses (below 4 mV) even during subjective night. This would be consistent with a loss of serotonergic release during the night suggesting the serotonergic fibres show diurnal and not circadian firing patterns, driven by environmental factors and not the circadian clock.

Solitarious animals showed peaks of ERG response amplitude, peak gradient, 50% repolarisation and half saturation intensity level earlier in the 24 h period than gregarious animals. For half saturation and response amplitude there was a lead of 30 min and for 50% repolarisation time there was a lead of 2 h for the first 24 h period. Under LD conditions this suggests that the sensitivity seen is phase shifted forward but makes no assumptions as to differences in the period of the rhythmicity under these conditions (assumed to be 24 h in this analysis). Gaten (unpublished) has previously shown that gregarious animals lead solitarious animals in rhythmicity of locomotor activity under LD conditions by approximately 4 h. This is in contrast to the current study and suggests locomotor activity is not driven directly by the same rhythmic process as the rhythms seen in this study. This difference is consistent with work in the German cockroach, *Blattella germanica* (Chang and Lee, 2001), where the circadian rhythms of locomotor activity and ERG amplitude were shown to be driven by a different pacemaker. This was established by severing the optic nerves, hence isolating the circadian clock present in the optic lobes. Locomotor activity persisted suggesting it was not reliant on the pacemaker in the optic lobes. Given the possibility of a

different anatomical location, it is not unlikely that the pacemaker driving locomotor activity is differentially timed relative to the pacemaker in the optic lobe.

Over the course of the experiment, including data from DD conditions, the gregarious ERG data produced a more significant fit to a 20 h rhythm, whilst the solitarious animals show more significant fit to a 24 h rhythm. This data should be interpreted with caution as it could be argued that much of the strength of the 20 h fit seen in the gregarious data is driven by the 49 h and 52 h time points which do not show the expected I/V relationship (Figure 12). However, this data suggest that gregarious animals show a shorter circadian period length under free-running conditions. This does not indicate the molecular central clock varies functionally between the two phenotypes, indeed Tobback *et al.* (2011) has previously shown that circadian expression of three circadian genes does not differ between the two phenotypes in the CNS under LD conditions. A perceived decreased circadian period could be due to social interaction producing a continuous forward phase shift; social interaction has been shown to produce a phase shift in *Drosophila* (Levine *et al.,* 2002); olfaction due to social interaction produced a significant effect on the phase of the animals. Crowded gregarious animals undergo significantly more continuous social interaction than isolated solitarious animals, and in this study they continued to do so throughout the examined period, although they were not undergoing social interaction whilst recorded. At the present time it is not possible to maintain a locust in the gregarised state without the use of chemosensory and visual, and/or tactile stimulation, which may contribute to social interaction induced phase shift (Levine *et al.,* 2002). It is likely, therefore, that the gregarious clock is being consistently advanced, a forward phase shift, as a result of these interactions in the order of 4 h a day. This effect could further explain the discrepancy between the DCMD results seen in Gaten *et al.* (2012) and the results of the current study. The DCMD experiments of Gaten *et al.* were carried out under constant low light conditions and are therefore comparable with the first day of constant dark. Indeed the shortened period length, described in the current study, of the gregarious rhythm accounts for the full 4 h difference seen in Gaten *et al.* (2012) between gregarious and solitarious animals. This social effect was hypothesised by Gaten *et al.* (2012) to potentially explain the earlier afternoon peak of the DCMD

activity in gregarious locusts, and as such the current study further supports this hypothesis.

The decreased variations and stronger fit to 24 h seen in the solitarious experiments throughout suggest a more predictable 24 h output from the clock, with greater significance in Cosinor factors. Gaten *et al.* (2012) expresses this as an increase in ultradian, shorter than 24 h, rhythms in gregarious animals interacting with the overall circadian rhythm and although the current study does not investigate the power of such short period length rhythms it does confirm a reduction in strength of 24 h rhythmicity. There are two possible mechanisms which likely interact to produce this difference. The overall outward appearance of the circadian clock is the result of many circadian oscillators, for example the lateral neurons in *Drosophila* (Helfrich-Forster, 2003; Dissel *et al., 2014*). The individual oscillators are synchronised, a key candidate for this synchronisation is PDF, this has been shown to be expressed by the LN and mutation of PDF loses oscillatory synchronisation disrupting circadian rhythmicity of output behaviour, in this case locomotor activity (Peng *et al.,* 2003). Lower levels of PDF in gregarious animals would likely result in a more varied and less consistent output to that seen experimentally in the current study, with reduced significance of rhythmic fits in both hiding and ERG assays. Such a difference in PDF expression should be detectable in gene expression assays and is investigated in a following chapter.

Solitarious animals could potentially gain more from accurate pre-empting of diurnal changes. Gregarious animals form part of a collective, a swarm of individuals. There is, therefore, less selection pressure associated with maintaining a tight clock, indeed it may be advantageous to allow greater temporal distribution of activities. Gregarious animals show a markedly different lifestyle, with swarms moving with the winds great distances in relatively short timeframes. Conversely the solitarious animals do not regularly move great distances (Ely *et al.*, 2011), only flying infrequently during the night. Gregarious animals are therefore more likely to rapidly change their environment. As such it is likely that the gregarious animals gain significantly from having a more adaptable clock, as one with lower synchronicity is likely to be, compared to the solitarious animals whereby a stricter more predictive clock is

preferable. The mechanism for such a difference is not clear; it might be a coincidental factor of the increased social interaction to which gregarious animals are exposed. In Drosophila a male-female couple shows different locomotor activity to isolated individuals (Fujii *et al.*, 2007), so it is not unreasonable to assume that in a swarm of locusts some might show differences in circadian rhythmicity due to the complex social environment gregarious animals inhabit. By contrast solitarious animals are in a very simple social environment with far fewer variables and hence may be expected to show more consistent responses. Alternatively it might be that the shorter 20 h period length discussed earlier is a consequence of a change in the synchronicity of individual oscillators in order to encourage an increase in temporal variability in behaviour.

3.4.3 Conclusions

This work shows a clear population density dependent difference in behavioural preferences in response to looming stimulus, both in behavioural type and in amplitude. This represents the first investigation into this phenotypic difference in *S. gregaria* and the first study to examine diurnal effects in this system. This study did not discover any clear diurnal effects in behavioural preference, but did reveal rhythmicity in angular response amplitude and suggests a divergence in the pathway responsible for rhythmicity in locomotor activity and loom detection which may be the result of a peripheral pacemaker.

Investigations into population density dependent differences in ERG responses showed a higher sensitivity in gregarious animals to an increase in light intensity, this was characterised by an increased light-on transient with an associated onset gradient. This is proposed to be in part responsible for the reduced habituation of downstream DCMD responses to repeat stimuli. This work also examined rhythmicity in ERG responses, showing a clear diurnal pattern in all examined characteristics of the waveform as predicted based on Horridge *et al.* (1981). Circadian patterns were detected in the second day in constant darkness and contrasting the data for gregarious and solitarious animals revealed a difference in period length. Gregarious animals show a shorter period length (20 h) in free running conditions, not previously described in the literature, compared to the 24 h period length of solitarious individuals. I propose this is likely related to the social environment; with greater social

interaction either having a causative role in the period length change via continuous forward phase shifting or reducing the selection pressure for a tight clock, resulting in a coincidental period length shortening as a result of reduced synchronisation.

Chapter 4:
Characterising the core circadian clock genes

4.1 Introduction

The fruit fly, *Drosophila melanogaster,* has been used extensively in circadian research as has the mouse, *Mus musculus*. In the interests of brevity these model organisms will be referred to in what follows as *Drosophila* and *Mus* respectively. First I describe the canonical *Drosophila* endogenous circadian clock; following that I discuss common variations including what is known of non model insects.

4.1.1 The endogenous circadian clock

At a biochemical level the circadian clock is defined by the daily cycle in transcription and degradation of 'timer' proteins (Figure 1). In *Drosophila* the primary proteins are Clock (CLK) and Cycle (CYC), which dimerise in the nucleus. The CLK-CYC dimer interacts with the E-box promoter region (CACGTG), promoting the transcription of associated genes (Kyriacou and Rosato, 2000). Among the genes activated are *period (per)* and *timeless (tim)*, which produce their associated proteins PER and TIM. These also dimerise at the Per-Arnt-Singleminded (PAS) domain of PER, and the PER interaction domain (PID) of TIM, forming a PER-TIM complex. The complex translocates to the nucleus where it inhibits the actions of CLK and CYC, this prevents further transcription of genes regulated by E-box regions, thereby reducing levels of PER and TIM. Reduced PER-TIM levels remove the blockade on CLK-CYC formation allowing the cycle to proceed. (Williams and Sehgal, 2001; Piggins and Guilding, 2011). The rhythmic transcription and breakdown of these genes and associated proteins is approximately 24 h in length (20 - 28 h).

Several elements affect the stability and expression of the core clock components described above. For example, *vrille* (*vri*) is an E-box regulated gene that is responsible for antiphasic regulation. VRI quickly accumulates in dimers, activating genes regulated by the V/P box (TTATGTAA) which repress the production of CLK. PAR domain protein 1ε (PDP1ε) is also E-box regulated, but it builds up more slowly than VRI and promotes CLK production. Clockwork Orange (CWO), a protein produced by a further E-box

regulated gene (*cwo*), is a repressor of E-box and therefore PER, TIM, VRI and itself (Kadener *et al.*, 2007; Matsumoto *et al.*, 2007). It accumulates over 6 h and may be a redundant off-trigger, ensuring that the E-box regulated genes cease to be expressed. Loss of CWO results in a counterintuitive *reduction* in levels of expression of E-Box regulated genes, revealing a lack of full understanding as to its role (Piggins and Guilding, 2011). Regulation of the constituents of this cycle is additionally carried out by kinase/phosphatase interactions (Christie *et al.*, 2013a & b). Specifically, TIM is regulated by the kinase Shaggy (SGG; ortholog of mammalian Glycogen synthase kinase 3), which regulates nuclear entry. The stability of PER is regulated by the kinase Doubletime (DBT; related to mammalian Casein Kinase 1, CK1). CLK is also regulated indirectly by DBT in that it piggy-backs with PER. PP2A is a complex heteromer phosphatise that converts pPER to PER, returning its stability to its original levels (Piggins and Guilding, 2011).

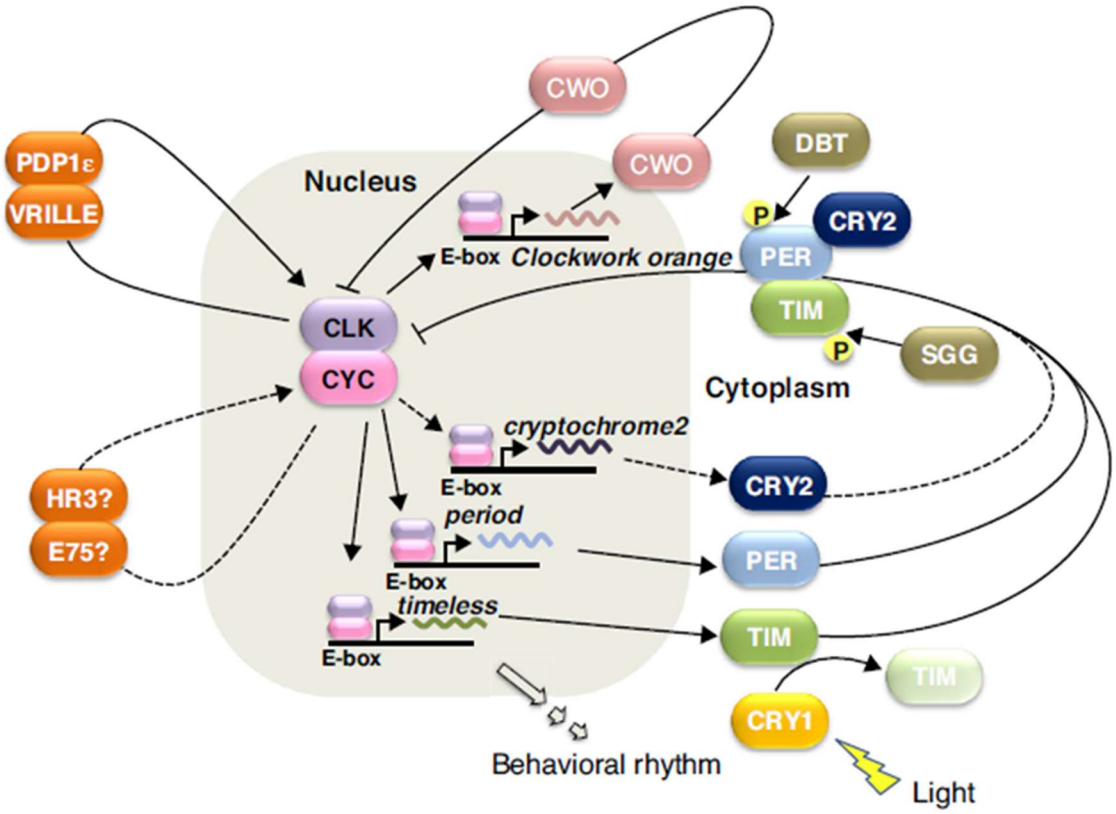
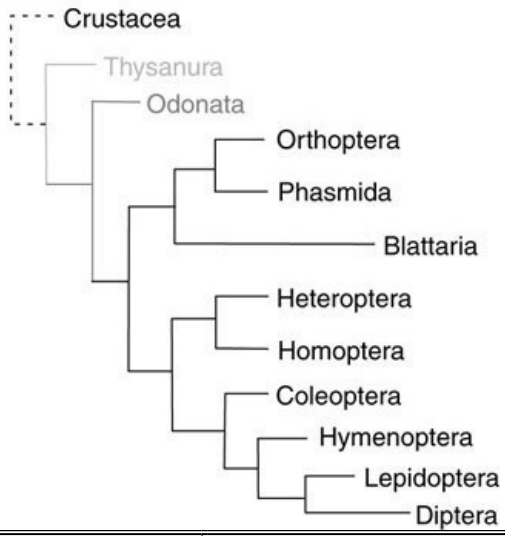


Figure 1: Diagrammatic view of the endogenous circadian clock (Tomioka and Matsumoto, 2015). Solid lines are confirmed in *Drosophila,* broken lines are hypothesised in other insects. The Clock-Cycle dimer interacts with the E-Box, promoting the production of Period and Timeless. The E-Box refers to a promoter region common to, amongst other genes, *per* and *tim*. These dimerise to form a complex that allows for the breakdown of Clock-Cycle dimers. Timeless is bound by Cryptochrome, a light activated protein, which in turn prevents the formation of Period-Timeless complex, with both proteins being broken down before having any effect on the Clock-Cycle dimer.

These systems function to regulate the expression of the core clock genes in order to maintain a 24 h cycle length, a key feature of rhythmic circadian expression. In addition the endogenous circadian clock must be entrainable. This function is carried out, in *Drosophila*, by Cryptochrome (CRY). CRY is responsible for light induced clock reset: binding to TIM when activated by light (Figure 1). Once bound the TIM-CRY complex degrades, reducing the levels of TIM and therefore of TIM-PER. This allows CLK-CYC formation and resets the clock. (Piggins and Guilding, 2011).

The mammalian endogenous circadian clock

The endogenous circadian clock of *Drosophila* differs in various ways from the mammalian clock of *Mus*, although the general schema is similar. In *Mus*, the primary oscillator consists of the mammalian homologs of CLK, CYC and PER. However, one striking difference is in the functionality of TIM and CRY. In *Drosophila* TIM forms a dimer with PER. In the mammalian clock this is the role of a light insensitive CRY protein (CRY-2; Griffin *et al.*, 1999; Kume *et al.*, 1999; Lee *et al.*, 2001) giving CRY-2 the name mCRY, while CRY-1 is referred to as dCRY. In *Mus* there are no CRY-1 proteins and two CRY-2 like proteins, mCRY-1 and mCRY-2, both of which are rhythmically expressed (Shearman *et al.*, 2000). The structure and function of CLK and CYC also differ. The transcriptional activity of CYC in *Drosophila* is due to the PolyQ region on CLK (Allada *et al.*, 1998), whereas in *Mus*, it is due to a transactivation domain (Bmal C-Terminal Region: BCTR) on the c-terminus of CYC (BMAL) (Kiyohara *et al.*, 2006). The presence of the BCTR domain distinguishes BMAL from CYC. The expression of CLK also differs between *Drosophila*-like clocks and mammalian-like clocks. In the former, expression cycles in a circadian fashion, driven by the cyclic repression by PER-TIM. In the latter, CLK expression is near constant under both LD and DD conditions (Kamae *et al.* 2010; Kamae *et al.*, 2012).



Species		Circadian Gene										
		clock	*cycle*	*period*	*timeless*	*timeout*	*cry-1*	*cry-2*	*doubletime*	*vrille*	*cwo*	*pdp1*
Diptera	*Drosophila melanogaster*	Y	Y	Y	Y	Y	Y	N	Y	Y	Y	Y
	Musca domestica	Y	Y	Y	Y	?	Y	N	?	Y	?	?
	Aedes aegypti	Y	Y	Y	Y	Y	Y	Y	?	?	?	Y
	Anopheles gambiae	Y	Y	Y	Y	Y	Y	Y	?	?	?	Y
Lepidoptera	*Antherea pernyi*	Y	Y	Y	Y	?	Y	Y	?	Y	?	?
	Bombyx mori	Y	Y	Y	Y	Y	Y	Y	Y	?	?	?
	Danaus plexippus	Y	Y	Y	Y	Y	Y	Y	Y	Y	?	Y
Hymenoptera	*Solenopsis invicta*	Y	Y	Y	N	Y	N	Y	?	Y	Y	Y
	Apis mellifera	Y	Y	Y	N	Y	N	Y	?	Y	?	Y
Coleoptera	*Tribolium castaneum*	Y	Y	Y	Y	Y	N	Y	?	Y	Y	Y
Hemiptera	*Acyrthosiphon pisum*	Y	Y	Y	Y	Y	Y	Y	?	Y	?	Y
	Riptortus pedestris	Y	Y	Y	?	?	Y	Y	?	Y	?	?
Orthoptera	*Gryllus bimaculatus*	Y	Y	Y	Y	?	Y	Y	Y	Y	Y	Y
	Rhyparobia maderae	?	?	Y	Y	?	?	Y	?	?	?	?
Thysanura	*Thermobia domestica*	Y	Y	?	Y	?	?	Y	?	?	?	?

Figure 2: Top, Phylogenetic tree of insect orders (Evans and Gundersen-Rindal, 2003). Bottom, core circadian genes found in insects of various orders on the basis of a database survey (modified from Tomioka and Matsumoto, 2015). Y = existence, N = absence, ? = to be determined.

Non-model insect endogenous circadian clocks

Most non-model insects express canonical clock genes (those seen in *Drosophila*) such as *Clk, cyc, per* and *tim* (Zhu *et al.*, 2008a; Cortes *et al.*, 2010; Rund *et al.*, 2011; Ingram *et al.*, 2012; Rund *et al.*, 2013), however Hymenopteran species, for example honeybees, do not express *tim* (Zhan *et al.*, 2011), instead timeout (*tim-2*) is rhythmically expressed. It is thought that timeless (*tim-1*) represents a duplication of timeout (*tim-2*) (Rubin *et al.*, 2006). CLK and CYC in many non-model insects show

mammalian like structure, with a highly conserved BCTR on CYC. In addition CYC proteins generally have a basic Helix Loop Helix (bHLH) domain and two PAS domains (Tomioka and Matsumoto, 2015). CLK proteins consist of a bHLH domain, two PAS domains and a PAC domain. Additionally PolyQ regions have been identified, although these are not as extensive as those seen in *Drosophila* (Tomioka and Matsumoto, 2015). Recent work has also identified many insects possess mCRY homologues (CRY-2) which do not have photoreception properties (Yuan *et al.*, 2007). All CRY homologs contain a DNA photolyase domain and a FAD binding domain. In the monarch butterfly c*ry*-2 has been shown to be co-expressed with *per* and *tim* with mutation of cry-2 causing arrhythmia (Merlin *et al.*, 2013). It is thought that the presence of *tim* in these systems acts to allow phase shift due to light, allowing entrainment to light stimuli (Zhu *et al.*, 2008b).

In *Gryllus bimaculatus* rhythmic output of the clock is abolished under RNAi knockdown of *per* or *Clk* (Moriyama *et al.* 2008; Moriyama *et al.*, 2012). Additionally, the same effect is seen in the periphery (Urya and Tomioka, 2010). By contrast knockdown of *tim* has little effect on peripheral rhythmicity. This suggests CRY is likely to function as a core circadian component, as in mCRY, at least in the periphery. RNAi knockdown of *Clk* in *S. gregaria* is lethal, whilst knockdown of *per* or *tim* results in reduced progeny (Tobback *et al.*, 2011). No work examined behavioural outputs whilst knockdown of any circadian genes was taking place.

In *S. gregaria* none of the clock genes discussed here have yet been fully sequenced, with short partial reads for *Clock* (HQ428033.2), *period* (HQ428031.1), *doubletime* (HQ428034.1) and *timeless* (HQ428032.1) (Tobback *et al.*, 2011; Tobback and Huybrechts, unpublished). The expression levels of these fragments have been examined, showing cyclic expression of *Clk*, *per* and *tim* under LD conditions (Tobback *et al.*, 2011). No expression level analysis has been carried out for any other circadian genes in this species.

Localisation of the endogenous circadian clock

The majority of circadian investigations in *Drosophila* have been carried out using forward genetic screens, identifying genes responsible for a particular phenotype, for

alterations in rhythmic behaviour phenotypes, coupled with immunohistochemistry to determine spatial patterns of protein expression. About 150 'clock neurons' in the central brain have been identified by their expression of the canonical clock genes (Helfrich-Forster, 2003). They are attributed to one of 6 groups of neurons: three lateral groups and three dorsal groups. The lateral neurons (LNs) are the dominant central clock for locomotor activity, being both necessary and sufficient in *Drosophila* (Kaneko and Hall, 2000; Grima *et al.*, 2004; Helfrich-Forster *et al.*, 2007). Two of these lateral neurons (LN_d & l-LN_v) express the neuropeptide Pigment-Dispersing Factor (PDF), while the third (s-LN_v) does not. PDF is thought to help synchronise the central clock of the brain (Peng *et al.*, 2003) with those in peripheral tissues, where circadian clock genes are also expressed (Cuticle deposition: Ito *et al.*, 2008; Malpighian tubules: Hege *et al.*, 1997). The dorsal neurons (DNs) are thought to contribute to behavioural rhythms, but only in LD conditions, and are unable to contribute in the absence of input from the LNs under constant conditions (Helfrich-Forster *et al.*, 1998; Blanchardon *et al.*, 2001; Veleri *et al.*, 2003).

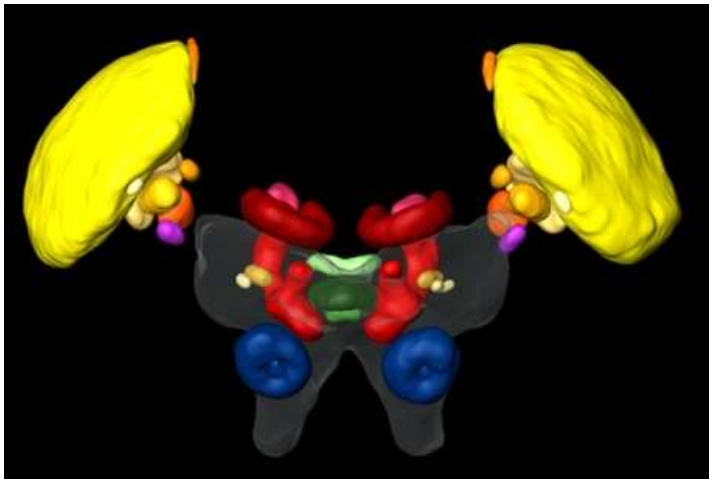


Figure 3: Surface reconstruction of the *S. gregaria* brain, anterior view (Jundi, *et al.*, 2010). Yellow & orange = optic lobes; grey = central brain; blue = antennal lobes; red & pink = calyx, primary and accessory.

Hemimetabolous insects such as cockroaches and crickets, in common with most multicellular organisms, are thought to have both a central clock and peripheral clocks. The current view is that the central endogenous circadian clock of such insects thus represents a more conserved ancient clock, prior to later additions and subtractions (Tomioka and Matsumoto, 2015). In the case of *Gryllus bimaculatus,* as well as other crickets and *Thermobia domestica*, the compound eyes are thought to be the primary

photo-entrainment site for the central clock, with sectioning of the optic nerves removing the ability to entrain (Nishiitsutsuji-Uwo and Pittendrigh, 1968; Page, 1978; Tomioka and Chiba, 1984). There is some evidence however that the ocelli may provide entrainment in some species, such as *Teleogryllus commodus* (Rence *et al.*, 1988). The central clock itself is thought to reside in cells of the optic lobe, as is the case in cockroaches, crickets and some beetles (Figure 3, yellow; Nishiitsutsuji-Uwo and Pittendrigh, 1968; Tomioka and Chiba, 1984; Abe *et al.*, 1997; Balkenohl and Weber, 1981). In crickets this has been narrowed down to the lamina or medulla of the optic lobe which, combined with the compound eye, produce a circadian rhythm (Tomioka and Chiba, 1992). Removal of the optic lobes in crickets does not immediately extinguish rhythmic behaviours; rhythmicity decays over several days suggesting that a peripheral clock is present (Tomioka, 1985) and influences for behavioural outputs. This peripheral clock is entrained primarily to the primary clock, although it is also capable of entraining to temperature cycles (Rence and Loher, 1975; Page, 1985; Tomioka and Chiba, 1989). The location of this secondary clock is not currently known. In contrast to hemimetabolous insects, holmometabolous insects such as those in Diptera and Lepidoptera*,* for example in the Chinese tasar moth, *Antheraea pernyi,* and the Cecropia Moth, *Hyalophora cecropia,* the central brain (Figure 3, grey) is thought to be the location of the central circadian clock (Sauman and Reppert, 1998; Truman, 1972).

4.1.2 Transcriptomics

Genetic techniques have developed rapidly since the turn of the century both in effectiveness and cost (Figure 4). Transcriptome sequencing (RNA-seq) has become a powerful system for generating large amounts of transcript data, especially in organisms lacking a full genomic sequence (Yang and Smith, 2013). Using this technique on such samples presents the challenge of de-novo transcriptome assembly, whereby the genetic sequences obtained must be accurately ordered and combined into continuous sequences known as contigs. Any failure here will affect the downstream analysis. However, the benefit of this approach is the opportunity to discover genes that remain hard to identify through other means such as degenerate PCR or Rapid Amplification of cDNA Ends (RACE). Once a comprehensive transcriptome

is assembled, expression level analysis becomes possible. Through this technique the expression levels of any expressed genes can be recorded more accurately, more easily and more cost effectively than carrying out individual qPCR experiments with each gene.

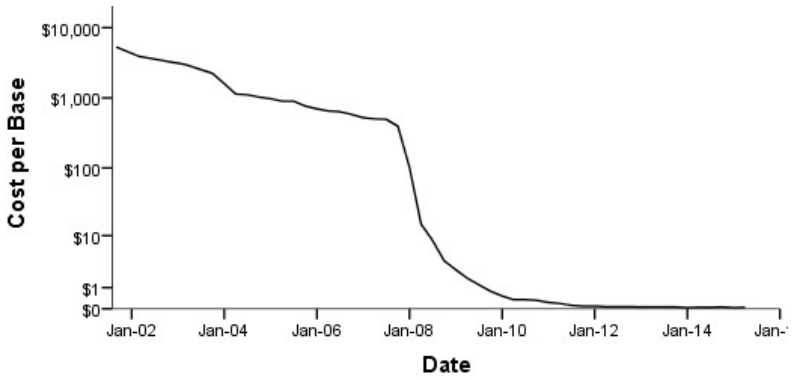


Figure 4: Cost of RNAseq experiments adapted from (Wetterstrand,2015) showing the rapid reduction in cost per base since next generation technology entered use in 2008. Since approximately 2012 the cost per base has become negligible with the majority of costs associated with sample preparation, quality control and data storage.

RNA-seq platforms output short read data of various read lengths and depths, dependent on the platform and the settings. Each read is effectively a random selection from the available pot of genetic material, here the extracted and purified RNA, so highly expressed genes are more likely to be completely and accurately sequenced. Poorly expressed genes will have fewer fragments sequenced and therefore may not be fully covered in the transcriptome. It is therefore important to utilise the correct experimental design to collect the required data. Long reads make for more accurate assembly of de-novo transcriptomes, while short reads allow for accurate gene expression analysis (Gibbons *et al.*, 2009). The depth of the read (number of reads) does not have any experimental tradeoffs, with greater read depth detecting more of the low expressed genes and providing a more robust value of gene expression, at an increased financial cost.

