Drastic times call for drastic measures:

how timing affects host-parasite interactions

Mary Lynn Westwood

A thesis submitted for the degree of

Doctor of Philosophy

Institute of Evolutionary Biology, The University of Edinburgh

2021

Authorship declaration

I composed this thesis. All analyses and writing are my own, with help from my supervisor Professor Sarah Reece and coauthors. Sarah Reece advised the writing, analysis, and experimental designs for all three data chapters. Nathan Bailey provided crickets from Oahu, and Marlene Zuk provided crickets from Mangaia. Aidan O'Donnell and Ronnie Mooney helped maintain the cricket colony and aided all experiments. Quentin Geissmann wrote the R script "Tempaural". I use the word "we" throughout the data chapters because they were written as papers.

7 January 2022

Publications arising from this thesis

Westwood, Mary L., *et al.* "A novel audio-to-circadian analysis pipeline reveals singing rhythms of male Pacific field crickets are clock controlled." In preparation.

Westwood, Mary L., *et al.* "Temporal escape from infection: evidence of circadian shifts in singing in a parasitised population of crickets." In preparation.

Westwood, Mary L., *et al.* "Testing possible causes of gametocyte reduction in temporally out-of-synch malaria infections." Malaria journal 19.1 (2020): 1-10.

Westwood, Mary L., *et al.* "The evolutionary ecology of circadian rhythms in infection." Nature ecology & evolution 3.4 (2019): 552-560.

Acknowledgements

My mom began night classes at the local community college when I was very little. During the day, she would stay home with my brother and I while my dad worked long hours as an electrician (he's still an electrician, and the hardest worker I know). My mom finally became a lawyer when I was in high school – simultaneously stealing from me the chance to become the first "Dr" in the family while teaching me that anything is possible if you put your mind to it. Thank you, Mom and Dad. You inspire me every day.

My brother was accepted to MIT for undergrad, and while I don't consider myself a competitive person, I'd be remiss not to mention the motivation he provided me to do something with my life. Thanks for that, Mitch.

It'd be impossible for me to thank all of the giant's shoulders I've stood upon, but there are some noteworthy individuals requiring acknowledgement. Sarah Reece – thank you for teaching me that problems are just that, problems to be solved. You've always been in my corner, and I feel lucky to have you as a mentor. Tom Rooney – I remember once in class you called on me to answer the four traits necessary for evolution by natural selection. I couldn't remember them all, but you still took me on for my first undergraduate research project, and then another, then another, and finally a Masters. Now that I have a PhD in Evolutionary Biology, I can confidently say my memory is just as bad as ever and please don't ask me to list anything off the top of my head (and thank you for taking a chance on me). Finally, Mr. Berry – your pop quizzes and owl pellets were the biological spark that set me off on this path. Thank you for that.

To the Reecearchers – thank you for being the most supportive, fun, and knowledgeable group of people I know. There's no better crew to spend overnight in Ashworth sampling mice with. To my collaborators – your willingness to share your time, expertise (and crickets) made this thesis possible. Thank you.

Finally, when I think back on the past four years, the moments which brought me the most joy were not academic achievements. They were the late night, too many pints kind of evenings spent with friends. If you're reading this and wondering if I'm talking about you – I definitely am. Thank you for helping me to keep sight of life outside of my laptop.

Lay summary

Approximately every 24-hours, the Earth completes a rotation on its axis, resulting in predictable cycles of day and night. The daily alternation of light and dark is so reliable that nearly all life on Earth has evolved to take advantage of it. These so-called circadian rhythms help organisms to maximise survival and fitness. In essence, they help organisms to perform the "right activities at the right time of day". One of the most common ecological interactions is that between hosts and parasites. Because both parasites and circadian rhythms are so ubiquitous, understanding how rhythms affect interactions between parasites and their hosts can help to explain the many diverse relationships seen in nature. In this thesis, I outlined how to examine evolutionary ecology from a chronobiological framework and why it matters to do so, asked questions about the role of rhythms in mating behaviours using the pacific field cricket *Teleogryllus oceanicus,* and about the role of rhythms in reproductive effort using the rodent malaria parasite *Plasmodium chabaudi.*

In Chapter 2, I wrote a perspectives paper which outlines the importance of integrating evolutionary ecology with chronobiology. I cover this topic from three complementary angles: (1) rhythms in parasite offense, (2) rhythms in host defence, and (3) parasite manipulation of host rhythms. Next, in chapters 3 and 4 I examined whether the cricket host of an acoustically-orienting parasitoid fly may have shifted the time-of-day it sings to avoid being parasitised. I did this first by verifying circadian rhythmicity and characterising singing rhythms (Chapter 3), then I compared singing rhythms between a parasitised and an unparasitised population (Chapter 4). We found that the parasitised population sings less at the time of day the fly is thought to be most actively host-seeking. This suggests that circadian singing rhythms may be a target of natural selection. In chapter 5, I focus on the rodent malaria *Plasmodium chabaudi*, which grows and develops in the hosts red blood cells over 24-hours, and each developmental stage is time-of-day specific. When mismatched to host time-of-day, both the sexual and asexual stages of *P. chabaudi* reduce numbers by ~50%. I tested whether the sexual stage parasites may be employing a strategy to reduce their numbers (i.e., conversion), or whether host immune rhythms may be responsible for reduced parasite numbers, in temporally mismatched infections. I did not find support for either hypothesis, and so I outline future directions in chapter 5.

The overarching goal of this thesis is to highlight the gains to be made from integrating evolutionary ecology with circadian biology, especially in the context of infection and host-parasite interactions. From both a basic to applied standpoint, using a chronobiological framework to interrogate evolutionary and ecological questions can help to hinder or even block the chain of transmission in human pathogens, make medical interventions evolution-proof, and resolve open questions concerning some of the most common ecological interactions.

Abstract

Earth's daily rotation causes predictable cycles of day and night, which all life has evolved to cope with. Circadian clocks (i.e. daily, biological timekeepers) are ubiquitous and allow organisms to schedule activities, from gene expression to physiologies to behaviours, according to the time-of-day they are best undertaken. Most research on circadian rhythms has focussed on uncovering the genes and molecular pathways involved in the workings of circadian clocks. However, there is increasing interest in the evolution and ecology of circadian rhythms – particularly, in how rhythms affect interactions between organisms. One of the most fundamental ecological interactions is that between parasites and hosts. In this thesis, I explore how circadian rhythms mediate infection through the lens of evolutionary ecology. My chapters consider how the rhythms of both hosts and parasites evolve in response to each other, with a focus on how rhythms mediate activities underpinning sexual reproduction. Specifically, I have outlined how to examine evolutionary ecology from a chronobiological framework and why it matters to do so, asked questions about the role of rhythms in mating behaviours using the pacific field cricket *Teleogryllus oceanicus,* and about the role of rhythms in reproductive effort using the rodent malaria parasite *Plasmodium chabaudi.*

First, I wrote a perspective paper (Chapter 2) demonstrating the value of integrating evolutionary ecology and chronobiology. This is the first paper detailing the role of rhythms as mediator to natural and sexual selection, including the development of hypotheses examined in Chapters 4 and 5. Further, I challenge conventional wisdom emerging in chronobiology that immune rhythms mediate susceptibility, and propose how parasite manipulation of host rhythms may explain unusual host behaviours that have so far defied explanation. Moreover, this paper is the first to consider a periodic environment from the parasites "point of view", because most work to-date has focussed on host rhythms in immune defence.

In chapters 3 and 4 focussed on a cricket-parasitoid fly system (*Teleogryllus oceanicus* – *Ormia ochracea*) to examine whether hosts can evolve altered rhythms in mate-seeking behaviours as a parasite avoidance strategy ("temporal escape"). I expected this as the parasitic fly locates its cricket host by following the sound of male crickets when they sing to attract female mates, and then homing in using visual cues. Thus, singing in male crickets is a sexually selected trait that individuals must balance with natural selective pressure from the fly. To begin to ask whether temporal escape could have evolved, I had to first make *T. oceanicus* into a tractable system for chronobiology and characterise its singing rhythm. Thus, in Chapter 3, I performed experiments to uncover to what extent singing in *T. oceanicus* is clock-controlled. To derive data to analyse in a robust circadian context, I developed a pipeline which combines machine learning and high performance computing. The circadian phase markers I extracted showed conclusively that singing is

circadian in *T. oceanicus* and variation amongst individuals suggests natural and sexual selective pressures may shape singing rhythms.

Next, in chapter 4 I performed an experiment to compare the circadian singing rhythms of an ancestral, unparasitised population of *T. oceanicus* (from the Cook Islands) with a population from the Hawaiian island of Oahu that has experienced sufficiently high parasitism by *O. ochracea* to evolve several forms of morphological defence. Specifically, I tested whether the timing of singing by males from Oahu differs from the singing rhythm of males from the Cook Islands, hypothesising that Oahu males should be less likely to sing at dusk because that is when the fly is thought to be most likely to host-seek. I found that while both populations have similar entrained and free-running periods, circadian phase markers vary between the populations. Males from Oahu sing nearly twice as much as Cook Island males, but Oahu males are much less likely to sing during the light phase and around dusk. While many other selection pressures will differ between the Oahu and the Cook islands and the population introduced to the Hawaiian islands has experienced a strong bottleneck, which may influence singing rhythms, the timing differences I observe are consistent with temporal escape as a parasite avoidance strategy.

In Chapter 5, I switched to malaria parasites to test whether host rhythms influence parasite investment into sexual reproduction. When out-of-synch with host rhythms, *P. chabaudi* parasites suffer a 50% reduction in the density of both asexual and sexual stages (termed "gametocytes") in the host's blood. I focused on asking whether reduced investment in gametocytes and/or increased mortality of gametocytes might explain their lower density in out-of-synch infections. I first analysed data from a previous experiment on reproductive effort (called the "conversion rate"), which is known to be plastically down-regulated when parasites experience stressful situations. Second, I carried out experiments to test whether a key aspect of the innate immune response (the inflammatory cytokine tumor necrosis factor, TNF) varies in its gametocytocidal efficacy according to host time-of-day and gametocyte age. I found that neither plasticity in conversion rate or rhythms in TNF-caused gametocyte mortality explain the reduction in gametocytes observed in out-of-synch infections and suggest alternative explanations. Gametocytes are required for between-host transmission of malaria parasites so understanding why it matters for gametocytes to be synchronized to host circadian rhythms might suggest novel approaches to blocking parasite transmission.

Decades of research into the molecular underpinnings of circadian clocks has highlighted the disconnect between progress in understanding the mechanisms driving rhythms and their evolutionary and ecological significance. Infections are ubiquitous in nature, so understanding how rhythms in parasite offense interact with rhythms in host defences are an excellent arena for integrating circadian biology with evolutionary ecology and may uncover novel strategies for controlling infections.

Table of Contents

1. General Introduction

The daily rotation of the Earth causes predictable cycles of day and night, which all life has evolved to cope with. From the Latin *circa dies* (meaning "about a day"), circadian clocks help organisms to anticipate the periodicity of light and dark and the environmental changes that accompany day and night. By allowing organisms to prepare in advance for the challenges and opportunities of periodicity in environmental conditions, circadian rhythms confer fitness benefits (reviewed in(Vaze and Sharma 2013). They appear so advantageous they are phylogenetically ubiquitous (Bell-Pedersen *et al.* 2005; Jabbur, Zhao, and Johnson 2021) and so commonplace they were long overlooked by science – indeed, it was not until the 18th century that the first observation of circadian rhythmicity was recorded. French astronomer Jean-Jacques d'Ortous de Mairan found that having placed a *Mimosa pudica* plant into a cupboard, its leaves continued to open and close with ~24h periodicity despite the absence of light cues (Mairan and Jj 1729). Indeed, many biological processes display rhythms, from gene expression to physiologies to behaviours. For example, 40% of the genes of mice exhibit daily rhythms in expression (R. Zhang et al. 2014).

The field of chronobiology took off in the mid-20th century; first as a field focussed on evolutionary and ecological questions, and but soon pivoting towards uncovering the molecular underpinnings of clocks. Consequently, the genes and molecular pathways driving the "canonical" circadian clock, the transcription-translation feedback loop (TTFL), are far better understood than the evolutionary and ecological forces shaping biological rhythms (Sharma 2003; Green *et al.* 2002). The relatively few studies probing the adaptive value of circadian clocks do reveal clear fitness benefits (e.g. (Dodd *et al.* 2005; Ouyang *et al.* 1998; Woelfle *et al.* 2004). The fitness benefits of clocks are generally classed as either extrinsic or intrinsic, depending on whether the

timing of a rhythm has evolved to align with rhythmicity in the external environment or other rhythms within the organism, respectively (Sharma 2003).

Testing for and quantifying fitness benefits of rhythms is challenging because it requires manipulating the timing of rhythms expressed by an organism in an ecologically relevant manner. However, the resonance hypothesis, which predicts that fitness is maximised when an individual's rhythm matches that of the external environment (Ouyang *et al.* 1998; Dodd *et al.* 2005), has been supported by studies using *Drosophila* (C. S. Pittendrigh and Minis 1972; Horn *et al.* 2019), in wild caught mosquito larvae (*Wyeomyia smithii*) (Emerson *et al.* 2008), mice (Spoelstra *et al.* 2016), and the fungus *Neurospora discreta* (Koritala *et al.* 2020). Other approaches to probing the benefits of rhythms involve altering the operation of clocks, for example disrupting the suprachiasmatic (SCN) clock of chipmunks increases mortality during the hibernation season (DeCoursey, Walker, and Smith 2000; DeCoursey *et al.* 1997) and disruption to the timing of the clock reduces lifespan and reproductive output of *Drosophila* (Beaver *et al.* 2002). Evidence for the value of intrinsic rhythms are less clear but consistent with observations that circadian clocks operate in organisms that live in non-rhythmic environments, such as cave-dwelling fish and millipedes (Cavallari *et al.* 2011). Further, *Drosophila* selected in arrhythmic conditions do not lose rhythms despite the fact that selectively neutral traits in this species are usually lost readily (Sheeba, Sharma, and Joshi 1999; Sharma 2003). Intrinsic benefits may also explain fundamental processes such as why cell division and metabolism occurs at different times of day; for example, yeast divide at night because mutation is more likely in the day (Chen *et al.* 2007).

While most studies have considered the consequences of an organism's rhythms in the context of rhythms in the abiotic environment or internally, how circadian rhythms of other organisms (i.e. the biotic environment) shape rhythms remain

poorly understood. Interactions within species, such as between males and females, and between species such as between predators and prey, and hosts and parasites, are fundamental aspects of ecology and often occur at specific times of day. For example, *Nicotiana attenuate* coordinates rhythms in flower movement in response to whether it will be pollinated by nocturnal hawkmoths or diurnal hummingbirds (Yon *et al.* 2017), guillemot fledglings fledge by jumping off cliffs at the time-of-day predation risk is likely to be lowest (Tinbergen & Daan 1979), and plant clocks upregulate anti-herbivore defences in the daytime to counter the predictable feeding rhythm of the cabbage looper caterpillar (Goodspeed *et al.* 2012). Furthermore, the greatest daily rhythm of biomass – the migration of planktonic copepods – is thought to be timed to optimise the trade-off between the simultaneous availability of food and risk of predation (Hays, Kennedy, and Frost 2001; Häfker *et al.* 2017).

1.1. Periodicity in host-parasite interactions

Parasites (which I use as a term to encompass parasites, pathogens and parasitoids), much like circadian rhythms, are ubiquitous in nature (Poulin 2007). They affect not only their hosts, but also have a profound impact on ecosystem functioning as a whole (Thomas *et al.* 2005). Moreover, many parasites relevant to human health (and the organisms that vector them) display daily rhythms. As such, how circadian rhythms govern interactions between hosts and parasites is a topic of growing basic and applied interest (Westwood *et al.* 2019). For example, parasites display rhythms in offence, (the traits underlying between host transmission and within-host survival (Reece, Prior, and Mideo 2017; Rijo-Ferreira *et al.* 2017)), whereas hosts display rhythms in defence (such as in behaviour and immune function) to either avoid, or mitigate, the costs of parasitism (Scheiermann *et al.* 2018; Martinez-Bakker & Helm 2015). Understanding how rhythms in parasites and hosts evolve, and possibly co-evolve, can inform control strategies and medical interventions. For instance, timing

treatment to coincide with rhythmicity in parasite activities that determine vulnerability to intervention could improve host outcomes (Ballesta *et al.* 2017), or delivering vaccines at specific times of day may improve their efficacy (Fortier *et al.* 2011). Thus, uncovering how and why rhythms have evolved, and how they govern interactions between hosts and parasites during infections is important, from understanding ecosystem functioning to improving human health.

In this thesis, I examine the evolution and ecology of circadian rhythms in infection using two systems: first, male *Teleogryllus oceanicus* crickets whose rhythmic sexual advertisement makes them vulnerable to infection via the lethal, acoustically-orienting parasitoid fly *Ormia ochracea* in Hawaii. Second, I use the rodent malaria parasite *Plasmodium chabaudi,* which displays robust daily rhythms in replication (within host red blood cells) that are aligned to the timing of host rhythms (O'Donnell *et al.* 2011). While the first system focusses on the effect of parasites on host rhythms, the latter focusses on the effect of host rhythms on parasites. Thus, these two distinct yet complementary systems represent two sides of the same coin.

1.2. Circadian rhythms: a primer

Circadian rhythms are governed by molecular clocks whose outputs ebb and flow with a periodicity of approximately 24-hours, reflecting the rotation of the Earth on its axis. Rhythms are driven by either transcriptional-translational feedback loops (TTFL), non-, or post-transcriptional oscillators. The genes underpinning TTFL clocks show little homology across divergent taxa (e.g. (Loudon 2012; Rubin *et al.* 2006; Zhu *et al.* 2008; Tei *et al.* 1997; Rutila *et al.* 1998), making it easier to characterise circadian rhythms by quantifying their outputs at the level of traits, rather than by examining DNA sequences. The criteria for a trait to be governed by a circadian clock include 1) a self-sustained period (i.e., duration) of approximately 24-hours, 2) entrainment to environmental time cues ("Zeitgebers"), and 3) temperature compensation to ensure

the clock ticks at the same pace across biologically relevant temperatures. The existence of non- and or post-transcriptional oscillators is a recent development in chronobiology and so how they operate is yet to be revealed (O'Neill *et al.* 2011). However, one possibility is explored by recent work that uncovered entrainable, ~24h, temperature-compensated cell-autonomous rhythms in $[Mg^{2+}]_i$ availability in eukaryotes. Thus, this non-transcriptional oscillator is capable of imparting circadian rhythmicity to cellular processes in which MgNTP hydrolysis is rate-limiting (Feeney et al. 2016).

Zeitgebers (derived from the German "zeit" meaning time and "geber" meaning giver) notify an organism's circadian machinery of environmental time. While the most common Zeitgeber is light, various others have been described such as temperature, humidity, feeding, and social interaction. Even in the absence of Zeitgebers, a true circadian rhythm oscillates with periodicity of ~24-hours; this is known as the "free-running" period. Thus, a common method for testing circadian rhythmicity is to test whether rhythms persist with ~24-hour periodicity after placing an organism into constant conditions (i.e., "free-running conditions"; for example, constant light). The approximate nature of the intrinsic period (i.e. close to but never exactly 24 hours) means that the clock entrains, or synchronises, daily with the rhythm of the Zeitgeber. This important feature of circadian clocks allows organisms' behaviour and physiology to maintain the appropriate phase relationship (or, timing) to the environment despite seasonal variation in photoperiod. The final feature of circadian clocks is that they are robust to variation in ambient environmental temperature. This is important, because it is easy to imagine the maladaptive nature of a clock that runs slower on cold than on a warm day. Taken together, these features allow organisms to adaptively anticipate changes in day and night, rather than rely on using simple phenotypic plasticity with which organisms must wait until transitions between day and night occur to change behaviours (often referred to as a "just in time strategy"). For

example, plants use their clocks to ready photosynthetic machinery in advance of sunlight, animals exhibit food anticipatory activity (e.g., increase activity, core body temperature, serum corticosterone, and duodenal disaccharides), and prey strategically retreat to shelter prior to the onset of predator activity (Michael *et al.* 2003; Dodd *et al.* 2005; Stephan 2002; Nelson & Vance 1979).

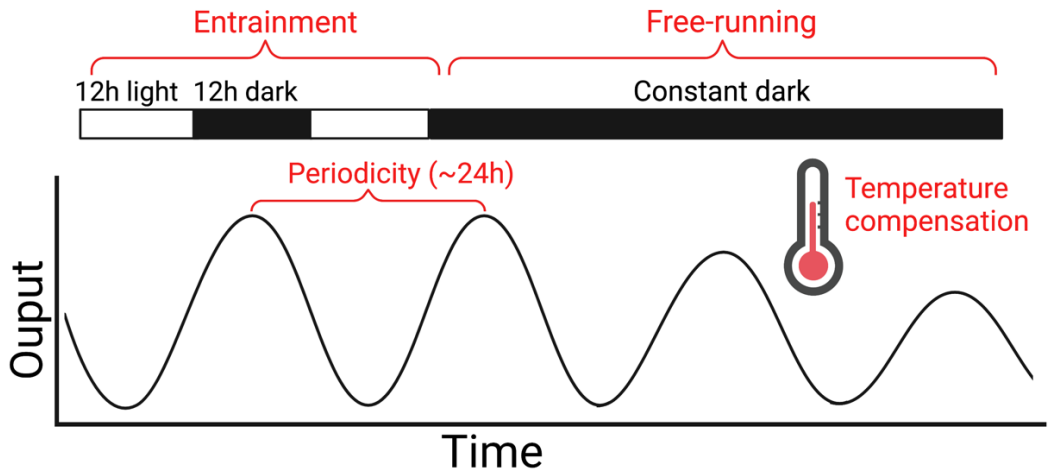


Figure 1.1. The three characteristics of circadian rhythms include (1) entrainment to a Zeitgeber (i.e., an environmental time cue), (2) self-sustained ~24-hour period under free-running (i.e., constant) environmental conditions, and (3) temperature compensation across a range of biologically realistic temperatures.

1.3. Role of rhythms in infection: hosts

Host defence against parasites most obviously includes the immune system. Mounting evidence reveals elements of the immune system are influenced by circadian clocks; indeed, clock genes are expressed in a variety of immune cells (Scheiermann, Kunisaki, and Frenette 2013; Curtis *et al.* 2014). For example, rhythmic production of pro-inflammatory cytokines TNF-α and IL-6 by macrophages and the mobilization of inflammatory monocytes are regulated by the clock (Keller *et al.* 2009; Lang *et al.* 2021). Observations that immune function is primed for defence during the active phase of an organism (i.e., during the daytime for diurnal animals and night-time for nocturnal

animals) and repair in the rest phase has led to the notion that immune defence has evolved to peak when parasite encounter rate is highest (Scheiermann, Kunisaki, and Frenette 2013; Labrecque and Cermakian 2015). This idea is challenged in Chapter 2 because the immune system operates within a broad set of constraints (e.g., the need to temporally couple/decouple compatible/dependent activities; (Sarah E. Reece, Prior, and Mideo 2017), parasite encounter does not always predictably occur during the active phase, and some immune "defences" are actually helpful to parasites.

Traditionally, rhythmicity in elements of the immune system have been studied in isolation from each other and in culture. Those which are studied *in vivo* are performed in model systems, and only mimic the initial steps of infection or homeostasis. Thus, it is unclear if rhythms continue to persist or matter during ongoing infection. For example, it may be beneficial to overrule clock control during the acute phase of infection because drastic measures are required to combat the parasite and increase the likelihood of survival. For example, hyper- and hypothermia are common responses to infection which simultaneously disrupt circadian rhythms in core body temperature and are known to promote survival (Evans, Repasky, and Fisher 2015; Earn, Andrews, and Bolker 2014; Romanovsky and Székely 1998; Liu et al. 2012). However, chronic infections may last so long that disrupting clock control for an extended period of time becomes too costly or disadvantageous to host survival. Continuing with the example of disrupted core temperature, mounting a fever requires a 10-12% increase in metabolic rate per 1°C increase in temperature (Kluger 1979). Thus, chronic disruption of circadian periodicity of core body temperature is likely to be too costly to maintain. The costs and benefits of overriding or maintaining clock control during infection likely depend on the severity of the disease and also the extent to which parasite exploitation of the host and the host's own immune defences (i.e., immunopathology) are responsible for the severity of infection.

Although immune function is only one of many ways host rhythms impact infection, other host defences have received markedly less attention. Behavioural and morphological defences are an often lower-cost ways to prevent infection, compared to using immune responses to combat invaders. For example, the time-of-day of hosts are active and when they feed interact to impact how well hosts fight parasites, as is the case for mice infected with the intestinal helminth *Trichuris muris*. Mice infected in the morning (during the resting phase) expel worms sooner and have stronger T-helper 2 responses than those infected in the evening (during the active phase) (Hopwood *et al.* 2018). However, when mice are fed during the daytime regardless of when they are active, this effect is reversed in an immune-independent way (Hopwood *et al.* 2018). Thus, clock control of a diversity of host behaviours and physiologies - that includes immune responses - likely operates in tandem to defend against infection. These ideas are introduced in Chapter 2 and developed in Chapters 3 and 4.

1.4. Role of rhythms in host defence: when natural and sexual selection face off

Cricket song is one of the most charismatic examples of a rhythmic, sexually-selected trait and has long been a subject of fascination to humans. From early Polynesians whose mythology described crickets as "embodiments of the souls of loved ones who… identify themselves with their calls" ((Werrer Loher and Orsak 1985), to Charles Dickens' novella "The Cricket on the Hearth" whose chapters are called "chirps", and even the more current inclusion of cricket song into the soundtrack of Ariana Grande's "Positions" as tribute to her late partner, Mac Miller.

While the conspicuous nature of cricket song has ignited human interest throughout history, it is indeed this same feature that enables predators and parasites to co-opt it for their benefit (Zuk and Kolluru 1998). Namely, towards homing in on possible prey and/or hosts. One such example is that of *T. oceanicus* crickets which are native to Australasia but were introduced in the Hawaiian Islands, possibly long ago by early

Polynesian settlers, or much more recently by ships in the 19th century (Tinghitella *et al.* 2011; Otte 1994; Kevan 1990). Only in the Hawaiian Islands does the range of *T. oceanicus* overlap with the parasitoid fly *Ormia ochracea*. The native range for *O. ochracea* is North America but it has also been introduced in the Hawaiian Islands (W. Cade 1975; Zuk, Simmons, and Cupp 1993; Lehmann 2003). Gravid female flies listen in on singing males to find potential hosts for their larvae, and once found, deposit them on and around males which hatch and burrow into males to consume them from the inside. The process from infection to death generally takes 7-10 days (W. Cade 1975; T. J. Walker and Wineriter 1991), and while singing males bear the highest risk of infection, "satellite" non-singing males and nearby females are sometimes (though rarely) infected (Zuk, Simmons, and Cupp 1993; Zuk, Rotenberry, and Tinghitella 2006).

T. oceanicus provides the most well-known demonstration of rapid evolution of host evasion in response to the costs of infection by *O. ochracea.* In 2003, a "flatwing" male *T. oceanicus* morph was first discovered on the Hawaiian Island of Kauai (Zuk, Rotenberry, and Tinghitella 2006). These males have feminised wings which lack the typical sound producing structures of "normal-wing" males, thus precluding them from singing (Zuk, Rotenberry, and Tinghitella 2006). Since then, numerous other wing "morphs" have been discovered which aid in protection against the fly **(**e.g., curly wing, small wing, purring, and rattling morphs; (Pascoal *et al.* 2014; Tinghitella *et al.* 2018; J. Rayner *et al.* 2019). The trade-off, however, is reduced attractiveness to females, which overwhelmingly prefer normal-wing song (Tanner, Swanger, and Zuk 2019; Tinghitella *et al.* 2021).

How normal-wing males balance the fitness benefits of singing to attract mates while minimising the fitness costs of fly infection has been a topic of discussion for decades. It is possible that a small number of normal-wing males persist due solely to their mating advantage, or normal-wing males may have evolved non-morphological defences

against infection. For example, Zuk, Simmons, and Cupp (1993) revealed that ancestral, unparasitised populations of *T. oceanicus* in Western Australia and French Polynesia are more likely to sing around dusk and dawn compared to populations on the Big Island of Hawaii (Zuk, Simmons, and Cupp 1993). Host seeking by *O. ochracea* is thought to be most likely around dusk (Kolluru 1999; William Cade 1979), so a potential explanation for the altered singing rhythm of Hawaiian males is "temporal escape". By reducing singing activity at the most dangerous times of day, males lower their risk of infection but are still able to attract females at other times. While temporal segregation of singing patterns by male crickets has evolved to maintain reproductive isolation across species, whether temporal escape from infection can evolve is unknown. For example, in contrast to Zuk, Simmons, and Cupp (1993), Kolluru (1999) found that Hawaiian *T. oceanicus* sing most often at dusk, coinciding with peak fly activity (Kolluru 1999).

In Chapter 4, I undertake an in-depth comparison of singing rhythms of male *T. oceanicus* from Hawaii with those from an ancestral population to test for evidence of temporal escape. To carry out this project, I first had to develop a high throughput and high-resolution method to collect singing data and analyse its patterns (see Appendix 8.1, Chapter 2) and ascertain whether singing by *T. oceanicus* is under the control of a circadian clock (Chapter 3).

1.4.1. Circadian clocks in crickets

To-date, research on circadian rhythms in crickets has centred around the molecular underpinnings of their clocks. For example, their circadian clocks appear similar to those in *Drosophila* and mammals (reviewed in (Panda, Hogenesch, & Kay 2002), operating via a canonical circadian clock, i.e. a transcription-translation feedback loop involving clock genes *period* (*per*) (Moriyama *et al.* 2008), *timeless* (*tim*) (Danbara *et al.* 2010), *Clock* (*Clk*) (Moriyama *et al.* 2012), and *cycle* (*cyc*) (Uryu, Karpova, and Tomioka 2013). Further, in keeping with Pittendrigh's observation that the circadian system in

certain organisms is composed of several oscillators (Pittendrigh 1981), crickets have bilaterally paired pacemakers located in two optic lobes which exchange circadian and light information (Loher 1972; Tomioka & Chiba 1986, 1992; Tomioka, Nakamichi, & Yukizane 1994).

Most research on the molecular and overt rhythms of crickets has focussed only on two model species, *Gryllus bimaculatus* and *Teleogryllus commodus* (see e.g., (Danbara et al. 2010; Hassaneen et al. 2011; Moriyama et al. 2008; Abdelsalam et al. 2008; Werner Loher 1972, 1974; Sokolove 1975; Sokolove and Loher 1975; Moriyama et al. 2012; Kenji Tomioka and Chiba 1986; Uryu, Karpova, and Tomioka 2013; K. Tomioka, Nakamichi, and Yukizane 1994; Kenji Tomioka and Chiba 1992; Nose et al. 2018; Moriyama et al. 2009) with a few exceptions (e.g., (Abe, Ushirogawa, & Tomioka 1997; Nowosielski & Patton 1963; Fergus, Decarvalho, and Shaw 2011). Thus, because crickets inhabit nearly all regions across the globe (up to 55° latitude; (Alexander & Otte 2009), research on a greater variety of species could reveal how the rhythms that govern their behaviour and physiology are shaped by their widely variable environments.

Circadian control of singing in *T. oceanicus* has long been suspected, but never formally demonstrated. Thus, in Chapter 3, I first developed a novel audio-to-circadian analysis pipeline to perform a robust interrogation of circadian singing parameters. Collecting continuous audio data for weeks to months at a time proved technologically challenging and computationally intensive. Although the final method employed to collect and analyse round-the-clock singing data is outlined in Chapter 3, significant time and effort was spent trialling a suite of other methods. The two primary previously trialled methods are described in Appendix 8.2, but briefly they include video tracking of wing movements as well as audio analysis using amplitude thresholds. Both of these methods proved less reliable and/or tractable than the cross validation and machine learning analyses deployed in Chapters 3 and 4. Nonetheless, lessons learned whilst trialling these

methods and those ultimately used in the analyses led to my learning a great deal, especially in the areas of bioacoustics, audio and video recording, machine learning, high performance computing and command line coding (BASH).

1.5. Role of rhythms in infections: parasites

Parasite fitness is composed of within-host survival and between-host transmission. As such, parasites are suggested to schedule their activities to take advantage of rhythmic transmission opportunities, rhythmic weaknesses in host immune defence, and rhythms in the availability of the host's resources that they exploit. One prominent example is the migration of microfilariae (the transmission form of filarial worms) from host organs to peripheral capillaries during the time of day vectors are active, thus increasing the likelihood of transmission (Hawking 1967). Notably, parasite species transmitted by nocturnally active mosquitoes migrate to the peripheral capillaries at night, and those transmitted by diurnal vectors display the opposite schedule for migration (Figure 1.5.1.). While capitalising on periodicity in transmission opportunities intuitively appears an obvious adaptive explanation for this rhythmic migration, the question of why microfilariae do not remain in peripheral capillaries all the time must be answered to conclude that rhythmic migration is adaptive. Instead of a focus on this question, effort has focused on speculating what time-of-day cue microfilariae use to schedule their migration (specifically, rhythmicity in oxygen tension is suggested to reflect the active versus rest phase of the host, which is aligned to the day-night cycle). A similar phenomenon occurs in the cercaria of various subspecies of Schistosome flatworms (spp.) that cause Bilharzia, which emerge from their snail intermediate host in the early morning or the late afternoon, depending on whether they seek diurnal livestock or nocturnal rodents for their next host (McMahon 1976; de Azevedo *et al.* 2011).

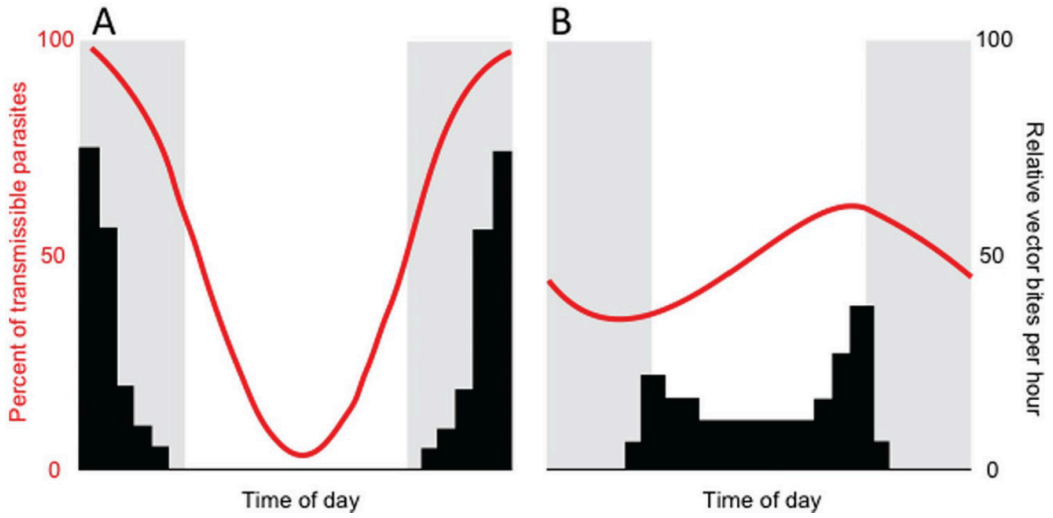


Fig. 1.5.1. Microfilariae migration from the hosts lungs to the peripheral circulation aligns with the activity rhythms of mosquito vectors. Red lines illustrate rhythms in the percentage of the maximum number of microfilariae observed in the peripheral blood of hosts, and the bars illustrate vector biting activity. (A) The nocturnally periodic form of *Wuchereria bancrofti* is vectored by night-biting *Anopheles* and *Culex* mosquitoes, and (B) the diurnally subperiodic form is vectored by day-biting *Aedes* mosquitoes. Figure taken from Reece *et al.* (2017) and originally adapted from Pichon and Treuil (2004).

1.5.1. Rhythms in malaria parasites

Explaining malaria parasite rhythms was the focus of a flurry of research several decades ago. The general consensus was that – like for microfilariae and Schistosomes - rhythmicity in transmission opportunities has selected for rhythmicity in activities undertaken within the host (Reece, Prior, & Mideo 2017). However, recent work over the last decade is challenging this view by revealing that rhythms experienced in the within-host environment have a direct impact on parasite fitness. For example, asexual stage malaria parasites capitalize on the daily variation in nutritional content of blood – driven by the host's feeding-fasting rhythm - to schedule their replication within red blood cells (Prior *et al.* 2018; Hirako *et al.* 2018). Coordinating asexual development with

daily rhythms in the availability of amino acids appears to maximise parasite exploitation of the host's resources as well as enabling sexual development to follow the best schedule to align with the activity of the mosquito vector (Prior *et al.* 2020). Whilst increasing evidence suggests malaria parasites, like *Trypanosoma brucei* which causes sleeping sickness (Rijo-Ferreira *et al.* 2017, 2020), possess the ability to keep time (with a clock or a via a just in time strategy), how they achieve this is unknown. Furthermore, whilst the benefits of aligning development with host and vector rhythms seem clear, this may not be the case because studies differ in whether there are negative consequences for parasites following the 'wrong' schedule.

Since the Hippocratic era, periodic recurrence of fever has been used as a diagnostic tool for malaria (*Plasmodium* spp.; (Kwiatkowski & Greenwood 1989). The spike in fever (every 24, 48, or 72h depending on the species) is caused by the synchronous bursting of parasites that have completed their development within the host's red blood cells (RBC). Progeny, both sexual and asexual, are released from RBC in this pyretic display which invade new RBC to develop into either a male or female transmission stage (term "gametocyte"), or an asexual stage (Fig. 1.6.1). Gametocytes do not replicate in the host and are responsible for transmission; they are the only stage infective to insect vectors. Sequential cycles of replication by asexual stages enables parasite density to rapidly increase within the host, ensuring within-host survival, causing the severe symptoms of malaria, and providing a source population for the production of gametocytes. Thus, while gametocytes represent parasite investment in reproduction, asexual stages are responsible for within host-survival. During each cycle of asexual replication (the intraerythrocytic developmental cycle, IDC), a small but variable proportion of parasites commit to sexual stage investment; this plastic trait is called the conversion rate (Carter et al. 2013; P. Schneider, Greischar, et al. 2018; P. Schneider and Reece 2021). The IDC schedule is also plastic, altering its timing and duration in response to changes in environmental factors such as temperature and perturbations to host rhythms.

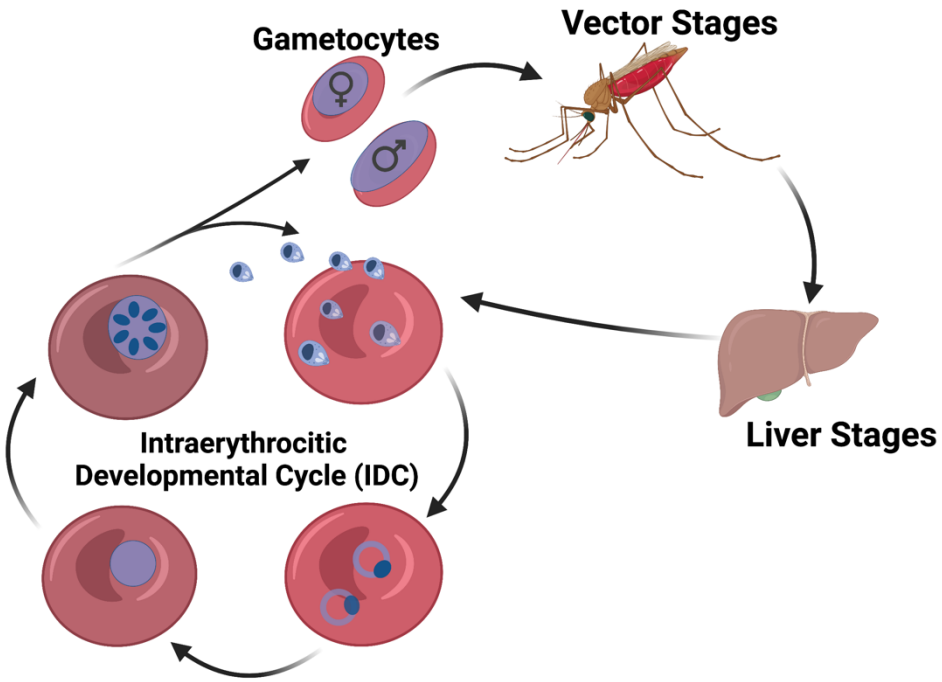


Figure 1.6.1. The malaria life cycle. Once transmitted to a host via the bite of an infected mosquito, malaria parasites undergo successive rounds of replication in the liver before migrating to the host's blood stream to invade red blood cells. Parasite replication in the blood (the intraerythrocytic developmental cycle) is rhythmic and culminates in the release of merozoites (asexual parasites) and gametocytes (sexual stage parasites). Asexual committed merozoites continue within-host infection whereas gametocytes are transmitted to mosquito vectors.

While the rhythms of human malaria parasites can be studied in controlled conditions using *in vitro* culture, this precludes examining parasite rhythms in an ecologically relevant setting. Thus, to interrogate the relationship between host and parasite rhythms, the rodent malaria *Plasmodium chabaudi,* provides a useful model system. When the schedule of *P. chabaudi's* IDC, is perturbed to become misaligned with the host's rhythms, the parasite incurs a ~50% reduction in both sexual and asexual stage parasites during the

acute phase of the infection (O'Donnell *et al.* 2011). This is interpreted as a fitness cost given that asexual stages are necessary for within-host survival and gametocytes are necessary for transmission.

The loss of asexual stages cannot be explained simply by a mis-timed IDC becoming exposed to a peak in a rhythmic host immune response it is particularly vulnerable to (Prior *et al.* 2020). Specifically, if this were to occur, a large loss of asexuals would be observed in the first few IDC following misalignment and the greater this impact, the faster the IDC would become realigned to host rhythms. Instead, a very modest reduction in asexuals occurs in the first cycle following misalignment and the overall 50% reduction in asexual stages is due to the cumulative impact of a small initial cost on exponential growth (O'Donnell, Mideo, & Reece 2013). Further, even following a 12 hour misalignment to host rhythms, the IDC becomes realigned rapidly; within 5-7 IDC (O'Donnell & Reece 2021). Instead, the costs of mismatch for asexual stages are subtle; >57% of *P. chabaudi's* genes are expressed with a 24 hour rhythm and when misaligned to host rhythms, the expression patterns of genes associated with important cellular processes (e.g., DNA replication and the ubiquitin and proteasome pathways) are disrupted (Subudhi *et al.* 2020). This disruption likely reflects the consequences of IDC stages being out of synch with the rhythmic nutrients they need from the host, as well as disruption due to the IDC becoming rescheduled to re-align with the host's rhythms (O'Donnell, Prior, & Reece 2020).

In contrast to the impact of host rhythms on asexual (IDC) stages, what explains the loss of sexual stages is unknown. This topic is the focus on Chapter 5. Given that asexuals are the source population for the production of gametocytes, then 50% fewer asexuals is naturally expected to result in 50% fewer gametocytes. However, the relationship between asexual density and gametocyte density is not that simple; conversion rate (the proportion of asexuals that produce gametocyte-committed progeny) is plastic and

adjusted in line with fluctuations in asexual replication. In Chapter 5, I test the prediction that parasites adopt reproductive restraint to help mitigate the negative impacts of misalignment to host rhythms. Furthermore, unlike for asexual stages, the loss of gametocytes in misaligned infections could be due to gametocytes at a vulnerable age encountering a more dangerous immune environment. Thus, in Chapter 5, I also test whether an interaction between gametocyte age and an element of the pro-inflammatory immune response that is both rhythmic in mice (Keller *et al.* 2009) and known to reduce the within-host survival of gametocytes (Long *et al.* 2008), can explain the loss of gametocytes in misaligned infections.

1.6. Thesis outline and aims

In this thesis, I examine how circadian rhythms modulate interactions between hosts and parasites. I do so from an evolutionary ecology perspective, and my specific contributions from each chapter include:

Chapter 2: providing a case for, and a framework to, integrate evolutionary ecology into chronobiology when studying rhythms in infections.

Chapter 3: uncovering and parameterising circadian singing rhythms in a cricket species that has been subject to strong natural selection by a parasitoid fly.

Chapter 4: revealing that singing rhythms of crickets from a parasitised population display qualities consistent with the evolution of a novel defence against parasitism.

Chapter 5: ruling out two hypotheses to explain the loss of sexual stage malaria parasites in infections that are temporally misaligned with host rhythms.