There are several computational solutions available for de-novo assembly, such as Trinity (Grabherr *et al.*, 2011), trans-ABySS (Robertson *et al.*, 2010), Bridger (Chang *et al.*, 2015) and SOAPdenovo (Xie *et al.*, 2014). Functionally similar, these packages utilize de Bruijn graphs to estimate transcripts based on the available reads (Yang and Smith, 2013). This process involves breaking the available reads down into smaller sub-reads, or kmers, which are then examined for overlaps. Smaller kmer

lengths require higher computational resources, but increase the chance of longer contigs being created at the cost of reduced accuracy and reduced ability to cope with repeated regions. Larger kmers make for smaller computational demands, allow longer repeated regions to be resolved but produce more small contigs. Trinity utilizes a single kmer length (25bp); TransABySS utilizes a range of kmer lengths; SOAPdenovo allows the use of different kmer lengths as set by the user to optimise for the unique requirements. All these programs attempt to correct assembly errors by selecting for highest expression of components, and as such are optimised to constructing references for gene expression analyses (Yang and Smith, 2013). A disadvantage of this correction process, particularly where multiple biological samples are combined into RNA pools, is that the variation within a locus is lost, with single contigs produced for each locus rather than each isoform being made available.

Once assembled it is possible to query the transcriptome, looking for contigs that match known genes. This process known as genomic mining is an effective method of gene discovery. Recently such analysis pathways have been used to uncover important circadian genes in many species using previously existing transcriptomes (Christie *et al.*, 2013a & b). This process involves first querying the database with known sequences. Any alignments with contigs within set parameters (frequently $p < 0.001$; Christie *et al.*, 2013a & b) are put through a process known as the Basic Local Alignment Search Tool (BLAST; NCBI), compared, and aligned to all known gene sequences. Contigs may either represent complete coverage of a gene open reading frame (ORF) or may consist of a partial match to known sequences of that gene. Identified contigs can then be verified by specific PCR; if the sequence is correct an amplicon of the expected size will be produced and visible on gel electrophysis.

4.1.3 Aims and hypotheses

This chapter focuses on gene discovery for the central clock genes in *Schistocerca gregaria*, in the central brain, the optic lobes (thought to be the location of the central clock), and in the meso- and meta-thoracic ganglia where key processes involved with population density phenotypic plasticity have been localised (Anstey *et al.*, 2009; Rogers and Ott, 2015). The main target genes are *Clock, cycle, period* and *timeless* as well as the regulatory genes *doubletime, cryptochrome,* and *vrille*. Other genes linked to the circadian clock were also queried; however these are not discussed in detail. In addition to gene discovery, this work lays the groundwork for the expression level analysis in the following chapter, forming a comprehensive annotated de-novo transcriptome of all genes expressed in the brain, optic lobes and thoracic ganglia over the course of a 24 h period. It is expected that the endogenous circadianclock of *S. gregaria* will be similar to that of *G. bimaculatus,* as discussed above, due to its evolutionary similarity.

4.2 Methods

4.2.1 General husbandry

All animals (*Schistocerca gregaria* Forskål) used in this work were reared at the University of Leicester, U.K. Second generation solitarious animals were obtained as 4th or 5th instar juveniles from a colony at the University of Cambridge. These nymphs were reared under further isolation in Leicester until adulthood. Both solitarious and gregarious animals were maintained under a 12 : 12 h light : dark regime, with light levels of 750-1500 lx during the photophase, and 0 lx during the scotophase. Cage temperatures rose to 36°C during photophase and fell to 25°C during scotophase and were identical for both phenotypes. Locusts received fresh wheat seedlings and bran flakes *ad libitum*. Solitarious animals were reared individually in 10 x 10 x 20 cm cages isolated from sight and smell of other animals. Gregarious individuals were kept at high density (100-300 per cage) in 50 x 50 x 50 cm cages. These conditions were identical to those used previously.

4.2.2 Experimental conditions

RNA was extracted from samples collected from animals held under 12:12 light-dark (12:12 LD) conditions with abrupt transitions in light intensity. Temperatures cycled in time with the lights, but showed more graded transitions from 25° during the scotophase to 36° during the photophase. Any manipulations that were required during photophase were carried out under low intensity red light. Food (wheat grass and bran) was maintained ad lib. Samples were taken every 3 h with the first sample 1 h after lights on (at ZT 1). 8 time-points were therefore available, with samples from 3 different animals at each time-point (Figure 5B). At least one male and at least one female were sampled at all time points.

4.2.3 Tissue dissection

Dissections were carried out under low intensity red light at 25°C. The anterior aspect of the head was removed by slicing downwards with a razor blade, and then the head was rapidly removed from the prothorax. Immediately thereafter the head was sliced horizontally at the level of the oesophagus, removing the sub-oesophageal ganglion. This left a block of dorsal head tissue, approximately 3 x 5 x 5 mm, containing the brain and eyes, which was deposited into a 0.5 ml aliquot of RNAlater (Sigma). Isolation of

this tissue into RNALater took no more than 15 s from first handling the animal, thus minimising the risk of RNA breakdown or transcription responses to stress. The thorax was then sliced horizontally dorsal to the ventral nerve cord allowing the removal of the meta- and meso-thoracic ganglia. These were stored in a separate aliquot of RNALater. All samples were stored at 4-8°C. The head was subsequently dissected further in RNALater, isolating the brain and optic lobes from the head and then separating the central brain from the optic lobes. Each biological replicate was stored separately at -80°C.

4.2.4 RNA extraction and sequencing

Extraction and sampling was pooled differently for brain + optic lobe samples and ganglia samples. In the brain + optic lobe samples both tissues were pooled for each individual, with three replicates per time point resulting in 24 samples. The ganglia samples were pooled with tissue from one individual per time point in each sample resulting in 3 samples per phenotype. Together this totalled 54 samples. Gender totals were tracked for each sample to allow gender balanced analysis of gene expression. These samples are also utilised in Chapter 5.

RNA extraction was carried out utilizing the Trizol reaction, as set out in Table 1 (Figure 5A). These samples underwent TURBO DNase (Life Technologies) treatment to remove any possible DNA contamination (Table 2; Figure 5A). Samples were then cleaned using the RNeasy mini kit (Qiagen) to remove any phenol contamination remaining from the Trizol extraction (Table 3; Figure 5A) and were then subjected to quality control testing using a bioanalyzer (Agilent, G2939A). RNA integrity (RIN) and RNA concentration were measured to quantify the quality and quantity of RNA in the sample. RIN values were not representative of quality for *S. gregaria* due to the ribosomal profile not matching any computational templates. As a result, the electropherogram of each sample was visually inspected for degradation (Figure 5A; Figure 6). The electropherogram is a histogram generated *in silico* of the molecular lengths present in a sample. It provides similar information to that obtained by running the sample on an electrophoresis gel but is more sensitive. A typical high quality RNA sample would show as two distinct peaks representing the two ribosomal subunits (Figure 6B), whilst a low quality RNA

sample would show reduced peaks, with increased noise at lower nucleotide lengths (Figure 6C)

Once the quality and quantity of RNA had been measured, the samples were sent for sequencing with the NextSeq platform (Illumina; Glasgow Polyomics, UK); Sampling parameters were: PolyA library preparation, paired end reads at 2x75bp with 25M reads. These parameters were optimised for good coverage, with the number of reads appropriate for expression level analysis. De-novo transcriptome assembly and gene discovery relied on the large number of reads to acquire a complete transcriptome. As previously discussed, optimisation for transcriptome assembly requires large read length (150+bp), whilst this study made use of 75bp reads. The expected total number of reads with all samples combined was in excess of 1.3×10^9.

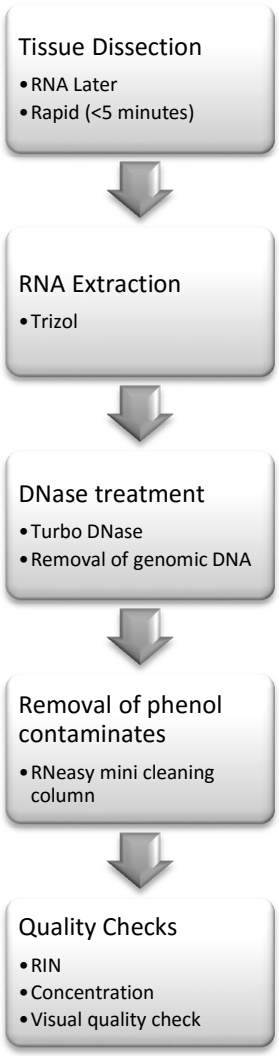


Figure 5: Sample preparation workflow from tissue dissection to quality checks prior to sequencing. Further details of protocols used are provided in the following tables: Trizol extraction (Table 1), Turbo DNase treatment (Table 2) and RNeasy mini cleaning column (Table 3).

Table 1: RNA extraction protocol in Trizol.

Treatment	Settings/volume	Duration
Homogenise in Trizol	200 µl	
Incubate	Room Temp.	5 min
Centrifuge	4°C; Max speed (>16000 RCF)	10 min
Isolate supernatant	Continue to use supernatant	
Add chloroform	40 µl	
Incubate	RT	3 min
Centrifuge	4°C; Max speed	15 min
Isolate aqueous phase	Continue to use aqueous phase	
Add isopropanol	100 µl	
Incubate	Room Temp.	10 min
Centrifuge	4°C; Max speed	10 min
Remove supernatant	Discard supernatant	
Add Ethanol	70/75%; 1 ml	
Vortex		Brief (<10 s)
Centrifuge	Max speed	Brief (<15 s)
Remove supernatant	Without disturbing precipitate	Repeat until dry
Allow to dry	Room Temp.	5 min
Resuspend in TRIS	20 µl; 1M	

Table 2: Turbo DNase protocol for RNA samples

Treatment	Settings/volume	Duration
Add Turbo DNase 10x Buffer	5 µl	
Add Turbo DNase	1 µl	
Incubate	37°C	30 min
Add Inactivation reagent	5 µl	
Mix	Inversion; Room Temp	5 min
Centrifuge	10,000G	1.5 min
Isolate supernatant	Continue to use supernatant	

Table 3: RNeasy Mini spin protocol

Treatment	Settings/volume	Duration
Add H_2O	50 µl	
Add RLT Buffer	350 µl	
Mix	pipetting	Brief (<15 s)
Add Ethanol	95-100%; 250 µl	
Mix	pipetting	
Transfer to RNeasy spin column		
Centrifuge	10,000 RPM	15 s
Discard flow through		
Add RPE	500 µl	
Centrifuge	10,000 RPM	15 s
Discard flow through		
Add RPE	500 µl	
Centrifuge	10,000 RPM	2 min
Add H_2O	50 µl	
Centrifuge	10,000	1 min
Replace Elute into column	50 µl	
Centrifuge	10,000 RPM	1 min

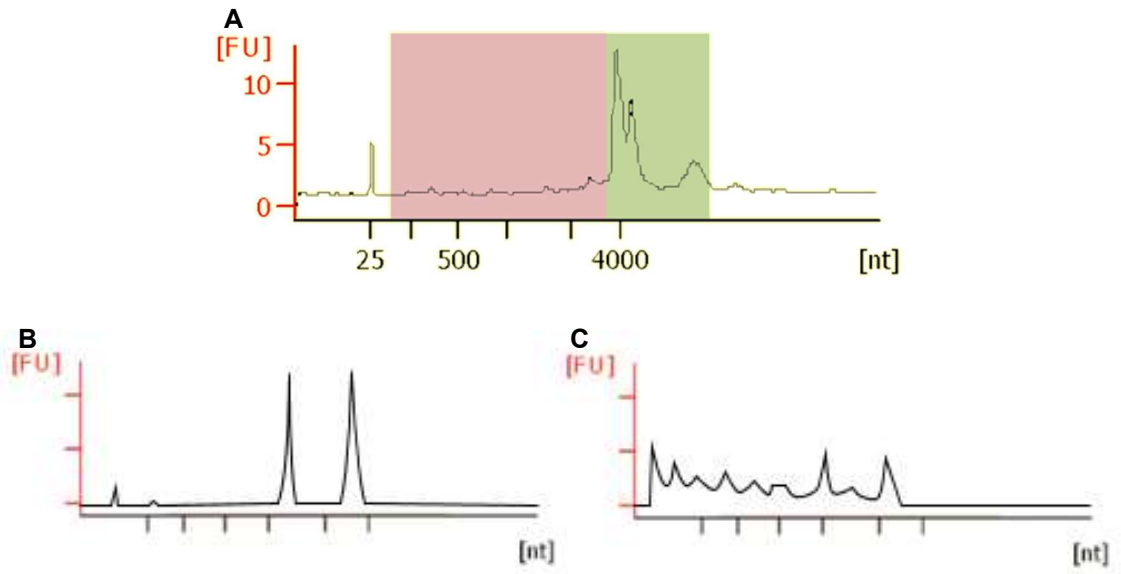


Figure 6: Example electropherograms. Sample G16B (A). Ideal high quality sample (B) and highly degraded sample (C). Both B and C represent hypothetical mammalian samples so the exact location of the ribosomal peaks differs from *S. gregaria.* Axis units, X: nucleotides (nt), Y: fluorescence units (FU). Sharp peaks in the green region represent ribosomal RNA and are indicative of high quality RNA as in B; peaks in the red region would indicate degradation of the ribosomal RNA as in C.

4.2.5 De-Novo transcriptome assembly

Sequencing resulted in approximately 1.3 x 10^9 reads, ranging from 25 to 75 bp in length in Fastq format. A multi-assembler approach was taken to forming the de-novo transcriptome, with assemblies generated by Trinity, Trans-ABySS and Bridger. The first assembly made use of all the available data, combining all files and removing low quality reads (Trimmomatic, AVGQUAL: 20; http://www.usadellab.org/cms/?page=trimmomatic). These data were normalised *in silico* (using normalisation in Trinity; https://trinityrnaseq.github.io/), a process in which kmers that were represented by more than 50 reads were discarded. This optimised the workflow, reducing the read count by approximately 80% with no loss in coverage. Reads shorter than 60bp were then removed (Trimmomatic) and the remainder were assembled by Trans-ABySS at kmer lengths of 21, 31, 41, 51 and 61. The resulting assemblies were combined by Trans-ABySS, removing any duplicates. Two further assemblies were carried out with Bridger and Trinity; in order to optimise the workflow each was carried out on a subset of the available data. The data was divided by phenotype to allow future work to distinguish any potential differences, for example isoform or splice variants without further assembly. Bridger assembly was carried out on a subset of the available data (gregarious animals only) using a kmer length of 31. No normalisation was carried out on this assembly, but only read lengths of 72-74bp were utilised (3' end trimmed if over 74). This was because Bridger struggles with different read lengths (software creator Juntao Liu, personal communication). Trinity assembly was carried out on a subset of available data (solitarious animals only), using kmer length 25. Reads were selected as described for the Bridger assembly above. All the assemblies produced were combined, producing a final assembly that made use of the benefits of each assembler and kmer length combination (Haznedaroglu *et al.*, 2012). The final assembled transcriptome required further processing to remove duplicates and prevent nonsense sequences from affecting the subsequent analysis reported in the following chapter. This processing is discussed in detail in the next chapter.

4.2.6 Gene discovery

The transcriptome was queried using BLAST looking for similarity between the transcripts produced and with known sequences (Table 6), For *Clk* and *cyc*, where BLAST (NCBI) analysis returned significant similarities, specific primers (Table 4) were produced against the sequences to verify the accuracy of the assembly of these genes. PCR was run under the protocol in Table 5, using Q5 High-Fidelity DNA Polymerase (NEB) with annealing temperatures specific to each primer pair (primer pair annealing temp = lower annealing temp +3°C). The reaction mixture was then run on an agarose electrophoresis gel and visualised.

Table 4: Specific Primer information

Target	Primer Name	Primer Sequence	Annealing Temp (°C)
CLK	JSGClkLF2	TGGACAAGCGCTGAAAAGTA	59
CLK	JSGClkLR2	AACCTGTATCTGATGTCTGGAAA	57
CYC	JSGCYCLF	TGTACATGGCACCAGCAGTTTTGT	68
CYC	JSGCYCLR2	ACAATATGAACAAAATGGGATTTC	64

Table 5: PCR protocol using Q5 high fidelity DNA polymerase. The annealing temperature was set at a value 3°C above the lower annealing temperature of the primer as shown in Table 4.

Treatment	Settings/Volume	Time
Master-mix	5X Q5 Buffer : 5 µl dNTP's : 0.5 µl Primer (F&R) : 1.25 µl Template DNA : 1 µl Q5 DNA polymerase : 0.25 µl H_2O : 15.75 µl	
Initial Denaturation	98°C	30 s
25-35 Cycles	98°C	10 s
	ANNEALING TEMP 50-72°C	30 s
	72°C	30 s/kb (expected size of fragment)
Final Extension	72°C	2 min
Hold (storage)	4°C	

4.2.7 Sequence analysis

The sequences were analysed with respect to conserved regions, using both NCBI BLAST (http://www.ncbi.nlm.nih.gov/) and SMART (http://smart.embl-heidelberg.de/); any conserved regions were compared to known sequences for the target gene in both invertebrates (*Drosophila*) and mammals (*Mus*). Phylogenetic analysis of the deduced sequence was carried out by MEGA6 (Tamuraka, *et al.*, 2013) using the neighbour-joining method (Saitou and Nei, 1987) with a bootstrap test with 1000 replicates. Evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are shown in number of AA substitutions per site. Due to time constraints this work focused on *clock, cycle, period, timeless, doubletime, cryptochrome* and *vrille*, but the transcriptome will provide opportunities for extensive future analyses.

4.3 Results

53 samples produced sufficient RNA in terms of quantity for the required sequencing. Of these, three samples: one solitarious ganglia (SA); and two brain and optic (S16A and G19B) samples showed moderate degradation. The remaining 50 samples were of high quality and all (including SA, S16A and G19B) were sequenced and included in the transcriptome described here.

Significant alignments were obtained to query sequences in 27 of the 28 queries (Table 6). The identity strength of these sequences ranged from 40% to 94%. The sequences of c*lock, cycle, period, timeless, doubletime, cryptochrome,* and *vrille* are examined in more detail and described below.

Table 6: Summary of 28 alignments. Left: query sequences from *Drosophila* where appropriate, otherwise *Mus*. Accession #: FlybaseID provided where available (starting FB...) otherwise NCBI accession number. Right: Alignment hits with query, longest appropriate contig provided with E value and %identity. E values represent the probability the alignment is due to chance, values below e-250 are displayed as 0.

Query	Species	Accession #	Contig ID	E	Identity (%)
Clock	*Drosophila*	FBpp0076500	k51.R1551152	2e-156	62
Cycle	*Drosophila*	FBpp0074693	comp8387_seq0	3e-177	65
Period	*Drosophila*	FBpp0070455	k61.J705482	2e-94	44
Timeless	*Drosophila*	FBpp0077256	k31.R13759193	0	53
Timeless (2)	*Mus*	BAA76390.2	c226810_g2_i3	0	42
Cryptochrome (1)	*Drosophila*	FBpp0083150	c229989_g1_i2	0	58
Cryptochrome (2)	*Mus*	AAD46561	c218781_g2_i1	0	71
Doubletime	*Drosophila*	FBpp0085106	comp12113_seq1	0	87
Vrille	*Drosophila*	FBpp0289297	comp7283_seq0	1e-48	55
Clockwork Orange	*Drosophila*	FBpp0081723	c226458_g4_i2	4e-36	44
casein kinase II α	*Drosophila*	FBpp0070043	comp1620_seq1	0	92
casein kinase II β	*Drosophila*	FBpp0089135	comp1835_seq0	4e-134	92
Psdp1	*Drosophila*	AAF04509	comp4097_seq2	5e-49	77
PDP1E	*Drosophila*	FBpp0076495	comp4097_seq2	2e-47	77
PP1A	*Drosophila*	FBpp0084026	comp809_seq1	0	94
PP1B	*Drosophila*	FBpp0071382	comp1193_seq0	0	93
PP2A Microtubule star	*Drosophila*	AAF52567.2	comp621_seq4	0	94
PP2A_WBTA	*Drosophila*	FBpp0084579	c150575_g1_i1	0	91
PP2A_WBTB	*Drosophila*	FBpp0288759	k51.J1586286	0	72
PP2A Twins	*Drosophila*	AAF54498.1	k61.J699880	0	83
SGG	*Drosophila*	FBpp0070450	k51.R1551264	0	86
SLIMB	*Drosophila*	FBpp0083434	k51.R1550706	0	82
PDHR	*Drosophila*	FBpp0099841	c229856_g1_i6	6e-148	55
PDF	*Drosophila*	AAF56593.1	Not Found	NA	NA
Nemo	*Drosophila*	NP 001261555	k51.S1691955	0	94
Jetlag	*Drosophila*	NP 608880	k41.R4700445	3e-48	40
Reverb	*Mus*	EDL16166	k31.R13788670	9e-46	74
Lark	*Drosophila*	Gi 62510825	k51.J1583537	1e-91	64

4.3.1 Clock

The gene encoding Clock (sgCLK) was assembled from the available dataset (Table 6; Figure 7). The assembled contig was 3384 nucleotides long with an ORF of 747 amino-acids length. The existing partial sequence from Tobback *et al.* (2011; HQ428033.2) was completely and accurately contained within the transcript (Figure 7: Left). Conserved motifs within the deduced protein sequence were seen as expected Specifically the HLH, PAS, PAS, PAC structure was similar to that seen in other species. The *S. gregaria* sequence contained a short PolyQ region, similar in length to the *Mus* PolyQ and much reduced compared to that in *Drosophila* (Figure 7: top). The sequence was successfully re-amplified by PCR producing a band of the anticipated size (Figure 7: top-right). Phylogenetic analysis grouped the deduced protein most closely with that from the cricket *G. bimaculatus*. The next closest match was with the silverfish, *T. Domestica* (Figure 7: bottom-right).

4.3.2 Cycle

The gene encoding Cycle (sgCYC) was assembled from the available dataset (Table 6; Figure 8). The assembled contig was 3131 nucleotides in length, with an ORF of 650 amino acids (Figure 8; left). There is no previously existing sequence for this species. The conserved motifs show a similarity with other known CYC/BMAL proteins, with a HLH, PAS, PAS, PAC, BCTR structure. sgCYC contains a BCTR domain in common with *Mus* and in contrast to *Drosophila* (Figure 8: top). It should therefore be referred to as a BMAL1-like protein. The phylogenetic analysis matched *S. gregaria* BMAL1-like protein most closely with that from *T. domestica*, the silverfish (Figure 8: bottom right). There is no published CYC protein sequence for *G. bimaculatus.* The sequence was successfully re-amplified by specific PCR, producing a band of the anticipated size (Figure 8: top right).

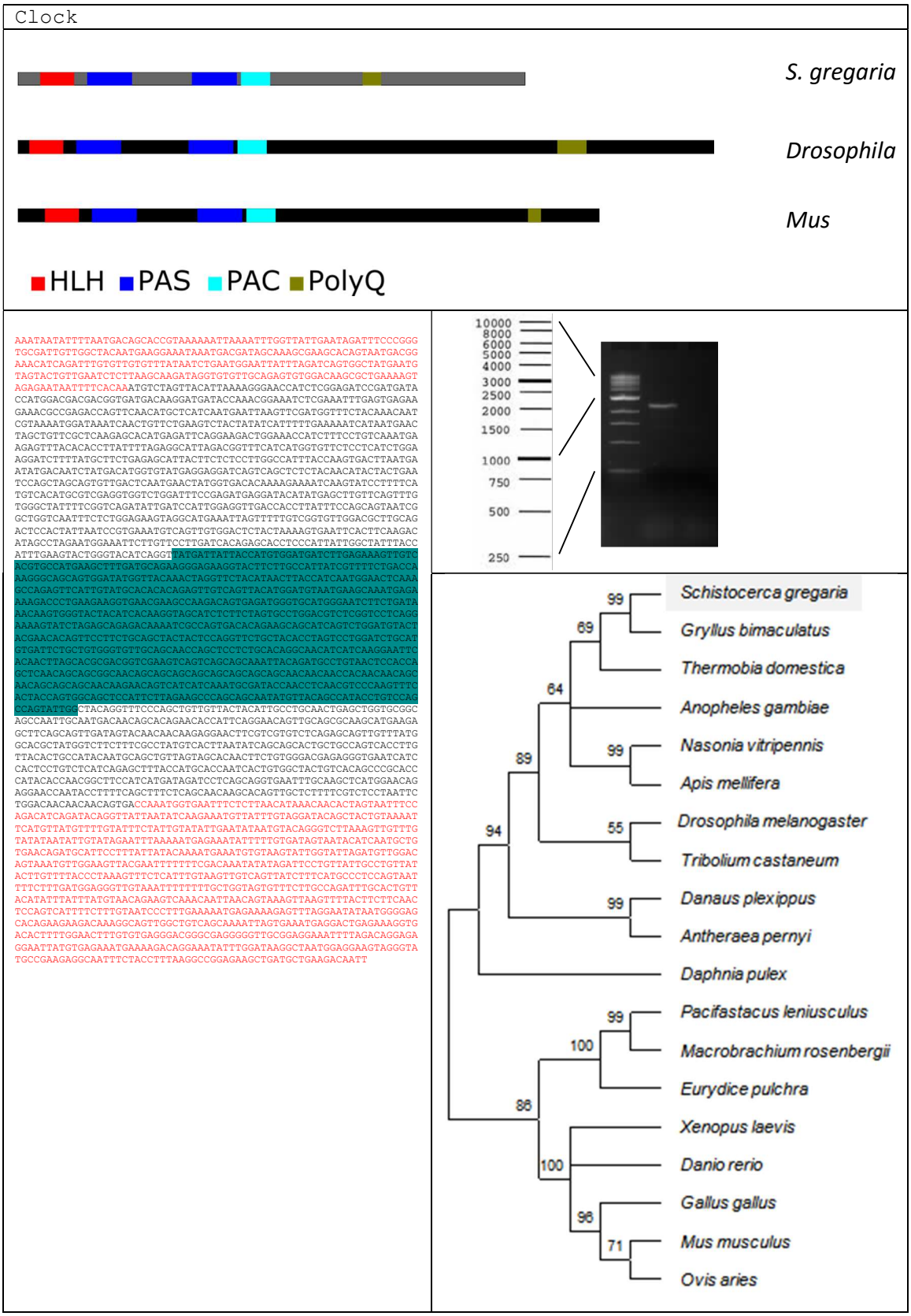
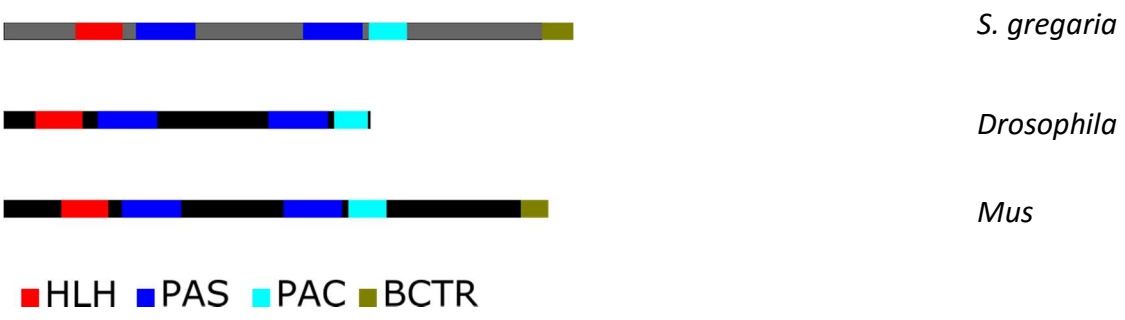


Figure 7: Analysis of Clock sequence. Top: comparison of conserved domains between *S. gregaria* (light grey), *Drosophila* and *Mus* (both black). Drawn to scale, the legend boxes are 20 AA long. Left: the deduced sequence for Clock, ORF in black, non-coding sequence in red, previously known partial sequence highlighted in teal. Right, top: PCR re-amplification of Clock from deduced sequence using gene-specific primers. Right, bottom: Phylogenetic tree, the inferred evolutionary relationships Obtained using the Neighbour-Joining method. The percentage of replicate trees in which associated taxa clustered in the bootstrap test (1000 replicates) is shown on each branch. Analysis involved 19 amino acid sequences. Analysis conducted in MEGA6.