2. Chapter 2: The evolutionary ecology of circadian rhythms in infection

This work has been published as:

Westwood, Mary L., *et al.* "The evolutionary ecology of circadian rhythms in infection." *Nature ecology & evolution* 3.4 (2019): 552-560.

2.1. Abstract

Biological rhythms coordinate organisms' activities with daily rhythms in the environment. For parasites, this includes rhythms in both the external abiotic environment and the within-host biotic environment. Hosts exhibit rhythms in behaviours and physiologies, including immune responses, and parasites exhibit rhythms in traits underpinning virulence and transmission. Yet, the evolutionary and ecological drivers of rhythms in traits underpinning host defence and parasite offence are largely unknown. Here, we explore how hosts use rhythms to defend against infection, why parasites have rhythms, and whether parasites can manipulate host clocks to their own ends. Harnessing host rhythms or disrupting parasite rhythms could be exploited for clinical benefit; we propose an interdisciplinary effort to drive this emerging field forward.

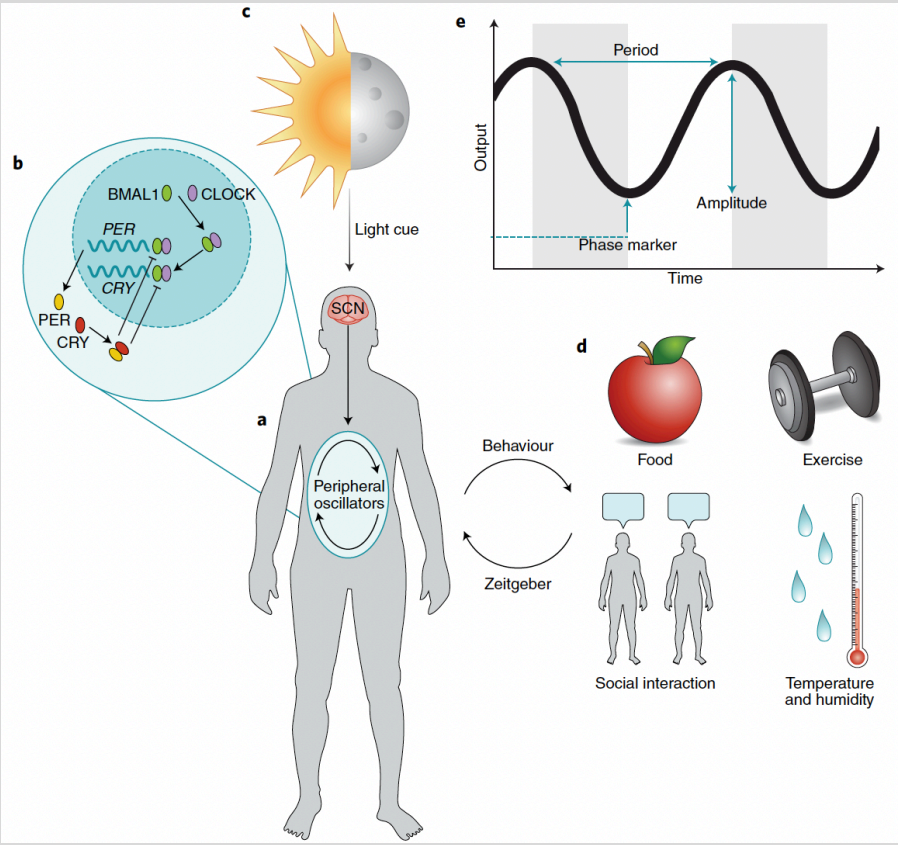
2.2. Introduction

Circadian rhythms have long been taken for granted by science. Indeed, the first observation of a clock-controlled behaviour (leaf opening and closing in *Mimosa pudica*) was not recorded until the 18th century (de Mairan 1729). Following the fundamental observation that organisms can adaptively anticipate daily rhythms in their environment, the field of "chronobiology" took off in the mid-20th century with a focus on evolutionary and ecological questions. However, the advent of genetic tools a few decades later shifted the remit to determining the molecular and genetic workings of circadian clocks. Yet,

despite their assumed major impact on fitness, circadian rhythms remain overlooked in evolutionary ecology (Sharma 2003; Green *et al.* 2002; Helm *et al.* 2017). Here, we propose that the integration of chronobiology and evolutionary ecology return to its roots to tackle a topic of growing and applied interest; the role of rhythms in host-parasite interactions. Note that we use the term "parasite" to collectively refer to all agents of infection (e.g. single-celled and multicellular eukaryotes, bacteria, viruses).

One of the most fundamental ecological interactions is that between hosts and parasites. Research from diverse taxa (plants, mammals, and insects) reveals that host clocks drive daily rhythms in immune defences, disease severity and spread (Scheiermann *et al.* 2018; Martinez-Bakker & Helm 2015). Parasites display daily rhythms in traits underpinning within-host survival and between-host transmission (Reece, Prior, & Mideo 2017; Rijo-Ferreira *et al.* 2017). Rhythms in parasite activities and in host responses to infection could provide an advantage to parasites, hosts, both, or neither. To what extent parasites and hosts are in control of their own and/or each other's rhythms is also poorly understood.

Understanding the evolution (and possibly, coevolution) of rhythms may enable vaccines and drugs to take advantage of rhythmic vulnerabilities in parasites or harness host rhythms to improve efficacy and reduce drug toxicity. For such interventions to be robust to parasite evolution, understanding how host-parasite interactions shape rhythms in hosts and parasites is necessary (Reece, Prior, & Mideo 2017). Key questions include how rhythms in diverse host traits contribute to defence, how parasites cope with exposure to their host's rhythms, and whether hosts and parasites can manipulate each other's rhythms for their own benefit. We discuss these three scenarios, identify systems to explore them, and offer ways in which this knowledge can be exploited to improve health. An evolutionary ecologist's introduction to chronobiology is provided in Boxes 1 and 2.



Box 1. What are circadian rhythms?

Biological rhythms are deemed to be controlled by circadian clocks if they meet several criteria (Johnson *et al.* 2004). First, their duration (period) must be approximately 24 hours. Second, they must persist (free-run) in conditions without time-of-day cues, which is usually assessed by observation in constant light or dark. Third, the phase of the oscillator or outputs are set (entrained) by a time-of-day cue (Zeitgeber) which is usually light. Fourth, unlike the rate of many chemical reactions, the speed of a circadian clock varies little over a biologically realistic range of environmental temperatures (temperature compensation). Together, these criteria allow organisms to fulfil a key feature of circadian rhythms: anticipatory, rather than reactionary, behaviour. For instance, plants ready photosynthetic machinery in anticipation of sunlight (Michael *et al.* 2003, Dodd *et al.* 2005) and animals exhibit food-anticipatory activity (e.g. increases in core temperature, activity, serum corticosterone, and duodenal disaccharides) prior to foraging (Stephan 2002). The workings of circadian clocks are sufficiently flexible to allow organisms to cope with gradual changes in photoperiod across seasons, but not flexible enough to instantly cope with changes in time zones (which is why travellers experience jet lag).

The mammalian circadian system is composed of the "central" clock in the brain (suprachiasmatic nucleus; SCN) and "peripheral clocks" in other organs and tissues (A). Clocks in nucleated cells are run by transcription-translation feedback loops (TTFL). For example, in animals the proteins CLOCK and BMAL1 act as activators and members of the PER and CRY families are repressors (Young & Kay 2001). (B). Retinal photoreceptors receive light cues which are carried through the hypothalamic optic tract and transmitted to the SCN, resulting in its synchronization/entrainment (C). Clocks in organs and tissues (peripheral clocks) can be entrained by feeding rhythms, and in taxa other than mammals, exercise, social cues, and abiotic rhythms in temperature and humidity may entrain clocks (D). Rhythms are often characterised by their period, amplitude, and markers for phase (E; grey bars illustrate nighttime for a rhythmic trait measured over 48 hours). They are described in relation to the time since the Zeitgeber (ZT) occurred (e.g. ZT6 refers to 6 hours after dawn) which usually differs from the actual time-of-day (Circadian Time; CT).

Box 2. Why have circadian rhythms evolved?

Circadian clocks appear so advantageous that nearly all eukaryotes have a circadian system in most cells (Dunlap 1999). Circadian clocks may confer two kinds of fitness benefit: coordinating behaviours with rhythms in the external environment (extrinsic adaptive value), and temporally compartmentalising incompatible processes (intrinsic adaptive value) (Green *et al.* 2002). For instance, intrinsic benefits are conferred when cell division in yeast is temporally constrained to the reductive phase of metabolism, minimising rates of genetic mutation (Chen *et al.* 2007). However, most studies of the fitness consequences of circadian rhythms have focussed on the benefits of synchronizing activities with rhythms in the abiotic environment: matching the period of day-night rhythms enables cyanobacteria to outcompete strains whose clocks run faster or slower (Ouyang *et al.* 1998) and enhances the survival of *Arabidopsis* (Dodd *et al.* 2005). Rhythms in the biotic environment (Sharma 2003) matter too. For example, the sea urchin *Centrostephanus coronatus* avoids predatory sheephead wrasse (*Pimelometopon pulchrum*) by foraging at night and retreating to shelter prior to the onset of wrasse activity (Nelson & Vance 1979).

Despite the diversity of extrinsic rhythms that could select for the scheduling of diverse processes, there are surprisingly few demonstrations that circadian clocks actually affect fitness. For example, fitness is greater in wild-type mice than mutant mice with shortened periods (Spoelstra *et al.* 2016), flies with clock mutations die more rapidly than wild types after infection with bacteria (Stone *et al.* 2012; Lee & Edery 2008), and circadian knockout plants flower later and are less viable than wild-type plants (Green *et al.* 2002). However, depending on ecological context, rigidly scheduling activities according to day and night is not always the best strategy. For example, nocturnal mice boost energy efficiency by switching to diurnality when challenged with cold and hunger (van der Vinne *et al.* 2014). Nursing honeybees, that remain in the hive are arrhythmic, because round-the-clock care is necessary for larvae; and, if needed, diurnal foraging bees can revert to arrhythmic nursing behaviour (Bloch & Robinson 2001). Shorebirds also display considerable plasticity in activity rhythms during breeding, likely explained by predator avoidance strategies (Bulla *et al.* 2016).

The above examples illustrate the gains to be made from integrating chronobiology with evolutionary ecology in general (Helm 2017). We propose that such an approach offers a novel advance to the study of host-parasite interactions and coevolution. Coupling the well-developed conceptual frameworks for unravelling how circadian oscillators operate, and probing the costs and benefits of phenotypically plastic traits that are relevant to infection, will explain why rhythms in immune defences and parasite traits occur.

2.3. Rhythms in host defence

The most patent defence against infection is the immune response, and a wealth of evidence reveals that circadian clocks play a role in orchestrating immune defences (Scheiermann *et al.* 2018). Circadian clock genes are expressed in many types of immune cell, and the immune and circadian systems are connected in multiple ways (Scheiermann, Kunisaki, & Frenette 2013; Curtis *et al.* 2014). For instance, the clock gene Bmal1 mediates the balance between pro- and anti-inflammatory responses (Zasłona *et al.* 2017). Rhythmic production of the pro-inflammatory cytokines *TNF*-α and IL-6 by macrophages is clock controlled (Keller *et al.* 2009), and mobilization of inflammatory monocytes is also regulated by the clock (Curtis *et al.* 2014). This phenomenon, termed "anticipatory inflammation", appears uncoupled to metabolic rhythms and may defend against incoming parasites (Nguyen *et al.* 2013). Similarly**,** in humans, proinflammatory cytokines peak in circulation during the day (active phase) (Haus & Smolensky 1999), whereas hematopoietic stem and progenitor cells, and most mature leukocytes, peak at night (Haus and Smolensky 1999; Haus *et al.* 2018). In nocturnal mammals, an inverse rhythm is often observed, with innate defences peaking at night (active phase) and repair mechanisms peaking during the day (resting phase) (Scheiermann, Kunisaki, & Frenette 2013).

Observations of immune rhythms have given rise to the notion that organisms invest in defence during the active phase when parasite encounter is assumed most likely, and repair during the resting phase (Labrecque & Cermakian 2015). Temporal segregation of immune responses may thus solve problems caused by having immune defences continually tuned to maximal (e.g. collateral damage via immunopathology; (Graham, Allen, Read 2&005). Also, energetic demands imposed by activity and metabolism may trade-off against immune defence (Kerr, Gershman, & Sakaluk 2010). Intuitively, "defence

only during the active phase" suggests the host is achieving the most "bang for the buck" by ensuring activities that are energetically costly, or likely to cause collateral damage, are only performed when most useful. However, this intuition may be naïve. First, it ignores the potential for constraints imposed by the need to temporally couple (or de-couple) certain immune rhythms with other internal rhythms (Reece, Prior, & Mideo 2017). This includes separating the timing of metabolism from defensive actions within immune cells themselves (Scheiermann *et al.* 2018; Labrecque & Cermakian 2015). Second, it assumes that a parasite encounter is rhythmic and predictably occurs in the active phase. This is clearly the case for food-borne parasites, but ingestion is not the only route into a host. Rather, the immune system functions within a broad set of energetic demands in which parasite defence is just one of many requirements. For example, rhythmic stomatal opening for gas exchange during the day is a well-used route into plants by bacterial pathogens (Roden & Ingle 2009). Consequently, *Arabidopsis* is better able to detect and defend against parasites in the morning than evening (Bhardwaj *et al.* 2011; Ingle *et al.* 2015). Given the wealth and diversity of data (illustrated in Table 1), meta-analyses are needed to test whether the timing (phase) of rhythms in immune effectors relates to nocturnal vs diurnal lifestyles and whether they function in front-line or secondary defences, or healing.

Infection in the active vs resting phase for diverse hosts (flies, plants, mammals) dramatically affects disease severity and mortality rates (Table 1), suggesting that the phase of immune rhythms upon infection matters. Most studies performed in plants (Table 1) point towards infection during the active phase resulting in greater resistance to infection and less damage to the plant. But the degree to which immune rhythms result in time-of-day differences in parasite control can be counter-intuitive. For example, mice mount higher clock-controlled proinflammatory responses against *Salmonella enterica Typhimurium* when challenged in their rest phase, but bacterial load is also higher and hosts have worse symptoms (Bellet *et al.* 2013). Furthermore, *Leishmania* parasites infect host neutrophils and macrophages, and the clock-controlled secretion of

chemoattractants by these immune cells facilitates their infection, making parasite invasion more successful at night when immune activity is highest (Kiessling *et al.* 2017). Thus, whether immune rhythms are sufficient to entirely explain divergent outcomes of time-of-day of infection is unclear (Table 1). Studies that separate the effects of immune rhythms on preventing infection from their role in dealing with ongoing infection will reveal the extent to which immune rhythms are beneficial and when they should be overruled to deal with a major threat. Additionally, most time-of-day immune challenges have used either bacteria or chemicals, raising the question of whether a more diverse array of challenges are needed to establish general patterns.

Host spp.	Challenge	ToD	Outcome in rest versus active phase	Ref
Mus musculus – house mouse (nocturnal)	*Salmonella typhmurium*	ZT4/16	Greater inflammation and bacterial load when infected in the rest phase	Bellet *et al.* 2013
	Leishmania major	Subjective day/night	Lower parasite burden and lower severity when infected in the rest phase	Kiessling *et al.* 2017
	Lipopolysaccharide (LPS) endotoxin	Subjective day/night	Lower concentrations of cytokines when infected in the rest phase	Gibbs *et al.* 2012
		ZT11/19	Higher mortality when challenged in the rest phase	Marpegan *et al.* 2009
		Subjective day/night	Greater inflammatory responses and lower bacterial burden when challenged/infected in the rest phase	Gibbs *et al.* 2014
	Streptococcus pneumoniae	ZT0/12		
	Murid Herpesvirus 4	ZT0/10	Greater viral replication when infected in the rest phase	Edgar *et al.* 2016
	Helicobacter pylori	ZT1/7/13	Lower lymphocyte numbers when infected in the rest phase	Druzd *et al.* 2017
	Vesicular stomatitis virus	ZT0/12	Higher mortality when infected in the rest phase	Gagnidze *et al.* 2016

Drosophila melanogaster – fruit fly (diurnal)	*Pseudomonas aeruginosa*	ZT1/5/9/13 /17/21/1	Lowest mortality when infected in the rest phase (especially ZT21)	Lee & Edery 2008
		Subjective day/night	Lowest bacterial burden when infected in the rest phase	
	Streptococcus pneumoniae	ZT7/19	Slowest rate of mortality when infected in the rest phase	Stone *et al.* 2012
	Escherichia coli	ZT0/6/12 /18	Infection at all ZT induces sleep the morning after infection and sleep was more prolonged after infection in the rest phase	Kuo *et al.* 2010
Anopheles stephensi - Asian malaria mosquito (nocturnal)	*Escherichia coli*	Morning/ evening	Lower bacterial growth and lower mortality when infected in the rest phase	Murdock, Moller-Jacobs, & Thomas 2013
Arabidopsis thaliana – thale cress (diurnal)	*Pseudomonas syringae*	ZT0/4/10 /16	Immune defences are highest when inoculation occurs early in the active phase Note photoperiod is 9 hours light:15 hours dark	Griebel and Zeier 2008
	Botrytis cinerea	Dawn/dusk	Larger lesions when inoculated in the rest phase	Hevia *et al.* 2015
		ZT0/3/6/9/ 12/15/18 /21/24	Greater susceptibility when inoculated in the rest phase	Ingle *et al.* 2015
	Pseudomonas syringae	Subjective day/night	Lower infiltration of bacteria when infected in the rest phase	Korneli, Danisman, & Staiger 2014
		Subjective morning /evening	Greater suppression of bacterial growth at the start of the rest phase when spray-inoculated, and greater suppression of bacterial growth at the start of the active phase when syringe-infiltrated	Bhardwaj *et al.* 2011

	Hyaloperonospora arabidopsidis	Dawn/dusk	Highest percentage of leaves with sporangiophores when infected in the start of the rest phase	Wang *et al.* 2011
Danio rerio zebrafish (diurnal)	*Salmonella typhimurium*	ZT4/16	Lower survival when infected in the rest phase	Du *et al.* 2017
Oreochromis niloticus – Nile tilapia (mostly diurnal)	LPS	ZT3/15	Greater humoral immune response when infected in the rest phase	Lazado, Skov, & Pedersen 2016
Phodopus sungorus - Siberian hamster (nocturnal)	LPS	ZT1/16	Shorter febrile response and more persistent locomotor activity when infected in the rest phase. Note, photoperiod is 16 hours light:8 hours dark	Prendergast *et al.* 2015

Table 1. Impact of immune challenge during the rest and active phases of hosts. A selection of studies identified as time-of-day immune challenges from PubMed searches for ""time of day" plus "immune and infection" and ""circadian rhythm" plus "immune and infection"". Articles were included if the study involved a time-of-day immune challenge; those without a time-of-day immune challenge were not included in the table. Time-of-day (ToD) is given as hours since lights on (ZT) for organisms in entrainment conditions, and as subjective day/night for those in constant light or dark conditions (i.e. corresponding to the light or dark portion of the cycle before experiencing constant conditions). Unless otherwise stated, entrainment conditions are 12 hour light:dark. Outcomes of challenge in the rest phase (daytime for nocturnal organisms, nighttime for diurnal organisms) are compared to challenge in the active phase in terms of virulence metrics and immune effectors measured.

That host circadian clocks impact on infection via traits other than immune responses has been largely overlooked. Rhythmicity in host activity may determine when hosts provide the best resources to their parasites and offer the most opportunities for onwards

transmission (Zuk, Rotenberry, and Tinghitella 2006; Levri and Lively 1996; Ponton et al. 2011). For example, a recent study of the intestinal helminth *Trichuris muris* demonstrates the role of host rhythms in foraging. Mice infected in the morning (resting phase) expel worms sooner and have a stronger T-helper 2 response than dusk-infected (active phase) mice, and this effect is reversed when mice are fed only in the day, in an immune-independent manner (Hopwood *et al.* 2018). Host feeding rhythms are relevant to gut microbiota, and a two-way feedback between host and microbe rhythms has been proposed (Johnson *et al.* 2017). Daily rhythms in host reproductive behaviours may make hosts vulnerable to infection. For example, the crepuscular and nocturnal singing activity of the cricket *Teleogryllus oceanicus* allows the acoustically-orienting parasitoid fly *Ormia ochracea* to locate hosts, but the flies are best able to hunt when darkness is incomplete (Zuk, Simmons, and Cupp 1993). A rhythmically expressed reproductive behaviour (singing) got the host into this mess, and it appears that natural selection has found two solutions (see Box 3).

In addition to immune responses, infected hosts often exhibit adaptive sickness behaviours consisting of endocrine, autonomic, and behavioural changes that perturb circadian rhythms (Clark, Budd, & Alleva 2008; Dantzer *et al.* 2008). For example, wild red colobus monkeys (*Procolobus rufomitratus tephrosceles)* decrease energetically costly activities, and rest frequently, while shedding whipworm eggs (Ghai *et al.* 2015). Fever, another common sickness behaviour, is sufficiently advantageous to offset the 10-12.5% increase in metabolic rate required for each 1°C increase in temperature (M. J. Kluger 1979) and has been conserved throughout more than 600 million years of vertebrate evolution (Evans, Repasky, and Fisher 2015). Fever enhances an organisms chance of survival by creating a hostile environment for parasites and a more active immune response (Evans, Repasky, and Fisher 2015; Matthew J. Kluger, Ringler, and Anver 1975; Schulman et al. 2005; Earn, Andrews, and Bolker 2014). Under normal circumstances, the so-called central (SCN) clock controls body temperature rhythms, but how the SCN and

inflammation interact to control temperature is unknown. Though many behaviours altered during infection are clock-controlled during health, the extent to which organisms become too sick to maintain normal behaviour or adaptively disrupt their rhythms is unclear. Additionally, clock-control could facilitate recovery of rhythms during the return to health.

Viewing the host as a collection of traits connected by the circadian system has the potential to uncover novel strategies to resist infection and reveal the circumstance in which immune rhythms reflect constraints or adaptations. Indeed, rhythmic metabolism of xenobiotic substances (e.g. drugs and vaccines) influences efficacy and toxicity in a time-of-day dependent manner(Levi & Schibler 2007). For example, halothane (a commonly used anaesthetic) administered to mice in the daytime results in low mortality (5%), but mortality increases (76%) if administered at night(Matthews, Marte, & Halberg 1964) and half of the best-selling drugs in the USA for humans target the products of genes that are rhythmically expressed (in mice) (R. Zhang et al. 2014). A better understanding of host rhythms could be harnessed to make drugs and vaccines more effective, as well as mitigating the negative effects of modern lifestyles that involve shift work and jet lag. However, for such interventions to be sustainable in the face of parasite evolution, understanding the ecology of rhythms from the perspective of parasites is also required.