Cycle



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ACGTTTCACGATGTGTTCGTTTTCGTTCTGTGGTATAAGTGACGAATATATCTCAAGTGTACGTGT
GAGTGACTCGTTTTCGTGGAATAGTTCACGTGATTCTGCAGCTGTGTCGTCGTGTCATTAAGGAAA
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CGGTCGTGCGTGCCGACGCGTCATGCGCTCCAAGCCTTCTTCCTTCAGAAATTCCTGTTCCACATC
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CGTCGACAGCAGCGGAGACCACTCGCGGAAACGCAAGAGCTCCTTCAGCAAAAACAGCGACATCGA
TGGCGACACTGGAGATGACGTCAAGTCCGTACGCACCAATGAGGACAGCAAGAAGCAGAACCACAG
TGAGATCGAGAAGAGGCGGCGGGACAAGATGAACACATACATTACGGAGCTGTCAGCAATGGTGCC
CATGTGTCATGCCATGTCCCGCAAGCTGGACAAGCTGACAGTGCTGCGCATGGCTGTGCAGCATCT
CAAGACCATCCGTGGTGCCGTCCACTCCTACACTGAGGGCCACTATAAGCCCTCCTTCCTCTCTGA
TCAGGAACTCAAGACCCTCATCTTACAGGCAGCTGAGGGATTCCTCTTTGTTGTTGGCTGTGATAG
AGGAAGGATTCTGTACGTCTCAGAATCTGTTTCTCAAATTTTAAGCTATTCACAGGGGGACCTATT
AGGCCAAAGTTGGTTTGATATCCTCCATCCCAAAGATGTAGCAAAAGTGAAAGAGCAGCTCTCTTC
TTCTGATCTTAGTCCCAGGGAAAGGCTGATTGATGCCAAAACTATGCTCCCAGTGAAGACAGATGT
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ACATTCGCCTGTAGTTAAAGAAGAAGCTGATACCACTACTGGTTGCCACCGGCGCAAAAAGCAGCA
ACCTCCAGATCGAAAATACTGTGTGATACAATGCACTGGATATCTCAAATCTTGGGCCCCTGCTAA
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TGTCGGTCGTGTCCAGCCACAAGTGACACAACGCCCACCACTGACAGGAATGAGACGACCCAATGT
TCGAACAATACAGTTCATATCAAGGCATGCTCTTGATGGCAAATTCCTTTTTGTCGACCAAAGGGC
TACCTTGGTACTTGGTTTCCTGCCTCAGGAGTTGCATGGTACGAGCATGTATGAGTACTACCATCA
TGAAGACATTGCACATCTTGCTGAATCACATAAAATAGCTTTGCAGGGTCCAGAAAAAGTGACAAC
TCAGGTATACCGATTTAAAAGCAAAGAGGGATCATTTATAAAACTTCAAAGTGAATGGAAATCCTT
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TCGAACTGTTGAAAGCAGTATACCACAGACTGAAGGGGCAAACCACTCACATAGTGGTTTTGATTT
CTTCACACAATCAAATGGAAACAGAGAAATGCAACGATTCATTAGTACTCATGTAGAAGCCAGCAA
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AGCAAATACAAGGTCTTCTGAAAGCAATGAGAGTTTCTGAAACACATTCCATGAGGAGCTATTGAA
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TTTTAAAACTCATTTTCAAAATAGAAAATGAAGAAATGGAATGTTCTCAGGGTTGTTATTGGTCTC
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CGAACAATGTATTTTATTACGAAATTTTAGTGTCAATTTTGAAGATATTCTTGTAAATATTTATGC
CTCTGTACTCAAAGACTTTTCAATGCATTCTCGACAGAAGCCTTGGGAAGAGAATCTGACATTATT
TGCCAGGCAAAAATGTTATTTGGACCAATGTTCAATGTGTGTTGTGCACTGATCTTGAAGATTTGT
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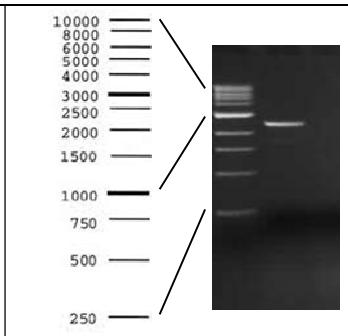

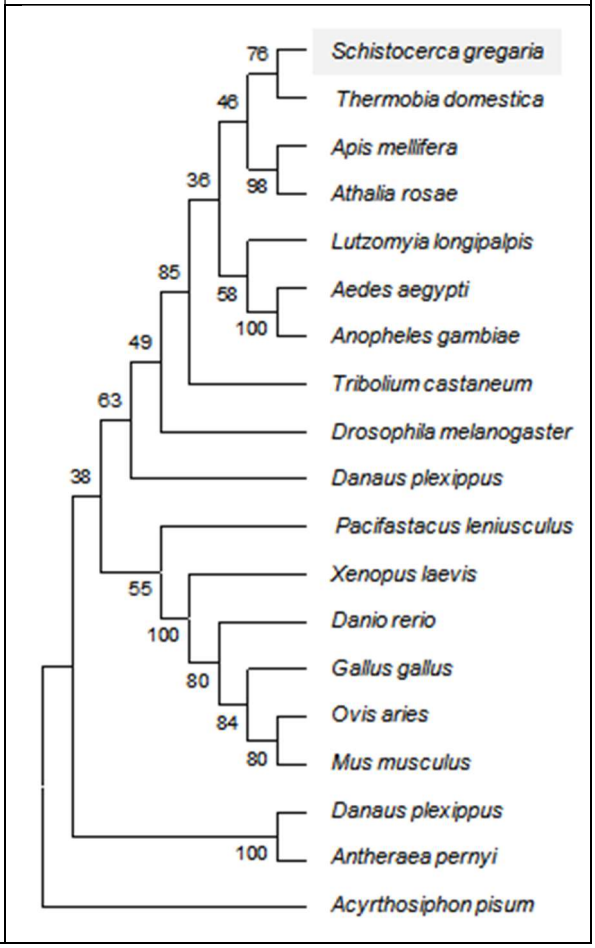

Figure 8: Analysis of Cycle sequence. Top: comparison of conserved domains between *S. gregaria* (light grey), *Drosophila* and *Mus* (both black). Drawn to scale, the legend boxes are 20 AA long. Left: the deduced sequence for Cycle, ORF in black, non-coding sequence in red. Right, top: PCR reamplification of Cycle from deduced sequence using specific primers. Right, bottom: Phylogenetic tree, the inferred evolutionary history using the Neighbour-Joining method. Percentage of replicate trees in which associated taxa clustered in the bootstrap test (1000 replicates) is shown on each branch. Analysis involved 19 amino acid sequences. Analysis conducted in MEGA6.

4.3.3 Period

The gene encoding Period (sgPER) was assembled from the available dataset (Table 6; Figure 9). The assembled transcript was 5774 nucleotides long, with an ORF of 1265 amino acids. The existing partial sequence from Tobback *et al.* (2011; HQ428031.1) was completely and accurately contained within the transcript (Figure 9: left). The conserved motifs were similar to other PER sequences, with a period-C conserved region similar to that of *Mus* (Figure 9: top). Phylogenetic analysis showed closest relationships to *G. bimaculatus* and the German cockroach, *Blattella germanica* (Figure 9: right). No protein sequence was available for *Thermobia domestica.*

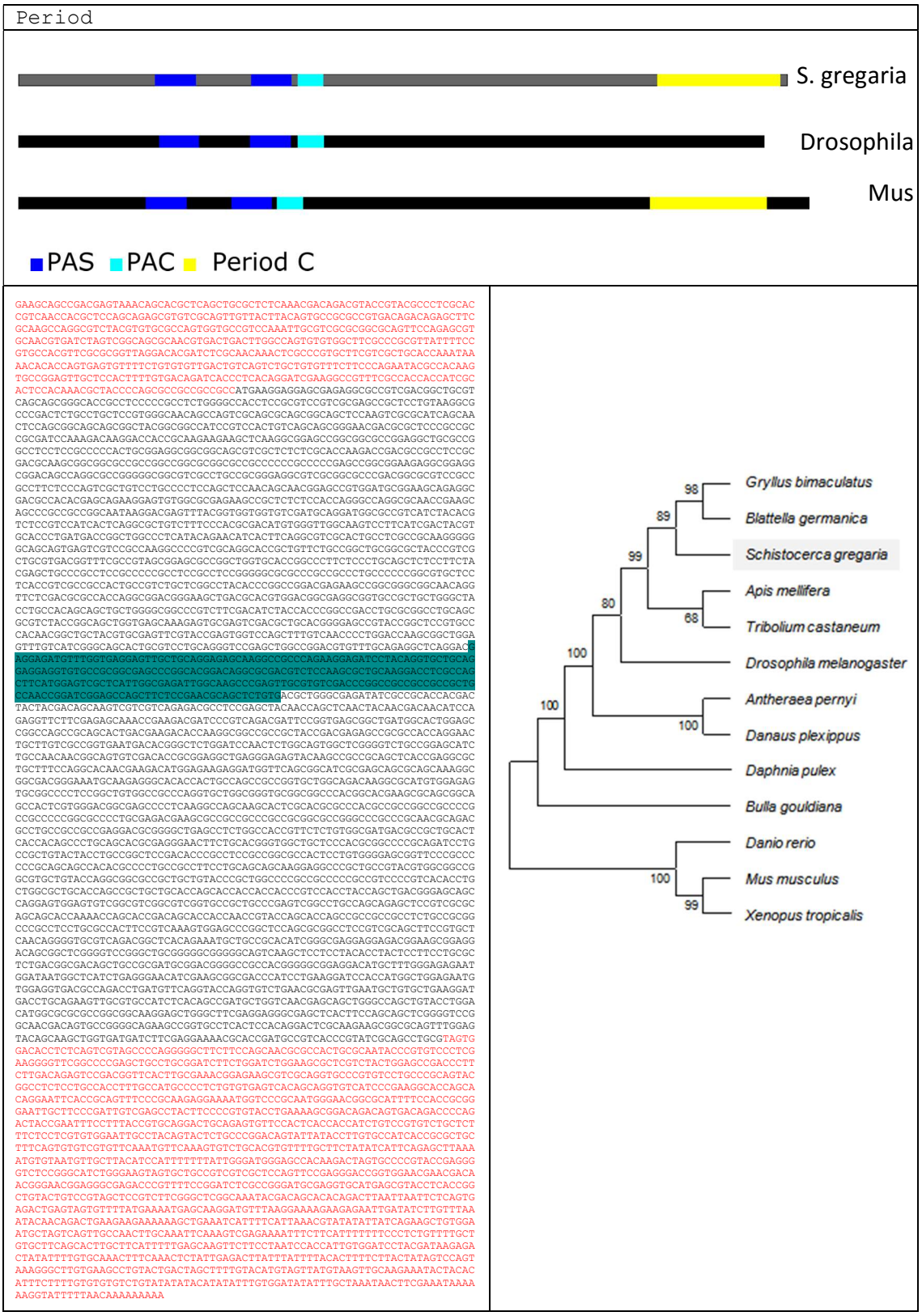

Figure 9: Analysis of Period sequence. Top: comparison of conserved domains between *S. gregaria* (light grey), *Drosophila* and *Mus* (both black). Drawn to scale, the legend boxes are 20 AA long. Left: the deduced sequence for Period, ORF in black, non-coding sequence in red, known partial sequence highlighted in teal. Right: Phylogenetic tree, the inferred evolutionary history using the Neighbour-Joining method. Percentage of replicate trees in which associated taxa clustered in the bootstrap test (1000 replicates) is shown on each branch. Analysis involved 13 amino acid sequences. Analysis conducted in MEGA6.

4.3.4 Cryptochrome 1 & 2

Two genes encoding Cryptochrome (CRY) were assembled from the available dataset (Table 6; Figure 10; Figure 11) and are here referred to as sgCRY-1 and sgCRY-2. sgCRY-1 was assembled into a transcript that was 6050 nucleotides long with an ORF of 540 amino acids (Figure 11: Left). sgCRY-2 was assembled into a transcript of 4785 nucleotides with an ORF of 566 amino acids (Figure 11: Right). Both paralogs contained conserved motifs for photolyase and an FAB binding domain also found in *Drosophila* CRY and *Mus* CRY-1 and CRY-2 (Figure 10: Top). sgCRY-1 was phylogenetically most similar to CRY in *Drosophila* and CRY-1 in species such as the butterfly, *Danaus plexippus* and moth *Antheraea pernyi* and is therefore a dCRY-like cryptochrome. sgCRY-2 was phylogenetically most similar to CRY-2 in species including *Danaus plexippus* and *Antheraea peryni* and together with them is related to *Mus* CRY-1 and CRY-2. It is therefore an mCRY-like cryptochrome (Figure 10: Bottom).

4.3.5 Timeless & Timeout

Genes encoding both Timeless (sgTIM-1) and Timeout (sgTIM-2) were assembled from the available dataset (Table 6; Figure 12; Figure 13). For Timeless (sgTIM-1) the assembled transcript contained 5774 nucleotides, with an ORF of 1265 amino acids. The existing partial sequence (HQ428032.1; Tobback *et al.*, 2011) was fully and accurately contained within the contig (Figure 13: left). sgTIM-2 was assembled into a contig of 4862 nucleotides with an ORF of 1454 amino acids (Figure 13: right). sgTIM-1 lacked the Timeless-C domain, and is thus most similar to TIM-1 in *Drosophila*. sgTIM-2 contained this domain, and is thus most similar to *Drosophila* TIM-2 and *Mus* TIM. sgTIM-1 was phylogenetically most similar to TIM-1 of *G. bimaculatus* (Figure 12: bottom). Timeout (sgTIM-2) was phylogenetically most similar to TIM-2 of the red flour beetle *Tribolium castaneum* (Figure 12: bottom). It should be noted that many genera (e.g. *Xenopus, Mus, Danio* and *Apis*) do not express two Timeless paralogs. In these cases the single expressed gene has either been described as “mammalian like” in reference to the single *Mus* Timeless sequence that clusters with TIM-2 sequences or alternatively the single paralog may cluster to *Drosophila* Timeless, TIM-1 and is thusly “D*rosophila* like”.

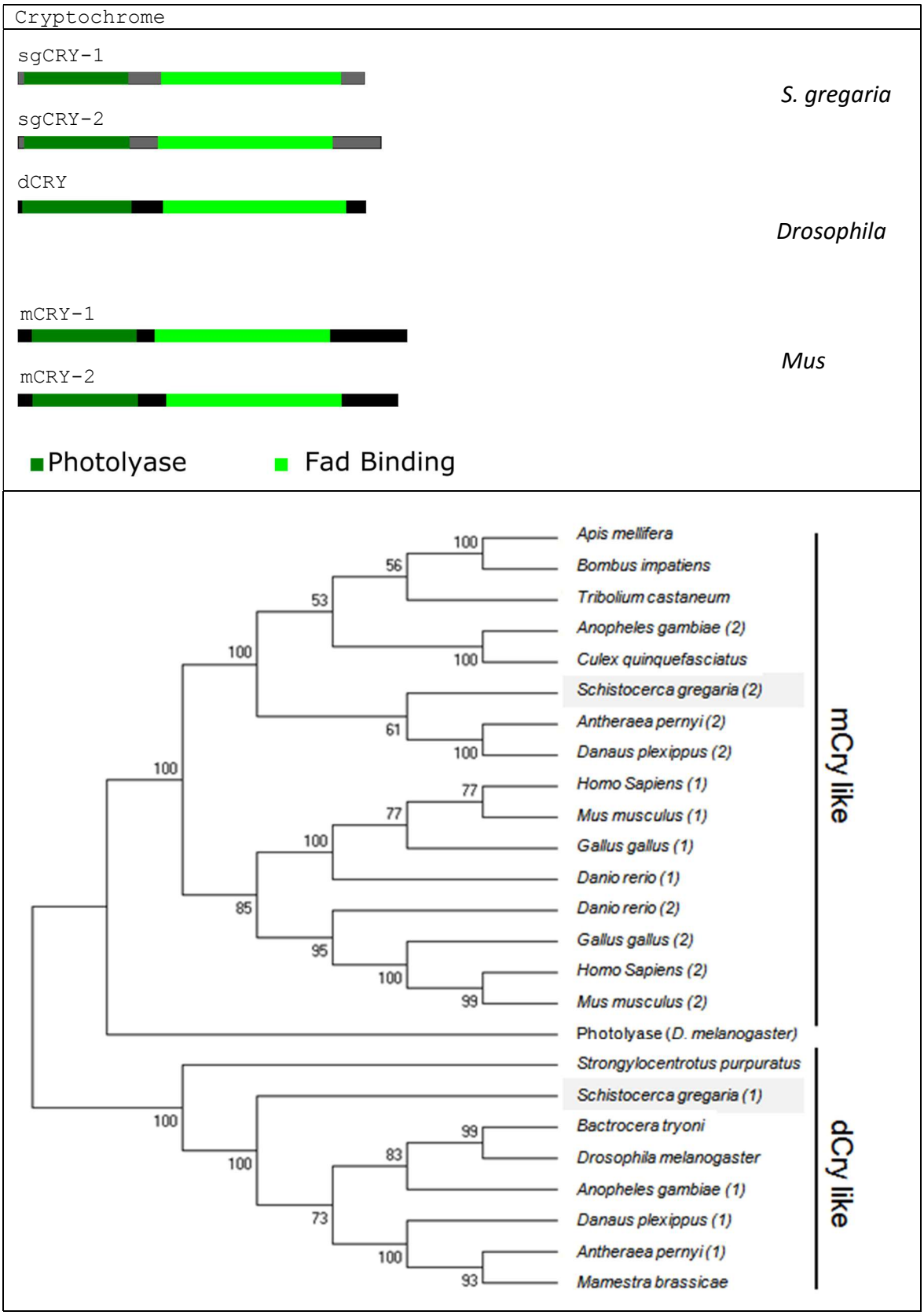

Figure 10: Analysis of sgCry-1 and sgCry-2 sequences. Top: comparison of conserved domains between *S. gregaria* (light grey), *Drosophila* and *Mus* (both black). Drawn to scale, the legend boxes are 20 AA long. Bottom: Phylogenetic tree, the inferred evolutionary history using the Neighbour-Joining method. Percentage of replicate trees in which associated taxa clustered in the bootstrap test (1000 replicates) is shown on each branch. Analysis involved 25 amino acid sequences (24 Cry, 1 Photolyase). Analysis conducted in MEGA6. Where more then one cry homolog exists in a species the entry is labelled (1) or (2) accordingly.

Cry-1	Cry-2
ACTATATAACATGGAGCACACTCCTAGAGAAGGCACAAGATTTGTGCTACGGAGCGTAGACCCACC CCGCATTGTTGCGAATGAGCCGATTGTACTTCTGTTGAAGCTTACATGTCGTCGAATATAACCTTG ATTCCGTTATATTAGATTTCTACTCGTGAGTTCACCTGACCAGTCAGCTGTTTAACACTTACGCTG CAAGTACAGCTGTTGGAATATTAAATGGAATTATAATTCCATGATTACAGTGTTGCCAGTGATTGA ACTCAAAATAAAAACGAAGCATCCAGTTTATTGATCACCAGCTGTATACTTATATAACTGTTAAAG CCGACACAGGGTATAGTGCCTTGAGAGTCCTGAATACGCCAGCAGTCCATACATTGTCGTAGCTAT GGCAGGAGGCATGAAACGGAAGGTTACTGTTCATTGGTTTCGGCATGGCTTGCGCTTACATGACAA CCCAGCCTTGCACGAAGCATTGAAAGAAAGCGAAGAATTTTATGCAGTATTCATTTTCGATGGAGA AAGCGCAGGCACAAAAAATGTAGGTTTCAACAGAATGAGGTTTCTGATCGAATCCCTCAAAGATAT TGACAGGCAGCTTAGAGCAGTTGGTGGACGATTGTATGTCTTTCACAACAACCCAGTCTCTGTGTT TAAAGGCTTGCATGAAGAGCTAGGTCTTTATAAACTTTGTTTTGAACAGGACTGTGAACCAATTTG GCGTCCCAGAGATAACGCTGTTAAAAAGTTTTGTGAAGAGCATGGAATAATTTGTGTTGAAAAAGT CTCTCACACACTTTGGGATCCTAATGTTGTTATTAAAACGAATGGAGGTACGCCCCCATTAACGTA CCAGATGTTCTTGCATACAGTTGGTGTTATTGGCCCACCTCCACGACCTGTGGACAATATAGACTG GACAGGTGTTACATTTGGAAAGCTCTCTGATTACCTTTCTACAGAATTGAAGGTTGTCGACAGTGT TCTAACACCTGAGAAATTTGGTGTGTTTCCTGAAATGGGCAGTGGGGACAAATGATCCGTTGGAT TGGTGGAGAAACACGAGCTCTGGAACACTTGAAAGAACGCCTGTCTCTAGAGGAAGCAGCATTCCG AGATGGTTACTATCTTCCGAATCAGTCAAAACCTGACCTCCTGGGGCCACCAACCAGTCAAAGTGC AGCACTTCGCTTTGGATGCCTCTCTGTTCGCAAGTTTTATTGGAGCATTCATGACTTGTTCCACTC TGTTTACAATGGCAATCTTCCTTTGAACCAAAAGATCACTGGCCAGTTAATATGGAGAGAGTATTT TTACACAATGTCAGTCAATAATGAGTTTTATGGTGAAATGGCAAACAATCCAATATGCCTGAACAT TCCATGGAAAGATGGATCTGAGAAGGGACATCTAGAGAGATGGCAAGAAGGGAAAACTGGATATCC ATTCATTGATGCTGCTATGCGACAGCTAAAGCTGGAAGGTTGGATTCACCATGTGGCTCGTAATGC TGTGGCCTGTTTTCTTACACGTGGAGATCTGTGGATCAGTTGGGAGGCAGGCTTGCAACACTTTTT GAAGTACTTGTTGGATGCTGACTGGTCAGTGTGTGCAGGCAACTGGATGTGGGTGTCCAGTTCTGC TTTTGAACAACTTCTTGATTGTTCACATTGTGTTTGCCCAGTTAACTATGGCAGGCGGCTCGATCC TTGGGGGGAGTATATAAAGCGTTACATACCTGAACTACAAAACTATCCGGTAGAATATTTATATGA GCCGTGGAAAGCACCACTTGAGGTGCAAGAACGAGCTGGCTGTGTTATTGGAAAGGACTACCCAGA GAGGATTGTGGATCATCAAGATGCTTCCAGAAGGAATAAACAATACATGCAGCAAGTCCGAGACAT GCTGATGAAAGCTCCTCCACATTGTTGCCCATCTAATGAGGAGGAGACCAGGCAGTTCATGTGGTT GCCAGAAAGCTGCATGGAACATGTACTGCATGCTTAGTAAACTTTTACTGTAAGGAAATGTTAATT AAAAATCTCCTTAGCATCTTAAAGTACCATTGTTCCTCGGCAGAATTTCCTTTGTATGTGTATGTA GAAATAGCTTCATAACCAAGTGGTCATTGCTGTAAGTTAAATTAAGTAAAGATGCACATTATCTGT TCTGTCCAGTATGAACACATCCTTTTGTCATTGACAGTTGAATGCAGAGTTGTTACTGTTTATTCA AAGTTTTTTTTGTAGTGATTCCACAGTTTTGTCAATATATAATCAGTGGTATTAAATAATTTATAAG ATTCGCAAACACGAGAAGGTGTAAAAAAAAAGTAGAAATACTGATCAGTGAGAGAAACTGGGCTG AATCATATTATGTACTTATTTACTTGTTTTGAGTTTAACTGTTACACCAAAGTTGCTTGAAAGCTA TGATCTAGGGGATGTCCTTGGAAAGTAGCTGTTTGTCTGGAGGAGGATTTAATATATTATTACTAA ATGTTTTTGGTCTGTAAGCCAGCTTCACATTTAGTGGACAGCTGTATATATCTTCATTATTAACTA ACACTAAGGGTCATTTAATAATTGAAGAATATTTCATCATATAAGTGTATTAACACTGCAAAAACA GGCCAAGCACAACTTTATGTACTTTTATGTGCCACTCCATATAAACACCCTGAACATTCACACTTT TGCCAACACCATCTAAGCAATTCCTTCAGATAATACGCACTTGCTTGGTCATTCAAAGTACAAAAA 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Figure 11: Deduced sequences for sgCry-1 and sgCry-2, ORF in black, non-coding sequence in red

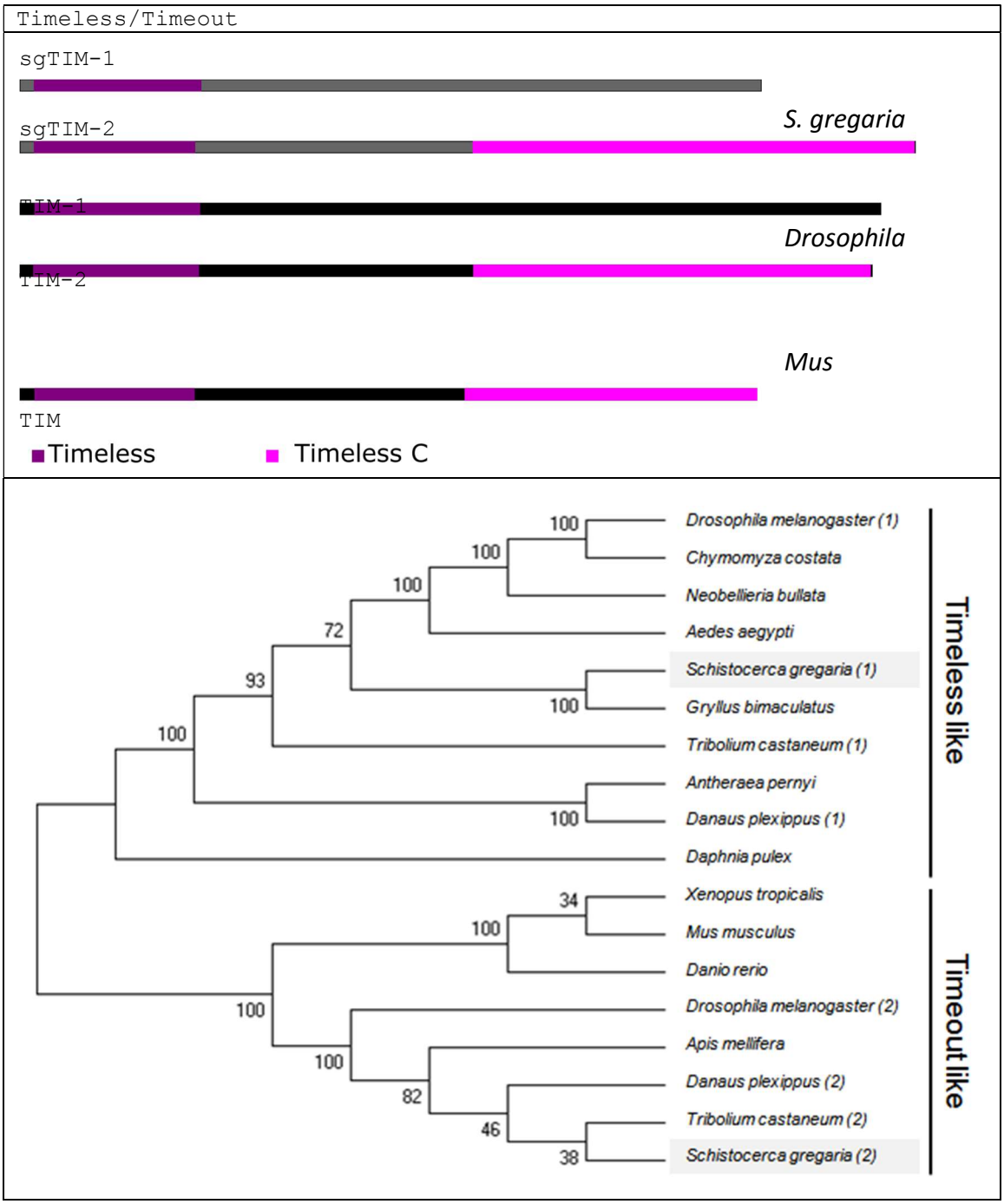


Figure 12: Analysis of sgTim-1 and sgTim-2 sequences. Top: comparison of conserved domains between *S. gregaria* (light grey), *Drosophila* and *Mus* (both black). Drawn to scale, the legend boxes are 20 AA long. Bottom: Phylogenetic tree, the inferred evolutionary history using the Neighbour-Joining method. Percentage of replicate trees in which associated taxa clustered in the bootstrap test (1000 replicates) is shown on each branch. Analysis involved 17 amino acid sequences. Analysis conducted in MEGA6.

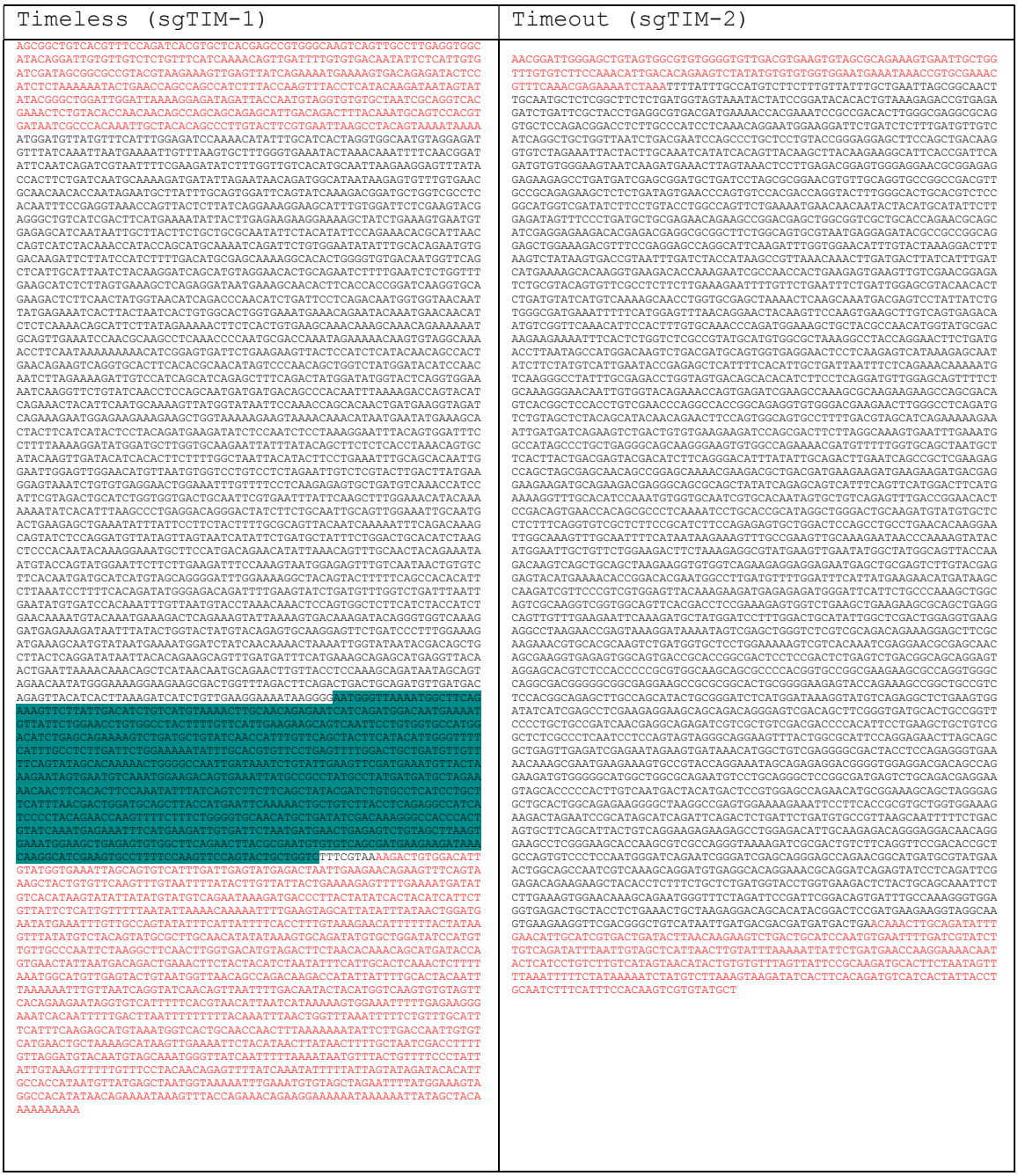


Figure 13: Deduced sequences for Timeless (TIM-1) and Timeout (TIM-2) as described in Figure 12, ORF in black, non-coding sequence in red, existing partial sequence is highlighted in teal.

4.3.6 DoubleTime & Vrille

The genes encoding two regulatory proteins, Doubletime (DBT) and Vrille (VRI), were assembled from the available datasets (Table 6). Doubletime showed similarity to homologues in *Drosophila* and *Mus* (Casein Kinase 1), with a conserved Pkinase domain (Figure 14: top). Vrille showed similarity to the available sequence in *Drosophila*, with a BRLZ domain present (Figure 14: bottom). However there is a 40% reduction in the size of the deduced protein compared to *Drosophila*.

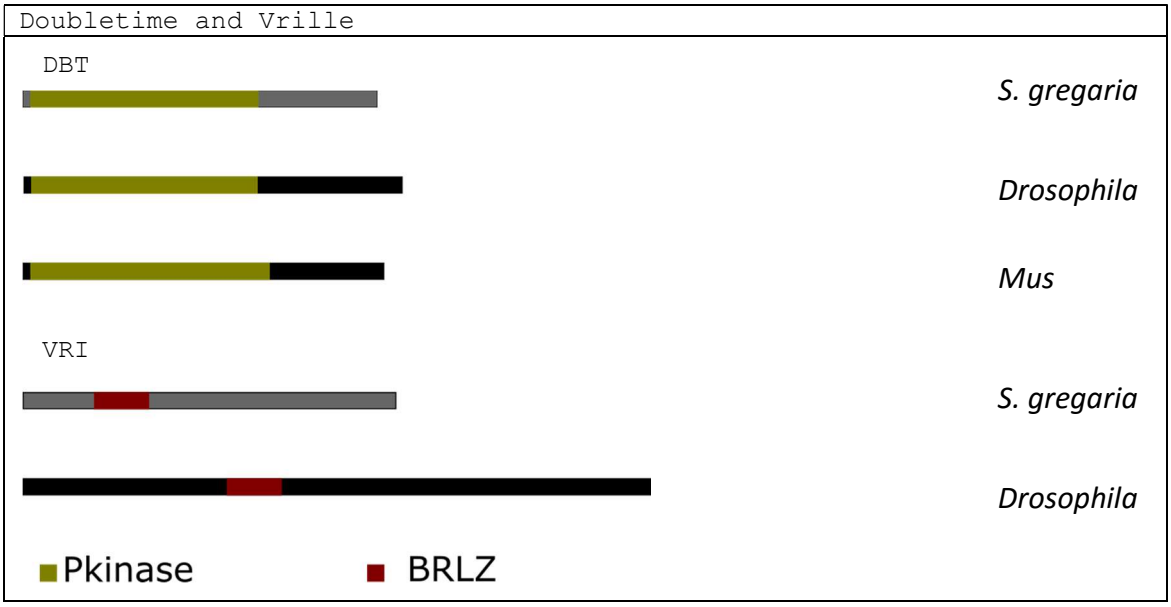


Figure 14: Analysis of Doubletime (Top) and Vrille (Bottom) deduced protein sequences. Comparison of conserved domains between *S. gregaria* (light grey), *Drosophila* and *Mus* (both black). Drawn to scale, the legend boxes are 20 AA long.

4.3.7 Additional circadian genes

Putative sequences were also produced for: *clockwork orange* (*cwo*), *casein kinase II alpha & beta* (*ckIIα* & *β*), *psdp1, pdp1e, pp1a, pp1b, pp2a, microtubulestar, pp2a_wbta, pp2a_wbtb, twins, shaggy (sgg), slimb, pdhr, nemo, jetlag, reverb* and *lark* (Table 6; Figure 15). In the interests of time and brevity these genes have not been investigated in depth here, but will provide the basis for a large amount of future work with this dataset.

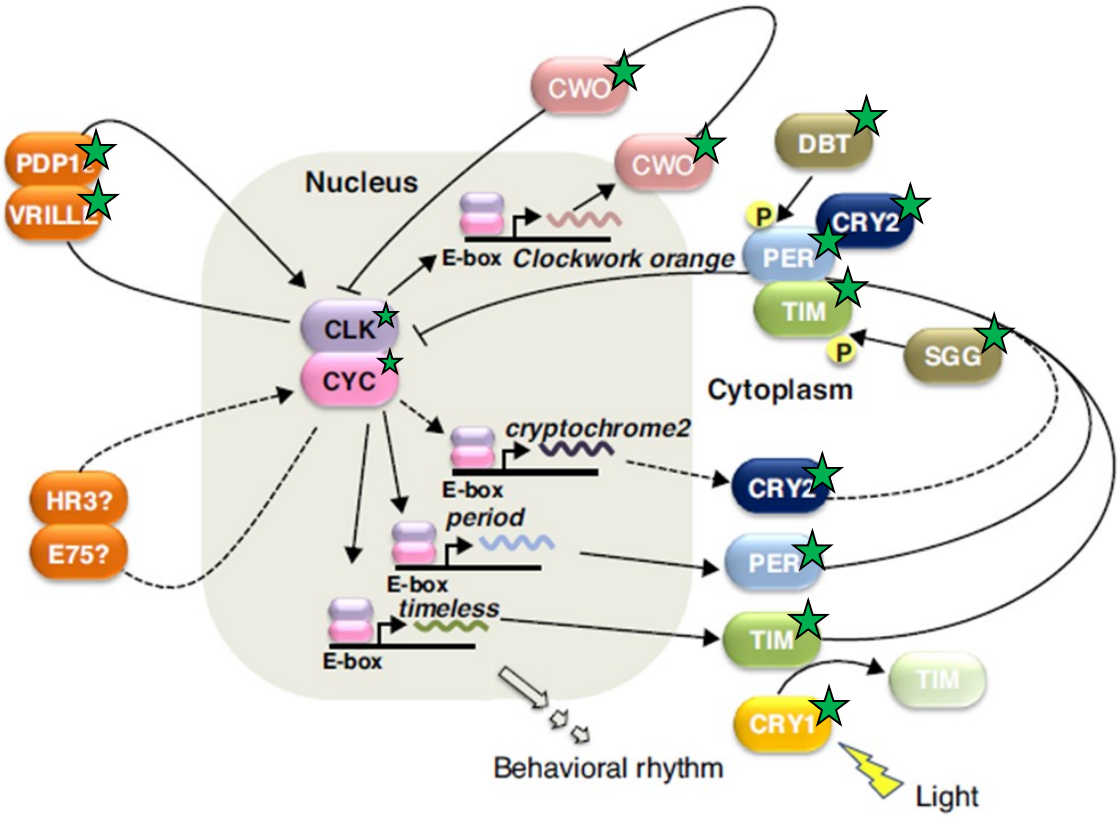


Figure 15: Proteins involved in the central endogenous circadian clock (Adapted from Tomioka and Matsumoto, 2015). Solid lines indicate pathways confirmed in *Drosophila,* dashed lines are pathways proposed in other insects. Green stars indicate that the gene encoding the protein has been deduced in the present work.

4.4 Discussion

The main aims of the work described in this chapter were to: (1) generate a transcriptome for use in later quantitative work; and (2) to use this transcriptome to discover the sequences of the central clock genes and regulatory genes involved in controlling and maintaining the central clock in *S. gregaria*. Surprisingly little was known about the circadian clock genes of this important model species of phenotypic plasticity, with only partial sequences available for a small selection of the key genes, all of which were contained within the sequences reported in this work. The detection of sequences based on all queried proteins was complete, with full length transcripts assembled for all those examined (Figure 15). This confirms the presence of the related pathways in *S. gregaria*, increasing the understanding as to the molecular working of the circadian clock in this organism, with sequences now available for more in-depth analysis. In addition this transcriptome presents the opportunity to study the structure and expression profiles of any gene of interest for future studies. Chapter 5 will make use of the transcriptome produced here to: (1) analyse the cyclic expression of the genes described here; (2) identify clock controlled genes (CCGs); and (3) examine differential patterns of expression brought about by population density dependent phenotypic plasticity.

4.4.1 Clock and Cycle

This work has deduced the complete sequence for *clock* and *cycle* from the generated CNS transcriptome of *S. gregaria*. sgCYC shows the expected bHLH, PAS and PAC functional domains; but of particular interest is the presence of the BCTR which suggests that the function of the protein is similar to that of BMAL in *Mus*. The BCTR of BMAL acts as the transcription region, whilst CYC lacks this region and the transcription activity is carried out by CLK, localised to the large PolyQ region in *Drosophila*. It is likely that this region is critical to the function of the gene, as deletion of this region in *Mus* abolishes the CLK-BMAL heterodimer's transcription activity (Moriyama *et al.*, 2013). The Clock deduced protein sequence reported here also contains the bHLH, PAS and PAC domains required for forming a heterodimer with CYC/BMAL. The 27 AA PolyQ region of the deduced *S. gregaria* CLK protein resembles the 24 AA region of *Mus* more closely than the 57 AA region of *Drosophila*. This

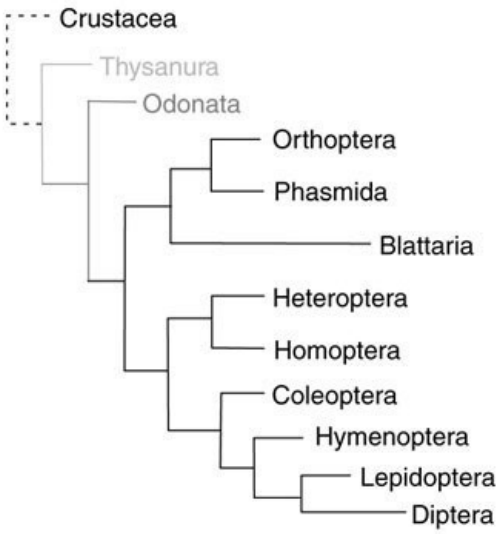
provides further evidence that the transcriptional activity of the sgCLK-sgCYC heterodimer is likely attributable to the BCTR on CYC/BMAL and not in the PolyQ region of CLK as is the case in *Drosophila.* Analysis of the expression levels of these genes over the time-points available will provide further evidence as to the similarity of the endogenous circadian clock to the mammalian system.

4.4.2 Cryptochrome

Drosophila expresses only one CRY homolog named dCRY or CRY-1. This protein is light-sensitive. When activated, it binds to TIM in TIM-PER dimers, leading to dimer degradation and a consequent reduction in TIM-PER dimers. *Mus,* however*,* expresses two CRY homologs, mCRY-1 and mCRY-2. These are distinct from dCRY and are not photosensitive. I show that *S. gregaria* expresses one CRY-1 homolog (sgCRY-1) and one CRY-2 homolog (sgCRY-2). This is similar to the results seen in *D. plexippus* (Zhu *et al.*, 2008a & b) and holds with the hypothesis that non-model insects posses a endogenous circadian clock that has retained ancestral characteristics that have been lost in *Drosophila* (Tomioka and Matsumoto, 2015), containing elements of both mammalian and *Drosophila* like clocks.

4.4.3 Conclusion: *G. bimaculatus* and *T. domestica* and the ancient clock.

Taken together the *S. gregaria* sequences described here are closely aligned to the known sequences of *Gryllus bimaculatus* and *Thermobia domestica* where they are available. *G. bimaculatus* is a cricket and was therefore expected to present the closest match and does so where available. The similarity of the deduced Cycle protein to that of mammalian BMAL is consistent with findings in *G. bimaculatus*, where the BCTR function domain has also been identified (Uryu *et al.*, 2013). All circadian genes with sequences available for *G. bimaculatus* have been identified in *S. gregaria* (Figure 16 Bottom). *T. domestica* represents the most primitive clock for which circadian biology has been studied as a member of the most evolutionary divergent order and the consistencies between *S. gregaria* and this species is a strong indication of the conversed nature of the circadian clock in insecta as a whole. The overall pattern of gene expression seen in Figure 16 (Bottom) suggests that some Diptera, Coleoptera and Hymenoptera exhibit a reduced suite of circadian clock genes while Orthoptera and Lepidoptera express the full ancient endogenous circadian clock.



Species		Circadian Gene										
		clock	*cycle*	*period*	*timeless*	*timeout*	*cry-1*	*cry-2*	*doubletime*	*vrille*	*cwo*	*pdp1*
Diptera	*Drosophila melanogaster*	Y	Y	Y	Y	Y	Y	N	Y	Y	Y	Y
	Musca domestica	Y	Y	Y	Y	?	Y	N	?	Y	?	?
	Aedes aegypti	Y	Y	Y	Y	Y	Y	Y	?	?	?	Y
	Anopheles gambiae	Y	Y	Y	Y	Y	Y	Y	?	?	?	Y
Lepidoptera	*Antherea pernyi*	Y	Y	Y	Y	?	Y	Y	?	Y	?	?
	Bombyx mori	Y	Y	Y	Y	Y	Y	Y	Y	?	?	?
	Danaus plexippus	Y	Y	Y	Y	Y	Y	Y	Y	Y	?	Y
Hymenoptera	*Solenopsis invicta*	Y	Y	Y	N	Y	N	Y	?	Y	Y	Y
	Apis mellifera	Y	Y	Y	N	Y	N	Y	?	Y	?	Y
Coleoptera	*Tribolium castaneum*	Y	Y	Y	Y	Y	N	Y	?	Y	Y	Y
Hemiptera	*Acyrthosiphon pisum*	Y	Y	Y	Y	Y	Y	Y	?	Y	?	Y
	Riptortus pedestris	Y	Y	Y	?	?	Y	Y	?	Y	?	?
Orthoptera	*Gryllus bimaculatus*	Y	Y	Y	Y	?	Y	Y	Y	Y	Y	Y
	Rhyparobia maderae	?	?	Y	Y	?	?	Y	?	?	?	?
	Schistocerca gregaria	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Thysanura	*Thermobia domestica*	Y	Y	?	Y	?	?	Y	?	?	?	?

Figure 16: Top, Phylogenetic tree of insect orders (Evans and Gundersen-Rindal, 2003). Bottom, core circadian genes found in insects of various orders on the basis of a database survey (modified from Tomioka and Matsumoto, 2015). Y = existence, N = absence, ? = to be determined. *S. gregaria* (from the present study) is highlighted in grey.

Chapter 5:
Differential and rhythmic patterns of gene expression

5.1 Introduction

In this chapter I use the assembled transcriptome (see Chapter 4) to examine differences in gene expression between gregarious and solitarious individuals in the ganglia and brain + optic lobes, and rhythmic expression in the brain + optic lobes under 12 h light : 12 h dark (LD) conditions.

5.1.1 Diurnal and circadian rhythms

The patterns of cyclic gene expression seen in an organism are generated by two main processes. Environmental factors, such as light intensity or temperature, can act directly on gene expression pathways, creating transcriptome rhythms that are entirely responsive in nature (Lemberger *et al.*, 1996). These rhythms are expressed only under cyclic environmental conditions. Where these natural rhythms, e.g. light-dark (LD), follow a daily pattern, the expressed patterns are best described as diel rhythms. Gene expression can alternatively be driven directly by the central molecular clock which is regulated by the endogenous cycling of *per-tim* and *Clk-cyc* (Bell-Pedersen *et al.*, 2005). Genes controlled in this way are known as 'clock-controlled genes' (CCG) and differ from environmentally triggered rhythms by persisting under constant conditions (e.g. constant dark, DD). Where the period of these intrinsic rhythms is approximately 24 h in length, these rhythms are termed 'circadian'. As the central molecular clock is synchronised to the environment under LD conditions the expression of a CCG will correlate with the expression of the central clock genes (*per* and *tim, or Clk* and *cyc*), although a lag period may be present. Many genes display properties of both processes. In these cases the pattern of gene expression may display circadian rhythmicity under constant conditions while also reacting to the changing environmental variables (e.g. AtSTP1 expression; Stadler *et al.*, 2003). Under LD conditions the patterns of expression of these genes might not be distinguishable from either of the previous two conditions.

The molecular circadian clock relies on the rhythmic expression of component genes. In *Drosophila*, *Clock* shows rhythmic expression, with *timeless* and *period* showing antiphasic rhythmic activity. *Cycle* does not show rhythmic expression. This pattern differs to that in other insects, including the mosquitoes *Aedes aegypti* and *Anopheles gambiae*. In these cases all four genes show rhythmic expression, with *per* and *tim* showing antiphasic expression to *Clk* and *cyc* (Meireles-Filho and Kyriacou, 2013; Figure 1A).

The field cricket *Gryllus bimaculatus* is the closest related insect to *S. gregaria* for which relatively complete circadian gene expression data exists. It shows expression similar to that of *A. Gambiae* (Figure 1), with rhythmic expression of *cyc*, *per* and *tim* (Moriyama *et al.*, 2012). In contrast to both *Drosophila* and *A. gambiae*, however, under normal conditions *G. bimaculatus* shows a constitutive expression of *Clk*. Rhythmic expression can occur when expression of *cyc* is repressed; suggesting that cyclic *Clk* expression may represent a secondary mechanism in this organism (Moriyama *et al.*, 2012). The only work relating directly to a circadian gene expression profile in *S. gregaria* is that of Tobback *et al.* (2011). Here partial sequences for *Clock*, *period* and *timeless* were produced from a database of Expressed Sequence Tags (EST) utilising Blast (NCBI). Expression levels of these genes in the brains of adult *S. gregaria* (of both genders pooled) were studied using real time qPCR with samples collected every 3 h over 24 h under LD conditions. This experiment showed that both gregarious and solitarious locusts have circadian expression of three core circadian genes, *Clock*, *period* and *timeless* (Figure 1, Bottom). *Clock* is highly expressed at ZT 0 while *period* and *timeless* come to a peak at 12 – 18 h. However, all three genes appear to follow a similar expression profile with a peak at ZT 0, dipping between ZT 3-9 before rising again through ZT 12-15. This, along with the non-specific RNAi knockdown results, whereby knockdown of *per* increases *tim* expression and *vice versa*, suggest limited accuracy in the results. RNAi knockdown of *clock* had a lethal effect, while knockdown of *period* and *timeless* caused a significant reduction in the number of offspring. No behavioural assays were carried out as part of this knockdown procedure, so it is unclear what effects, if any, there might have been on circadian rhythms of behaviour.

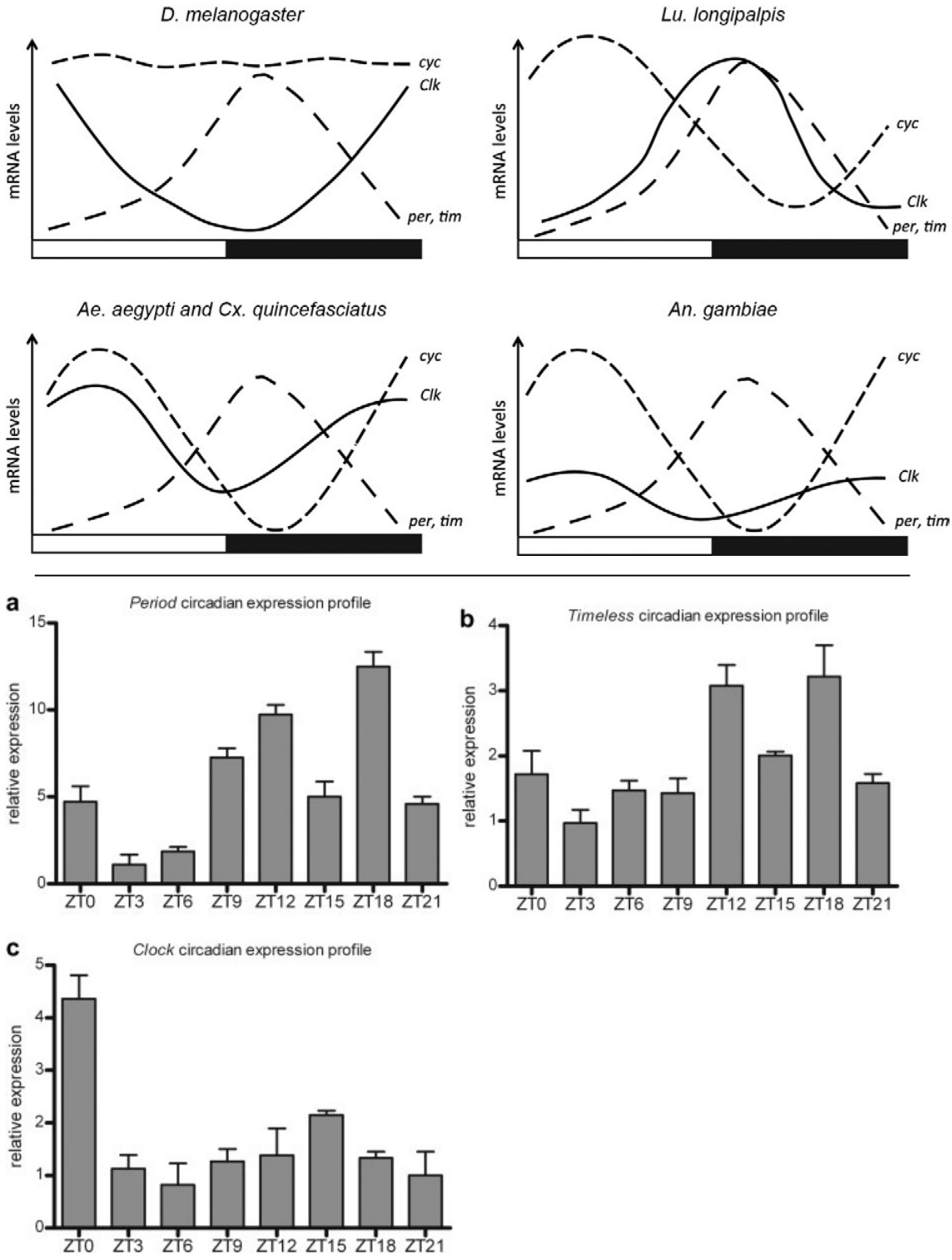


Figure 1: Top: Gene expression of *Clock* (*Clk*), *cycle* (*cyc*), *period* (*per*) and *timeless* (*tim*) in *Drosophila*, *Lutzomyia longipalpis, Aedes aegypti* and *Culex quinquefasciatus*, and *Anopheles gambiae* (Adapted from Meireles-Filho and Kyriacou, 2013). Bottom: Expression of *per* (a), *tim* (b) and *clk* (c) in a combined dataset of 4 gregarious and 3 solitarious adult locust brains. The bar shows the mean and the whiskers indicate SE. (Tobback *et al.*, 2011)

Genome-wide profiling of all expressed genes via DNA microarray in *Anopheles gambiae* under both LD and DD conditions revealed rhythmic gene expression in many pathways including olfaction, visual transduction and immune responses (Rund *et al.*, 2011). Under LD conditions over 1200 genes were identified with a period length of 20 - 28 h in the whole head. This represents 9.7% of the tested gene set. Ptitsyn *et al.* (2011) carried out a DNA microarray analysis of *A. aegypti* under LD conditions and also showed a range of processes to be rhythmic in gene expression including growth, development and immune response. It is therefore likely that similar pathways will be rhythmically expressed in *S. gregaria*.

5.1.2 Phenotypic change

Phenotypic plasticity refers to the ability of an individual animal to change its phenotype based on the prevailing environmental situation. In the case of complex phenotypes, as seen in the density dependent phenotypic plasticity in the locust, which are formed of multiple traits, plasticity can be viewed as a number of traits tending to one or the other extreme phenotype. As such there are two possible mechanisms at play. First, the triggering environmental factor acts via a single or small number of loci, for example a single gregarising factor such as a hormone or neurotransmitter, that act to regulate the relevant traits to produce the phenotype (Bradshaw, 1965; Schlichting, 1986). Alternatively, the environment may act via a number of loci each independently varying a subset of the affected traits. This may be the result of a single environmental factor, such as temperature, or various environmental factors that may co-occur, each effecting a smaller change that makes a part of the overall phenotypic change (Via and Lande, 1985; Via, 1987).

While little is known of the *S .gregaria* circadian clock, an EST based oligo-microarray has been carried out comparing gene expression in the CNS of gregarious and solitarious adults (Badisco *et al.*, 2011). Sampling did not take account of the time of day with samples collected between 10am and 4pm (L. Badisco, personal communication), it is therefore likely that this study represents a bias towards expression patterns during the day. The microarray study revealed differential expression between gregarious and solitarious samples of CNS tissue and was limited to genes included in the EST database. Key genes differentially expressed in this study

included helicase and a probable cytochrome which were more highly expressed in gregarious samples, and genes involved with RNA processing and translation which were more highly expressed in solitarious samples. In total 214 genes were differentially expressed out of 20755 represented on the microarray. A list of 16 differentially expressed genes confirmed by qPCR in that study is provided for reference (Table 1).