Box 3. Case studies illustrating the role of circadian rhythms in parasite offence, host defence, and host manipulation

Host-parasite system: *Teleogryllus oceanicus* (Pacific field cricket) & *Ormia ochracea* (parasitoid fly)
What we know: *O. ochracea* deposit larvae which burrow into the host and emerge 7-10 days later, resulting in host death. A flatwing morph that is physically incapable of calling has evolved to evade the risk of parasitism by acting as a silent, satellite male (Zuk, Rotenberry, & Tinghitella 2006).
A more nuanced form of parasite evasion? In addition to the flatwing morph, natural selection may have found another solution. Some males condense singing activity to the darkest part of the night (Zuk, Simmons, & Cupp 1993) which may hamper the fly's ability to use visual cues to home in on hosts. Parasite evasion (via a flatwing phenotype or phase-shifted calling) trades off against attracting females, potentially constraining selection on these strategies. Moreover, multiple activities need to be coordinated for successful reproduction (e.g. locomotion, foraging, spermatophore production). Given that many of these traits are clock-controlled, could altering the timing outputs of the clock be a streamlined way of phase-shifting all related activities and minimizing the costs of parasite evasion?

Host-parasite system: Carpenter ants *& Ophiocordyceps* spp. and *Pandora* spp. (fungi)
What we know: *O. unilateralis s.l.* induces workers of its carpenter ant host, ordinarily active during the night-time, to wander out of the ant nest during the day-time. Hosts then summit vegetation and adopt a mandibular death-grip in elevated positions. This manipulated behaviour is highly time-of-day and species-specific and occurs within a 3-hour window at dawn or in the mid-late morning, depending on the species (deBekker *et al.* 2015; Hughes 2011). Clinging to vegetation, the ant dies whilst the fungus completes its life cycle by growing a spore-producing stalk out of the dorsal region of the ant's thorax (Hughes *et al.* 2011).
A case for coevolution and ecosystem specificity? The jigsaw puzzle of how the fungus controls the ant is still being pieced together. Clocks may play a central role because infection alters the expression of host clock homologues *period* and *cycle* (deBekker *et al.* 2015). Host manipulation also appears to involve altering host chemosensory abilities, potentially via rhythmic secretion of enterotoxins (deBekker *et al.* 2017), all achieved from the fungus's primary location in muscle tissues (Fredericksen 2017).

Host-parasite system: Mammals *& Plasmodium* spp*. (malaria parasites)*
What we know: Malaria parasites synchronously burst from the host's blood cells every 24, 48, or 72 hours depending on the parasite species (Garcia *et al.* 2001). When out of synch with the host's circadian rhythms, parasites incur an approximately 50 percent reduction in the densities of both asexual stages (necessary for in-host survival), and sexual stages (responsible for transmission; O'Donnell 2011) before they become rescheduled to be in synch with host feeding rhythms (Prior *et al.* 2018; Hirako *et al.* 2018).
Three worlds collide: a complex system of interactions? Why aligning the phase of parasite rhythms with the host's rhythms is important remains mysterious, but recent work suggests that parasites are also selected to coordinate with the time-of-day their mosquito vectors are active (Schneider *et al.* 2018; Pigeault *et al.* 2018) (see Rund *et al.* 2011 for information on *Anopheles* circadian rhythms). If differently phased rhythms for asexual replication are required to provide the best matches to host and vector rhythms, parasites face a trade-off between maximizing in-host survival and between-host transmission. Such a tension could be exploited by novel drug treatments to coerce parasites into a loss of fitness. Further, mosquito nets have induced a shift in *Anopheles gambiae* biting activity, ultimately resulting in a change in host-parasite timing (Riji-Ferreira *et al.* 2017; Souogoufara *et al.* 2014; Rund *et al.* 2016). The epidemiological consequences of this are unknown.

2.4. Rhythms in parasite offence

Scheduling activities to take advantage of daily rhythms in transmission opportunities could be a general explanation for rhythms in parasites. The most well-known example concerns the transmission forms (microfilariae) of different species of filarial worms. They move from the host's organs to the capillaries during the day or night, depending on whether they are transmitted by day- or night-biting insect vectors (Hawking 1967). In addition to the activity patterns of vectors, rhythmic interactions with hosts also matter. For example, the larval stage of the blood fluke *Schistosoma japonicum* emerge from their invertebrate host to seek a mammalian host at different times of day. Flukes emerge in the afternoon when the preferred host is nocturnal or in the morning if seeking a diurnal host (Mouahid *et al.* 2012). Parasites that have free-living stages are also subject to rhythms in the abiotic environments. The coccidian parasite *Isospora* sheds from its host in the late afternoon to minimise UV exposure and desiccation risk whilst undergoing a developmental transition necessary to infect new hosts (Martinaud, Billaudelle, & Moreau 2009). However, key questions remain about the adaptive nature of these rhythms. For example, why aren't microfilariae located in the peripheral capillaries all day long? Is a cost associated with this location, which is only worth paying at times of day when vectors are active?

In contrast to the role of parasite rhythms in transmission, their role in within-host survival has received less attention. Many host rhythms (in addition to immune rhythms) present opportunities and constraints for parasites. *Trypanosoma brucei* (which cause sleeping sickness) display circadian clock-driven rhythms in the expression of metabolic genes (Rijo-Ferreira *et al.* 2017). These rhythms correlate with time-of-day sensitivity to

oxidative damage, thereby suggesting the need to cope with redox challenges caused by rhythmic digestion of food by hosts. In contrast, rhythms in the development of asexually replicating malaria parasites capitalise on daily variation in the nutritional content of blood caused by host immune responses and feeding patterns (Prior *et al.* 2018; Hirako *et al.* 2018). Whether malaria parasites cannot complete their developmental cycle until the host makes nutrients available, and/or use nutrients rhythms as a time-of-day cue to set the pace of their development, is unknown (Reece & Prior 2018) (see Box 3).

Clocks in parasites or hosts could have fitness consequences for one or both parties, or neither. Fitness consequences for both hosts and parasites suggests that clocks could coevolve. Clock coevolution is suspected for the plant-pollinator system *Petunia axillaris* and *Manduca sexta* (Fenske *et al.* 2018), in which nocturnal scent emission by *P. axillaris* coincides with foraging activity in the hawkmoth *M. sexta*. Both traits are clock-controlled, and appear so well synchronized that, even in the absence of floral scent emission, *M. sexta* exhibits a burst in foraging activity at the same time that floral scent emission is expected to be greatest. However, foraging behaviour also remains sensitive to the environment, as evidenced by absence of activity when the moth is subjected to light at night. If rhythms in different organisms do coevolve, then they should use the same Zeitgeber, but how robust should their timing systems be to fluctuations in the environment? If the rhythm of one party is more readily disrupted (masked) by environmental change, or faster at tracking seasonal changes in photoperiod, then the relationship may be disrupted to the gain of hosts or parasites. Exploring the degree and consequences of plasticity in rhythms is pertinent because climate change is interfering with the ability of interacting species to synchronise (Kharouba *et al.* 2018).

The situation is further complicated when interactions between both host and parasite clocks shape disease trajectories. For example, in a plant-fungus system (*Arabidopsis thaliana* and *Botrytis cinerea*, respectively), when both parties are in the same

photoperiod schedule, primary plant defences peak in the morning, and the fungus produces the biggest lesions when inoculated at dusk (Hevia *et al.* 2015). The authors were able to separate the contributions to pathogenicity by host and parasite clocks using reverse lighting schedules for fungus and plants: fungus at dusk produced more severe infections than fungus at dawn, regardless of time-of-day for recipient plants (Hevia *et al.* 2015). Furthermore, this suggests *B. cinerea* anticipates and exploits weaknesses in plant defence at dusk rather than attempting to overwhelm dawn defences (see section "Rhythms in host defence"). Separately assigning the contributions of rhythms in hosts/vectors and parasites to virulence and transmission is necessary to understand whose genes control which rhythms, and hence how they can be shaped by selection.

If parasite rhythms are adaptive, then disrupting them could reduce disease severity as well as transmission. However, understanding the timing mechanisms of parasite rhythms is necessary to disrupt them (Reece, Prior, & Mideo 2017). Unravelling how parasite rhythms are controlled is a considerable challenge. Parasites might allow the host to inadvertently schedule their activities for them, in which case the genes encoding parasite timing mechanisms belong to hosts. Alternatively, parasites might keep time using a circadian clock (with the properties described in Box 1), as demonstrated for *T. brucei* and *B. cinerea.* Given the diversity in clock genes across taxa, searching genomes for known clock genes often yields "absence of evidence" not "evidence of absence." Instead, round-the-clock transcriptomics or proteomics, paired with bioinformatics approaches to mine for known core clock-related functional domains and sequence patterns may find candidates. However, simpler time-keeping strategies exist, though they do not necessarily have the advantages of temperature compensation or anticipation. For example, cell division cycles are often controlled by hourglass mechanisms that rely upon threshold concentrations of substances, independently of periodic phenomena (Schneider, Rund, et al. 2018; Pigeault et al. 2018). Alternatively, organisms can react directly (via "tracking") to temporal changes in the environment. Note, this differs from

masking, a chronobiological phenomenon in which the expression of a clock-controlled rhythm is suppressed by a change in the environment without having a direct effect on the period or phase of the underlying rhythm (Schneider, Rund, et al. 2018). A response that directly tracks time-of-day cues may suit parasites with multi-host lifecycles if each host type provides a different time-cue.

Given that rhythms in *T. brucei* metabolism and plasticity in development during the asexual cycle of *Plasmodium spp.* enables these parasites to tolerate drugs, there is an urgent need for proximate and ultimate explanations of their rhythms. The *T. brucei* clock is entrained by temperature cycles, but if other parasites use Zeitgebers to set their clocks, or respond directly to time-of-day cues, that are readily perturbed, it should be possible to reduce parasite fitness by interfering with their rhythms. Further, reports of changes to the biting time of mosquito populations that transmit malaria suggests that insecticide-treated bed nets are imposing selection on vector rhythms (Sougoufara *et al.* 2014; Rund *et al.* 2016; Rijo-Ferreira *et al.* 2017). Given that rhythms of parasites and mosquitoes each affect malaria transmission in lab experiments (P. Schneider, Rund, et al. 2018; Pigeault et al. 2018), what are the likely epidemiological consequences? Recent work suggests that mosquitoes are more susceptible to infection when they feed in the daytime and parasites are more infectious at night (P. Schneider, Rund, et al. 2018). Thus, day-biting could increase the prevalence, but not burden, of malaria in mosquitoes. However, in the longer term, if parasites evolve to invert their rhythm but mosquitoes do not, both prevalence and burden may increase.

2.5. Parasite manipulation of host rhythms

Rhythms in host processes offer opportunities that parasites could exploit. Could parasite fitness be increased by coercing hosts into altering their rhythms? Although many striking examples of parasite manipulation of host phenotypes (i.e. changes to host traits that benefit parasites) are known (Thomas, Rigaud, & Brodeur 2010), the notion of “parasite

manipulation of host clocks" is largely unexplored (De Bekker, Merrow, & Hughes 2014). A pre-requisite for parasite manipulation is that a phenotypically plastic host trait is targeted; and circadian clocks are flexible. Because clocks control much of the host's behaviour and physiology (Ko & Takahashi 2006) and clocks throughout a given host involve the same players in the canonical clock (the TTFL), manipulation of the host's time-keeping may be an efficient way to simultaneously alter many aspects of the within-host environment. Alternatively, parasites interests may be served by bolstering circadian rhythms of their hosts during sickness to ensure they forage and interact with conspecifics, as usual.

As outlined in the section "Rhythms in host defence," separating the effects of being sick *per se* from host defence and parasite manipulation is challenging. Recently, a combination of culture and comparison of infection models has revealed that *T. brucei* alters expression rhythms of clock genes in host mice (Rijo-Ferreira *et al.* 2018). Specifically, infected hosts are more active in the resting phase (phase-advanced) because the clock runs faster (shorter period). Effects at organismal, cellular, and molecular levels suggests the behaviour is not just a result of sickness (Rijo-Ferreira *et al.* 2018). However, it is not clear how *T. brucei* achieves this, and whether the parasite benefits from altering host rhythms. One target of circadian disruption by viral parasites is the gene *Bmal1*, a core clock gene. Herpes and influenza A virus replication and dissemination within the host is enhanced in infections where *Bmal1* is knocked out (Edgar *et al.* 2016). However, it remains unclear if virus replication is maximised by simply disturbing rhythmicity in host cell cycles or if this is a case of immune manipulation since *Bmal1* appears involved in innate host defence (Edgar *et al.* 2016). Having observed changes to host clocks, the proceeding step is to decipher the ecological context behind these effects.

The above examples lend proof-of-principle to the idea that parasites can manipulate host clocks and could be a general explanation for examples of host manipulation. Hairworms

(Nematomorpha) are a well-known case of temporally linked behavioural manipulation. They infect various arthropods, notably crickets, and cause the host to wander in an erratic manner until a body of water is encountered. The host commits suicide by jumping in water, and the adult hairworm emerges. Infected hosts are found wandering only in the early part of the night (Thomas *et al.* 2002), and uninfected hosts are rarely motivated to jump into water. Infected crickets differentially express an array of proteins, some of which are linked to visual processes and circadian clocks (Biron *et al.* 2006). Culturing isolated host cells with parasite products and quantifying the expression of clock genes (following Rijo-Ferreira *et al.* 2018) could illuminate this case of parasite manipulation. For systems without relevant insect cells lines, or cases where manipulation is likely to be tissue/cell type specific, a transcriptomics approach may be useful (Hughes *et al.* 2017). Round the clock expression data can be mined for putative core clock genes and their phase, amplitude and period assessed in control and manipulated hosts. This, however, is likely to be extremely challenging for host species whose timekeeping does not rely on a canonical circadian clock.

Another putative case for clock manipulation concerns the New Zealand freshwater snail (*Potamopyrgus antipodarum*) infected with *Microphallus* trematodes (Lively 1987) (Trematoda: Microphallidae). Uninfected adult snails forage primarily at night on the upper surfaces of rocks in the shallow-water margins of lakes. These snails retreat to under rocks at sunrise, which likely reduces their risk of predation by waterfowl, which are the definitive host for *Microphallus*. Infected snails, however, show delayed retreating, potentially making them more likely to be consumed (Levri & Lively 1996). Crucially, the apparent manipulation only occurs when the parasite is mature. Snails infected with immature (non-transmissible) stages exhibit the same risk-averse retreating behaviour as uninfected snails (Levri & Lively 1996). In addition, snails infected with other species of sterilizing trematodes, which are not trophically transmitted, do not exhibit the same risky behaviour as those infected with *Microphallus* (Levri 1999), thereby

eliminating the possibility that the *Microphallus*-induced behavioural change is a simple artefact of parasitic castration. Finally, *Microphallus*-infected snails spend more time foraging on the top of rocks, even when food was removed whereas uninfected snails retreated to shelter (Levri 1999). Taken together, the data suggest that *Microphallus* induce a change in snail behaviour that increases trophic transmission, potentially via manipulation of clock-controlled activity rhythms.

There are many ways that parasites could interfere with clock-controlled host behaviours. A blunt instrument would be to alter perception/detection of the Zeitgeber that sets the time of the host's clock, which is usually light. For example, *Microphallus* could interfere with photoreception to reduce the sensitivity of snails to dawn, causing their clocks to phase delay and forage at higher light intensities than un-manipulated snails. Alternatively, parasites could induce the host to ignore its clock (mask) or alter clock regulation of hormones that relay time-of-day information around the host. For example, baculoviruses appear to perturb the circadian rhythms of their caterpillar hosts by disrupting hormones that control climbing behaviour. In the baculovirus (*Lymantria dispar* nucleopolyhedrovirus), a single gene inactivates 20-hydroxyecdysone (Hoover *et al.* 2011) (a host hormone regulated by a circadian oscillator), motivating the caterpillar to climb high atop their host plants. Here, they liquefy and disseminate the virus to caterpillars below, as well as infecting birds who consume the corpses (Goulson 1997). Similar to the manipulation of caterpillar hosts, many species of parasitic fungi (*Ophiocordyceps spp*. & *Pandora spp.*) alter the daily behavioural rhythm of a variety of ant species (de Bekker *et al.* 2015, 2018) (See Box 3).

Parsing out whether temporal disruption is a host response or clock manipulation is nearly, if not entirely, impossible without uncovering the mechanism of manipulation. The lack of insight into the mechanisms parasites use to interfere with their hosts has stalled progress in the field of "host manipulation by parasites" (Herbison, Lagrue, & Poulin

2018). This gap could be filled by harnessing the tools and conceptual framework developed in chronobiology. Many of the examples above have employed an ecological approach, yet a chronobiological approach can help elucidate both proximate and ultimate explanations.

2.6. Conclusion

Over the past few decades, the focus of chronobiology has been to elucidate the mechanistic underpinnings of biological rhythms. We propose that now is the time to integrate this knowledge into parasitology, evolutionary ecology, and immunology (see Box 2). Indeed, the role of biological rhythms in infectious disease is a growing topic that holds promise for improving human and animal health. History clearly illustrates that attempts to control parasites are usually met with counter-evolution (in the form of drug resistance, vaccine escape, and host shifts). A comprehensive understanding of how rhythms affect parasite invasion and exploitation of a host (or vector) offers novel ways to disrupt the chain of transmission and treat disease. Further, clock coevolution may occur in host-parasite-vector interactions, resulting in complex arms races best understood through the lens of chronobiology coupled with evolutionary ecology. Chronobiology supplies a myriad of tools to help elucidate rhythmic phenotypes and reveal to what extent host and parasite genes are responsible for rhythms in disease phenotypes. Adding an evolutionary ecology framework will ensure this information is generalisable and used to make interventions as evolution-proof as possible.

3. Chapter 3: A novel audio-to-circadian analysis pipeline reveals singing rhythms of male field crickets are clock controlled

3.1. Abstract

Circadian rhythms are ubiquitous in nature and endogenous circadian clocks drive the daily expression of many fitness-related behaviours. However, little is known about whether such traits are targets of selection imposed by natural enemies. In Hawaiian populations of the nocturnally active Pacific field cricket (*Teleogryllus oceanicus)*, males sing to attract mates, yet sexually-selected singing rhythms are also subject to natural selection from the acoustically-orienting and deadly parasitoid fly, *Ormia ochracea*. Here, we use *T. oceanicus* to test whether singing rhythms are scheduled by circadian clocks and thus possible targets of selection imposed by flies. Singing rhythms fulfilled all criteria for endogenous circadian clock control, including being driven by photoschedule, self-sustained periodicity of approximately 24 hours, and being robust to variation in temperature. Furthermore, singing rhythms varied across individuals, suggesting genetic variation on which natural and sexual selection pressures can act. Sexual signals and ornaments are well-known targets of selection by natural enemies, but our findings indicate that the circadian timing of those traits' expression may also be of key importance.

3.2. Introduction

The daily rotation of the Earth causes predictable cycles of day and night, which nearly all life has evolved to cope with. Circadian clocks (i.e. daily, biological timekeepers) are ubiquitous and allow organisms to schedule activities, from gene expression to physiologies to behaviours, according to the time-of-day they are best undertaken. Most research on circadian rhythms has focussed on uncovering the genes and molecular pathways involved in the workings of circadian clocks. However, there is increasing

interest in the evolution and ecology of circadian rhythms – particularly, in how rhythms affect survival and reproduction (Greives *et al.* 2015; Hau *et al.* 2017; Rubin *et al.* 2017; Westwood *et al.* 2019; Hozer *et al.* 2020). Overt and rhythmic sexual signals provide an opportunity to examine these questions as they often put the signaller at risk of predation and/or parasitism, and so, are subject to natural selection as well as sexual selection. A well-studied case in which a rhythmic mating behaviour is subject to both natural and sexual selection concerns the Pacific field cricket, *Teleogryllus oceanicus*. These crickets are introduced in Hawaii, where they are subject to the lethal, acoustically-orienting parasitoid fly *Ormia ochracea*. Strong natural selection against "normal-wing" singing males has led to the evolutionary spread of distinct male forms which silence or reduce their song, protecting them against the fly (e.g. flatwing, curlywing, small-wing, and purring phenotypes) (Zuk, Rotenberry, and Tinghitella 2006; Pascoal et al. 2014; Robin M. Tinghitella et al. 2018; J. Rayner et al. 2019). Pre-existing satellite behaviour (i.e., employing a silent strategy whilst intercepting females attracted to singing males) likely facilitated the spread of these mostly silent/altered wing morphs throughout the Hawaiian Islands (Nathan W. Bailey, McNabb, and Zuk 2007). However, the loud, long-range calling song is much more conspicuous to females, which also show preference for normal-wing song. Thus, conferring normal-wing males a mating advantage and explaining, at least in part, their persistence in the wild (Nathan W. Bailey and Zuk 2008; Robin M. Tinghitella and Zuk 2009; Robin M. Tinghitella et al. 2021).

While the singing of normal-wing males renders them vulnerable to parasitism, *O. ochracea* may not be positively phonotactic (i.e., attracted to sound) throughout the entirety of the night. Indeed, multiple studies suggest *O. ochracea* phonotaxis to *T. oceanicus* song peaks around dusk (William Cade 1979; Kolluru 1999) and trails off prior to sunrise. However, evidence shows Hawaiian *T. oceanicus* have curbed singing around both dawn and dusk (Zuk, Simmons, and Cupp 1993) (compared to unparasitized, ancestral populations), though notably Kolluru (1999) found Hawaiian *T. oceanicus* activity

peaks only at dusk, coinciding with the time of greatest fly phonotaxis (Kolluru 1999). Regardless of the precise timing of fly phonotaxis, whether and how a restricted singing window can evolve depends on how it is controlled. If the onset of singing is a direct response to experiencing dusk (i.e. behavioural plasticity) then delaying singing requires either the evolution of usage of a different cue such as even lower light intensity, or the evolution of a delay between cue and response. This could occur if selection acts on existing genetic variation for photosensitivity or lag in response to light intensity. In contrast, if the singing window is controlled by a circadian clock (Werrer Loher and Orsak 1985) how singing behaviour responds to clock outputs could change. Because clocks and their outputs control much of an organism's physiology and behaviour (e.g. >80% and >40% of protein-coding genes show daily, rhythmic expression in male baboons and mice, respectively; (R. Zhang et al. 2014; Mure et al. 2018), alterations to clock mechanics may be constrained if singing is a cue for, or a tightly linked aspect of traits that have to occur in advance of mating. For instance, if spermatophore formation precedes the onset of singing by a fixed amount of time (such as in *T. commodus*; (Werner Loher 1974) a phase delay in the onset of singing may be difficult or impossible without an accompanied shift in the onset of spermatophore formation. Under such scenarios, the extrinsic consequences of singing (e.g. parasitism risk and mate attraction) trade off with each other as well as with the intrinsic consequences (e.g. readying a spermatophore). Further complexity occurs when closely related species share a common landscape. For example, crickets of the genus *Laupala* (sympatric species *L. cerasina* and *L. paranigra*) exhibit significant daily temporal differences in singing (and thus mating), which likely reduces interspecific acoustic interference and mating (Danley *et al.* 2007).

Understanding how singing rhythms can evolve requires knowledge of the extent of their circadian regulation, their sensitivity to variation in abiotic conditions (such as temperature), and their variation between individuals within a population. Research on circadian rhythms in crickets has largely mirrored that of chronobiology, with early work

focussing on the ecology of rhythms (W. Loher and Edson 1973; Werner Loher 1974; Werner Loher and Weidenmann 1981; Werrer Loher and Orsak 1985) and a subsequent shift in focus towards determining molecular clock mechanisms (Lupien *et al.* 2003; Moriyama *et al.* 2008, 2009; Danbara *et al.* 2010; Moriyama *et al.* 2012; Uryu, Karpova, & Tomioka 2013). Though still not fully understood, the molecular underpinnings of clocks in crickets appear similar to those in *Drosophila* (which have been studied extensively) and mammals (reviewed in (Panda, Hogenesch, & Kay 2002) operating via a canonical circadian clock, i.e. a transcription-translation feedback loop involving clock genes *period* (*per*) (Moriyama *et al.* 2008), *timeless* (*tim*) (Danbara *et al.* 2010), *Clock* (*Clk*) (Moriyama *et al.* 2012), and *cycle* (*cyc*) (Uryu, Karpova, & Tomioka 2013). Understanding how molecular clocks operate opens the door towards using this information to answer questions pertaining to the evolutionary ecology of circadian rhythms, particularly how circadian rhythms govern interactions between individuals (e.g., males and females, predators and prey, and hosts and parasites).

Early studies pertaining to circadian rhythmicity in *T. oceanicus* singing address some, but not all, requirements for a rhythm to be deemed circadian (Werrer Loher and Orsak 1985). Rhythms are deemed under the control of an endogenous circadian oscillator when they meet all four of the following requirements: (1) the duration of the rhythm has a "period" of approximately 24-hours (24h), (2) the rhythm persists ("free-runs") under constant environmental conditions, (3) the timing ("phase", e.g. onset and offset) of the rhythm is set ("entrained") by an environmental time-cue ("Zeitgeber"), and (4) the pace of the clock is unaffected by a biologically realistic range of temperatures ("temperature compensation"), which is usually examined under free-running conditions. Verifying these characteristics in the form of behavioural assays requires observing the behaviour for multiple consecutive days under both a standard photo-schedule (12h Light : 12h Dark; LD) and a reversed photo-schedule (12h Dark : 12h Light; DL), as well as constant light (LL) or constant darkness (DD) to determine the free-running period and its stability over a

range of temperatures. Without verification of each of these characteristics, an observed rhythm may simply be the direct response of an organism to a change in the external environment and not the product of a cellular autonomous circadian oscillator. For example, Tan & Robillard (Tan & Robillard 2021) observed some time-of-day variation in singing activity across 11 cricket species but were unable to parameterise rhythms or determine whether an endogenous oscillator is involved.

Here, we ask whether the singing rhythm of *T. oceanicus* is circadian, and we assess individual variation in rhythmic parameters. This provides a rare opportunity to examine circadian rhythms in the context of natural selection and rapid evolution in the wild. To do this we develop a novel audio-data-to-circadian analysis pipeline for the extraction (via 'Tempaural', a bespoke R package we implement in the 'Rethomics' analysis framework), processing (through machine learning), and analysis of around-the-clock continuous audio data. We then deploy our method to analyse data from three experiments, revealing that singing rhythms are under endogenous circadian clock control, driven by photoschedule, and robust to variation in temperature. Furthermore, individual variation underlies differences in parameters that characterise singing rhythms.

3.3. Methods

Animals, rearing and experimental conditions

Experimental subjects were taken from laboratory stock populations established in 2012 from females collected in Lai'e (W. T. Schneider, Rutz, et al. 2018) At the time of establishment, approximately 50% of males in the population expressed the flatwing phenotype. For the purpose of this experiment, we excluded flatwing males and hereafter, "adult male" refers to the normal-wing phenotype. We housed both stock and experimental animals in 9L plastic boxes with egg cartons for shelter and fed Burgess™ Excel Junior and Dwarf rabbit pellets with water available *ad libitum*. Rearing conditions consisted of a LD photoschedule (lights-on at 06:00 UTC and lights-off at 18:00 UTC), and

temperature was maintained at 25°C. To house crickets during the experiment, we used either Panasonic MIR-154-PE Cooled Incubators or LEEC SFC3C R/H Ultrasonic Humidity Cabinets. Males in each experiment were within three days post-eclosion and physically and acoustically isolated from all other males for the duration of the recordings. During experiments, all recordings were made from individual crickets with food and water *ad libitum* and egg carton for shelter.

Experimental designs

We conducted three experiments to test whether *T. oceanicus* singing rhythms fulfil the criteria for control by an endogenous clock. Our first experiment (experiment 1) was designed to verify singing is nocturnal and characterise its basic daily patterns. We then carried out two further experiments to test whether singing rhythms persist in the absence of a time-of-day cue, and if rhythms are robust to temperature variation under both constant (experiment 2) and entrainment (experiment 3) conditions. Specifically, experiment 2 tests whether the period of a given rhythm is maintained over a range of temperatures, and experiment 3 probes whether entrainment (when e.g. the onset and offset of a rhythm occurs in relation to the Zeitgeber) occurs in a manner independent of temperature.

Experiment 1: Characterising the prevalence and timing of singing across days

Adult males (n=4) were recorded continuously for at least 8 days under photoperiod-reversed (DL) conditions relative to standard rearing conditions, and constant temperature (25°C) (Figure 1). We used these audio recordings to estimate the period of singing under predictable environmental conditions, to determine the proportion of time spent singing each day, and to determine how much singing occurs during the light versus dark phase.

Experiment 2: Fundamental circadian properties

Adult males (n=14) were haphazardly assigned to one of three temperature treatment groups (22°C, n=5; 25°C, n=4; 28°C, n=5) (see Figure 1) all in constant darkness (DD) and recorded continuously for at least 8 days. Audio recordings from these crickets revealed whether singing rhythms free run (i.e., persist in the absence of rhythmic environmental cues) and are temperature compensated (i.e., an approximately 24h period is maintained despite the different temperatures).

Experiment 3: Temperature compensation under entrainment

Adult males (n=17) were haphazardly assigned to one of three treatment groups: 22°C (n=8), 25°C (n=6), or 28°C (n=8) (see Figure 1). We recorded each cricket under standard LD conditions, then at lights on (Zeitgeber Time, ZT0) on day 9, we switched to photoperiod-reversed (DL) conditions and recorded each cricket for a further 8 days. By reversing ("phase-shifting") the photoschedule during the experiment, we tested whether crickets are able to entrain to an altered photoperiod, and whether entrainment is temperature compensated.

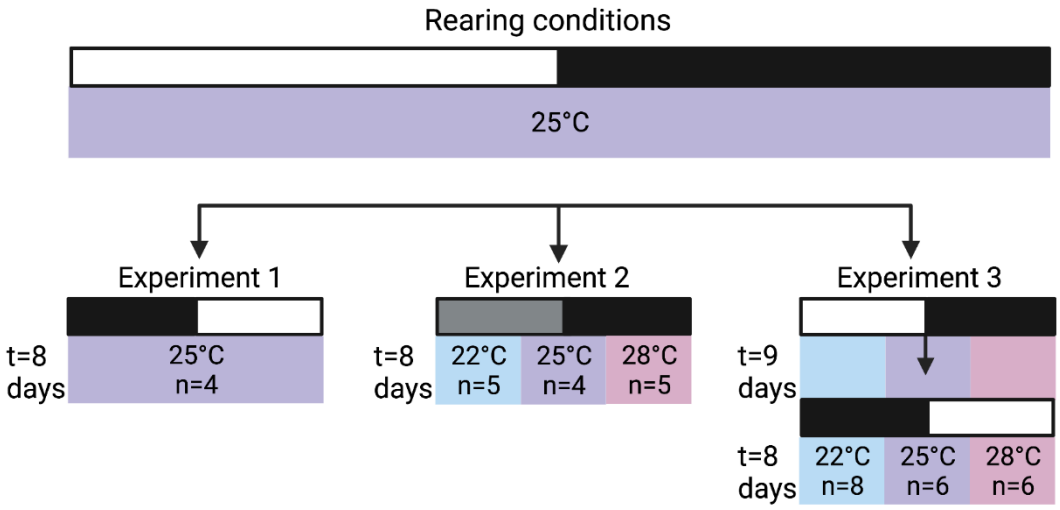


Figure 1. Rearing and experimental conditions. Individuals were removed from rearing conditions and placed into incubators where they were recorded for at least 7 days per photoschedule regime. Crickets in experiment 1 were recorded under photoperiod-

reversed conditions (DL) relative to rearing conditions, crickets in experiment 2 were recorded in constant darkness (DD), and crickets in experiment 3 were recorded under standard (LD) and then photoperiod-reversed conditions (DL). Temperature for each group is indicated by colour (blue=22°C, purple=25°C, and pink=28°C) and photoschedule is illustrated by the white (light), black (dark), and grey/black (subjective day/night during constant darkness) bars. Subjective day/night refers to the portion of each circadian cycle that corresponds to lights on/off in rearing conditions.

Audio recordings

We collected continuous audio data using recorders (Sony™ ICD-UX560 Digital Voice Recorders equipped with Integral™ Micro Secure Digital eXtended Capacity cards) set to a sampling rate of 44.1 kHz and a 16-bit resolution (stereo MP3 file format). Recorders were adhered to the inside of each plastic box and fitted with an external power supply cord. We transferred audio files to external hard drives (Western Digital 4TB Elements Portable Hard Drive – USB 3.0) prior to analysis on a personal computer (2017 Apple MacBook Pro using macOS Catalina) and the University of Edinburgh's high performance computing cluster.

Identifying singing and characteristics of singing rhythms

Crickets produce acoustic signals by rhythmically opening and closing their forewings, rubbing the scraper and file together (i.e. "stridulation") (Pfau & Koch 1994), and bouts of singing generally last from several seconds to minutes (W. T. Schneider, Rutz, et al. 2018). Calling song produced by normal-wing *T. oceanicus* has a dominant frequency between 4-5kHz and is characterised by a long chirp followed by a series of short chirps (Balakrishnan & Pollack 1996). Using the unique spectral properties of *T. oceanicus* song (see Appendix Chapter 3 Table I), we developed a random forest model using k-fold cross validation to predict whether a cricket sang during each consecutive 60s audio clip (hereafter referred to as a "clip") throughout its entire recording window (8-18 days per individual, depending

on the experiment). Using an algorithm to detect singing allowed us to efficiently process the audio files that exceeded 1TB (>12,000 hours) across all experiments. In order to facilitate and standardise the analysis of this large amount of data, we developed "Tempaural", an R package that interfaces bioacoustic data with the Rethomics framework. Temporal is freely available at https://github.com/rethomics/tempaural.

To generate our random forest model (Breiman 2001), we first randomly extracted 557 60s clips from 7 representative crickets spanning three different experimental conditions (i.e. LEEC or Panasonic incubator; 22°C, 25°C, or 28°C) and length of time spent in an incubator (8-18 days) (see experimental designs), thus accounting for incubator type, temperature, and cricket age in the training and validation of the model. Saved clips were tagged with a random string to ensure anonymisation and randomisation. We then manually listened to all clips and classified them as "singing" as a binary response variable (the cricket was heard singing at least once during the clip, this includes short chirps lasting ~1s; singing=1) or "background" (the cricket was not heard singing during the clip; singing=0).

Next, we initially extracted 19 audio features (e.g., descriptive statistics of the frequency in the 3-6 kHz spectrum) from each clip using the bioacoustics R package "Seewave" (Sueur 2018) (see Appendix: Chapter 3 Table I for the full list of features). These predictors were iteratively pared down to five which returned a high level of accuracy on the training set (Appendix Chapter 3 Table I). We used the Caret package in R to split the data into training and validation sets (75% and 25% respectively) (Kuhn 2008) and trained a set of models (classification and regression tree, k-Nearest Neighbors, and random forest) using k-fold cross validation (k=10) of which the random forest model performed best (accuracy = 0.978, kappa = 0.918). We estimated the performance of the model on our validation set, which returned a very high level of accuracy (accuracy=0.985, CI (0.949, 0.998), kappa = 0.948) (Appendix Chapter 3 Tables II-III and Figure I). We then applied this model to

score on all consecutive 60s clips as “singing” (1) or “not singing” (0). We averaged the values across clips from the simultaneous recordings of individuals in the same treatment groups to generate a continuously distributed variable for analysis and presentation.

Statistical and circadian analysis

We used R v4.0.1 (R Core Team 2020) for all analyses, except for the derivation of phase markers (onset, peak, and offset) which we obtained using ClockLab software (ActiMetrics, Wilmette, IL, USA). Prior to circadian analysis, we removed the first 72h from each individual’s dataset to allow for acclimation to experimental conditions. Further, we removed the first 4 days post-photoperiod-reversal to allow for transient cycles (i.e., the time necessary for a rhythm to reach a stable phase-relationship with the central circadian pacemaker) (Colin S. Pittendrigh and Daan 1976).

By examining double-plotted actograms for each cricket we carried out initial visual inspection of singing for the duration of each experiment. As “singing” was quantified as either a “0” or “1” for each minute, the legend scale (from 0-1) on Figure 3 B-D and Figure 4 can be interpreted as the mean “singing”. We excluded 1 cricket from experiment 2 at 25°C due to no singing, and we excluded 2 crickets from experiment 3 at 28°C (one for death, and the other for not singing). Recording equipment failure resulted in the loss of ~24h of data from 2 crickets in experiment 2 and ~12h of data from 1 cricket in experiment 3, though these crickets were retained in the final dataset because the remainder of their recordings were unaffected. Not every individual sang every day (or to a degree in which onset, peak, and offset were detectable); these individuals were retained in the dataset, and onset, peak, and offset were calculated only for those days in which they could be confidently estimated.

Free-running and entrained periods were calculated using Lomb-Scargle (LS) periodograms (Ruf 1999) via the Rethomics workflow (Geissmann *et al.* 2019). Mean

singing activity, free-running periods (FRP), and entrained periods were compared using t-tests and Kruskal-Wallis tests (Kruskal & Wallis 1952). Circular data (onset, peak, and offset) were modelled using Bayesian projected normal circular regression models compared by the "Watanabe-Akaike information criterion" (WAIC) (Watanabe & Opper 2010) using the R package "bpnreg" (v. 2.0.2). A change in 2 WAIC (ΔWAIC=2) was chosen to select competitive models. The most parsimonious of the competitive models was chosen for interpretation, and coefficients were considered significant if the high posterior density (HPD) estimates varied from zero (Cremers, Mulder, & Klugkist 2018). Finally, individual variation in phase markers was estimated via angular variances (V_m) (Jammalamadaka and Sengupta 2001) also using the R package "bpnreg".

3.4. Results

Experiment 1: Temporal characterisation

When placed under photoperiod reversed conditions at constant temperature, crickets sing significantly more often in the dark than the light phase (t = 5.35, p = 0.013, df = 3, 95% C.I. [1146.46, 4494.54]; Fig. 2A). Indeed, crickets sing, on average, at least once per minute during 68% of the dark phase (mean = 0.68 ± 0.18 S.D.) in contrast to only 1% of the light phase (mean = 0.01 ± 0.01 S.D.). In total, the most reserved cricket sang at some point during each minute for as little as 9.32h per day (S.E. = 0.00021) compared to the most vociferous cricket which sang at least once per minute for upwards of 12.7h per day (S.E. = 0.00022). Onset of singing began about an hour into the dark phase (mean onset = ZT13.06 ± 1.67h S.D.), peaked nearly 5 hours later (mean peak = ZT17.61 ± 1.95h S.D.) and tapered off just before the start of the light phase (mean offset = ZT23.91 ± 2.46h). Further, the entrained LS period estimate was close to 24h (mean period = 24.4h, S.E. = ± 0.15 Fig. 2B). These results show, as expected, males sing overwhelmingly during the dark phase and vary greatly in how often they sing on average per day.

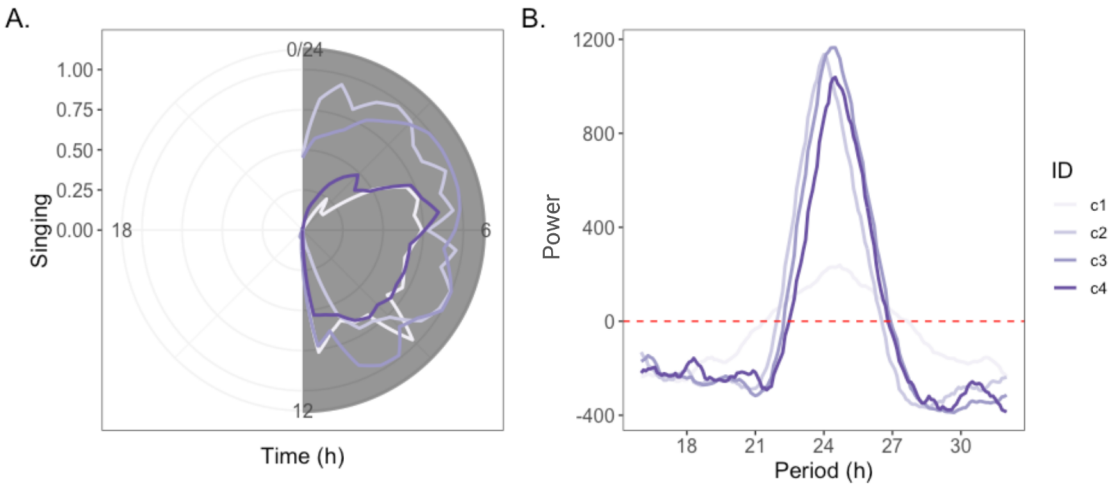


Figure 2. A. Group polar coordinate plot for photoperiod reversed crickets. Purple lines represent singing activity averaged and wrapped across 24h for each individual. Shaded grey and white areas indicate dark and light phases, respectively. Polar coordinates (0/24, 6, 12, and 18) represent time (ZT; in hours), and distance from the centre of the plot (indicated on the upper left quartile of the leftmost plot) illustrates average singing value (0 = no singing and 1 = singing recorded in at least part of a clip) for each cricket at a given 30-minute window across consecutive days of recording. **B.** LS periodograms for individual crickets under entrained, photoperiod reversed conditions. Period estimate (in hours) is shown on the x-axis and associated statistical power on the y-axis. The horizontal, red dashed line indicates the significance threshold. Solid lines illustrate the average power per treatment. For **A** and **B,** colours represent n = 4 individual crickets (c1-c4).

Experiment 2: Fundamental circadian properties

Free-running periods (FRP) are characteristically close to, but never exactly, 24h (Colin S. Pittendrigh and Daan 1976). In keeping with this, FRP for each temperature group is slightly longer than 24h (mean ± S.E.: 22°C = 25.0 ± 0.16; 25°C = 25.2 ± 0.01 28°C = 25.1 ± 0.13; Fig. 3A; Appendix Chapter 3 Figure II) and does not differ significantly between temperature groups (Kruskal-Wallis, chi square = 1.6, p = 0.45, df = 2) (Fig. 3A), giving an

overall FRP of 25.1 ± 0.08 S.E. Because FRPs are not precisely 24h, circadian rhythms drift over successive days while under constant conditions. For *T. oceanicus,* the elongated FRP delays the onset of singing each day, pushing onset further into subjective night and offset into subjective day (Fig. 3 B-D; see Appendix Chapter 3 Figure II for individual LS periodograms). These results show that singing rhythms in *T. oceanicus* are endogenous, close to 24h and temperature-compensated.

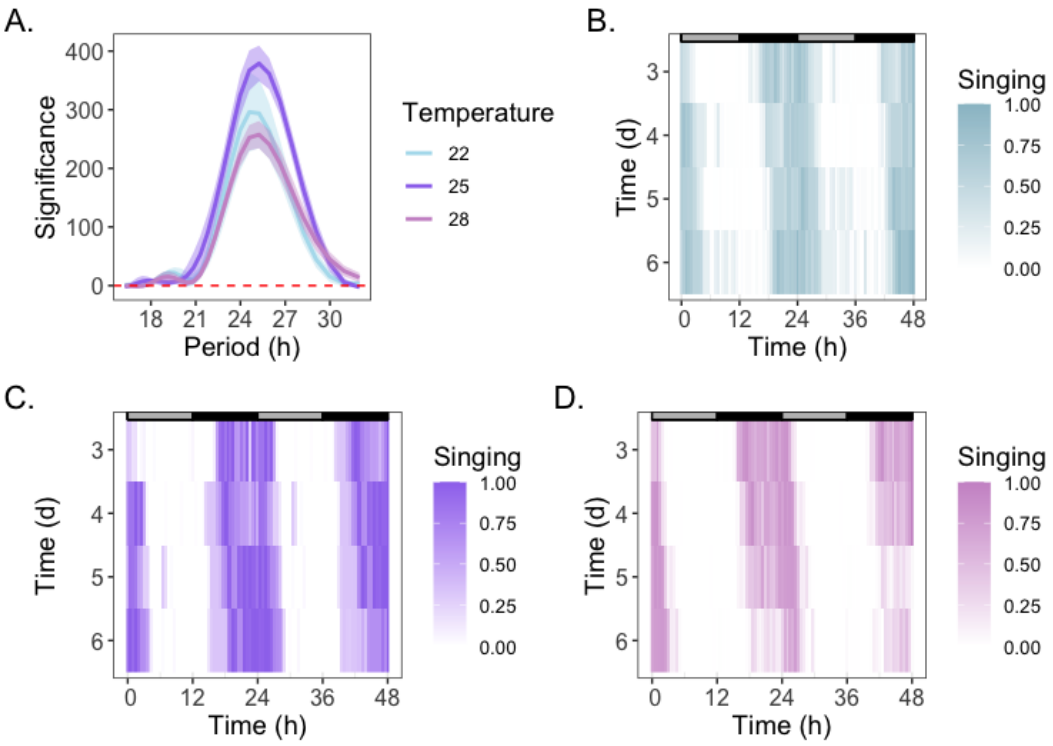


Figure 3. A. Average Lomb-Scargle periodograms for each temperature treatment group under free-running conditions. Period estimate (in hours) is shown on the x-axis and associated statistical power minus the significance threshold is on the y-axis. The horizontal, red dashed line indicates the significance threshold. Solid lines illustrate the average power per treatment, and shaded areas around each line indicate the standard error. **B-D.** Double-plotted (i.e., 48h) actograms averaged across all individuals within each temperature treatment (B, 22°C = blue, C, 25°C = purple, and D, 28°C = pink) showing

singing rhythms under free-running conditions (constant dark). Subjective light and dark phases are indicated by gray and black bars (respectively) situated at the top of each plot. Time in days is shown on the y-axis and time in hours is on the x-axis. Legends indicate singing as depth of colour.