A more comprehensive analysis of phenotypic gene expression differences has been carried out via RNAseq on *Locusta migratoria* (Chen *et al.*, 2010), although again no consideration for time of day was taken. This work analysed gene expression in whole-body samples (minus contents of alimentary canals) of animals from 6 different developmental stages: eggs, 1st and 2nd instars (combined), 3rd instar, 4th instar, 5th instar, and adults. At each developmental stage differentially expressed sequences were identified. Greater expression was seen in gregarious adults for genes with functional roles in 'intracellular structure', 'catalytic activity', 'lipid and glycan metabolism pathways' (Gene Ontology terms). By contrast, solitarious adults showed up-regulation of genes associated with 'cellular processes' and 'multicellular organismal processes'. Detailed analysis was undertaken on the 4th instar data, creating a functional network of differentially expressed genes between phenotypes. This revealed an overall up-regulation of pathways involved in 'signal transduction' in gregarious animals, in contrast to solitarious animals which showed an up-regulation in pathways associated with 'DNA synthesis', 'RNA synthesis/transcription' and 'RNA translation'. Chen *et al.* (2010) suggest that these differences in gene expression reflect differences in 'biological investment', indicating the increased priority of 'environmental interaction' in the gregarious phenotype compared to solitarious animals, which have a molecular focus on metabolism and biosynthesis (Figure 2;Chen *et al.*, 2010). This analysis fits with recent work comparing the anatomical volume of various brain regions, with gregarious adults showing a larger proportion of brain volume in regions associated with sensory processing compared to solitarious *S. gregaria* (Ott and Rogers, 2010). Chen *et al.* (2010) compared their work to previous ESTs stating more than double the number of differentially expressed genes were identified. Additionally the RNAseq technique was shown to be more

advanced in gene expression profiling, avoiding problems with low redundancy of sequencing reads and eliminating cloning bias.

Table 1: Selected contigs identified as being differentially expressed in the CNS of gregarious (G) and solitarious (S) *S. gregaria*. Adapted from Badisco *et al.* (2011). The numbers represent the number of tags referring each contig and indicate relative expression levels. Highlighted contigs were shown by qPCR to be differentially expressed.

EST ID	G	S	Annotation
Crowded>Isolated			
LC.228.C1.Contig298	9	0	Fasciclin(-like) precursor
LC.308.C2.Contig390	8	0	No annotation
LC.393.C1.Contig477	8	0	Similar to CG12163 (Cys-protease inhibitor)
LC.1955.C1.Contig2112	7	0	Similar to T-complex protein 1 subunit gamma
LC.4273.C1.Contig4391	7	0	No annotation
LC.446.C2.Contig534	5	0	Similar to 37694 protein (Leonardo protein)
LC.129.C1.Contig185	6	1	Slit homologue
LC.1849.C1.Contig2006	6	1	Signal peptidase complex subunit 2
LC.392.C1.Contig474	5	1	Probable cytochrome P450
Isolated>Crowded			
LC.1602.C1.Contig1749	0	15	RNA helicase Ddx1
LC.587.C1.Contig691	0	11	G-protein gamma subunit
LC.1473.C1.Contig1619	0	7	No annotation
LC.312.C1.Contig394	0	7	Glutamine synthetase
LC.4308.C1.Contig4427	0	7	No annotation
LC.1603.C1.Contig1750	0	5	No annotation
LC.733.C2.Contig853	1	5	Similar to 7B2 precursor

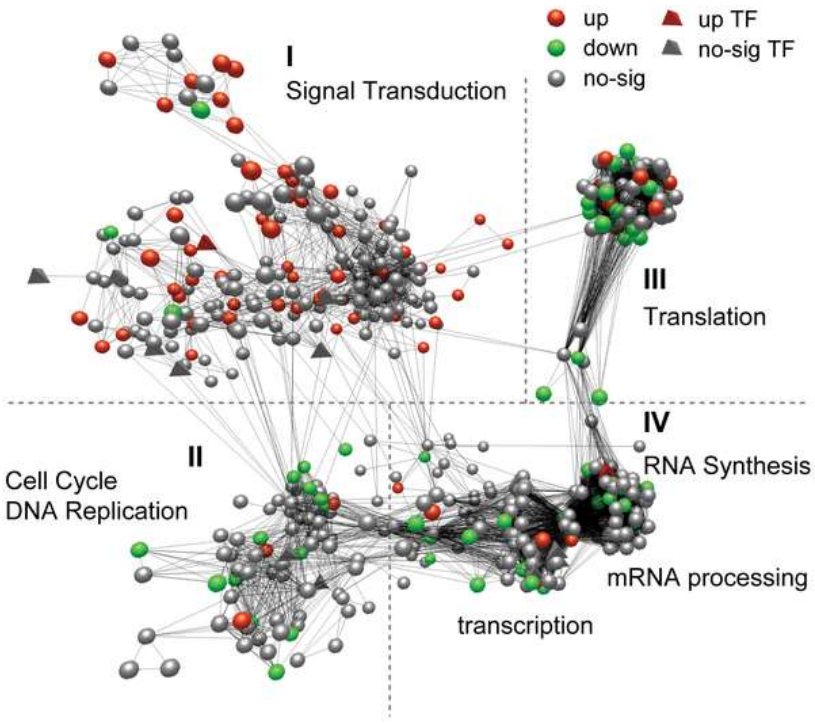


Figure 2: Functional network of differently expressed orthologs in *L. migratoria* (4th instar) (Chen *et al.*, 2010). Circular nodes represent proteins, triangular nodes represent transcription factors. Red indicates up-regulation and green indicates down-regulation in gregarious samples relative to solitarious samples.

Circadian clock and population density dependent phenotypic plasticity

The population density dependent phenotypic change seen in gregarious and solitarious *S. gregaria,* as discussed previously, has only briefly been examined on a molecular level. With the exception of work by Badisco *et al.* (2011) described above, the majority of molecular studies have focused on transitions between phenotypes and related neuropeptides such as neuroparsin (e.g. Claeys *et al.*, 2006) or neurotransmitters such as serotonin (e.g. Rogers *et al.*, 2014).

General locomotor activity and DCMD activity both show peaks later in the day in solitarious animals (E. Gaten, unpublished; Gaten *et al.*, 2012), whilst the only study to look at the expression of circadian clock genes in the two phenotypes concluded that the expression was not significantly different between the two phenotypes, although no data were provided to back up this assertion (Tobback *et al.*, 2011). The cricket, *G. bimaculatus*, does not show population density dependent phenotypic plasticity, but it does switch from expressing diurnal behaviour in the final instar to expressing nocturnal activity as an adult (Tomioka and Chiba, 1982; Nishinokubi and Tomioka, 2000). *G. bimaculatus*, does not show a difference in expression of the central clock

genes, *Clk*, *per* and *tim* associated with this behavioural change (Tomioka and Chiba, 1982; Tomioka and Abdelsalam, 2004). The change in behaviour is therefore not the result of a phase shift of the central molecular clock, where the timing of the peaks of gene expression of the core clock genes change between phenotypes, but is likely rather the result of changes to the output pathways of the clock (Tomioka and Chiba, 1982), with a possible role of serotonin in modulation of this change (Nishinokubi and Tomioka, 2000). It is possible that differences in the circadian expression of solitary and gregarious locust behaviour could also arise in the absence of changes in the central clock genes.

Vitellin and Vitellogenin

Solitarious female locusts lay more, smaller, eggs while gregarious animals lay fewer, larger, eggs (Injeyan and Tobe, 1981a). Levels of Vitellin, a key protein contained in the yolk of the eggs, does not differ overall between the phenotypes, however due to the difference in the number of eggs the content of each oocyte does differ, with less Vitellin per oocyte in solitarious females (Injeyan and Tobe, 1981a). Expression of *vitellogenin*, a precursor of vitellin which is present in the haemolymph, has been linked to juvenile hormone (JH) levels in *Tribolium castaneum*, with increased JH leading to increased Vitellogenin production (Sheng *et al.,* 2011), the inverse interaction has been shown in *Apis mellifera* with increased JH reducing *vitellogenin* expression (Hartfelder and Engels, 1998). In *S. gregaria* JH titres differ between the two extreme phenotypes, with solitarious adults showing higher levels of JH compared to gregarious adults (Injeyan and Tobe, 1981b). It is likely that *vitellogenin* expression also differs between the two extreme phenotypes but no direct analysis of gene expression of *vitellogenin* has previously been carried out in *S. gregaria*, and it is unclear if *vitellogenin* expression can be expected to be increased or decreased due to the higher JH titre. In addition to Vitellin production and transportation, Vitellogenin has also been shown to reduce oxidative stress in the honey bee, *A. mellifera* (Nelson *et al.*, 2007; Amdam *et al.*, 2012), promoting longer life. Solitarious animals live longer, with an average adult life expectancy of 65 days compared to gregarious animals which have an adult life expectancy of 48 days (Uvarov, 1966), if *vitellogenin* is

more highly expressed in solitarious locusts there is the possibility that it has the same effects as those seen in *A. Mellifera*.

Immunity and stress

Disease resistance has been shown to increase with population density in a wide range of animals e.g. the mealworm beetle, *Tenebrio molitor* (Barnes and Siva-Jothy, 2000) and the gypsy moth, *Lymantria dispar* (Reilly and Hajek, 2008). This is likely related to the increased exposure to transmissible diseases in crowded conditions. In contrast animals in a low population density are better off investing energy in reproduction and growth. This density dependent prophylaxis was originally examined in caterpillars (Wilson and Reeson, 1998;White and Wilson, 1999), but more recent work has been carried out in *S. gregaria* (Wilson *et al.*, 2002). Here the resistance of gregarious animals to the pathogenic fungus *Metarhizium anisopliae* was increased compared to that of solitarious animals. The increased resistance was the result of increased antimicrobial activity, not behavioural changes (Wilson *et al.*, 2002). As such it is likely that pathways associated with immunity and antimicrobial activity will be over-expressed in gregarious samples compared to solitarious samples. The haemolymph of arthropods contains serine proteinase inhibitors (Kanost, 1999), which proteins primarily act to reduce the proteinase activity of invading pathogens and parasites. Some inhibitors are specific to fungal or bacterial activity, while others regulate endogenous proteinases responsible for activity such as coagulation or cytokine activity. The expression of serine proteinase inhibitors can therefore be seen as a genetic indication as to the immunocompetance of an insect (Kanost, 1999).

5.1.3 Hypothesis

This chapter will examine the expression of core circadian clock genes in CNS tissues of solitarious and gregarious adult locusts. The results of the previous chapter confirm similarity to the *G. bimaculatus* clock in terms of structure and sequence consensus. The cyclic expression of the molecular clock is therefore expected to be similar to that of *G. bimaculatus*, with *cyc*, *per* and *tim* all showing rhythmic expression and *Clk* showing constitutive expression under LD conditions.

Gene expression differences between solitarious and gregarious animals are expected to include an increase of vitellogenin, translation/transcription and DNA synthesis in solitarious samples with an increase in signal transduction and immune pathways in gregarious animals. The limited results of Badisco *et al.* (2011) should be confirmed and extended through the use of a considerably more sensitive technique.

5.2 Methods

This chapter makes use of the same tissue samples as described in Chapter 4. Animal husbandry, dissection, sample sequencing and assembly construction are described in more detail in Chapter 4. These methods are briefly recapped below.

5.2.1 General husbandry

All animals (*Schistocerca gregaria* Forskål) used in this work were reared at the University of Leicester, U.K. Both solitarious and gregarious animals were maintained under a 12 : 12 h light : dark regime, with light levels of 750-1500 lx during the photophase, and 0 lx during the scotophase. Cage temperatures rose to 36°C during photophase and fell to 25°C during scotophase. Locusts received fresh wheat seedlings and bran flakes *ad libitum*. Solitarious animals were reared individually in 10 x 10 x 20 cm cages isolated from sight and smell of other animals. Gregarious individuals were kept at high density (100-300 per cage) in 50 x 50 x 50 cm cages.

5.2.2 Tissue dissection, RNA extraction and sequencing

Dissections were carried out under low intensity red light at 25°C under LD conditions at 3 h intervals from ZT1, resulting in 8 time points. Extraction and sampling was pooled differently for brain + optic lobe samples and ganglia samples. In the brain + optic lobe samples both tissues were pooled for each individual, with three replicates per time point resulting in 24 samples per phenotype. The ganglia samples were pooled with tissue from one individual per time point in each sample resulting in 3 samples per phenotype. Together this totalled 54 samples. Gender totals were tracked for each sample to allow accurate comparison.

RNA extraction was carried out utilizing the Trizol reaction. These samples underwent TURBO DNase (Life Technologies) treatment followed by clean up in the RNeasy mini kit (Qiagen). Samples were analysed via bioanalyzer (Agilent, G2939A) prior to sequencing with the NextSeq platform (Illumina; Glasgow Polyomics, UK); Sampling parameters were: PolyA library preparation, Paired end reads at 2 x 75 bp with 25 M reads.

5.2.3 De-Novo transcriptome assembly and annotation

Sequencing resulted in approximately 1.3 x 10^9 reads ranging from 25-75 bp in length in Fastq format. A multi assembler approach was taken to forming the de-novo transcriptome, with assemblies generated by Trinity, Trans-absys and Bridger. All the assemblies produced were combined. This combined assembly was processed with CDhit-est (http://weizhongli-lab.org/cd-hit), a program which removes duplicate contigs, keeping the longest available. This reduced assembly was then processed with transdecoder (https://transdecoder.github.io/), which identifies ORFs and identifies the protein sequences produced. This allowed Blast2Go (Conesa *et al.*, 2005) to annotate the transcriptome ready for gene expression analysis.

5.2.4 Gene expression analysis

All p values are adjusted to take repeated tests into consideration (q Values). The type of correction is False Detection Rate (FDR) and was carried out by the relevant software as part of the analysis or using the R statistical package.

Expression analysis was carried out using the Tophat and Cufflinks workflow (Trapnell *et al.*, 2012). Reads were mapped to the annotated *S. gregaria* transcriptome using Tophat (https://ccb.jhu.edu/software/tophat/index.shtml). These data were then analysed using Cufflinks (http://cole-trapnell-lab.github.io/cufflinks) which outputs the Fragments Per Kilobase of transcript per Million mapped reads (FPKM) for each assembled transcript. This value represents the proportion of reads in that sample that map to each transcript and is used to plot the raw expression of a gene. Differential expression made use of CuffDiff (part of the Cufflinks package) which compares FPKM in two samples.

Differentially expressed transcripts (q Value < 0.05) underwent Gene Ontology (GO) enrichment analysis. The transcripts over-expressed in either gregarious or solitarious animals compared to the other, known as clusters, were analysed. This identified GO terms, gene pathways, which appear in the clusters in a higher proportion than they are present in the reference set, the annotated transcriptome. The probability that expression of those transcripts given expression of associated transcripts is due to chance is output as the q value.

Rhythmic expression was tested using JTK_Cycle (Hughes *et al.*, 2010) and RAIN (Thaben and Westermark, 2014). JTK_Cycle follows a non-parametric method whereby the expression is expected to be symmetrical, referring to the shape of a peak. A symmetrical expression profile will rise to the peak at the same rate that it falls from it. This method is useful for analysing patterns of expression of the central circadian genes which show symmetrical expression, but is of limited use in detecting saw-tooth expression patterns. RAIN utilises umbrella methods to allow detection of non-symmetrical expression (Thaben and Westermark, 2014). For this reason RAIN was used to assess general rhythmicity within the transcriptome.

5.3 Results

From the data collected, three different comparisons were carried out. First, phenotypic differences in gene expression in the ganglia were examined. Second, phenotypic differences in gene expression in the brain + optic lobes of male animals were examined. Third, differential expression in the brain + optic lobes of female animals was compared to that of the male animals; pathways differentially expressed in both cases were identified. Finally, gene expression in the brain + optic lobes of both genders combined was examined with regard to circadian/diurnal rhythms.

5.3.1 Phenotype-specific differences in gene expression in thoracic ganglia

The samples generated from the pooled meso- and meta-thoracic ganglia were analysed to detect any differences in expression between solitarious and gregarious animals. Three samples were analysed for each phenotype. Each of these samples contained ganglia collected from each of the eight time points (i.e. 8 sets of ganglia per sample). As the tissues were gender balanced no effect due to gender was expected.

The reads from these samples were mapped to the annotated transcriptome generated in Chapter 4. This allowed analysis for differential expression using CuffDiff. Of the 121,481 transcripts in the total assembly (formed of ganglia and brain + optic lobe tissues) 103,292 were expressed in the ganglia of either the gregarious or solitarious animals. Of these 2120 were significantly differentially expressed after controlling for repeated tests (FDR; q Value < 0.05; Figure 3). Three main groupings of expression are present in Figure 3: genes highly expressed in gregarious compared to solitarious are distributed close to the x axis, those highly expressed in solitarious animals compared to gregarious are distributed close to the y axis, those that are not significantly differentially expressed are seen a group extending diagonally. 1272 transcripts were more highly expressed in solitarious ganglia, 848 in gregarious ganglia. Three key differentially expressed pathways were visually identified based on the most highly expressed genes (Figure 3, red regions): Vitellogenin, immunity and immune response, and DNA/RNA synthesis, processing and translation.

Vitellogenin was over-expressed in solitarious ganglia (Table 3) with a 2-fold increase (range of foldchange: 1.95 - 2.86) in expression. DNA/RNA polymerases, translation

initiation/elongation factors were over-expressed in solitarious samples (Table 2). Many genes associated with immune response were over-expressed in gregarious samples (Table 4; Table 5). These include: lysozymes, caspase (Table 4), serpins, heat shock proteins (70 and 90), defensin and locustin (Table 5). Pigment dispersing factor (PDF) was not significantly differentially expressed between the two extreme phenotypes in the ganglia (Fold change: -4.43175; T-stat: -2.4118; $p = 0.005$; $q = 0.080$).

GO enrichment analysis of the over-expressed genes in solitarious ganglia found 'lipid transporter' was the only pathway over-represented. The majority of associated genes were annotated with Vitellogenin (Table 6). No pathways associated with DNA/RNA polymerases or similar functionality were identified by this analysis, consequently the increased expression described should be interpreted in terms of individual gene expression rather than whole pathway regulation.

GO enrichment analysis of the over-expressed genes in gregarious ganglia identified many over-represented pathways including hydrolases, carbohydrate metabolic processes and immune processes (full list in Table 6). This shows an increase in catabolic, metabolic and prophylactic processes in the gregarious ganglia.

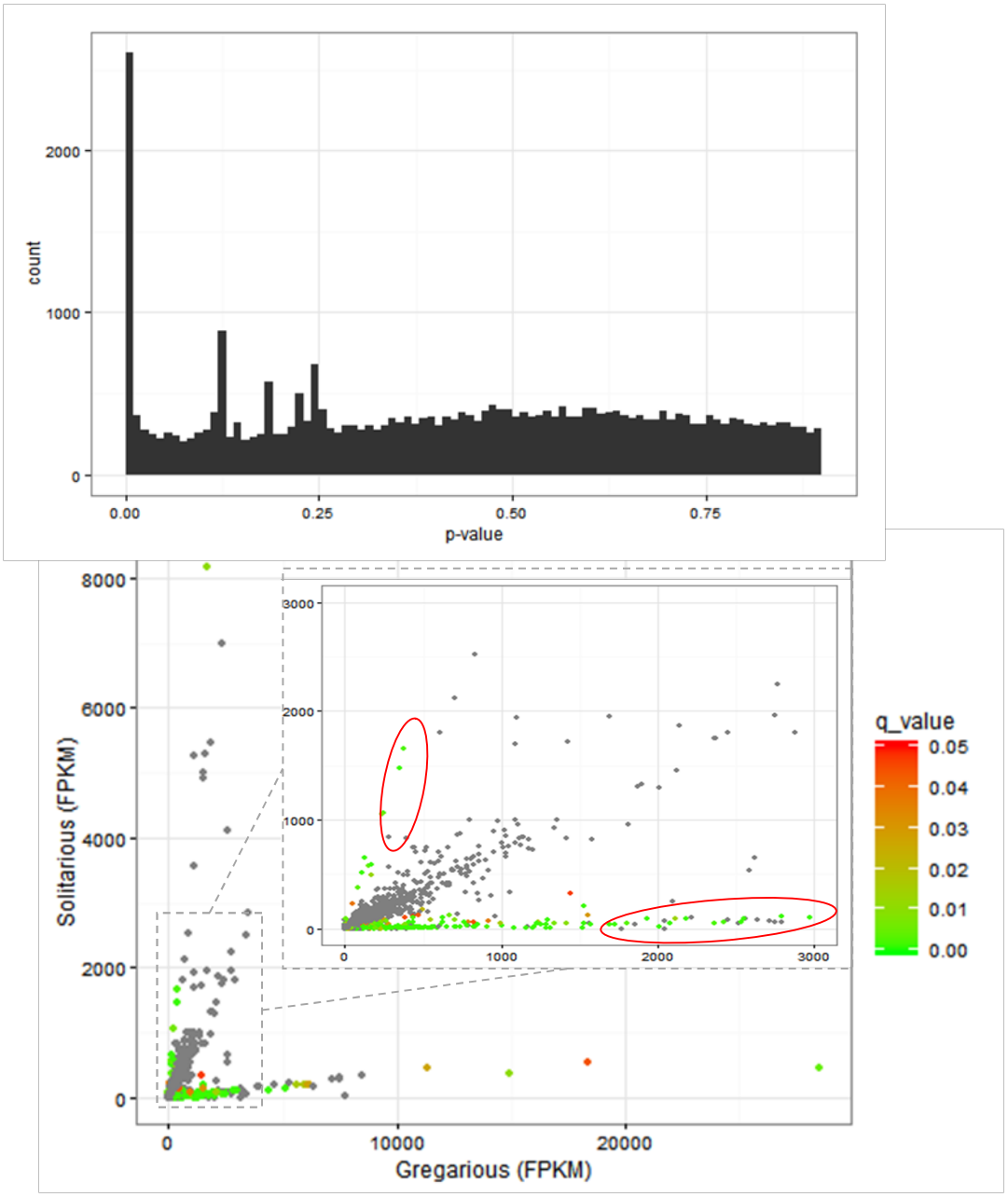


Figure 3: Top: histogram of p-values for differential expression of transcripts between solitarious and gregarious ganglia. Bins are 0.01 in width. There is a large peak in the 0 - 0.01 bin indicating genes that are differentially expressed, with an otherwise flat distribution of non-significant p values. Bottom: Expression of transcripts in gregarious (x-axis) and solitarious (y-axis). Inset: the data in the dashed box magnified to clearly show the distribution at the lower expression levels. Red selections, differentially expressed transcripts within these regions were manually examined as described; Grey, non-signficant; Red-green, raising significance from 0.05 to 0.

Table 2: Differential expression of DNA/RNA polymerases and translation initiation/elongation factors in the ganglia in *S. Gregaria*. Number of reads referring to each transcript (FPKM) for Gregarious (G) and Solitarious (S) animals, Fold change (FC) between phenotypes, statistical values (Test Statistic and q Value) and associated annotation details. Where expression = 0 in one sample (S or G) but still tested FC and Test stat read "NA".

Contig ID	G	S	FC	Test stat	q Val	Accession Gene Species
k31.S14021460	0	1.54	NA	NA	0.001690	B4HIM0 DNA polymerase *Drosophila sechellia*
c174854_g4_i1	0	0.99	NA	NA	0.003846	E9RHN9 DNA polymerase *Phraortes illepidus*
c62827_g1_i1	0	1.22	NA	NA	0.001690	S4PAK8| Fragment DNA-directed RNA polymerase *Pararge aegeria*
c170913_g3_i1	0	1.86	NA	NA	0.001690	U4U4W4 DNA-directed RNA polymerase *Dendroctonus ponderosae*
k21.S47981822	0	0.87	NA	NA	0.001690	V5YTZ0 DNA-directed RNA polymerase *Triops granarius*
c318083_g1_i1	0	1.18	NA	NA	0.001690	A0A0A9YFB5 DNA-directed RNA polymerase *Lygus hesperus*
k21.R47889846	2.34	0	NA	NA	0.001690	B8YJC8| Fragment Eukaryotic translation elongation *Stephos longipes*
k31.S14014487	0	1.33	NA	NA	0.001690	A0A067RES0 Eukaryotic translation initiation factor *Zootermopsis nevadensis*
c166524_g2_i1	0	1.06	NA	NA	0.002801	I4DR57| Fragment Eukaryotic translation initiation factor *Papilio polytes*
comp104274_seq0	1.01	0	NA	NA	0.002801	B4J6D5 Eukaryotic translation initiation factor *Drosophila grimshawi*
comp145624_seq0	0.96	0	NA	NA	0.023379	B4PNN4 Eukaryotic translation initiation factor *Drosophila yakuba*
c274093_g1_i1	0	2.31	NA	NA	0.001690	B0WAM5 Eukaryotic translation initiation factor *Culex quinquefasciatus*
c226380_g1_i1	0.07	6.76	6.54	3.93	0.026673	A0A0A9Y517 Eukaryotic translation initiation factor *Lygus hesperus*
c214291_g1_i1	0	1.46	NA	NA	0.003846	A0A026X3M3 Eukaryotic translation initiation factor *Cerapachys biroi*
k41.S4727663	0	3.15	NA	NA	0.001690	U5EXY4| Putative Eukaryotic translation initiation factor *Corethrella appendiculata*
k21.S47999622	0	1.24	NA	NA	0.003846	E0VT44| Putative Transcription elongation factor *Pediculus humanus*

Table 3: Differential expression of Vitellogenin in the ganglia of *S. gregaria*. Number of reads referring to each transcript (FPKM) for Gregarious (G) and Solitarious (S) animals, Fold change (FC) between phenotypes, statistical values (Test Statistic and Q Value) and associated annotation details.

Contig ID	G	S	FC	Test stat	q Val	Accession Gene Species
Comp187761_seq0	86.4	376.8	2.12	2.45	0.003846	V9HZ08 Vitellogenin *Bemisia tabaci*
comp10504_seq0	122.1	649.2	2.41	2.86	0.001690	Q9XZD3 | Fragment Vitellogenin *Romalea microptera*
comp245406_seq0	107.6	517.8	2.27	2.77	0.001690	A0A0A9YM76| Fragment Vitellogenin *Lygus Hesperus*
comp233580_seq1	238.3	1049.3	2.14	2.30	0.006904	A0A0A9XTE2| Fragment Vitellogenin *Lygus hesperus*
comp226762_seq0	242.7	1065.5	2.13	2.46	0.002801	A0A077LSH0| Fragment Vitellogenin1 *Zootermopsis nevadensis*
comp234894_seq0	372.4	1656.4	2.15	2.34	0.002801	A0A077LSH0| Fragment Vitellogenin1 *Zootermopsis nevadensis*
comp46350_seq0	150.0	581.0	1.95	2.34	0.004872	A0A059WHP4| Fragment Vitellogenin 2 *Triatoma infestans*

Table 4: Differential expression of caspase, lysozyme and lysosomal protease in the ganglia in *S. Gregaria.* Number of reads referring to each transcript (FPKM) for Gregarious (G) and Solitarious (S) animals, Fold change (FC) between phenotypes, statistical values (Test Statistic and Q Value) and associated annotation details. Where expression = 0 in one sample (S or G) but still tested FC and Test stat read "NA".

Contig ID	G	S	FC	Test stat	Q Val	Accession Gene Species
c200904_g1_i1	63.40	22.75	-1.48	-1.91	0.016804	L0AV20 I-type lysozyme *Coptotermes formosanus*
c212342_g1_i1	190.80	30.30	-2.65	-2.85	0.001691	H2EQQ0 C-type lysozyme *Schistocerca gregaria*
c217162_g1_i1	5.41	0.65	-3.05	-2.39	0.009922	I1XB31 C-3 lysozyme *Periplaneta americana*
comp10730_seq0	7.41	1.32	-2.45	-1.82	0.041521	L0AV20 I-type lysozyme *Coptotermes formosanus*
comp19470_seq2	1.42	0	NA	NA	0.001691	A0A087TX10|Fragment Lysosomal aspartic protease *Stegodyphus mimosarum*
comp9527_seq0	9.91	1.81	-2.46	-2.35	0.006905	A0A067RI85 Lysozyme 1 *Zootermopsis nevadensis*
k21.R47582794	627.81	98.09	-2.68	-2.84	0.001691	H2EQQ0 C-type lysozyme *Schistocerca gregaria*
c217395_g2_i11	17.48	3.26	-2.42	-2.47	0.001691	W5JU83 Caspase short class *Anopheles darlingi*
c217395_g2_i4	19.00	3.51	-2.44	-2.39	0.006905	W5JU83 Caspase short class *Anopheles darlingi*
c217395_g2_i7	9.12	1.93	-2.24	-2.01	0.011584	A0A067R0M8 Caspase-1 *Zootermopsis nevadensis*

Table 5: Differential expression of serpins, heat shock proteins, defensin and locustin in the ganglia in *S. Gregaria*. Number of reads referring to each transcript (FPKM) for Gregarious (G) and Solitarious (S) animals, Fold change (FC) between phenotypes, statistical values (Test Statistic and Q Value) and associated annotation details. Where expression = 0 in one sample (S or G) but still tested FC and Test stat read "NA".

Contig ID	G	S	FC	Test stat	Q Val	Accession Gene Species
comp27806_seq0	0.84	0	NA	NA	0.001691	F4W666 Heat shock 70 kDa protein *Acromyrmex echinatior*
comp30902_seq0	2.45	0	NA	NA	0.001691	F4W666 Heat shock 70 kDa protein *Acromyrmex echinatior*
comp53368_seq0	1.65	0	NA	NA	0.001691	A0A0B4L0J4 Heat shock protein 70 *Lissorhoptrus oryzophilus*
comp91679_seq0	1.67	0	NA	NA	0.001691	G8Z376 Heat shock protein 90 *Panonychus citri*
k31.R13757873	0.25	9.13	5.21	3.11	0.008897	F4W666 Heat shock 70 kDa protein *Acromyrmex echinatior*
c199895_g2_i1	558.85	17.75	-4.98	-4.01	0.001691	Q6TAX6 Defensin *Bombus ignitus*
c49653_g1_i1	1520.53	12.13	-6.97	-5.37	0.001691	Q25313| Putative Defense protein *Locusta migratoria*
comp181679_seq0	332.25	7.72	-5.43	-5.89	0.001691	Q25313| Putative Defense protein *Locusta migratoria*
comp247_seq0	53.41	5.24	-3.35	-3.09	0.002801	M4X2T6 Defensin *Pyrrhocoris apterus*
comp41_seq0	702.40	15.57	-5.50	-6.09	0.001691	Q25313| Putative defense protein *Locusta migratoria*
k31.S14189412	821.35	16.87	-5.61	-6.22	0.001691	Q25313| Putative defense protein *Locusta migratoria*
c210508_g1_i1	45.19	9.30	-2.28	-2.51	0.002801	E0VLN2| Putative Serine proteinase inhibitor, *Pediculus humanus*
c218285_g1_i1	35.31	12.90	-1.45	-1.79	0.048917	L7WRS0| Fragment Proteinase inhibitor serpin *Locusta migratoria*
c219934_g1_i3	21.08	0.54	-5.29	-4.09	0.001691	L7WRS0| Fragment Proteinase inhibitor serpin *Locusta migratoria*
k41.J4712423	18.53	2.26	-3.04	-2.36	0.004873	D6W6R5 Serine protease P44 *Tribolium castaneum*
k41.J4782138	182.42	2.72	-6.07	-3.69	0.001691	D6W6R5 Serine protease P44 *Tribolium castaneum*
k41.S4888686	55.20	18.97	-1.54	-1.98	0.017714	A0A067RHT4| Fragment Serine protease snake *Zootermopsis nevadensis*
k41.S4925963	8.88	1.32	-2.75	-2.57	0.001691	A0A067QHJ6 Serine protease easter *Zootermopsis nevadensis*
comp320_seq0	68.47	6.43	-3.41	-3.97	0.001691	P83428 Locustin *Locusta migratoria*

Table 6: Over-represented pathways identified by GO enrichment analysis. Top: over-represented terms over-expressed in solitarious samples. Bottom: over-represented terms over-expressed in gregarious samples.

Label	Q Value	Description	# in cluster	# in assembly
Over-expressed in Solitarious ganglia				
GO:0005319	0.018578	lipid transporter activity	8	45
Over-expressed in Gregarious ganglia				
GO:0008810	4.95E-17	cellulase activity	13	18
GO:0030245	4.95E-17	cellulose catabolism	13	18
GO:0000272	4.95E-17	polysaccharide degradation	13	18
GO:0042302	1.87E-06	structural constituent of cuticle	23	293
GO:0016798	2.98E-06	hydrolase activity, acting on glycosyl bonds	22	280
GO:0010466	1.53E-05	negative regulation of peptidase activity	11	77
GO:0030414	1.53E-05	peptidase inhibitor activity	11	77
GO:0010951	2.29E-05	negative regulation of endopeptidase activity	13	117
GO:0042832	2.86E-05	defence response to protozoa	4	5
GO:0004470	3.88E-05	pyruvic-malic carboxylase activity	6	19
GO:0004471	3.88E-05	(S)-malate:NAD+ oxidoreductase (decarboxylating)	6	19
GO:0005576	6.54E-05	extracellular	25	440
GO:0003796	6.54E-05	muramidase activity	6	21
GO:0004553	1.17E-04	O-glucosyl hydrolase activity	19	288
GO:0042742	1.17E-04	defense response to bacterium	5	14
GO:0004869	1.22E-04	cysteine protease inhibitor activity	6	24
GO:0008233	1.38E-04	proteinase activity	39	921
GO:0045087	2.23E-04	nonspecific immune response	7	40
GO:0005991	2.65E-04	trehalose metabolism	4	9
GO:0004555	2.65E-04	alpha,alpha-trehalase activity	4	9
GO:0003987	2.65E-04	acetyl CoA synthase activity	4	9
GO:0019427	2.65E-04	acetyl-CoA biosynthetic process from acetate	4	9
GO:0016208	2.65E-04	AMP binding	4	9
GO:0003674	2.92E-04	molecular function	5	18
GO:0006108	3.09E-04	malate metabolism	7	44
GO:0002376	3.65E-04	immune system process	6	31
GO:0005975	4.17E-04	carbohydrate metabolic process	28	609
GO:0016787	4.69E-04	hydrolase activity	89	2971

5.3.2 Phenotype-specific differences in gene expression in the brain + optic lobes: male

Of the 121,481 transcripts in the total assembly, 96051 were expressed in the pooled brain + optic lobes of either the gregarious or solitarious males. Of these, 1605 were significantly differentially expressed after controlling for repeated tests (FDR; q Value < 0.05; Figure 3). The three differentially expressed pathways identified in the ganglia were re-examined.

Vitellogenin was over-expressed in solitarious males compared to gregarious males, with a 2.9 fold increase in expression (2.4 - 2.94), a single Vitellogenin annotated transcript showed lower expression (Table 7). The expression of this single transcript was comparatively low compared to the other differentially expressed transcripts. GO enrichment analysis of the over-expressed transcripts in solitarious males include 'lipid transporter', as in the ganglia. Additional pathways over-expressed were identified as haem-binding as well as oxidoreductase, monooxygenase and O-glucosyl hydrolase activity (Table 8).

No heat shock proteins were differentially expressed, however defensin, locustin, serpins, caspase and lysozymes were all over-expressed in gregarious males (Table 9). PDF was not significantly differentially expressed between the two phenotypes in the brain + optic lobes of males (Fold change = -0.654799; T-stat = -0.489693; p = 0.187; q = 0.742). GO enrichment analysis of over-expressed transcripts in solitarious males showed transcripts associated with haem-binding, oxidoreductase, monooxygenase and O-glucosyl hydrolase activity are over-represented (Table 10). Functional groups over-represented in transcripts over-expressed in gregarious males include immune processes, catalytic activity and hydrolase activity.

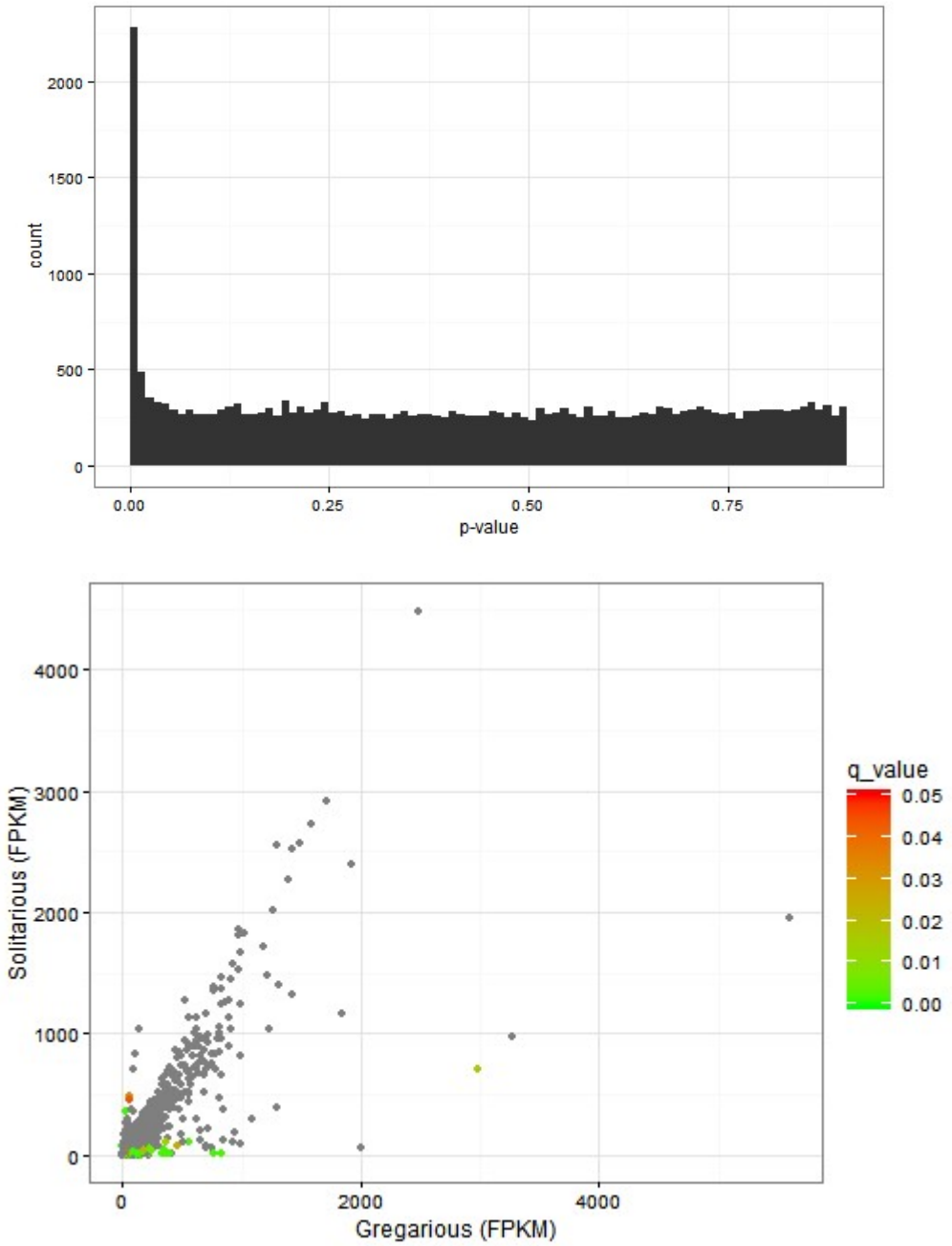


Figure 4: Top: histogram of p-values for differential expression of transcripts between solitarious and gregarious brain + optic lobes. Bins are 0.01 in width. There is a large peak in the 0 - 0.01 bin indicating genes that are differentially expressed with a flat distribution of non-significant p values. Bottom: Expression of transcripts in gregarious (x-axis) and solitarious (y-axis). Grey, non-significant; Red-green, raising significance from 0.05 to 0.

Table 7: Differential expression of Vitellogenin in the brain + optic lobes of male *S. gregaria*. Number of reads referring to each transcript (FPKM) for Gregarious (G) and Solitarious (S) animals, Fold change (FC) between phenotypes, statistical values (Test Statistic, P value and Q Value) and associated annotation details.

Contig ID	G	S	FC	Test stat	Q Val	Accession Gene Species
comp127017 seq0	63.61	490.90	2.95	0.67	0.033097	B1B5Z4 Vitellogenin *Lethocerus deyrollei*
comp187348 seq0	57.73	449.48	2.96	0.71	0.036264	B1B5Z4 Vitellogenin *Lethocerus deyrollei*
comp247633 seq0	59.92	455.81	2.93	0.69	0.043968	B1B5Z4 Vitellogenin *Lethocerus deyrollei*
comp9630 seq0	3.17	1.33	-1.26	-1.10	0.049816	A0A067QLY6 Vitellogenin *Zootermopsis nevadensis*
k41.S4900544	0.51	2.79	2.45	1.29	0.047589	A0A067R0P6 Vitellogenin *Zootermopsis nevadensis*

Table 8: GO term overrepresentation analysis transcripts over-expressed in solitary male brain + optic lobes

Label	Q Value	Description	# in cluster	# in assembly
GO:0016705	0.0011001	oxidoreductase activity	11	448
GO:0045281	0.0011001	succinate dehydrogenase complex	2	2
GO:0000104	0.0011001	succinic acid dehydrogenase activity	2	2
GO:0005506	0.0013441	iron binding	12	577
GO:0005319	0.0020689	lipid transporter activity	4	45
GO:0020037	0.0039953	haem binding	11	577
GO:0019478	0.0046677	D-amino acid catabolic process	2	5
GO:0004497	0.0071381	monooxygenase activity	9	447
GO:0006869	0.0071381	lipid transport	4	72
GO:0008273	0.009058	calcium, potassium:sodium antiporter activity	2	8
GO:0008408	0.0181591	3'-5'-exonuclease activity	3	45
GO:0004553	0.0384469	O-glucosyl hydrolase activity	6	288

Table 9: Differential expression of serpins, defensin and locustin in the brains + optic lobes of male *S. gregaria*. Number of reads referring to each transcript (FPKM) for Gregarious (G) and Solitarious (S) animals, Fold change (FC) between phenotypes, statistical values (Test Statistic, P value and q Value) and associated annotation details.

Contig ID	G	S	FC	Test stat	q Val	Accession Gene Species
c49653_g1_i1	168.62	7.60	-4.47	-2.56	0.002095	Q25313| Putative Defense protein *Locusta migratoria*
comp181679_seq0	162.99	8.36	-4.29	-2.91	0.002095	Q25313| Putative Defense protein *Locusta migratoria*
comp41_seq0	358.66	16.08	-4.48	-2.58	0.002095	Q25313| Putative Defense protein *Locusta migratoria*
k31.S14189412	401.23	18.87	-4.41	-2.83	0.002095	Q25313| Putative Defense protein *Locusta migratoria*
comp320_seq0	7.78	1.63	-2.26	-1.46	0.021811	P83428 Locustin *Locusta migratoria*
c216493_g1_i1	3.50	1.15	-1.61	-1.33	0.020884	L0AV20 I-type lysozyme *Coptotermes formosanus*
comp10730_seq0	2.77	0.69	-2.00	-1.28	0.017883	L0AV20 I-type lysozyme *Coptotermes formosanus*
c217395_g2_i11	9.49	2.14	-2.15	-1.67	0.002095	W5JU83 Caspase *Anopheles darlingi*
c217395_g2_i4	11.30	2.41	-2.23	-1.85	0.002095	W5JU83 Caspase *Anopheles darlingi*
c217395_g2_i7	4.66	1.09	-2.10	-1.62	0.005293	A0A067R0M8 Caspase-1 *Zootermopsis nevadensis*
k41.J4778916	0.79	0.09	-3.08	-1.72	0.033097	W8BN88 Caspase *Ceratitis capitata*
c178582_g1_i1	162.17	63.27	-1.36	-1.08	0.043968	A0A067QVT0 Serine proteinase *Zootermopsis nevadensis*
comp22519_seq0	0.89	0.22	-2.04	-1.49	0.017883	A0A067QL71 Serpin| dipetalogastin *Zootermopsis nevadensis*
comp3506_seq0	3.39	0.67	-2.34	-2.11	0.002095	A0A067R0A9 Serine proteinase *Zootermopsis nevadensis*
comp3506_seq1	3.59	0.72	-2.33	-2.24	0.002095	A0A067R0A9 Serine proteinase *Zootermopsis nevadensis*
comp3810_seq0	5.82	1.56	-1.90	-1.99	0.002095	A0A067QHJ6 Serine protease *Zootermopsis nevadensis*
comp3810_seq1	4.84	1.48	-1.71	-1.76	0.002095	A0A067QHJ6 Serine protease *Zootermopsis nevadensis*
k31.S14144771	24.20	7.37	-1.71	-1.47	0.007865	A0A067QVT0 Serine proteinase *Zootermopsis nevadensis*
k41.S4833988	89.15	29.82	-1.58	-1.49	0.005293	A0A067QVT0 Serine proteinase *Zootermopsis nevadensis*
k41.S4888686	39.95	11.56	-1.79	-1.60	0.005293	A0A067RHT4|Fragment Serine protease *Zootermopsis nevadensis*
k41.S4925963	2.81	1.15	-1.28	-1.15	0.043968	A0A067QHJ6 Serine protease *Zootermopsis nevadensis*

Table 10: Top 50 results (of 98) of GO enrichment analysis transcripts over-expressed in gregarious male brain + optic lobes.

Label	q Value	Description	# in cluster	# in assembly
GO:0055114	7.94E-11	oxidation-reduction process	142	2389
GO:0016491	3.58E-07	redox activity	104	1805
GO:0003824	7.09E-04	catalytic activity	81	1636
GO:0042302	8.03E-16	structural constituent of cuticle	45	293
GO:0004497	5.58E-09	monooxygenase activity	44	447
GO:0005506	1.24E-04	iron binding	40	577
GO:0020037	2.23E-04	haem binding	39	577
GO:0016705	1.99E-04	oxidoreductase activity	33	448
GO:0016758	3.03E-05	transferase activity	28	309
GO:0005576	3.53E-03	extracellular	28	440
GO:0016757	2.51E-02	glycosyltransferase activity	28	523
GO:0050660	9.65E-06	flavin adenine dinucleotide binding	27	270
GO:0022857	8.52E-03	transmembrane transporter activity	19	275
GO:0052689	1.25E-03	carboxyesterase activity	18	207
GO:0016614	9.99E-06	oxidoreductase activity	17	118
GO:0003774	3.83E-04	motor activity	17	166
GO:0016459	3.83E-04	myosin complex	17	166
GO:0006030	6.58E-04	beta-1,4-linked N-acetylglucosamine metabolism	15	142
GO:0008061	7.52E-04	chitin binding	15	146
GO:0030170	2.36E-02	pyridoxal phosphate binding	15	221
GO:0009058	4.50E-03	biosynthesis	14	161
GO:0016788	5.95E-03	esterase activity	11	112
GO:0051287	1.24E-02	NAD binding	11	124
GO:0008483	8.73E-04	aminotransferase activity	10	71
GO:0006520	4.39E-03	cellular amino acid metabolic process	10	90
GO:0016810	3.56E-03	hydrolase activity (not peptide bonds)	9	72
GO:0022891	4.39E-03	substrate-specific transmembrane transporter activity	9	75
GO:0004499	4.20E-07	dimethylaniline monooxygenase activity	8	15
GO:0004075	2.07E-05	biotin carboxylase activity	8	24
GO:0050661	8.55E-03	nicotinamide adenine dinucleotide phosphate binding	8	68
GO:0006032	1.29E-02	chitin breakdown	8	73
GO:0004568	1.29E-02	chitodextrinase activity	8	73
GO:0010466	1.52E-02	negative regulation of peptidase activity	8	77
GO:0030414	1.52E-02	peptidase inhibitor activity	8	77
GO:0070330	4.56E-07	aromatase activity	6	7
GO:0004470	3.64E-04	pyruvic-malic carboxylase activity	6	19
GO:0004471	3.64E-04	(S)-malate:NAD+ oxidoreductase	6	19
GO:0031090	5.18E-04	organelle membrane	6	21
GO:0043231	4.50E-03	intracellular membrane-enclosed organelle	6	34
GO:0006108	1.43E-02	malate metabolism	6	44
GO:0004617	1.88E-04	phosphoglycerate oxidoreductase activity	5	10
GO:0006546	2.79E-04	glycine catabolic process	5	11
GO:0006564	3.81E-04	L-serine biosynthesis	5	12
GO:0042742	6.71E-04	defense response to bacterium	5	14
GO:0003674	1.65E-03	molecular function	5	18
GO:0033961	1.65E-03	cis-epoxide hydrolase activity	5	18
GO:0002376	1.52E-02	immune system process	5	31
GO:0045087	3.57E-02	nonspecific immune response	5	40
GO:0016500	4.20E-02	protein-hormone receptor activity	5	42

5.3.3 Phenotype-specific differences in gene expression in the brain + optic lobes: female

Of the 121,481 transcripts in the total assembly 101432 were expressed in the brain + optic lobes of either the gregarious or solitarious females. Of these 3241 were shown to be significantly differentially expressed after controlling for repeated tests (FDR; $q < 0.05$). Described below are GO terms identified as over-represented in gregarious or solitarious females, these are compared to the results for male animals described above.

GO enrichment analysis of transcripts over-expressed in solitarious females compared to gregarious females includes 'succinate dehydrogenase' (Table 11), but not Vitellogenin ('lipid transport'). Of the 12 GO terms over-represented in male solitarious samples, 6 were also over-represented female solitarious samples (Figure 5, white).

GO terms identified by GO enrichment analysis of transcripts over-expressed in gregarious females includes metabolic pathways (e.g. pyruvic-malic carboxylase activity) and immune response pathways (e.g. defence response to bacterium) (Table 12). Of the 98 Go terms over-represented in male gregarious samples, 61 were also over-represented in female gregarious samples (Figure 5, grey).

Table 11: GO enrichment analysis of transcripts over-expressed in solitarious female brain + optic lobes.

Label	q Value	Description	# in cluster	# in assembly
GO:0016705	1.76E-08	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	27	448
GO:0005506	2.81E-08	iron binding	30	577
GO:0004497	1.37E-07	monooxygenase activity	25	447
GO:0020037	2.54E-07	haem binding	28	577
GO:0005929	2.67E-04	cilium	4	10
GO:0042073	2.34E-03	intraflagellar transport involved in cilium organization	4	17
GO:0045281	4.97E-03	succinate dehydrogenase complex	2	2
GO:0000104	4.97E-03	succinic acid dehydrogenase activity	2	2
GO:0005230	5.69E-03	extracellular ligand-gated ion channel activity	9	155
GO:0000445	1.18E-02	THO complex part of TREX complex	2	3
GO:0008092	1.86E-02	cytoskeletal protein binding	4	33
GO:0019898	1.91E-02	extrinsic to membrane	4	34

Table 12: Top 50 results (of 98) of terms identified by GO enrichment analysis of transcripts over-expressed in gregarious female brain + optic lobes compared to solitarious females in the sample tissue.

Label	q Value	Description	# in cluster	# in assembly
GO:0016491	3.91E-08	redox activity	178	1805
GO:0055114	5.70E-08	oxidation-reduction process	220	2389
GO:0030414	9.56E-08	peptidase inhibitor activity	22	77
GO:0010466	9.56E-08	negative regulation of peptidase activity	22	77
GO:0070330	4.20E-07	aromatase activity	7	7
GO:0002376	1.20E-06	immune system process	13	31
GO:0004499	3.87E-06	dimethylaniline monooxygenase (N-oxide-forming) activity	9	15
GO:0009058	1.00E-05	biosynthesis	29	161
GO:0045087	2.72E-05	nonspecific immune response	13	40
GO:0006189	2.72E-05	'de novo' IMP formation	8	14
GO:0042302	3.05E-05	structural constituent of cuticle	41	293
GO:0010951	9.07E-05	negative regulation of endopeptidase activity	22	117
GO:0006564	9.07E-05	L-serine biosynthesis	7	12
GO:0003796	9.07E-05	muramidase activity	9	21
GO:0004497	1.05E-04	monooxygenase activity	53	447
GO:0004075	2.69E-04	biotin carboxylase (component of acetyl CoA carboxylase) activity	9	24
GO:0004617	3.46E-04	phosphoglycerate oxidoreductase activity	6	10
GO:0006164	3.46E-04	purine nucleotide biosynthetic process	10	31
GO:0004867	4.41E-04	serine protease inhibitor activity	14	61
GO:0003937	4.41E-04	IMP cyclohydrolase activity	4	4
GO:0004643	4.41E-04	5'-phosphoribosyl-5-amino-4-imidazolecarboxamide formyltransferase activity	4	4
GO:0004639	4.41E-04	SAICARs activity	4	4
GO:0008745	4.55E-04	murein hydrolase activity	13	54
GO:0006546	4.94E-04	glycine catabolic process	6	11
GO:0008483	4.94E-04	aminotransferase activity	15	71
GO:0004760	4.94E-04	hydroxypyruvate:L-alanine transaminase activity	6	11
GO:0009253	5.91E-04	peptidoglycan catabolism	13	56
GO:0003824	6.95E-04	catalytic activity	139	1636
GO:0008453	7.85E-04	L-alanine:glyoxylate aminotransferase activity	6	12
GO:0004364	7.85E-04	glutathione S-transferase activity	18	101
GO:0004375	9.03E-04	glycine:H-protein-lipoyllysine oxidoreductase (decarboxylating, acceptor-amino-methylating)	5	8
GO:0050661	1.03E-03	nicotinamide adenine dinucleotide phosphate binding	14	68
GO:0019700	1.32E-03	phosphonate catabolism	4	5
GO:0042832	1.32E-03	defence response to protozoa	4	5
GO:0047304	1.32E-03	(2-aminoethyl)phosphonate--pyruvate aminotransferase activity	4	5
GO:0052689	2.69E-03	carboxyesterase activity	27	207
GO:0031090	3.11E-03	organelle membrane	7	21
GO:0030170	3.20E-03	pyridoxal phosphate binding	28	221
GO:0052655	3.51E-03	L-valine transaminase activity	3	3
GO:0009374	3.51E-03	vitamin H binding	3	3
GO:0009082	3.51E-03	branched chain family amino acid anabolism	3	3
GO:0052656	3.51E-03	L-isoleucine aminotransferase activity	3	3
GO:0004736	3.51E-03	pyruvate:carbon-dioxide ligase (ADP-forming)	3	3
GO:0052654	3.51E-03	L-leucine aminotransferase activity	3	3
GO:0009056	3.51E-03	degradation	3	3
GO:0019428	3.51E-03	allantoin synthesis	3	3
GO:0005319	4.40E-03	lipid transporter activity	10	45
GO:0006520	4.47E-03	cellular amino acid metabolic process	15	90
GO:0008152	4.47E-03	metabolism	421	5997
GO:0008448	5.75E-03	N-acetylglucosamine-6-phosphate deacetylase activity	4	7

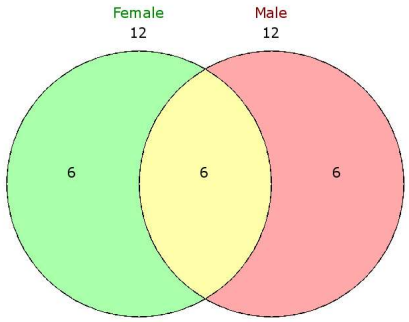
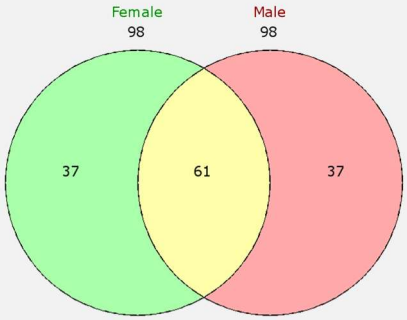
Over-expressed in Gregarious	over-expressed in Solitarious
cellular amino acid metabolic process	
cis-epoxide hydrolase activity	
pyruvic-malic carboxylase activity	
(S)-malate:NAD+ oxidoreductase (decarboxylating)	
chondroitinase I activity	
branched-chain amino acid metabolic process	
MCAT activity	
branched-chain-amino-acid transaminase activity	
3-ketoacyl-acyl carrier protein synthase activity	
defense response to bacterium	succinic acid dehydrogenase activity
extracellular	monooxygenase activity
oleoyl-acyl carrier protein thioesterase activity	iron binding
myristoyl-[acyl-carrier-protein] hydrolase activity	oxidoreductase activity
3-hydroxypalmitoyl-ACP dehydratase activity	haem binding
acyl-ACP-hydrolase activity	succinate dehydrogenase complex
acyl-CoA:malonyl-CoA C-acyltransferase (decarboxylating, oxoacyl- and enoyl-reducing and thioester-hydrolysing)	Over-expressed in Gregarious
acetyl-CoA:acyl-carrier-protein S-acetyltransferase activity	
enoyl-[acyl-carrier-protein] reductase (NADPH, B-specific) activity	
palmitoyl-[acyl-carrier-protein] hydrolase activity	
beta-1,4-linked N-acetylglucosamine metabolism	
iron binding	
chitin binding	
beta-ketoacyl-acyl-carrier protein(ACP) reductase activity	
oxidoreductase activity	
NAD binding	
molecular function	
intracellular membrane-enclosed organelle	3-ketoacyl-acyl carrier protein synthase activity
activation of NF-kappaB-inducing kinase activity	defense response to bacterium
glycine methyltransferase activity	extracellular
cellular amino acid metabolic process	oleoyl-acyl carrier protein thioesterase activity
cis-epoxide hydrolase activity	myristoyl-[acyl-carrier-protein] hydrolase activity
pyruvic-malic carboxylase activity	3-hydroxypalmitoyl-ACP dehydratase activity
(S)-malate:NAD+ oxidoreductase (decarboxylating)	acyl-ACP-hydrolase activity
chondroitinase I activity	acyl-CoA:malonyl-CoA C-acyltransferase (decarboxylating, oxoacyl- and enoyl-reducing and thioester-hydrolysing)
branched-chain amino acid metabolic process	acetyl-CoA:acyl-carrier-protein S-acetyltransferase activity
MCAT activity	enoyl-[acyl-carrier-protein] reductase (NADPH, B-specific) activity
branched-chain-amino-acid transaminase activity	palmitoyl-[acyl-carrier-protein] hydrolase activity

Figure 5: Venn diagrams showing number of over-represented GO terms in female (green), male (red) and both (orange). Over-represented GO terms in both male and female samples (yellow in venn diagrams) are listed: over-expressed in Gregarious (grey) and Solitarious (white).