Experiment 3: Temperature compensation under entrainment

Across all temperature treatment groups, crickets sing during the dark phase under standard lighting conditions (days 3-8, Fig. 4) and in photoperiod reversed conditions, following several days of adjustment (days 13-17, Fig. 4). Specifically, upon photoperiod reversal on day 9, crickets begin to shift singing patterns (delaying onset and offset) until re-aligned with their new photoschedule (days 13-17, Fig. 4).

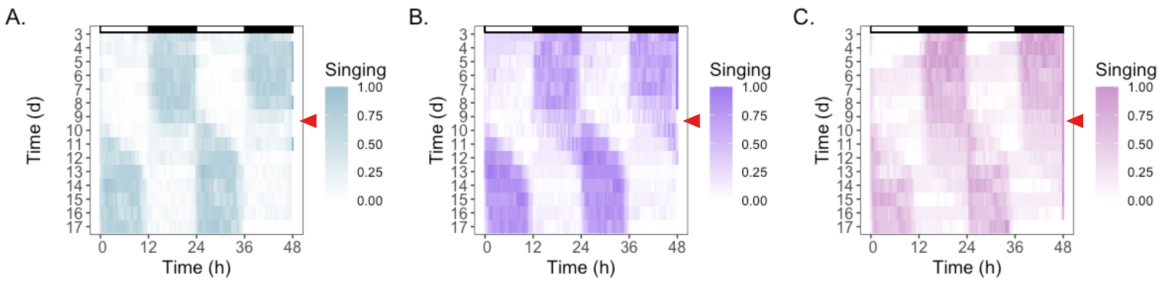


Figure 4. A-C. Average double-plotted actograms for each of three temperature treatments (**A.** 22°C = blue, **B.** 25°C = purple, and **C.** 28°C = pink) showing singing rhythms under entrained conditions (LD, white and black bars on top of plot) and following photoperiod-reversal at ZT0 on day 9 (red arrows). Time in days is shown on the y-axis and time in hours is on the x-axis. Legends indicate singing as depth of colour. Days 0-2 are removed to allow for acclimation to experimental conditions.

The process of entrainment and the resulting rhythms follow similar patterns across temperatures (Fig. 5; Tables 1-2). The most parsimonious model for onset and peak included both regime and temperature as main effects (ΔWAIC = 0 & 1.30, respectively;

Table 1). However, because the HPD for each temperature treatment contained 0 for both phase markers, but not for regime, we interpret regime as the main driver in any observed variation in both onset and phase. Specifically, upon phase shift onset and peak were phase advanced by ~1hr and ~1hr 18 min, each (mean = 0.97 ± 0.25h S.D. and mean = 1.30 ± 0.30 S.D. for onset and peak, respectively). Similarly, the most parsimonious model for offset included only regime (ΔWAIC = 0; Table 1) which resulted in a phase advance of just over 2h upon phase shift (mean = 2.08 ± 0.34h S.D.). Overall, we find that while temperature may increase model fit for some phase markers, it does not significantly contribute to explaining any observed variation, though we did find moderate evidence for a phase advance (~2h) in each of the three phase markers upon photoperiod reversal. However, this may be an artefact of experimental design and attributable to transient cycles.

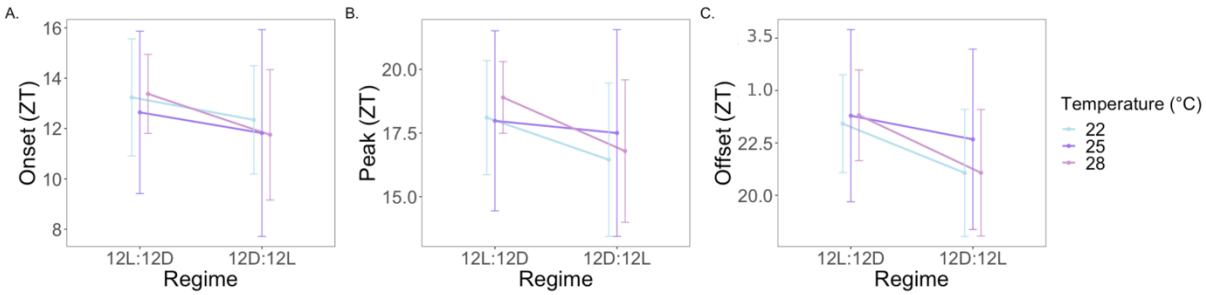


Figure 5. Phase markers (y-axis; **A.** Onset, **B.** Peak, and **C.** Offset in Zeitgeber time (ZT); mean ± S.D.) for each temperature treatment (legend; 22°C = blue, 25°C = purple, and 28°C = pink) across both lighting regimes (x-axis; LD and DL).

Response	Covariates	WAIC	pWAIC	ΔWAIC	lppd	WAIC*w*
Onset	Regime+temperature	425.14	11.01	0.00	-201.56	0.50
	Regime*temperature	425.32	17.43	0.18	-195.23	0.46
	Regime	430.60	5.52	5.46	-209.78	0.03
	Temperature	438.89	7.87	13.75	-211.58	0.00

	Null	444.93	2.66	19.79	-219.81	0.00
Peak	Regime*temperature	490.57	14.07	0.00	-231.22	0.57
	Regime+temperature	491.87	8.84	1.30	-237.10	0.30
	Regime	493.62	4.43	3.05	-242.38	0.12
	Temperature	516.65	6.53	26.07	-251.79	0.00
	Null	517.85	2.15	27.28	-256.77	0.00
Offset	Regime	517.68	5.06	0.00	-253.79	0.49
	Regime+temperature	518.12	9.58	0.43	-249.48	0.40
	Regime*temperature	520.71	14.86	3.02	-245.50	0.11
	Null	551.40	2.26	33.71	-273.43	0.00
	Temperature	552.39	6.82	34.70	-269.38	0.00

Table 1. Phase markers ("onset", "peak", and "offset"; "response") are modelled by "regime" (LD or DL) and "temperature" (22°C, 25°C, and 28°C) ("covariates"). WAIC, estimated number of parameters (pWAIC), ΔWAIC ($WAIC_{model} - WAIC_{min\ model}$), log pointwise predictive density (lppd) and WAIC *w* (WAIC weight) are shown for each model. Models are ordered in descending fit (best-fitting model at the top for each response).

Finally, average *T. oceanicus* singing rhythms under entrained conditions (i.e., for both LD and DL lighting regimes) are characterised by a period estimate of 24.72 ± 0.16h S.E., a mean onset of ZT12.48 (± 2.77h S.D.), peaking at ~ZT17.47 (± 3.07h S.D.), and mean offset of ZT22.37 (± 3.39h S.D.). These parameters varied between individuals, with the angular variances (V_m; Fisher 1995) ranging from 0.17-0.88 for the onset, 0.13-0.86 for the peak, and 0.30-0.94 for the offset (Table 2) (Batschelet 1981; Jammalamadaka and Sengupta 2001; Zar 2010). Further, singing prevalence over a circadian cycle averaged at 8.26 ± 3.83h (mean ± S.D.; Table 2) and varied greatly across individuals (coefficient of variation, C.V. = 46%) (Appendix Chapter 3 Figures III-IV).

Regime	Temp (°C)	Onset (ZT)	Peak (ZT)	Offset (ZT)	V_m Onset	V_m Peak	V_m Offset	Singing prevalence
LD	22	13.24±2.33	18.11±2.24	23.42±2.33	0.34	0.32	0.34	8.16±4.85
	25	12.64±3.22	17.98±3.54	23.80±4.11	0.60	0.70	0.87	9.25±4.63
	28	13.38±1.57	18.90±1.41	23.82±2.16	0.17	0.13	0.30	9.31±3.99
DL	22	12.34±2.15	16.45±3.01	21.07±3.03	0.29	0.53	0.54	7.22±2.90
	25	11.82±4.11	17.50±4.06	22.67±4.30	0.88	0.86	0.94	7.94±2.86
	28	11.75±2.59	16.79±2.80	21.07±3.02	0.41	0.47	0.54	8.08±4.26

Table 2. Phase markers ("onset", "peak", and "offset", mean ZT ± S.D.), angular variances (V_m onset and V_m offset), and singing prevalence per day ("singing prevalence", the average number of minutes per day that a cricket sang at least once; mean h ± S.D) by temperature ("temp", °C) and regime (LD and DL) as determined in experiment 3.

3.5. Discussion

Our experiments verify circadian control of singing, and coupled with our quantification of between-individual variation in the timing of circadian parameters (i.e., onset, peak, and offset) and the marked variation in singing prevalence, highlight circadian singing rhythms as a potential target for both natural and sexual selection (Westwood *et al.* 2019). Specifically, as expected, crickets sing primarily during the dark phase (Fig. 2A), and singing free runs under constant environmental conditions (Fig. 3A-D), is entrainable by a Zeitgeber (light; Fig. 4A-C), has a periodicity of approximately 24h under entrained conditions (experiment 1: 24.4 ± 0.15h S.E. Fig. 2B and experiment 3: 24.72 ± 0.16h S.E., see Appendix Chapter 3 Figure II-IV for individual LS periodograms) and a slightly longer FRP (25.1 ± 0.079h S.E., Fig. 3A), and is temperature compensated under a biologically realistic range of temperatures under constant conditions (Fig. 3A). Further, we find no evidence for an influence of temperature on phase markers under entrained conditions (Fig. 5, Table 1), though each phase marker advanced upon phase shift by ~1-2h (Table 2).

We found that individuals vary in the quantity of time spent singing and in the timing of their rhythms (C.V. = 46%; Table 2). Further, *post hoc* correlation analyses reveal a moderate positive correlation between peak and singing prevalence (r = 0.53, p = 0.044), indicating that the further into the dark phase an individual peaks in singing, the greater their overall singing prevalence (see Appendix Chapter 3 Figure V). While onset was not significantly correlated with singing prevalence (r = -0.41, p = 0.13; Appendix Chapter 3 Figure V), it did show a slightly negative trend, whereas offset showed a slightly positive trend (r = 0.37, p = 0.2; Appendix Chapter 3 Figure V), possibly suggesting that a wider singing window (i.e., earlier onset and later offset) results in greater singing prevalence per day. This, coupled with the significant positive relationship between peak and onset, could suggest crickets experience a "warming up" period at the onset of singing (as found in bush crickets and katydids) (Josephson & Halverson 1971; Heller 1986).

Both normal-wing (singing) and flatwing (silent) males exhibit satellite behaviour in this species (i.e., behaviour in which non-calling males intercept females attracted to calling males; (Zuk et al. 2018; Zuk, Rotenberry, and Tinghitella 2006). As such, variation in singing prevalence between individuals may be indicative of an individual's propensity towards satellite tendencies versus commitment to singing. However, juveniles reared in conditions mimicking populations with high levels of singing males are less likely to exhibit satellite behaviour (Nathan W. Bailey, Gray, and Zuk 2010), and since our population contains ~50% singing males, satellite behaviour may not be as prevalent in our population as others showing very high proportions of flatwings (e.g. in Kauai, where upwards of 90% of males are flatwing) (Zuk *et al.* 2018). However, because singing is energetically costly (Prestwich & Walker 1981; Hoback & Wagner 1997; Hack 1998) and condition-dependent (Holzer, Jacot, and Brinkhof 2003; Hunt *et al.* 2004; Judge, Ting, & Gwynne 2008; Houslay *et al.* 2017), the marked individual variation may simply be a result of rearing environment, physiological condition, and/or stochastic developmental

trajectories. Interestingly, our observed mean nightly singing prevalence was much higher than previously reported for Hawaiian *T. oceanicus* (Kolluru 1999). However, Kolluru (1999) removed adult male crickets from the field and observed their singing in the laboratory under ambient lighting conditions, and thus differences in singing prevalence may be due to the likely poorer condition of wild crickets or disturbances from the data collection methods. Further, while the wild crickets collected by Kolluru (1999) were not age-controlled, all of the crickets in our experiments were placed into experimental conditions within 1-3 days of eclosion and so singing prevalence may reduce as individuals age.

We found that, in general, male *T. oceanicus* sing between ~ZT13 and ~ZT23, peaking ~ZT17.5. Our results support and develop those of Zuk, Simmons, & Cupp (Zuk, Simmons, and Cupp 1993) who observed that wild *T. oceanicus* sing primarily during the dark phase in the Hawaiian Islands. Further, they found that unparasitised *T. oceanicus* populations begin to sing earlier and continue singing later (i.e., they appear to have a wider singing window) than do the Hawaiian populations (Zuk, Simmons, and Cupp 1993). Crickets in our experiment rarely sang during the light phase (e.g., crickets in experiment 1 sang only during ~1% of the light phase whereas they sang ~68% of the dark phase), fitting with the notion that selection may have acted on singing rhythms such that individuals in parasitised populations reduce (or, have nearly eliminated) singing at "risky" times-of-day. Future work comparing these two populations from a circadian framework could elucidate the extent to which selection has resulted in temporally distinct circadian singing patterns.

We reveal that nocturnal singing is not simply a phenotypically plastic response to dusk/darkness, but is scheduled by an endogenous circadian clock. Clocks give their owners the ability to anticipate when day/night will occur and so, prepare in advance (Aschoff 1965). Anticipating night-time could be useful for coordinating rhythmic mating

behaviours between males and females (Werrer Loher and Orsak 1985) or for timing conspicuous singing behaviour when parasitism and/or predation risk is low (Zuk, Simmons, and Cupp 1993). Interestingly, mean onset (~ZT13) occurs about an hour past the start of the dark phase (ZT12) – a finding apparently in contrast with the anticipatory nature of circadian rhythms (though, in line with previous findings in the wild (Zuk *et al.* 2018). However, as our lighting system was either on or off (i.e. did not gradually change to mimic dawn and dusk), nuance in anticipation may have been missed. Another possibility is that anticipatory activities occur in advance of the onset of singing, such as a warm-up period or spermatophore production (Josephson and Halverson 1971; Werner Loher 1974; Heller 1986). Further work could ramp light intensity up and down to mimic dawn and dusk to pinpoint the relationships between onset and offset with dusk and dawn, and across the suite of reproductive behaviours crickets engage in.

Singing rhythms appear robust to a range of temperatures under free-running conditions (Fig. 3), and entrained conditions (Table 1, Fig. 5), though we did find slight evidence for a modest phase advance upon photoperiod reversal (Table 2), possibly due to prolonged transient cycles. The variation in temperature we exposed the crickets to (22-28°C) approximates the annual variation in temperature in Hawaii where monthly temperatures range from a mean low of 22.8°C to a mean high of 27.4°C (National Weather Service, National Oceanic and Atmospheric Administration, monthly summarised data (mean min-mean max°C) in Honolulu, HI from 1950-2021). Thus, our experiments examining temperature compensation represent ecologically relevant treatments, and suggest crickets regain the appropriate phase relationship to the Zeitgeber regardless of temperature. However, for some organisms (e.g. *Neurospora*, *Drosophila*, and mice) temperature can act as an additional Zeitgeber to light (Y. Liu et al. 1998; Sidote et al. 1998; Refinetti 2010). Imposing temperature cycles that align with or oppose light dark cycles could parse out the relative contributions of light and temperature as Zeitgebers,

and inform how organisms respond to e.g. climate change, especially in the face of additional selection pressures imposed by infection.

To characterise rhythms from continuous audio recordings, a vast quantity of data are generated that precludes manual scoring. Therefore, we also present a novel audio-to-circadian analysis pipeline, capable of extracting useful parameters from which to train machine learning algorithms, which can then process large quantities of data. The application of machine learning techniques towards bioacoustic analysis is gaining traction (Aide *et al.* 2013; L. Zhang *et al.* 2017) and our pipeline can be used for any sound-producing species, whether for circadian analysis or simply for detecting signal in noise. Further, the pipeline may be applied to organisms not typically considered to acoustically advertise, including the detection of vibrational signals recorded on contact microphones. This could be especially useful in investigating singing rhythms in flatwing males, who do not produce song per se (W. T. Schneider, Rutz, et al. 2018), but have been shown to exhibit singing prevalence similar to normal-wing males (Rayner, Schneider, & Bailey 2020). However, whether flatwing males stridulate consistently throughout the night and/or maintain the same phase relationship with light as do normal-wing males remains unresolved.

In summary, we demonstrate that singing rhythms in *T. oceanicus* meet all four requirements necessary to be deemed under the control of an endogenous circadian oscillator. Our findings are largely in agreement with past prevalences towards elucidating the timing of singing (Werrer Loher and Orsak 1985; Zuk, Simmons, and Cupp 1993; Kolluru 1999) with some interesting differences in observed singing prevalence. Our work adds to this literature by interrogating singing from a robust circadian framework, which is important to show that the phase relationship of a behaviour (upon which selection is likely to act) is indeed heritable and not simply a plastic response (i.e., a reactionary or "just in time" response) to the environment.

4. Chapter 4: Circadian shifts in singing in a parasitised population of crickets suggests temporal escape from infection

4.1. Abstract

Circadian clocks provide organisms with an elegant solution to organising daily activities with predictable rhythms in the environment. For acoustically advertising species, the timing of reproductive advertisement also affects the risk of parasitism. For Hawaiian *Teleogryllus oceanicus* crickets, "normal-wing" males (males who produce the ancestral calling song) sing to advertise to females, which also attracts the deadly, acoustically-orienting parasitoid fly *Ormia ochracea. T. oceanicus* males generally sing from dusk until dawn, but since fly phonotaxis peaks at dusk, we hypothesised that males may evade the fly by delaying singing activity until after dusk. Thus, "temporal escape" minimises infection risk while still allowing males to attract females for much of the night. To test whether the singing schedule of males exposed to fly-selection is consistent with temporal escape, we compared singing rhythms, and their circadian control, between normal-wing males from the Hawaiian Island Oahu to males from an ancestral, unparasitised population from Mangaia in the Cook Islands. We find that singing in both populations is nocturnal and consistent with control by a circadian clock. Multiple aspects of the temporal organisation of singing within the dark phase vary between the populations and in a manner consistent with temporal escape. Particularly, when entrained, Oahu males sing ~5% less (or, 2.5-fold less) during the two hours following darkness, and sing 7% more during the darkest part of night, than males from Mangaia. These population differences are also likely governed by circadian clocks, because whilst free-running, Oahu males are also 1.2-fold less likely to sing in subjective light that Mangaia males. Almost all circadian singing parameters are more variable in the Mangaia population particularly when free-

running (~2-4-fold and ~3-7-fold higher whilst entrained and free-running, respectively), suggesting stronger recent selection on the rhythms of Oahu males. While bottlenecks associated with colonisation of the Hawaiian islands as well as fly-driven selection could impact on the timing and variability of singing schedules between populations, the precise differences suggest that by restricting their singing activity to the night-time, Oahu males may be temporally escaping detection by the fly, providing a rare demonstration of how circadian clocks mediate the intersection between natural and sexual selection.

4.2. Introduction

Parasites are ubiquitous in nature and the strong selective pressures they exert has led to the evolution of myriad host defensive strategies (Wilson & Cotter 2013). Most research on how hosts combat infection centres around immune responses which are a potent defence against parasitism. However, because immune function is costly and often acts as either a secondary or tertiary defence (Schmid-Hempel 2003; Cotter *et al.* 2008), primary defence strategies (such as certain behaviours and morphologies) are important, often lower-cost components, of host defence (Elliot & Hart 2010; Barbosa & Caldas 2007; Hart 1994). The evolution of such strategies necessarily depends upon the mode in which the host is detected by the parasite; for example, the parasitoid Tachinid fly *Ormia ochracea* listens in on male Pacific field crickets (*Teleogryllus oceanicus)* as they sing to attract mates (Sabrosky 1953). Acoustic-orientation by female *O. ochracea* is facilitated by their finely tuned hearing organs that more closely resemble those of Orthopterans than of other flies (W. Cade 1975).

In Hawaii, where both the Pacific field cricket and the fly are introduced (Tinghitella *et al.* 2011; Eldredge & Evenhuis 2003; X. Zhang *et al.* 2021), the lethal infection caused by *Ormia ochracea* has selected for the evolution of "flatwing" males that are morphologically incapable of producing sound (Zuk, Rotenberry, and Tinghitella 2006), and a suite of other phenotypes which produce sound distinct from the ancestral "normal-

wing" song (e.g., curly wing, small wing, and purring phenotypes; (Pascoal *et al.* 2014; Tinghitella *et al.* 2018; Rayner *et al.* 2019). However, despite the cost of infection being certain death, normal-wing singing males persist in Hawaiian populations, at least in part, because they are more attractive to females than divergent phenotypes with altered songs (Tinghitella *et al.* 2021). This illustrates an evolutionary challenge for acoustically advertising hosts; namely, the host must simultaneously balance the benefits of reproduction against the costs of threats to survival (W. Cade 1975). The fluid phenotypic makeup of *T. oceanicus* populations throughout the Hawaiian Islands represents a species in evolutionary flux (Tinghitella *et al.* 2021) – rapidly evolving morphological defence strategies which have yet to reach stable frequencies with each other and with normal-winged males. However, whilst novel morphologies have attracted much attention, the potential for the evolution of defences in normal-wing males, that have less impact on mating success than novel morphologies, has been largely overlooked (Westwood *et al.* 2019).