5.3.4 Rhythmic expression

Rhythmic expression data was generated from brain + optic lobe tissues pooled for each animal taken at 3 h intervals from ZT01 under LD conditions. The expression patterns of *clock, cycle, cry-1, cry-2, period* and *timeless* over 24 h of LD were plotted separately for male and female gregarious and solitarious animals in Figure 6. JTK_Cycle (Hughes *et al.*, 2010) was used to classify the rhythmicity of the plots as significant (solid lines in Figure 6) or non-significant (dotted lines in Figure 6). For each gene the significance of rhythmicity was calculated using all four datasets with RAIN (Thaben and Westermark, 2014). The mean pattern was computed to visualise this data (blue dashed line in Figure 6). The patterns described here relate to the RAIN analysis (blue dashed line in Figure 6). The expression of *clock* peaked at dawn (ZT01) and dipped at dusk (ZT13). The expression of *cycle* showed no clear rhythm. The expression of *cry-1* did not show significant rhythmicity, but followed the same pattern as *cry-2*. The expression of *cry-2* peaked during the night (ZT19) and dipped prior to dusk (ZT10). The expression of *period* peaked at dusk (ZT13) and dipped at dawn (ZT01). The expression of *tim-1* peaked during the night (ZT16-22) and dipped during the day (ZT4-10). RAIN analysis was used to fully assess the rhythmicity of the core clock genes and other rhythmic genes. Of the rhythms described above, *Clk*, *per*, *cry-2* and *tim-1* were significantly rhythmic after adjustment for multiple tests (FDR), whilst *cyc* and *cry-1* were not significant (Table 13).

Of the 33143 genes which showed expression in all brain + optic lobe samples, 2366 were shown to be significantly rhythmic. GO enrichment analysis of these rhythmic genes shows pathways such as cell division and DNA synthesis as well as biosynthetic processes (top 45 of 51 pathways in Table 14).

Table 13: RAIN analysis of *clock, cycle, cry-1, cry-2, period* and *timeless* gene expression.

Gene	Adjusted p-value
clock	0.0036
cycle	0.24
cry-1	0.69
cry-2	4.73E-08
period	8.79E-07
tim-1	0.017

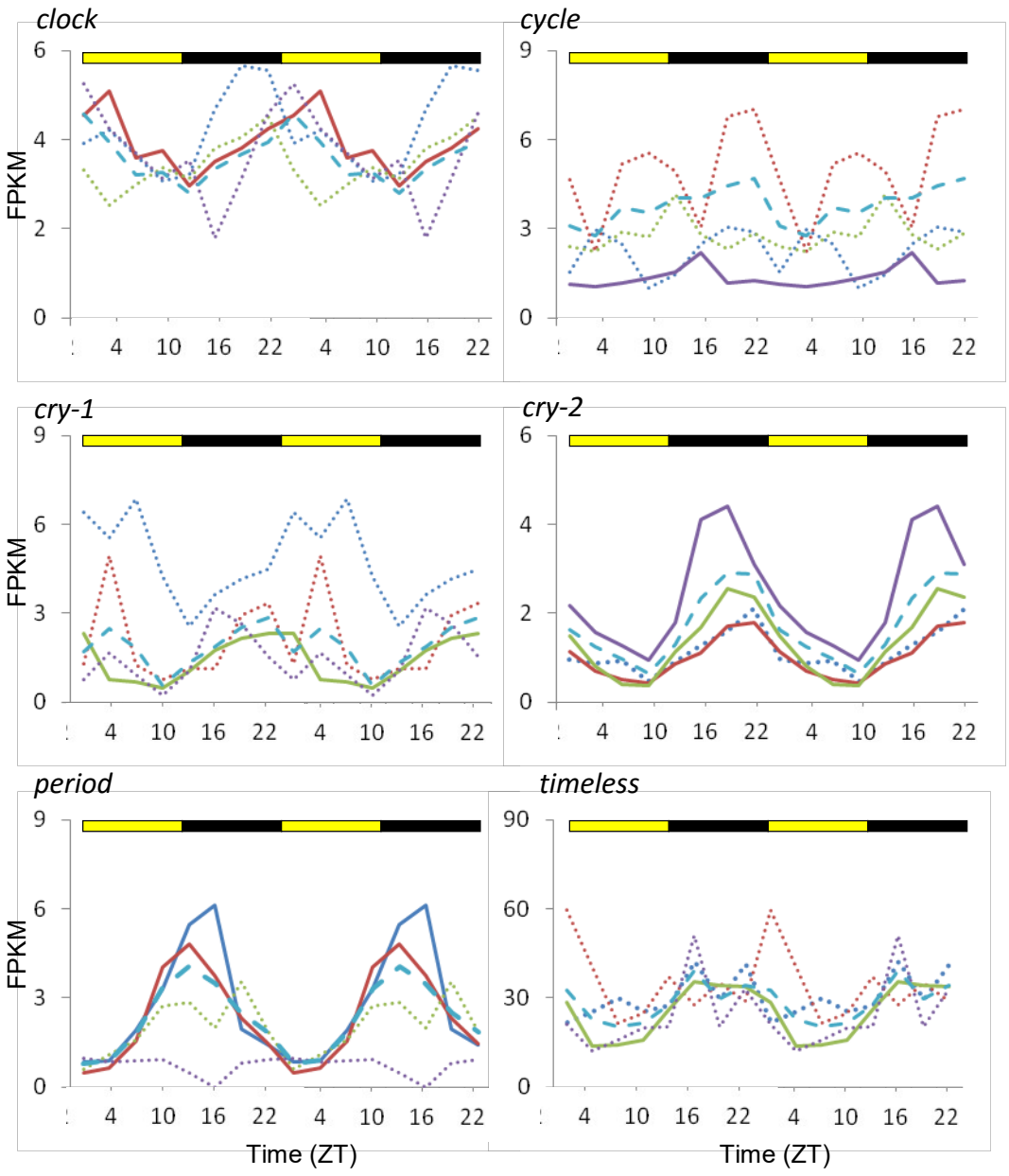


Figure 6: Expression levels of *clock, cycle, cry-1, cry-2, period* and timeless in the four available datasets: male gregarious (blue); female gregarious (red); male solitarious (purple); female solitarious (green). Data is double-plotted; Solid lines indicate significantly rhythmic expression pattern as determined by JTKcycle; dotted lines indicate non- rhythmic expression. Dashed light blue lines are averages over the four datasets. (Significance is shown in Table 13). Yellow and black bars indicate light and dark conditions respectively. X axis: time (ZT), Y axis: Fragments Per Kilobase of transcript per Million mapped reads (FPKM).

Table 14: Top 45 results (of 51) of terms identified by GO enrichment analysis of transcripts showing rhythmic expression in gregarious female brain + optic lobes as identified by RAIN.

Label	Q Val	Description	# in cluster	# in assembly
GO:0046872	1.33E-29	metal binding	420	4324
GO:0050113	3.39E-11	MOO activity	16	28
GO:0019310	3.39E-11	inositol degradation	16	28
GO:0005634	6.48E-07	cell nucleus	197	2366
GO:0016310	1.72E-04	phosphorylation	134	1605
GO:0006281	2.36E-04	DNA repair	40	323
GO:0004674	2.95E-04	protein serine/threonine kinase activity	42	352
GO:0051276	6.06E-04	chromosome organisation	12	46
GO:0008440	6.23E-04	Ins(1,4,5)P3 3-kinase activity	8	20
GO:0016301	6.47E-04	phosphokinase activity	126	1555
GO:0004703	6.47E-04	G-protein coupled receptor kinase activity	4	4
GO:0006355	6.47E-04	regulation of transcription, DNA-templated	101	1183
GO:0004644	6.47E-04	10-formyltetrahydrofolate:5'-phosphoribosylglycinamide N-formyltransferase activity	4	4
GO:0004641	6.47E-04	AIRS activity	4	4
GO:0004683	1.18E-03	calmodulin regulated protein kinase activity	7	17
GO:0006468	1.82E-03	protein amino acid phosphorylation	101	1223
GO:0006289	2.28E-03	nucleotide-excision repair	14	74
GO:0004637	2.28E-03	phosphoribosylamine-glycine ligase activity	4	5
GO:0016567	2.28E-03	protein ubiquitylation	25	189
GO:0034061	2.59E-03	deoxyribonucleate nucleotidyltransferase activity	5	9
GO:0004672	2.60E-03	protein kinase activity	98	1198
GO:0003913	2.76E-03	DNA photolyase activity	7	20
GO:0005096	3.20E-03	GTPase activator activity	21	150
GO:0035556	4.00E-03	intracellular signaling chain	47	480
GO:0004077	4.97E-03	biotin:apocarboxylase ligase activity	4	6
GO:0000439	6.40E-03	core TFIIH complex	7	23
GO:0016772	7.01E-03	transferase activity	111	1439
GO:0004842	7.06E-03	ubiquitin-protein ligase activity	23	184
GO:0009113	9.56E-03	purine nucleobase biosynthetic process	4	7
GO:0019343	1.53E-02	cysteine biosynthetic process	4	8
GO:0016810	1.53E-02	hydrolase activity	12	72
GO:0019344	1.53E-02	cysteine biosynthetic process	4	8
GO:0005694	1.53E-02	chromosome	19	149
GO:0043547	1.53E-02	upregulation of GTPase activity	39	407
GO:0004386	1.53E-02	helicase activity	47	518
GO:0006406	1.53E-02	mRNA export from cell nucleus	4	8
GO:0003937	1.67E-02	IMP cyclohydrolase activity	3	4
GO:0004643	1.67E-02	5'-phosphoribosyl-5-amino-4-imidazolecarboxamide formyltransferase activity	3	4
GO:0006189	1.68E-02	'de novo' IMP formation	5	14
GO:0006351	2.10E-02	cellular transcription, DNA-dependent	56	659
GO:0004122	2.23E-02	L-serine hydro-lyase (adding homocysteine)	4	9
GO:0003700	2.45E-02	sequence-specific DNA binding transcription factor activity	47	534
GO:0000166	2.77E-02	nucleotide binding	286	4403
GO:0051301	3.70E-02	cell division	12	82
GO:0071897	3.70E-02	DNA synthesis	14	104

5.4 Discussion

This chapter examined gene expression of the core circadian clock genes as well as differential expression between gregarious and solitarious animals. Cyclic expression of core clock genes was expected to be similar to that of *G. bimaculatus* with cycling of *cyc*, *per* and *tim*. Gene expression differences between solitarious and gregarious animals were expected to include an up-regulation of Vitellogenin, translation/transcription and DNA synthesis in solitarious samples with an up-regulation of signal transduction and immune pathways in gregarious animals.

5.4.1 Differential expression: Solitarious vs. Gregarious

Across the two different tissue sets, thoracic ganglia and brain + optic lobes, immune response was consistently over-expressed in gregarious animals (Figure 5; Table 6). Since the two sample sets are not independent (each thoracic ganglia sample has an equivalent brain + optic lobe sample from the same animal) this consistency shows this is not a single tissue specific difference, but does not provide stronger evidence of phenotypic variation in immune response. This overall increase in immune response in gregarious animals is consistent with investigations by Wilson *et al.* (2002), whereby gregarious animals were shown to have greater resilience against fungal infection. This population density linked immunity is known as "density-dependent prophylaxis" and has been seen in other species (see Introduction) which do not otherwise show density dependent polyphenism. It is therefore unclear if the mechanism of up-regulation is the same as the mechanism by which behavioural gregarisation occurs or a separate result of higher population density. The mechanism leading to gregarisation in *S. gregaria* is triggered by either tactile stimulation of the hind leg or by combined olfactory and visual cues (Rogers *et al.*, 2003; Anstey *et al*., 2009). This has been shown to produce an increase in serotonin during gregarisation (Anstey *et al*., 2009), although this is only transient and serotonin is found in higher concentration in solitarious ganglia (Rogers *et al.*, 2003). No transcripts associated with serotonin synthesis, metabolism, or reception were shown to be differentially expressed within either the ganglia or brain + optic lobe samples in the current study and no pathways associated with neurotransmitters were identified in GO enrichment analysis. In addition to the density-dependent prophylaxis seen in both the ganglia and brain + optic lobes, heat

shock proteins (HSP) were over-expressed in the ganglia of gregarious animals. Involved in stress response, these proteins have roles in folding, assembly, localisation, secretion, regulation and degradation of proteins (Feder and Hofmann, 1999). Additionally they have been shown to be over-expressed in gregarious *L. migratoria* (Wang *et al.*, 2007: in head, thorax, leg and whole body samples) and *S. gregaria* (Badisco *et al.*, 2011: whole CNS).

The gene encoding Vitellogenin is over-expressed in solitarious ganglia and solitarious male brain + optic lobe (Table 3; Table 7). Vitellogenin is associated with higher levels of JH, as seen in solitarious animals (Injeyan and Tobe, 1981b), as such this over expression of *vitellogenin* was as expected. No annotation for a gene encoding JH was made in the assembly in the current study so no conclusion can be drawn as to the interaction between the expressions of the two substances directly from the current study, however previous work has shown JH titres to be increased in solitarious animals (Injeyan and Tobe, 1981b). The expression levels of male solitarious (400 – 500 FPKM) was only half that seen in either solitarious or gregarious female (1000 – 2000 FPKM). Although solitarious locusts have been shown to have increased fecundity (Injeyan and Tobe, 1981a), the current study does not show an increase in expression of *vitellogenin* in female solitarious animals. As such it is likely that the increased fecundity seen is the result of increased JH and downstream effects other than Vitellogenin. The gene encoding Vitellogenin has been shown to be more highly expressed in Queen Honey bees, which show increased fecundity and longevity. Vitellogenin has been shown to have antioxidant actions, promoting long life (Corona *et al.*, 2007; Amdam *et al.*, 2012).

Within the ganglia dataset, DNA/RNA synthesis, transcription factors and associated genes were identified as being over-expressed in solitarious animals, although GO enrichment analysis does not identify the relevant pathways as over-represented as a proportion of the assembly. Such up-regulation was not seen in the brain + optic lobes. Up-regulation of DNA/RNA synthesis and processing in solitarious animals was seen by Badisco *et al.* (2011) in combined CNS samples. My results indicate that DNA/RNA synthesis and processing is over-expressed in the ganglia but not the brain + optic lobes. This may indicate increased cell growth and protein production in the ganglia of

solitarious animals. It is possible this is due to a change in biological investment, with solitarious animals favouring production of proteins that act to reduce oxidative stress as suggested by Badisco *et al.* (2011) and seen in the current study. In addition to Vitellogenin as discussed above, Oxidoreductase activity and succinate dehydrogenase activity were over-represented in transcripts over-expressed in solitarious brain + optic lobes (Figure 5: Top Right). These processes are associated with metabolism and reducing oxidative stress and have been shown to be over-expressed in *L. migratoria* from the 3rd instar (Chen *el al.*, 2010). By contrast, gregarious animals favour increasing production of the proteins which show reduced active time due to oxidative stress, such as hydrolases and transferases (Liebler, 2008; Sanchez *et al.*, 2008). This may counter the effects of oxidative stress seen in these pathways while avoiding the cost of increased protection from factors such as Vitellogenin and Oxidoreductase allowing for investment in processes with short term gains such as reproduction.

The data presented here show some agreement with those of Badisco *et al.* (2011). Of the 89 annotated genes identified as differentially expressed in Badisco *et al.* (2011), 48 were also annotated in the current study. Of these 17 (35%) were significantly differentially expressed in the ganglia (Table 15) whilst a further 12 (25%) showed non-significant differential expression that was of similar fold change to the previous study. Some of the differences between the studies may be due to the different experimental designs. For example, the current study had a higher throughput and consequently adjusted p values were more stringent, therefore similar fold changes would result in less significance as seen compared to previous work. Meanwhile the increased breadth of the current study resulted in the identification of significantly more differentially expressed transcripts. The annotation schemes also differed significantly, reducing the number of co-annotated genes: the current study annotated to the full non redundant protein sequences database, whereas Badisco *et al.* (2011) annotated to known *S. gregaria* and *L. migratoria* databases. Additionally the reference set to which differentially expressed genes were compared by Badisco *et al.* (2011) was not a full representation of the *S. gregaria* transcriptome, although it did include all known *S. gregaria* transcripts available at the time. By contrast the reference set in the current study was representative of all genes expressed in the brain, optic lobe and

thoracic ganglia by either phenotype at any of the time points. Consequently the GO enrichment analysis in the current study represents a more accurate image of overall differential expression between gregarious and solitarious individuals. In addition the current work is more comprehensive; with an order of magnitude more transcripts identified than in previous work on this species. Further work using this current dataset may focus on the strength of the annotation. Annotating directly with the *S. gregaria* and *L. migratoria* EST databases would allow more direct comparison between the current study and that of Badisco *et al.* (2011). This current work reveals similarities between differential expression patterns in *S. gregaria* and *L. migratoria*. Pathways such as immune response, DNA/RNA synthesis and oxidoreductase activity have been identified as differentially expressed between gregarious and solitarious in both species. The population density dependent phenotypic change is thought to have evolved separately (Song, 2005) and as such the level of similarity seen in differential expression is unexpected, with many pathways over-expressed in both species. Many non-locust grasshoppers show locust-like traits, such as population density colour change (e.g. *Melanoplus differentialis*; Dingle and Haskell, 1967), morphometrics (*Rhammatocerus schistocercoides*; Pierozzi and Lecoq, 1998) or behaviour such as swarming *(*R. *Schistocercoides*; Lecoq and Pierozzi, 1996a). It is therefore likely that density dependent phenotypic change is the consequence of multiple separately evolved traits all triggered by increases in density due to the shared selective pressures despite the disparate populations.

Table 15: Annotated transcripts identified as differentially expressed in solitarious (S) or gregarious (G) animals in both the current study (ganglia tissue) and the microarray by Badisco *et al.* (2011).

Annotation	Over-expressed in: (S/G)
transaldolase	S
peroxiredoxin	S
glutathion-s-transferase	S
putative cytochrome p450	S
cytochrome p450 cyp6bk17	S
x-prolyl aminopeptidase (aminopeptidase p) soluble	S
reverse transcriptase	S
proteasome (macropain) alpha 2	S
dead box atp-dependent rna helicase	S
alcohol dehydrogenase	S
alcohol dehydrogenase	S
heat-shock protein 70	G
putative defense protein	G
putative defense protein	G
heat-shock protein 90	G
SGP3 serine protease inhibitor	G
pacifastin-related 4a - pp4a	G
cation efflux protein zinc transporter	G

5.4.2 Rhythmic gene expression: circadian and diurnal

This work makes use of samples from 8 time points under LD conditions. The patterns of rhythmic expression reported above can therefore be described only as 'diurnal'. However, it is reasonable to assume the rhythms seen in the core circadian genes are circadian in nature.

In *S. gregaria, sgClk, sgper, sgcry-2* and *sgtim-1* were rhythmically expressed. Tobback *et al.* (2011) had previously seen rhythmic expression in *Clk, per* and *tim-1* examined with partial sequences. This current work confirms this rhythmic expression and additionally shows that *sgcry-2* shows the same rhythmic expression as *sgper* and sg*tim-1*. This suggests that sgCRY-2 does serve a similar function to that of mammalian cry. Expression of *sgtim-1* is less rhythmic (higher p-value) so it is likely that this serves as a secondary mechanism. This has been seen previously in *G. bimaculatus* in which *tim-1* was not rhythmically expressed until *cry-2* was knocked down (Tobback *et al.* 2011). Under this situation *tim-1* was then rhythmically expressed and took over circadian functionality. Rhythmic expression was not seen in sg*cyc* in *S. gregaria* in the current study. This was unexpected and indicates a similar expression profile to that seen in *Drosophila* (Figure 1) which is not normally seen outside of Diptera. Taken together, analysis of the core circadian clock genes indicates that the *S. gregaria* circadian clock is more primitive than the *Drosophila* clock, with elements such as cyclic *cry-2* in addition to *tim-1*. Comparing the expression profiles of the central genes in gregarious and solitarious animals, there is no major phase shift between the phenotypes such as could explain the shift in overall behaviour patterns, whereby gregarious animals are active during the day and solitarious animals are active around dusk (E. Gaten, unpublished; Ely *et al.*, 2011). This is similar to the situation in *G. bimaculatus* where behavioural output from the clock shifted due to developmental phenotypic change without a change in the expression profile of the central clock (Tomioka and Chiba, 1982; Nishinokubi and Tomioka, 2000). Similarly *Laupala cerasina* and *L. paranigra* have been shown to mate at different times of time whilst *period* gene expression did not differ (Fergus and Shaw, 2013). I therefore propose the differences in circadian activity between gregarious and solitarious animals is due differences downstream of the endogenous clock.

RAIN analysis of expression in the brain + optic lobes showed 4917 transcripts to be rhythmically expressed under LD conditions. Of these 2366 (1669 unique) were successfully annotated. 33,143 transcripts were tested and therefore 14.8% of expressed transcripts were rhythmic. This compares to the 9% seen in *A. aegypti* (Ptitsyn *et al.,* 2011). As in the current study, Ptitsyn *et al.* (2011) also included diurnal rhythms in their analysis and therefore the difference seen in the proportion of rhythmic genes between *Ae. aegypti* and *S. gregaria* must either be due to a difference between the species or an improvement in the technology and techniques since that work was carried out. Indeed, the increased sensitivity of RNAseq over the microarray technique used by Ptitsyn *et al.* (2011) may explain the majority of the increase. In addition, RAIN identifies approximately twice as many rhythmic genes as JTK_Cycle (Thaben and Westermark, 2014), and it is the latter of the two techniques which is most comparable to that used by Ptitsyn. Of the rhythmic GO terms that are over-represented there are terms related to DNA synthesis and cell growth. This is similar to the genes identified by Ptitsyn *et al.* (2011) in *Ae. aegypti*. Additionally kinases and GTPases were identified as over-represented in the rhythmic genes, which is consistent with results in *Drosophila* (Hughes *et al.*, 2012). This suggests that *S. gregaria* has rhythmic control of cell growth (DNA synthesis, cell growth and GTPases). Additionally it is likely the GTPase expression is responsible for diurnal patterns in signal transduction such as olfaction and vision.

5.4.3 Conclusion

This work has identified transcripts differentially expressed between phenotypes in more detail than was previously available, both in biological terms e.g. tissues and gender, but also in terms of technical precision and depth. Previous work by Badisco *et al.* (2011) identified 214 differentially expressed genes in the total CNS. This current work identifies 1605 - 3241 dependent on tissue and gender. I have shown that Vitellogenin is over-expressed in solitarious ganglia and brain + optic lobes (in males), and I propose this protein may have an oxidative stress protection role similar to that seen in *A. mellifera*. This would represent a change in biological investment a predicted by previous work on *L. migratoria*, with solitarious animals favouring lengthening the life of the individual whilst gregarious animals showed over expression of processes with short term gains such as catalytic processes. In addition I show increased expression immune processes in gregarious tissues, providing molecular evidence for density dependent prophylaxis in *S. gregaria*.

This work has shown the expression profiles of genes involved in the central circadian clock in *S. gregaria* as well as genes that are diurnally rhythmic under LD conditions. C*lock, period, cry-2* and *tim-1* were rhythmically expressed in this study providing evidence that the *S. gregaria* clock shows properties of both mammalian and *Drosophila* like circadian oscillators. Signal transduction and DNA synthesis/cell growth pathways were identified as rhythmically expressed, suggesting diurnal patterns in growth and environmental interaction (olfaction and vision).

Chapter 6:
General Discussion

This study set out to investigate circadian rhythms in *Schistocerca gregaria,* and differences in these rhythms associated with density dependent phenotypic plasticity. Swarming gregarious and cryptic isolated solitarious animals have previously been shown to express different diurnal patterns of behaviour. This study examines rhythmicity in: hatching; emergence of hatchlings; electroretinogram responses; looming stimulus avoidance behaviour; and gene expression of core genes. In addition, this study identifies and structurally characterises, in terms of conserved regions, the molecular machinery of the endogenous circadian clock in the context of known vertebrate and invertebrate circadian genes.

6.1 Diurnal patterns of hatching and emergence

Hatching and emergence are developmental processes vital to the success of an animal. Both processes have been shown to have diurnal and circadian control in a variety of species (See Chapter 2). *Gryllus bimaculatus* shows rhythmic hatching, with 88-97% of hatchings taking place within 3 h of dawn (Itoh and Sumi, 2000), similarly 94% of *Anolis sagrei* hatchings took place within 1 h of dawn (Nash *et al.,* 2015). Rhythmicity of eclosion in *Drosophila* has been seen to differ between two populations, dependant on geographical location; the northern population eclosed an average of 2.8 h earlier than those in the southern population (Lankinen, 1993). Emergence has previously been shown to be rhythmic in offspring of gregarious *S. gregaria* (Padgham, 1981), however no work has examined time of emergence in offspring of solitarious animals. Work carried out concurrently to the current study showed circadian rhythmicity of hatching in offspring of both solitarious and gregarious animals (Nishide *et al.*, 2015a), with gregarious offspring hatching prior to solitarious offspring.

I have demonstrated a strong rhythm in both hatching and emergence, with hatching occurring 2 - 3 h prior to dawn with gregarious eggs hatching prior to solitarious eggs. The difference between gregarious and solitarious hatching is consistent with work

carried out by Nishide *et al.* (2015a & b), however the current study shows later hatching relative to the work by Nishide *et al.* (2015a). I propose that this temporal difference in hatching is due to the difference in thermocycle between the two studies, with the current study using a more natural gradual temperature change compared to the abrupt temperature changes used in Nishide *et al.* (2015a).

Emergence had previously only been investigated in gregarious hatchlings (Padgham, 1981), with hatching occurring in the hours surrounding dawn. The current study confirms this in gregarious hatchlings and additionally shows a similar pattern in solitarious hatchlings. Solitarious hatchlings are shown to emerge earlier than gregarious hatchlings and also show stronger inherent sibling synchronicity, by contrast gregarious eggs hatch earlier than solitarious eggs in agreement to Nishide *et al.* (2015a). The period of time taken between emergence and hatching is longer in gregarious animals by 80 min when compared to solitarious animals (Figure 1). Taking this phase shift into account, the distribution of solitarious hatching is similar to that of gregarious hatching, but the distribution of emergence of solitarious hatchlings from the substrate is narrower than solitarious hatching as well as both behaviours in gregarious animals (Figure 1). I propose the differences seen between hatching and emergence in terms of phase shift and sibling synchronicity are likely to be due to the conspecific attraction and repulsion that is characteristic of the gregarious and solitarious phenotypes respectively. I suggest that solitarious hatchlings within the substrate, having hatched with a similar distribution to gregarious hatchlings, show increased activity in response to their close proximity to conspecifics. This activity encourages them to emerge sooner than gregarious animals which do not show increased activity due to proximity to conspecifics. Behavioural differences during emergence are not inconsistent with the differences seen in hatchling behaviour shortly after emergence. Hatchlings rapidly show a difference in locomotor activity regardless of conspecific activity with gregarious hatchlings showing increased activity once emerged, as is seen in adult individuals (Ellis and Pearce, 1962; Uvarov, 1966). Taken together with the current study this provides evidence that the behavioural differences between phenotypes are present at eclosion.

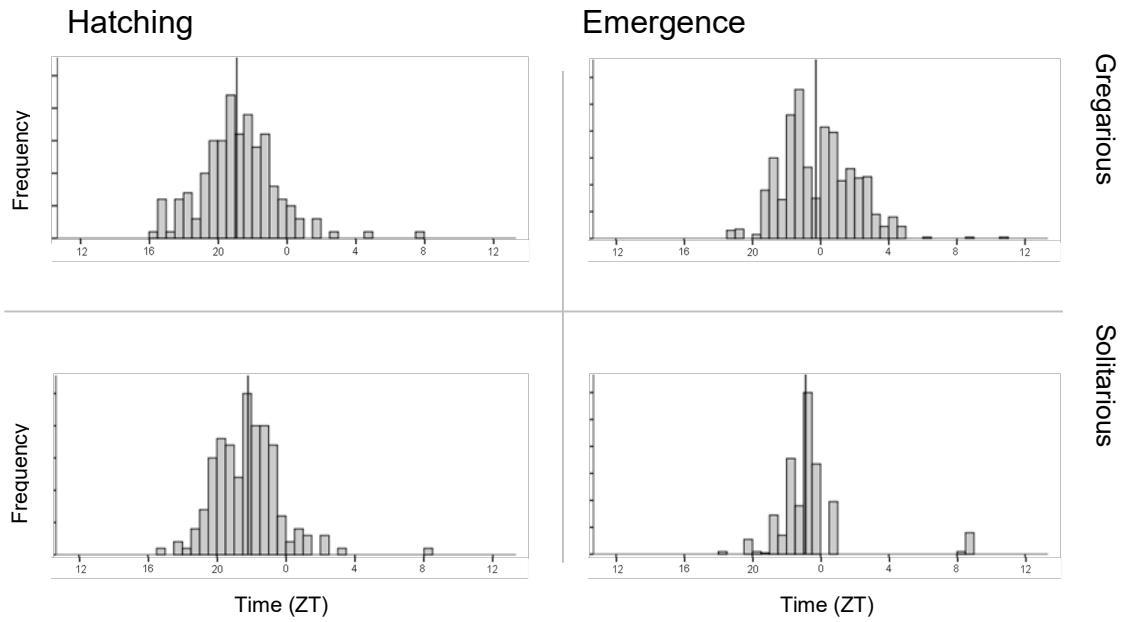


Figure 1: Comparing hatching (Left) and emergence (Right) time histograms for eggs derived from gregarious (Top) and solitarious (Bottom) females. Median: solid vertical lines. Bins are 30 min in width.

6.2 Retinal and behavioural responses to visual stimuli

Circadian rhythms have been seen in *S. gregaria* in the electrophysiological outputs of the visual system, the well studied DCMD (Descending Contralateral Movement Detector; Gaten *et al.,* 2012). DCMD has been associated with behaviours such as collision avoidance or glide-diving during flight (Gray *et al.*, 2001), and hiding, jumping and startle behaviours when the animal is at rest (Gabbiani *et al.*, 1999), although its role in this behaviour is poorly understood as DCMD is unable to code for all the information required by the behaviour (Hassenstein and Hustert, 1999). In order to further understand the circadian rhythms in DCMD and how they impact the behaviour of the animal I investigated retinal sensitivity and hiding behaviour at a number of time-points in both solitarious and gregarious animals. I have demonstrated increased visual sensitivity and increased behavioural responses in gregarious animals compared to solitarious animals, with greater amplitude ERG responses compared to solitarious animals and higher rotational amplitude of hiding responses.

Gregarious animals were more likely to make an active hiding manoeuvre than solitarious animals in response to a looming stimulus. This behavioural preference did not differ in a rhythmic fashion in the current study as was expected based on the rhythmicity of DCMD sensitivity (Gaten *et al.,* 2012). The rotational amplitude of hiding responses also did not differ in a rhythmic fashion, but with only 4 time-points analysed, this is not conclusive. The gregarious data are consistent with those collected in gregarious *L. migratoria* (Hassenstein and Hustert, 1999), showing active hiding to the majority of stimuli (> 60%, Figure 2, left). The solitarious data represent the first analysis of this behaviour in this phenotype and show a reduction in the amount of active hiding, with an increase in startle behaviour, when compared to gregarious responses (Figure 2, left). The mechanism for this difference is unclear, but it is likely to be a related to the increased cryptic camouflage seen in solitarious animals in contrast to gregarious animals. This is the case in animals such as anole lizards, whereby animals with greater camouflage show a greater startle response, with less movement in response to a potential threat (Heatwole, 1968). The spatiotemporal properties of the DCMD do not differ significantly between phenotypes in the areas the stimuli were presented to in this study (Rogers *et at.* 2010). However it is possible the behavioural

startle preference is due to the photosensitivity of the eye differing as described below, although care must be taken not to assume the startle response represents a lesser response to the stimuli. Here the lower response amplitude of solitarious ERGs, could lead to reduced avoidance behaviours.

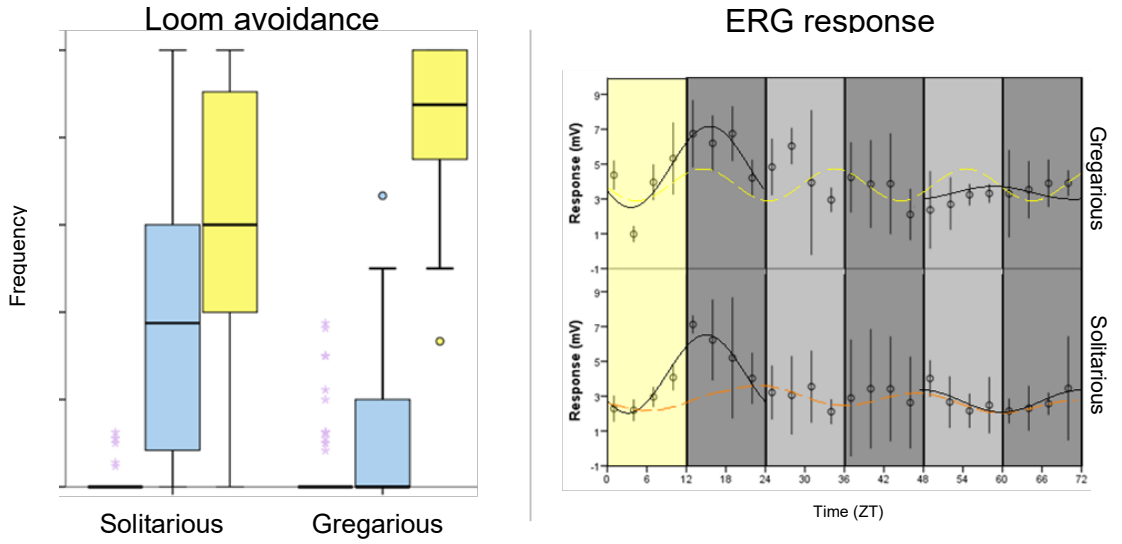


Figure 2: Percentage of responses categorised as startle (Blue), hide (Yellow) or jump (Purple), for gregarious and solitarious animals all time points pooled (Left). ERG response amplitude to light stimulus with 24 h cosinor fits (Black and orange) and 20 h cosinor fits (Yellow) in gregarious and solitarious animals. Black fits are over 24 h periods onlyb, coloured over full 72 h period.

ERG responses were diurnally rhythmic under LD and the second day in DD for both gregarious and solitarious animals, however the rhythmicity was not significant during the first day of DD (Figure 2; Right). The rhythm in the second day of DD suggests the ERG response is under circadian control. I show the period length to differ between solitarious and gregarious responses with solitarious responses being more closely fit by a 24 h rhythm and gregarious responses being more closely fit by a 20 h rhythm. This is consistent with the possible effect of social interactions that gregarious animals undergo as part of the crowding condition; this would result in a constant tendency to shift the circadian clock forward and would result in an apparent shortening of the period.

Social interaction has been shown to be a significant influence on the circadian clocks of humans (Stern and McClintock, 1998), rodents (Mrosovsky, 1988), fish (Kavaliers, 1980), bees (Toma *et al.*, 2000) and *Drosophila* (Levine *et al.*, 2002). In *Drosophila,* phase shift due to social interaction is driven by chemosensory pathways and results in high synchronisation between grouped individuals (Levine *et al.*, 2002).

Given chemosensory stimulation has been shown not to influence *S. gregaria* density dependant phenotypic variation (Rogers *et al.*, 2003) it is likely that any influence on the circadian clock is through a different mechanism. Therefore, experiments comparing two groups of gregarious animals should be carried out to determine if chemosensory stimulation is required for the social interaction effect on circadian period length in the Desert Locust, as described above. One crowded with conspecifics, as here, would be exposed to both tactile and chemosensory stimulation, compared with a second group gregarised using only tactile stimulation. By excluding the chemosensory stimulation in the second group it will be possible to establish if chemosensory cues are causative of a forward phase shift in the circadian clock. This would clarify if the period length differences described in the current study are due to population density phenotypes directly or coincident due to the social interactions experienced in one phenotype as I propose. Contrasting the current study and the existing work on rhythmicity in DCMD (Gaten *et al.,* 2012) shows that the rate of firing in DCMD does not align with response amplitude (or any other measure) in the recorded ERGs. Gaten *et al*. (2012) do make reference to stronger ultraduirnal rhythms in their data, for example 8 h and 12 h, in gregarious animals. These rhythms were not detected by the analysis in the current study. It is therefore highly likely that the two rhythms are not the result of a single oscillator and as such the rhythms seen in the retina do not drive the rhythmicity of DCMD. Multiple oscillators are known to exist in species such as the cockroach, *Leucophaea maderae*, where the optic lobes were shown to drive the ERG rhythm (Wills *et al.*, 1985). Serotoninergic fibres driven by the optic lobe oscillators, with increased firing during the subjective night, originate in the proximal rim of the optic lobe and run into the eye via the lamina (Nassel, 1988). I suggest, therefore, that the optic lobe oscillator is the primary driver of the ERG rhythms seen in the current study, whilst a separate oscillator, for example in the central brain, is likely the primary driver of DCMD rhythmicity.

By visually comparing the ERG data from LD conditions to *period* expression in the optic lobes and brain under LD conditions (see Chapter 5) it is clear that response amplitude closely matches expression level. ERG response peaks shortly after *period* expression (Figure 3). This provides further evidence for an endogenous oscillator,

although care should be taken not to confuse correlation with causation. This is further evidenced by the rhythmic ERG response amplitude seen in the second day of constant dark conditions (Figure 2 right). Although no expression data are available for this condition, this free-running rhythm is indicative of endogenous influence as opposed to diurnal influences.

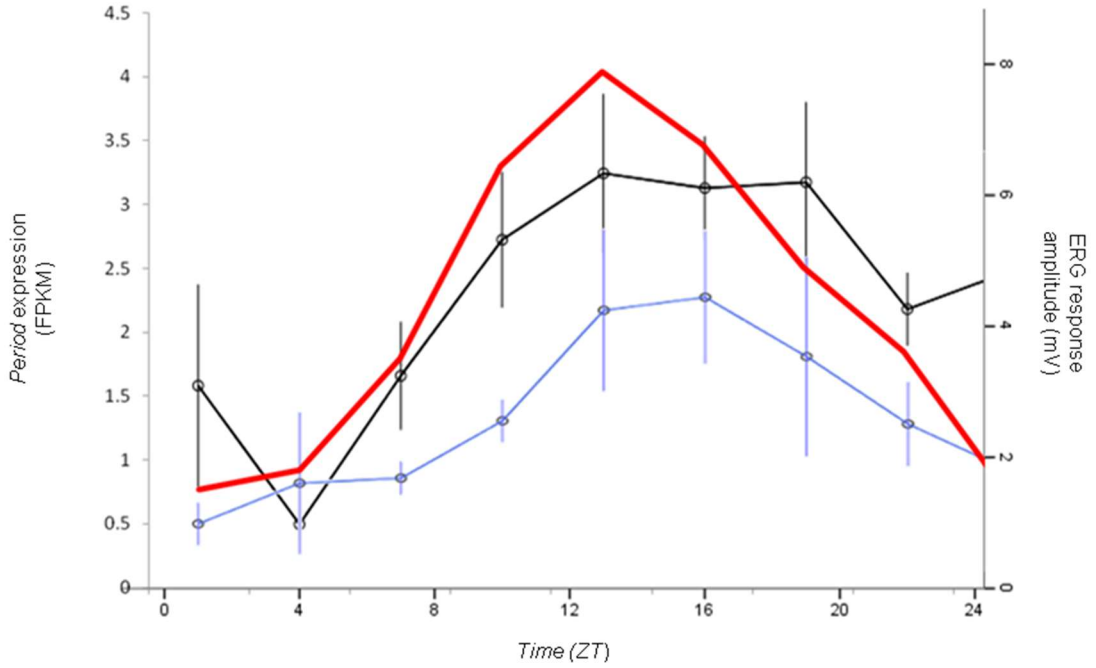


Figure 3: Average expression of *period* (Red, left axis) and ERG response to light intensities between 250-750 lx (gregarious, black; solitarious, blue: Right axis) shows similarity in gregarious animals. Solitarious animals show a similar peak although lower and 20 min earlier as discussed in Chapter 3.

6.3 Characterising the core circadian clock genes

Previous work on the core circadian clock genes in *S. gregaria* was lacking, with only partial sequences known for a limited number of genes. The transcriptome assembled in the current study from ganglia, brain and optic lobe samples provided high coverage of all the investigated genes. Full sequences for *clock, cycle, period, timeless, timeout, cryptochrome-1, cryptochrome-2, doubletime* and *vrille* were deduced from the available transcripts. The presence of both *cry-1* and *cry-2* is consistent with work in the field cricket, *Gryllus bimaculatus* (Moriyama *et al.*, 2012; Table 1). The existing partial sequences for the core circadian genes in *S. gregaria* assessed by Tobback *et al.* (2011) provided limited understanding of their functionality. I provide evidence that the structure of *clk* and *cyc* are indicative of a mammalian like functionality, whereby sgCYC (more correctly BMAL) contains a region associated with transcription activity (BCTR) and sgCLK shows a marked reduction in the length of its PolyQ region when compared to the homologue in *Drosophila.* In contrast to this, the presence of both *timeless* and *timeout* suggests similarity to *Drosophila.*

I have identified a full set of core circadian and related regulatory genes, making *S. gregaria* the most well understood of the Orthoptera in terms of the structural characteristics of these genes (Table 1). This work provides a firm foundation for future molecular work focusing on the circadian clock, and the knowledge required to manipulate the circadian clock to further investigate the interactions involved. Using the sequences described, it is now possible to carry out genetic manipulations, either creating non-functional mutants of the circadian genes, either through RNAi knockdown (as in Sugahara *et al.* 2015) or interference using non-functional transgenes (as in Gao and Zhang, 2007), or introducing *S. gregaria* sequences to *Drosophila* mutants deficient in that gene (as in Padgett *et al.*, 1993). Recovery of rhythmicity in the later case would be informative as to the functional similarities and differences between the circadian genes in the two species. This would be similar to work by Zehring *et al.* (1984) whereby rhythmicity of a *per* mutant in *Drosophila* was recovered using germline manipulations. Given the findings in the current work it would be expected that *clock* and *cycle* would not be able to recover *Drosophila* rhythms given their structural differences. However, *tim-1* and *cry-1* would be

expected to have similar functionality. In addition to the circadian genes described, this work provides a comprehensive transcriptome of genes expressed in the central nervous system (midbrain, optic lobes and thoracic ganglia). This may be queried to enable rapid identification of genes of interest for future studies and allows similar analysis to that carried out on the target genes in this study, with data for structural homogeny and gene expression available.

Table 1:. Clock genes found in insects of various taxa on the basis of a database survey, modified from Tomioka and Matsumoto, 2015. Y = existence, N = absence, ? = to be determined. *S. gregaria* is highlighted in grey.

Species		Circadian Gene										
		clk	*Cyc*	*per*	*tim-1*	*tim-2*	*cry-1*	*cry-2*	*dbt*	*vri*	*cwo*	*pdp1*
Diptera	*Drosophila melanogaster*	Y	Y	Y	Y	Y	Y	N	Y	Y	Y	Y
	Musca domestica	Y	Y	Y	Y	?	Y	N	?	Y	?	?
	Aedes aegypti	Y	Y	Y	Y	Y	Y	Y	?	?	?	Y
	Anopheles gambiae	Y	Y	Y	Y	Y	Y	Y	?	?	?	Y
lepidoptera	*Antherea pernyi*	Y	Y	Y	Y	?	Y	Y	?	Y	?	?
	Bombyx mori	Y	Y	Y	Y	Y	Y	Y	Y	?	?	?
	Danaus plexippus	Y	Y	Y	Y	Y	Y	Y	Y	Y	?	Y
Hymenoptera	*Solenopsis invicta*	Y	Y	Y	N	Y	N	Y	?	Y	Y	Y
	Apis mellifera	Y	Y	Y	N	Y	N	Y	?	Y	?	Y
Coleoptera	*Tribolium castaneum*	Y	Y	Y	Y	Y	N	Y	?	Y	Y	Y
Hemiptera	*Acyrthosiphon pisum*	Y	Y	Y	Y	Y	Y	Y	?	Y	?	Y
	Riptortus pedestris	Y	Y	Y	?	?	Y	Y	?	Y	?	?
Orthoptera	*Gryllus bimaculatus*	Y	Y	Y	Y	?	Y	Y	Y	Y	Y	Y
	Rhyparobia maderae	?	?	Y	Y	?	?	Y	?	?	?	?
	Schistocerca gregaria	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Thysanura	*Thermobia domestica*	Y	Y	?	Y	?	?	Y	?	?	?	?

6.4 Differential and rhythmic patterns of gene expression

I identify 1,605-3,241 transcripts differentially expressed between gregarious and solitarious samples of brain and optic lobe (between same-gendered samples), and ganglia (gender balanced) samples. Previous work by Badisco *et al.* (2011) identified 100 genes more strongly expressed in gregarious CNS and 114 in solitarious CNS of an Expressed Sequence Tag (EST) library of 20,755; this represented approximately 0.5% of the tested genes in each case. I identify 1,272 genes that were more highly expressed in solitarious ganglia, 848 in gregarious ganglia of a total 103,292 expressed genes. This represents 1.2% and 0.8% respectively. This represents both an order of magnitude more differentially expressed genes then previous work, but also provides a clearer view of the extent of expression difference between the phenotypes. Although

none of the five Gene Ontology (GO) terms identified by Badisco *et al.* (2011) were also explicitly identified in the current study using comparable methods, the identified GO terms are similar and the difference is probably a result of the incomplete reference set used in the previous work. This suggests that the work by Badisco *et al.* (2011) was accurate but incomplete. The current study represents a more complete comparison of the transcriptome in each phenotype.

I provide evidence of up-regulation of immune genes in all gregarious samples when compared to the solitarious samples. Previously the increased resistance of gregarious *S. gregaria* has only been shown by challenging with a pathogen (Wilson *et al.*, 2002). This work represents the first to provide a molecular basis for this difference. In addition, transcripts associated with stress response were identified as differentially expressed in the ganglia: heat shock proteins (HSP) 70 and 90 were more highly expressed in gregarious samples. This has previously been reported by Badisco *et al.* (2011) in full CNS samples, however I provide evidence that this is a local difference, with differential expression limited to the thoracic ganglia and absent from the brain and optic lobe. Work in rat brains following ischemic trauma suggest an increase in HSP 70 expression in regions undergoing damage, with prolonged high expression in highly damaged regions and transient expression in tissue which recovers (Welsh *et al.,* 1992). This suggests gregarious animals are undergoing tissue sustained tissue damage in the ganglia, likely due to oxidative stress as suggested by a reduced expression of genes associated with preventing oxidative stress (See Chapter 5).

I provide evidence that gregarious brain + optic lobes have increased expression of intracellular structure catalytic activity, lipid and glycan metabolism pathways. These pathways have not previously been identified as differentially expressed in *S. gregaria*. Additionally, I identify processes associated with metabolism as more highly expressed in solitarious brain + optic lobe samples. I show Vitellogenin to be more highly expressed in male (but not female) solitarious samples. Vitellogenin is highly expressed in the head and thorax of queen honey bees, which show increased fecundity and longevity relative to drones, which express low levels. Specifically Vitellogenin has been shown to have antioxidant actions, promoting long life (Corona *et al.*, 2007). It is likely to have a similar effect in *S. gregaria*, where the solitarious phenotype shows

both increased fecundity and longer life (Uvarov, 1966; Injeyan and Tobe, 1981a), although further work should be carried out to establish the validity of this hypothesis. Vitellogenin has been detected in the adult haemolymph 4 days after the final moult in solitarious adults and 6 days after the final moult in gregarious animals, this correlated with higher levels of juvenile hormone (JH) in solitarious adults, in the corpora allata (Injeyan and Tobe, 1981b). In *Locusta migratoria* removal of the corpora allata, the location of JH synthesis, prevents vitellogenin synthesis; the addition of JH or an analog was able to recover synthesis (Chen *et al.*, 1979).

Vitellogenin levels also differed in the oocytes, with detection at the 6th day in solitarious females and 8th days gregarious females (Injeyan and Tobe, 1981b). This difference reduced over the course of oocyte maturation, with a final titre similar between gregarious and solitarious overies. However, due to the increased number of ovarioles the vitellin content per oocyte was lower in solitarious eggs (Injeyan and Tobe, 1981a).

The current study only investigated *adult* CNS tissue samples, so the developmental expression differences between the two phenotypes can only be speculated upon. It is however likely that similar broad traits are conserved between developmental stages. With gregarious animals more likely to show higher investment in processes related to interacting with the environment and solitarious animals showing higher investment in processes related to long term survival. This is similar to results in *Locusta migratoria* (Chen *et al.,* 2010), and it is therefore likely that the different investment between phenotypes is most marked before the final moult (4th instar in *L. migratoria*), resulting in the characteristic pigmentation and morphological differences seen only in animals crowded or isolated prior to the final moult. Given the behavioural differences already present in the hatchlings (See chapter 2) it is likely that gene expression differs in the earliest stages, as is seen in *L. migratoria*; gene expression differences are seen in the eggs, and early instars (1+2) by Chen *et al.* (2010). The mechanism of the gene expression differences discussed both in this work, in *S. gregaria,* and by Chen *et al.* (2010), in *L. migratoria,* are unknown, although they are likely to be similar in the two species. It is possible that a combination of methylation and environmental triggers play a role. Differential methylation between phenotypes has been seen in both

species (Amarasinghe *et al.*, 2015 & Wang *et al.,* 2013) and has been implemented in consolidation of long term gregarisation state. Amarsinghe *et al.* (2015) described methylation differences between the two extreme phenotypes of *S. gregaria*, with long term isolation or crowding leading to significant differences on the methylation-sensitive amplified fragment length polymorphisms detected. Short term treatments did not, however, show significant methylation differences. Chen *et al.* (2010) identified over 90 genes that showed significant methylation differences between the two extreme phenotypes in *L. migratoria*, including pathways associated with cytoskeleton organization, molecular motors, kinases, receptors, vesicle transfer, signal transduction proteins and RNA-DNA binding.

I show that under LD conditions the central clock genes *clk*, *cry-2* ,*per* and *tim-1* are expressed rhythmically in the brain + optic lobes of *S. gregaria* but that *cyc, cry-1* and *tim-2* are expressed at relatively constant levels. This is comparable to the situation in *G. bimaculatus* (Tobback *et al.*, 2011)), where rhythmicity is seen in *per* and *tim-1* but not *cyc.*In *G. bimaculatus* however, *clk* was also arrhythmic in both the brain and optic lobe. The rhythmicity I describe in *per* and *tim-1* suggests they are functionally similar to the expression of homologs in both *Drosophila* and *G. bimaculatus*, however work in *G. bimaculatus* shows that rhythmic timeless expression is not necessary for circadian behavioural output (Danbara *et al.*, 2010). This is likely to be due to the presence of a cycling *cry-2* homologue. In mammalian circadian clocks two *cry-2* homologs (m*cry-1* and m*cry-2*) are responsible for the roles associated with *tim* in *Drosophila*, with *tim* not showing rhythmic expression. Cyclic expression of *cry-2* is consistent with mammalian type functionality of *cry-2* and the evidence in this current study is that *cry-2* shows strong rhythmicity. Having both *Drosophila* and mammalian like functionality is seen in many insects as seen by the wide scale existence of both *cry-1* and *cry-2* homologs as well as *tim-1* and *tim-2* throughout invertebrates (Table 1). It is likely the cyclic *Clk* expression seen in the current study is the result of the non-cyclic *cyc* expression. Cyclic *Clk* expression has been shown in *G. bimaculatus* in the absence of cycling *cyc* (Uryu *et al.*, 2013).

The two investigated phenotypes of *S. gregaria*, solitarious and gregarious, did not show large differences in either expression pattern or total expression of central clock

genes. This is despite a large shift in behavioural timings seen in general activity levels, both in the lab and in the field. Activity is seen to be phase shifted by approximately 6 h (E. Gaten, unpublished) and the activity of DCMD, a loom sensitive visual interneuron, shows a phase shift of 4 h (Steedman, 1990; Gaten *et al.*, 2012;). The differences seen in these two measures are not the result of differences at the central clock level with no phase shift of that magnitude being present (current study).

I identify rhythmicity in a large selection of genes (1669 uniquely annotated genes) including those associated with DNA synthesis and growth. This represents approximately 15% of the expressed transcriptome in the brain and optic lobes and presents opportunities to investigate the genetic basis for rhythmic behaviours. For example, transcripts associated with intracellular signalling are rhythmically expressed. It is likely to be related to the rhythmicity seen in ERGs in Chapter 3 (Figure 3) and future analysis of the described expression data should aim to deduce the timing of peak GTPase expression and other expression pathways associated with signal transduction in the two phenotypes and compare that to the ERG response profile and the DCMD firing rate profile described in Chapter 3. It is expected that the expression profile will correlate with DCMD firing rate, as this pathway is associated with signal interpretation.

There is consensus in expression profiles between this current study and RNAseq work on *L. migratoria* (Chen *et al.*, 2010). Whilst this reinforces the similarity between the two species it was unexpected to see such consistent similarity across the two species belonging to two different subfamilies. *S. gregaria* belongs to the subfamily Cyrtacanthacridinae and *L. migratoria* belongs to Oedipodinae and they are therefore thought to have evolved density dependent phenotypic change separately through either convergent, or partially convergent, evolution (Song, 2005; Pener and Simpson, 2009; See Chapter 1). Many non-locusts show locust-like traits, such as population density colour change (e.g. *Melanoplus differentialis*; Dingle and Haskell, 1967), or behaviour such as swarming *(Rhammatocerus schistocercoides*; Lecoq and Pierozzi, 1996). The detection of population density is also not consistent across locust species, for example behavioural change can be triggered by tactile stimulation of the hind legs (*S. gregaria*;

Rogers *et al.*, 2003) or the antenna (*Chortoicetes terminifera*; Cullen *et al.*, 2010). This suggests density dependent phenotypic change is a complex selection of traits which although triggered by a single environmental variable, population density, have evolved separately due to local selection pressures.

6.5 Future work

This current work has provided two main tools that will facilitate future work on circadian rhythmicity and population density dependent expression differences in *S. gregaria*. Firstly, the assembled transcriptome and samples available will enable identification of candidate genes, including those not currently annotated, associated with the population density dependent polyphenism and compare the expression in the two extreme phenotypes. Some possible avenues for investigation include expression of COP9 signalosome complex subunit 7A which is required for phenotypic transition in *L. migratoria* (Tong *et al.*, 2015). Additionally Corazonin has been shown to have a critical role in gregarious characteristics (Sugahara *et al.*, 2015) and is expected to show a difference in expression between the two extreme phenotypes. Secondly the time series samples provide the basis to investigate novel splicing events as seen in *Drosophila* (Hughes *et al.*, 2012) where novel splice variants have been seen in *clk, tim-1, cwo, pdp1, CKIIa* and *CKIIb* in addition to thousands of other splicing events across the genome.

6.6 Concluding remarks

This thesis has revealed population density dependent differences in circadian rhythms in *S. gregaria* in hatching, emergence and electroretinogram assays whilst also showing differences in behavioural responses to looming stimuli. I have generated a de-novo transcriptome of the brain and optic lobes, and the ganglia which has revealed the sequences for key central circadian clock genes and allowed inferences as to their functionality. In addition I have provided further evidence as to the extent of the transcriptomic differences seen as a result of the population density dependent plasticity and the extent to which the transcriptome is controlled by the circadian clock. Finally my work provides the foundation for future molecular work on *S. gregaria*, both in terms of differential expression and rhythmic expression.

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