Male *T. oceanicus* are nocturnal, singing between dusk and dawn (Westwood *et al.* in prep Chapter 3; (Zuk, Simmons, and Cupp 1993). *O. ochracea* phonotaxis (i.e., attraction to sound) to cricket song is greatest around dusk, presumably because this is when audio, and potentially also visual cues, are both available for host seeking (Walker 1993). Extensive playback experiments reveal Hawaiian *O. ochracea* are most responsive to *T. oceanicus* singing around sunset and steadily become less responsive in the hours approaching dawn (Fig. 1, adapted from (Kolluru 1999). For example, unlike at dusk, *O. ochracea* is not attracted to broadcast song between the hours of 03:00 HST (approx. ZT21) and dawn. Similarly, *O. ochracea* are most attracted to *Gryllus integer* calling song around dusk, and markedly decrease responsiveness in the hours preceding dawn (W. H. Cade, Ciceran, and Murray 1996; William Cade 1979). However, whilst the study by Kolluru (1999) is the most extensive, fly phonotaxis rhythms are unresolved (William Cade 1979; Zuk, Simmons, and Cupp 1993).

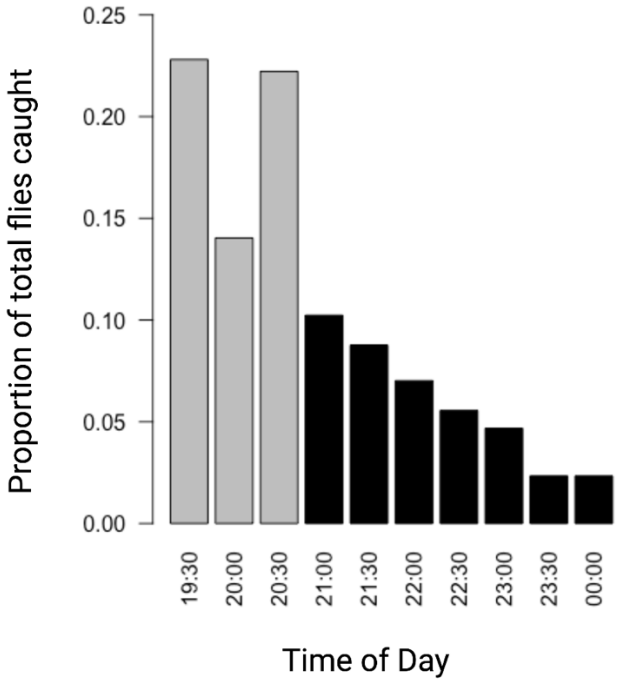


Figure 1. Reproduced and adapted from (Kolluru 1999). Proportion of Hawaiian *O. ochracea* attracted to synthesized normal-wing *T. oceanicus* calling song across 28 broadcast nights. Sunset and sunrise occurred at approximately 19:00 and 06:00 (HST) respectively. Gray bars highlight the greatest attraction of *O. ochracea* to male cricket song in the two hours following sunset.

Given that the risk of parasitism risk is highest during the hours around dusk, avoiding singing during this window may enable normal-wing males to evade infection via "temporal escape". Indeed, observations suggest the singing rhythms of normal-wing males from the Big Island of Hawaii differ from those of ancestral, unparasitised populations (Zuk, Simmons, and Cupp 1993). Here, we test whether the singing rhythm of normal-wing male *T. oceanicus* from Oahu, where there has been intense fly driven selection, is consistent with the evolution of a "temporal escape strategy" (Westwood *et al.* 2019). Similar to temporal escape, "temporal segregation" is common in acoustically-

signalling species (Greenfield 1997). Temporal segregation of songs avoids acoustic interference by sympatric heterospecifics, which may affect female recognition of suitable mates (or, indeed, a female's ability to locate mates). For example, crickets of the genus *Laupala* (sympatric species *L. cerasina* and *L. paranigra*) exhibit different timing for singing (and thus mating) (Danley *et al.* 2007), and male *Neoconocephalus spiza* katydids switch from nocturnal to diurnal singing patterns when the presence of sympatric congenerics is high (Greenfield 1988). Thus, ecological interactions between species are capable of shaping singing rhythms.

The extent to which temporal escape from parasitism (and temporal segregation to avoid mating interference) can change singing rhythms may depend on how reproductive activities are scheduled. We have recently demonstrated that an endogenous circadian oscillator schedules singing by male *T. oceanicus* (Westwood *et al.* in prep; Chapter 3). Namely, singing entrains to a Zeitgeber (light), free-runs under constant conditions with ~24h periodicity and is temperature compensated over a biologically realistic range of temperatures. How timing information from this endogenous oscillator is translated to singing activities is unknown, but could be subject to selection, whereas the clock itself must maintain accurate time-keeping because it schedules rhythms in many fitness-traits (Tomioka 2014). Due to the need for clocks to keep time, in certain circumstances, clocks are simply over-ridden; when it is cold and food is scarce, nocturnal rodents cannot afford the energetic costs of foraging at night and switch to diurnal activity, which enables them to conserve energy by remaining in a warm burrow during the coldest part of the circadian cycle (Hut *et al.* 2011; van der Vinne *et al.* 2014).

Coming from a largely aseasonal environment, it is unlikely *T. oceanicus* has required a flexible schedule over its evolutionary history. Thus, timing changes are most likely to occur by selection changing the relationship between the phase angle of the clock and singing. Indeed, our experiments suggest *T. oceanicus* from Oahu exhibit a condensed

singing schedule, singing less during the light part of the circadian cycle and during the first part of the night, than males from an ancestral population that has not experienced selection by *O. ochracea*. Encountering a novel parasite (the fly, *O. ochracea*) is only one of many environmental changes *T. oceanicus* has likely encountered during its colonisation of the Hawaiian islands, but our results suggest temporal escape could explain what appears to be directional and stabilising selection on circadian control of singing rhythms of Hawaiian males.

4.3. Methods

Animals, rearing and experimental conditions

Crickets from the Hawaiian island of Oahu were taken from laboratory stock populations established in 2012 from females collected in Lai'e, Koolauloa District, in the NE Oahu (21°38′55″N 157°55′32″W; (W. T. Schneider, Rutz, et al. 2018). Rearing conditions are described in N. W. Bailey and Macleod 2014 and are the same as those described below with only a minor discrepancy (these crickets were housed in 16L plastic boxes). At the time of establishment, approximately 50% of males in the population expressed the flatwing phenotype, reflecting substantial parasite pressure. For the purpose of this experiment, we excluded flatwing males and hereafter, "adult male" refers to the normal-wing phenotype. T*. oceanicus* is endemic throughout the Cook Islands, including Mangaia. Mangaia crickets were sourced from a colony initiated in 2019 from eggs laid by females from populations on Mangaia (kindly donated by Marlene Zuk, University of Minnesota). Neither *O. ochracea* nor the flatwing phenotype have been recorded in Mangaia (Heinen-Kay *et al.* 2019).

Colony rearing conditions consisted of groups of ~20 individuals housed in clear 9L plastic boxes with egg carton for shelter, *ad libitum* Burgess™ Excel Junior and Dwarf rabbit pellets for food, and water, at 25°C and in a 12h Light:12h Dark (lights-on at 06:00 UTC and lights-off at 18:00 UTC; LD), photoschedule. Males were removed from rearing

conditions and placed into experimental conditions within three days post-eclosion to standardise age and mating status. We individually housed males from Mangaia (n=11) and Oahu (n=9) in 9L plastic boxes with food and water as described for rearing conditions. Each cricket was kept in its own incubator (Panasonic MIR-154-PE Cooled Incubators or LEEC SFC3C R/H Ultrasonic Humidity Cabinets) for the duration of the experiment to ensure acoustic-isolation of each male.

Audio recordings

Following Westwood *et al.* in prep (Chapter 3), continuous audio recordings were made for each individual male using Sony™ ICD-UX560 Digital Voice Recorders equipped with Integral™ Micro Secure Digital eXtended Capacity cards) set to a sampling rate of 44.1 kHz and a 16-bit resolution (stereo MP3 file format) and connected to an external power supply cord. To ensure males did not physically interfere with the recorders, we secured them to the inside wall of the box using a strip of Velcro®. Once recordings were complete, we transferred the audio files to external hard drives (Western Digital 4TB Elements Portable Hard Drive – USB 3.0) and subsequently analysed recordings on both a personal computer (2017 Apple MacBook Pro using macOS Catalina) and through the University of Edinburgh's high performance compute cluster.

Detecting singing in continuous audio files

In a process called stridulation, *T. oceanicus* males rhythmically open and close their forewings, rubbing sound-producing structures against each other (i.e., the "file" and the "scraper") and producing their characteristic song (Pfau & Koch 1994). Singing bouts span several seconds to minutes (W. T. Schneider, Rutz, et al. 2018) and a bout is comprised of a long chirp followed by a series of short chirps with a dominant frequency range of 4-5kHz (Balakrishnan & Pollack 1996). We first segmented each continuous audio file into sequential 60s "clips". We then extracted 19 audio features (e.g., descriptive statistics of the frequency in the 3-6 kHz spectrum; Appendix Chapter 4 Table I) (Sueur, Aubin, &

Simonis 2008) which were iteratively pared down to five features which returned a high level of accuracy on the training set (Appendix Chapter 4 Table I). Using these, we generated a random forest model with >98% accuracy to produce a dataset containing to-the-minute resolution of whether or not each cricket sang at any point during each clip, for the entirety of the experiment (i.e., ~662,400 minutes continuous audio total) (Westwood *et al.* in prep; Chapter 3). Note, that this model detects whether or not a cricket sang for any amount of time during a given 60s audio clip, not how long it was singing for during each clip. Thus, each clip for each individual is assigned either a 0 (singing did not occur at any point during the clip) or a 1 (singing did occur at some point during the clip) and we termed this metric "singing minutes"; note, this parameter reflects the occurrence rather than the duration of singing throughout clip. For example, for a treatment group, a mean value of 0 reflects that no males sang in a given clip, 0.5 indicates half of the males sang at least once in that clip, and a value of 1 is returned if all males sang during that clip. As well as comparing "singing minute" values between the populations we also calculated "singing prevalence" as the sum of the number of minutes during which a given male sang (i.e., total clips when singing = 1) relative to the number of minutes throughout a window of interest, or relative to the total number of minutes a male sang during its average circadian cycle in a particular photoschedule.

Experimental design

We made continuous audio recordings whilst each cricket spent multiple days cycling through three sequential photoschedules. First, crickets were placed in incubators (Fig. 2A) held constant at 25°C (+/- 1C) with a photoschedule mirroring rearing conditions (LD) for 9 days (Days 0-8 on Fig. 2B). We used this condition to parameterise singing rhythms and singing prevalence in entrained conditions. Second, we reversed the photoschedule at ZT0 (DL) on day 9 for 6 days (Days 9-14 on Fig. 2B) to verify that singing entrains (i.e., its timing becomes aligned) to the timing of the new light:dark cycle. Third, we released the males into free-running conditions (constant darkness; DD) at ZT0 on day 15 for 8

days (Days 15-22 on Fig. 2B) to parameterise singing rhythms and singing prevalence in constant conditions. The focus of the DL and DD regimes were to probe circadian clock control of singing rhythms across the populations (Pittendrigh 1960). Males were recorded for longer during the initial LD photoschedule than in DL and DD to allow for initial acclimation to incubator conditions following rearing conditions.

We predicted that, compared to males from Mangaia (the ancestral population), males from Oahu should avoid singing during the daily window when infection risk is highest; which certainly includes dusk (Kolluru 1999) and may also include dawn (Zuk, Simmons, and Cupp 1993). If males from Oahu exhibit a symmetrically condensed singing window, we do not expect the timing of peak singing to differ to the peak for males from Mangaia, but if Oahu males avoid either dawn or dusk, then their peak of singing may be earlier or later (respectively) than Mangaia males. We also expected that a restricted singing window for Oahu males might result in singing occurring during a smaller number of hours throughout the circadian cycle (i.e. a lower overall singing prevalence) compared to males from Mangaia. Because males from both populations experience the same 24h photoschedule in the wild, we did not expect the duration (period) of singing rhythms to vary between the populations in LD entrained conditions, but their free-running periods in constant conditions could differ. If any population differences in the characteristics of singing rhythms are underpinned by endogenous circadian clocks, we expected to observe the same qualitative patterns in both entrained (LD) and constant (DD) conditions and a gradual inversion of rhythms over several days when the photoschedule switches from LD to DL.

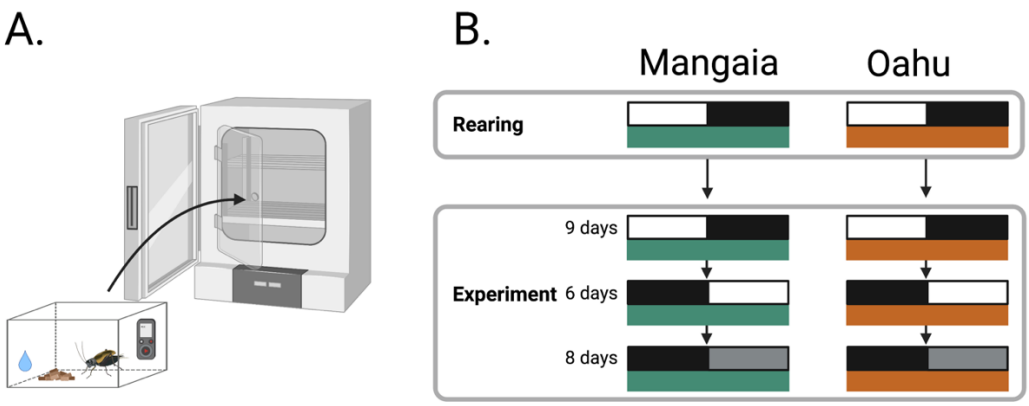


Figure 2. A. Individual males were housed in clear plastic boxes with shelter and food/water *ad libitum* and placed into separate incubators for recordings. Audio recorders were adhered to the inside of each box and collected continuous recordings for the duration of the experiment. **B.** Continuous audio recordings were made for males from the Oahu and Mangaia populations throughout several circadian cycles of exposure to the following photoschedules: LD (white and black bar), DL (black and white bar), free-running conditions (DD; black and grey bar) where black represents subjective night and grey represents subjective day.

Statistical and circadian analysis

Prior to analyses, data from all individuals for the first 48h after entering the experiment were removed from analyses to allow for incubator acclimation, and data for the first 48h after the transition to DD were removed to account for transient cycles (i.e., the time it takes an overt rhythm to reach a stable phase relationship with the central clock; (W. Cade 1975). We used all data from the remaining circadian cycles of each time series in LD and DD to estimate the characteristics of the singing rhythm for each male over an average circadian cycle. The characteristics of entrained singing rhythms were estimated from LD rather than DL data because there were too few circadian cycles in DL following entrainment to accurately estimate parameters. One individual died on day 14 (during DL) for the Mangaia population and one on day 21 (during DD) for the Oahu population.

Neither individual exhibited unusual rhythms prior to death so were retained in the dataset until death. Another individual from the Oahu population did not sing for the entirety of its recording from all photoschedules, and so was excluded from the data set.

Estimating period, rhythm parameters, and variation

Derivation of phase markers (onset, peak, and offset) were obtained using ClockLab software (ActiMetrics, Wilmette, IL, USA), and all other circadian analyses in R were performed using the Rethomics workflow (Geissmann *et al.* 2019). We calculated the entrained period for each male during the LD photoschedule, and their free-running period (FRP) during constant conditions (DD) using chi-square periodogram analyses. The significance threshold was set to $p<0.05$ and the most significant period estimates for each male in each condition were used for analyses.

Three males from the Mangaia population were excluded (2 from the LD and 1 from the DD periodogram analyses) because their data did not return a significant period estimate (see Appendix Chapter 4 Figures I-II for individual periodograms for each photoschedule). Not every individual sang every day to a degree in which phase markers were detectable; these individuals were retained in the dataset, and phase markers were calculated only for those days in which they could be confidently estimated. Mean phase markers were calculated for photoschedules and populations via circular means, and individual variation in phase markers was estimated via angular variances (V_m) (Jammalamadaka & Sengupta 2001).

Singing during different parts of the circadian cycle

As well as comparing timing parameters between populations we further characterise singing rhythms by testing whether singing prevalence varies during the light versus dark phase of the circadian cycle. Assigning singing to light or dark is straightforward in entrained conditions but more complex in free-running conditions. To make analogous

comparisons for singing in subjective "light" and "dark" phases we estimated the subjective "lights-on" and "lights-off" for each male by applying its free running period to the timing of lights-on and lights-off based on the previously experienced DL photoschedule. For example, because constant darkness began at ZT0 (on day 15), we estimated that subjective "lights-off" during "day 15" occurs after the half of a males' FRP, and subjective "lights-on" on "day 15+1" occurs after a full FRP, then the next subjective "lights off" occurs half the FRP following subjective "lights-on", etc (calculations for determining subjective dusk and dawn can be found in Appendix Chapter 4 Equation I).

Further, to investigate population differences in singing prevalence during different parts of the night, we quantified singing during the first two hours after lights-off as a proxy for "dusk" (ZT12-13) or before lights-on as a proxy for "dawn" (ZT22-23), and during a proxy for the darkest part of the night ("darkest"; ZT14-21) for LD entrained conditions. Again, we repeated this for free-running conditions (using timing relative to subjective "lights-off" and "lights-on") to probe for clock control of these patterns. Multiple circadian cycles within each photoschedule are condensed to generate an average circadian cycle for each photoschedule by determining their circular means.

Modelling

We used R v1.1.463 (R Core Team 2020) for all analyses except for the workflow (Geissmann *et al.* 2019). We compared period estimates, singing minutes, singing prevalence, and rhythm parameters, across populations and photoschedules separately by comparing competing models. To compare singing prevalence between populations and photoschedules we employed a Kruskal-Wallis test (Kruskal & Wallis 1952). Generalised linear mixed models were compared using the "Akaike information criterion - corrected" (Hurvich & Tsai 1993). AICc was used to avoid overfitting due to small sample sizes, and a change in 2 AICc (ΔAICc=2) was chosen to distinguish between models. Effects of photoschedule and population were modelled using Poisson error family, and to

control for variation between males in singing prevalence across the circadian cycle, we apply a log-transformed "offset" term to each model (note - this is distinct from circadian offsets) and fit “ID” and “observation” as a random effect to account for overdispersion. To account for circular data, phase markers (onset, peak, and offset) were modelled via Bayesian projected normal circular regression and compared using the “Watanabe-Akaike information criterion” (Watanabe and Opper 2010) with a ΔWAIC=2 selected to distinguish between models.

4.4. Results

Singing rhythms in both populations are consistent with circadian control

Visual inspection of double-plotted actograms of singing rhythms throughout the experiment (Fig. 3), verify nocturnality for singing rhythms in both Oahu and Mangaia males. Specifically, following photoschedule-reversal from LD to DL (red arrow), singing entrains, and following release into free-running conditions from DL to DD (blue arrow), singing rhythms persist but with a phase delay (i.e., the onset and offset of the rhythm are delayed each day; Fig. 3).

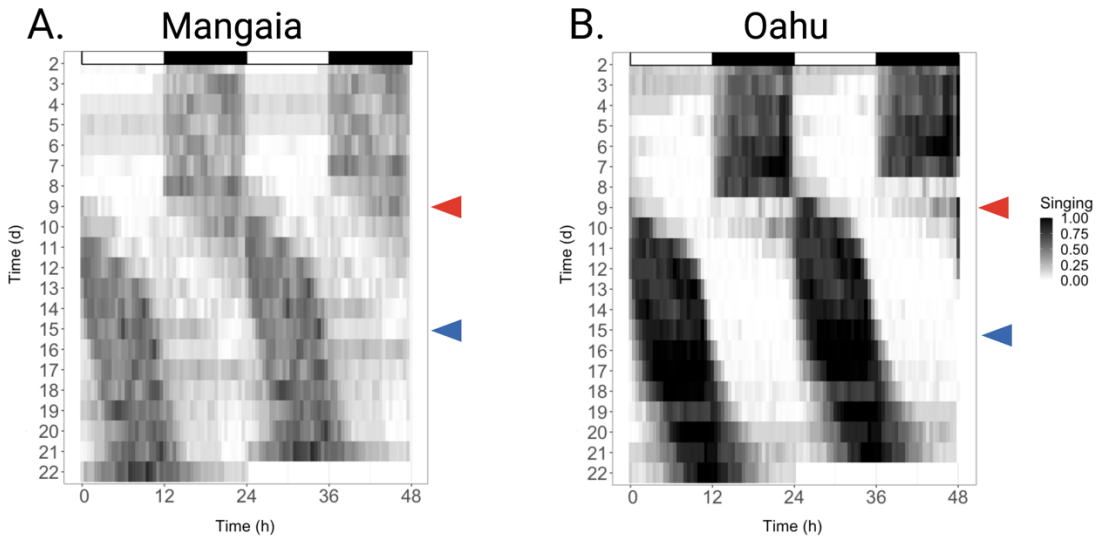


Figure 3. Average-double plotted actograms for each population, **A.** Mangaia and **B.** Oahu. The y-axis follows the duration of the experiment from top to bottom, and the x-axis

denotes time of day. The legend on the right indicates the mean number of individuals that sang (1) or not (0) during a given minute (0-1; "Singing"). For example, a value of 0.5 indicates half of all individuals in that population were scored as "singing" for that minute. White and black bars on top of each plot indicate the initial LD photoschedule, with the first 48h removed (i.e., days 0 and 1) for acclimation to experimental conditions, photoschedule -reversal (DL; red arrows) occurred at ZT0 on day 9 (red arrows), and free-running conditions (DD) began at ZT0 on day 15 (blue arrows).

A clock-controlled rhythm exhibits a duration close to ~24h in entraining light-dark schedules as well as a duration within a few hours of 24h when free running in constant conditions. Thus, we estimated period during entrained (LD) and free-running (DD) conditions (Table 1, Fig. 4).

Photoschedule	Population	Period Estimate
LD	Mangaia	24.1 ± 0.17
	Oahu	24.0 ± 0.011
DD	Mangaia	24.8 ± 0.76
	Oahu	24.9 ± 0.33

Table 1. Chi-square period estimates ("Period Estimate", mean ± S.E. in hours) for both populations ("Population") in entraining and free running conditions (LD and DD).

As expected, photoschedule drove differences between the observed period (ΔAICc = 0; Table 2). Namely, the FRP was on average, ~44min (i.e. 0.735h, 95% C.I. [0.397, 1.43]) and ~56min (i.e. 0.938h, 95% C.I. [0.688; 1.15) longer for Mangaia and Oahu males, respectively, than their entrained periods (Table 1). Furthermore, the addition of "population" to the model including only "photoschedule" decreased model fit (ΔAICc = 2.55) and the addition of the interaction further reduced fit (ΔAICc = 4.85). Thus, population differences are unlikely to contribute any biologically relevant variation in

entrained and free-running periods. Taken together, the actograms (Fig. 3) and period estimates indicate clock control of singing in both populations supporting Westwood *et al.* (in prep; Chapter 3).

Model description: Period estimate ~	df	log(*L*)	AICc	ΔAICc	AICc *w*
Photoschedule	3	-17.87	42.57	0.00	0.73
Photoschedule + population	4	-17.84	45.12	2.55	0.20
Photoschedule *population	5	-17.60	47.42	4.85	0.06
Null	2	-29.35	63.10	20.53	0.00
Population	3	-29.31	65.45	22.88	0.00

Table 2. Degrees of freedom (df), log-Likelihood (log(*L*)), AICc, ΔAICc ($AICc_{model} - AICc_{min\ model}$), and AICc *w* (AICc weight) for each linear model ordered in descending fit (best-fitting model at the top and models competitive with the best are bolded and italicized). The response variable for each model is "period estimate", "photoschedule" corresponds to 12L:12D (entrained) or 12D:12D (free-running), and "population" refers to the Oahu and Mangaia populations.

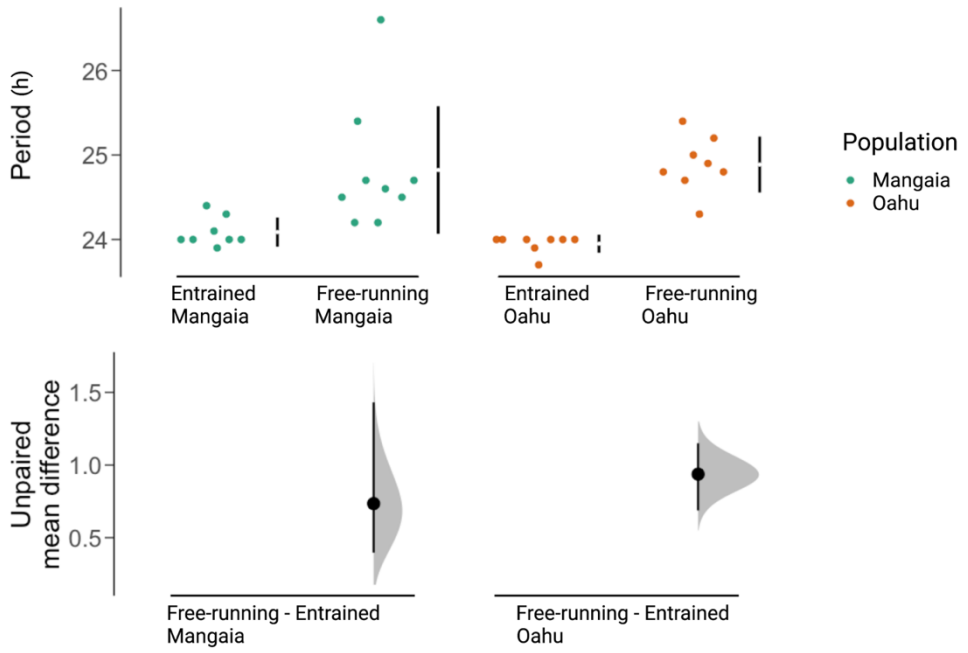


Figure 4. Cumming estimation plot for the entrained (LD) and free-running (DD) period estimates for each population (Mangaia and Oahu; x-axis, top row). On the top row, points represent individuals and are coloured by population (Mangaia=blue-green, Oahu=dark orange), and the mean ± S.D. is displayed as the vertical line with the gap indicating the mean. On the bottom row, the unpaired mean differences in period (or, FRP) for each population are shown. The black dots indicate the unpaired mean differences ("Unpaired mean difference", y-axis), and the 95% C.I. computed through bootstrap resampling is displayed as the grey graded sampling distribution.

Singing prevalence varies between populations

Oahu males sang at least once per minute for a total of ~ 9h 40m during an average circadian cycle in the entrained (LD) photoschedule and singing prevalence increased to ~10h 10m per cycle when free-running (in DD). In contrast, Mangaia males sang at least once per minute for ~5h during an average entrained circadian cycle, which increased to ~7h 55m per cycle when free-running. Note, that singing prevalence reflects how often, not necessarily how much, males sang. For example, given the different singing

prevalences observed in LD, overall singing effort during a circadian cycle could be equal if Mangaia males sing during approximately 10-fold more seconds per clip than Oahu males (i.e. if Oahu males sing for ~5s per clip and Mangaia males for ~50s).

The population estimates for singing prevalence suggest that during LD, Oahu males sang during almost twice as many minutes as Mangaia males, however, this was less pronounced during DD where Oahu males sang only 1.25-fold more often than Mangaia males. This difference was driven by a non-significant trend for Mangaia males to sing more during DD (mean entrained singing = 4.17 ± 4.27 S.D. and mean free-running singing = 5.63 ± 4.38 S.D.; Kruskal-Wallis chi-squared = 1.60, df = 1, p-value = 0.205; Fig. 5). Oahu males maintained the same level of singing in both photoschedules (Kruskal-Wallis chi-squared = 0.011029, df = 1, p-value = 0.916). However, despite mean singing prevalence per circadian cycle differing between the populations by several hours in both entrained and free-running conditions, these differences returned borderline statistical significance (unpaired mean difference for LD = 4.16 hours, 95% C.I. [-0.035, 8.02]; unpaired mean difference for DD = 2.86 hours, 95% C.I. [-0.495, 5.69]; Fig. 5).

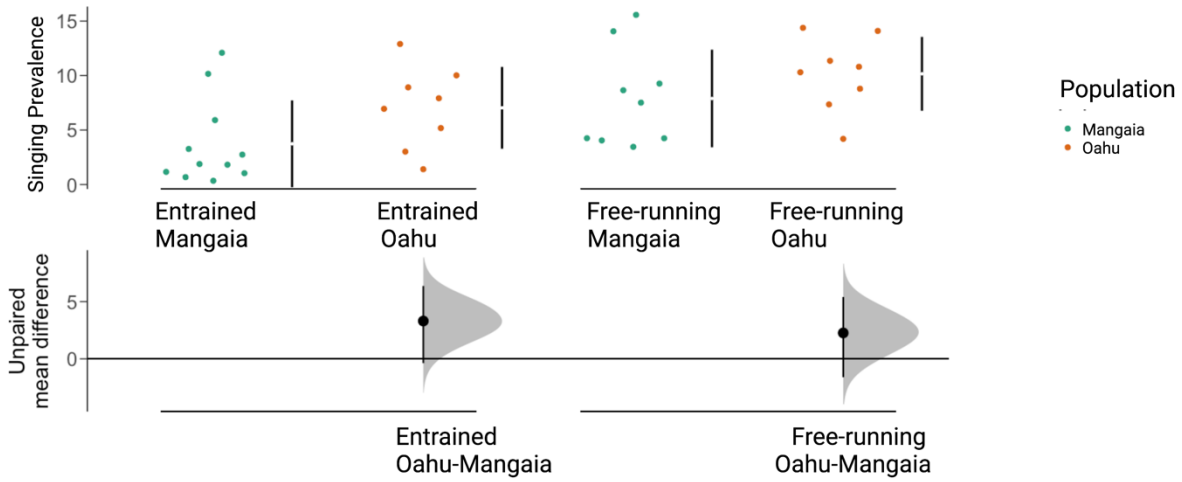


Figure 5. Cumming estimation plot for "Singing prevalence" (y-axis, top row) under entrained (LD) and free-running (DD) photoschedules for each population ("Mangaia" and "Oahu"; x-axis, top row). Singing prevalence is recorded as the number of hours within an average 24h cycle during which singing was recorded for each male. On the top row, points represent individuals and are coloured by population (Mangaia=blue-green, Oahu=dark orange), and the mean ± S.D. is displayed as the vertical line with the gap indicating the mean. On the bottom row, the "Unpaired mean difference" in "Singing prevalence" between populations for each photoschedule are shown (black dots). The black dots indicate the "unpaired mean difference" (y-axis), and the 95% C.I. computed through bootstrap resampling is displayed as the grey graded sampling distribution.

Singing schedule is driven by phase and may vary between populations in DD

Due to the strong trend for Oahu males to sing throughout more of the circadian cycle than Mangaia males, all subsequent analyses control for variation in singing prevalence exhibited by each male and so, we compare relative levels of singing across different times of day, within and between populations. To begin characterizing the singing schedule of each population we tested whether singing during the light and dark phases of the circadian cycle in entrained and free-running conditions differed. The best fit model for both photoschedules included only phase (ΔAICc = 0.00 each; Table 3) but the model including the interaction of phase and population was competitive under free-running conditions (ΔAICc = 1.81; Table 3). We find that while males in both populations sang less in the light (/subjective "light") than the dark (/subjective "dark") phases, this difference was much more apparent in entrained versus free-running conditions (55- and 4-fold fewer singing minutes in the light/subjective "light", respectively; Fig. 6). Furthermore, while singing levels in the dark and subjective "dark" phases vary little between populations and photoschedules, singing levels in the light and subjective "light" phases trend towards Mangaia increasing singing relatively more (3.6-fold) than Oahu (2.8-fold)

males (log(marginal mean difference) Mangaia-Oahu = 0.75 ± 0. 0.37 S.E., p =0.1005; Fig. 6).

Response	Covariates	df	log(*L*)	AICc	ΔAICc	AICc *w*
Entrained singing (LD) ~	***Phase***	4	-266.24	546.9	0.00	0.75
	Population+phase	5	-266.23	544.34	2.66	0.20
	Population*phase	6	-266.22	547.16	5.47	0.05
	Null	3	-344.25	694.95	153.27	0.00
	Population	4	-344.25	697.27	155.58	0.00
Free-running singing (DD) ~	***Phase***	4	-268.78	546.94	0.00	0.60
	Population*phase	6	-266.82	548.74	1.81	0.24
	Population+phase	5	-268.71	549.55	2.62	0.16
	Null	3	-395.97	798.46	251.53	0.00
	Population	4	-395.84	800.56	253.63	0.00

Table 3. Degrees of freedom (df), log-Likelihood (log(*L*)), AICc, ΔAICc ($AICc_{model} - AICc_{min\ model}$), and AICc *w* (AICc weight) for each generalized linear model ordered in descending fit (best-fitting model at the top and models competitive with the best are bolded and italicized). The response variable for each model is "singing", "phase" corresponds to either the "light" or "dark" phase, and "population" refers to Oahu or Mangaia.

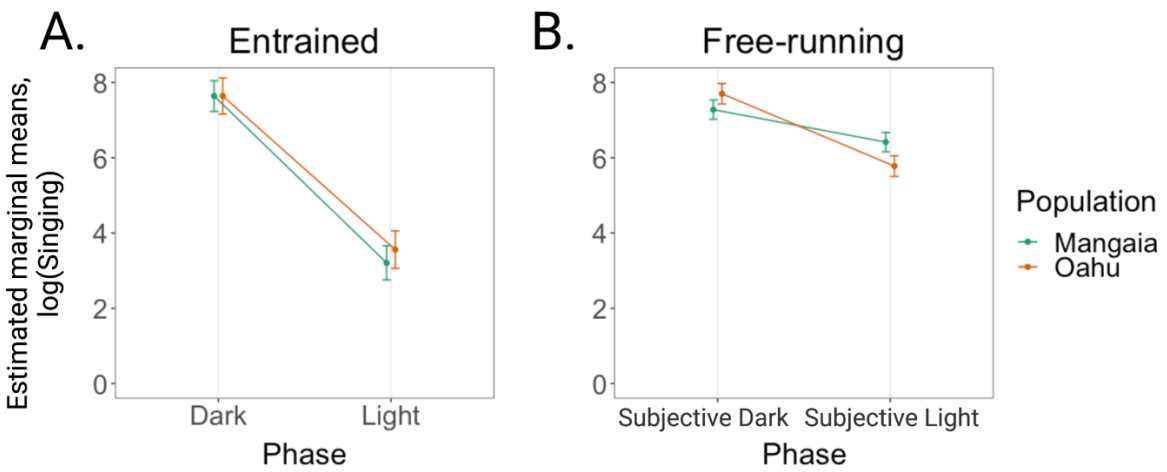


Figure 6. Model estimated marginal means of the log-transformed singing during the **A.** entrained and **B.** free-running photoschedule. Phase is shown on the x-axes, colours indicate population (Mangaia=blue-green, Oahu=dark orange) and vertical lines are S.E.

Singing levels during different parts of the night

Given that most singing occurs during the dark phase and risk of infection by *O. ochracea* is likely to be highest around dusk and lowest around dawn (Kolluru 1999), we next compared whether the populations exhibit different levels of singing during different parts of the night (Fig. 7).

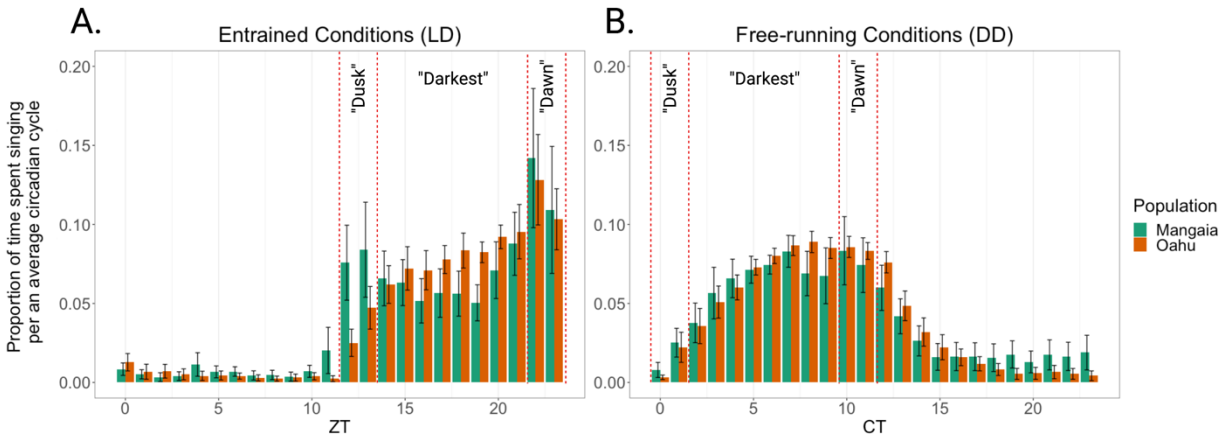


Figure 7. Bar plot showing the mean ± S.E. proportion of time singing during each hour for an average circadian cycle under **A.** entrained (time shown in Zeitgeber time, ZT; x-axis) and **B.** free-running conditions (time shown in circadian time, CT; x-axis). Colours in the legend indicate population (Mangaia=blue-green, Oahu=dark orange). The first set of red dashed lines surround the two ZT hours approximating "dusk" (ZT12-13; the first two hours of lights-off), the second set of red dashed lines surround the two ZT hours approximating "dawn" (ZT22-23; the last two hours of lights-off), and the space between (from "dusk" until "dawn") represent the "darkest" hours of the night (ZT14-21). Prior to release into DD, males were entrained to DL, and so rhythms in DD appear reversed to

those whilst entrained to LD.

We tested whether singing differed between the populations during different parts of the night (using our proxies for dusk, darkest part of the night, and dawn; "bin" in Table 4; Fig. 8). In entrained conditions, the model containing the interaction between "population" and "bin" was the best fit (ΔAICc = 0.00; Table 4). We found that while males from Mangaia undertake 85.2% of their singing during the dark phase, Oahu males undertake relatively more (90.8%) (Fig. 8B). Specifically, Mangaia males sang 2.41-fold (or 4.5%) more at "dusk" compared to Oahu males, though this was of borderline statistical significance (log(marginal mean difference) Mangaia-Oahu = 0.876 ± 0.459 S.E., p = 0.058; Fig. 8A-B). In contrast, this pattern reversed during the "darkest" hours, during which Oahu males sang 1.9-fold (or 7%) more than Mangaia males (log(marginal mean difference) Mangaia-Oahu = -0.661 ± 0.273 S.E., p=0.016; Fig. 8A-B). Finally, during "dawn", although there was a 3% difference between population singing prevalences, this was not significant (log(marginal mean difference) Mangaia-Oahu = -0.515 ± 0.446 S.E., p=0.250; Fig. 8A).

Under free-running conditions, the model containing only "bin" was the most parsimonious and best fit, and while the model with both "bin" and "population" as main effects was competitive (ΔAICc = 1.51; Table 4), it was 36% less likely to be the best approximating model (AICc *w*= 0.31 compared to AICc *w*= 0.67 for the full model; Table 4). Accordingly, post hoc comparisons reveal both populations sang in approximately the same manner across subjective "dusk", "darkest", and "dawn" whilst free-running ("dusk" log(marginal mean difference) Mangaia - Oahu = -0.21 ± 0.80 S.E., p = 0.787; "darkest" log(marginal mean difference) Mangaia - Oahu = -0.52 ± 0.73 S.E., p = 0.482; "dawn" log(marginal mean difference) Mangaia - Oahu = -0.582 ± 0.74 S.E., p = 0.434). Thus, while population may help to explain a small but non-significant amount of variation, the main driver of variation in singing across the dark phase under free-running conditions is "bin".

Response	Covariates	df	log(*L*)	AICc	ΔAICc	AICc *w*
Dark phase singing during LD ~	***Bin*population***	8	-1346.06	2708.78	0.00	0.93
	Bin+population	6	-1351.32	2715.03	6.25	0.04
	Bin	5	-1352.56	2715.39	6.61	0.03
	Population	4	-1359.86	2727.90	19.12	0.00
	Null	3	-1361.14	2728.39	19.62	0.00
Subjective "dark" phase singing during DD ~	***Bin***	5	-349.86	711.06	0.00	0.67
	Bin+population	6	-349.33	712.57	1.51	0.31
	Bin*population	8	-349.27	717.96	6.90	0.02
	Null	3	-373.46	753.44	42.38	0.00
	Population	4	-373.22	755.31	44.26	0.00

Table 4. Degrees of freedom (df), log-Likelihood (log(*L*)), AICc, ΔAICc ($AICc_{model} - AICc_{min\ model}$), and AICc *w* (AICc weight) for each generalized linear model ordered in descending fit (best-fitting model at the top and models competitive with the best are bolded and italicized). The response variable for each model is the time spent singing during the dark (or subjective "dark") phase for entrained and free-running conditions, respectively. Covariates are "bin" (i.e., for LD: "dusk" (ZT12-13), "darkest part of the night" (i.e., "darkest", ZT14-21), or "dawn" (ZT22-23); and the subjective/free-running equivalents calculated for each male), and "population" refers to Oahu or Mangaia.

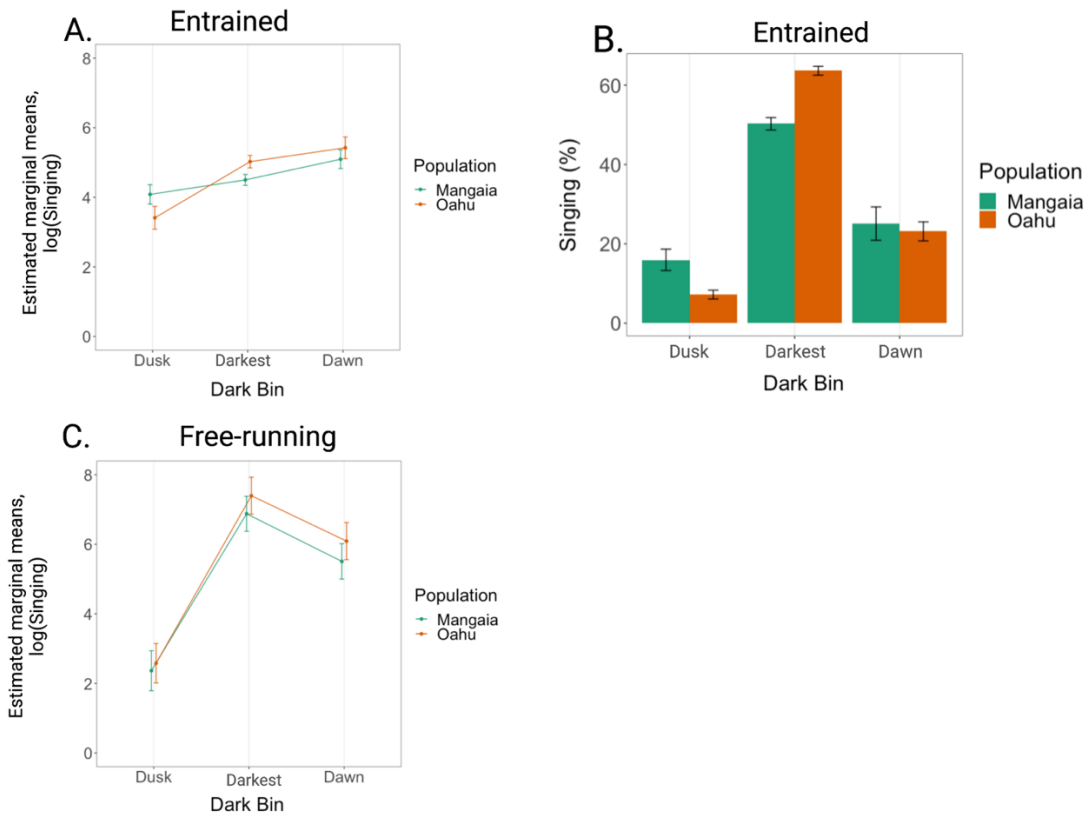


Figure 8. **A.** Model estimated marginal means of the log-transformed dark phase singing during LD, **B.** distribution of singing prevalence during the night for an average circadian cycle in LD, and **C.** model estimated marginal means of the subjective log-transformed "dark" phase singing during DD. Data are binned into three groups: "dusk" (ZT12-13), "darkest" (ZT14-21), and "dawn" (ZT22-23), or the free-running/subjective equivalents. Colours indicate population (Mangaia=blue-green, Oahu=dark orange) and vertical lines are S.E.

Phase markers for singing rhythms

Thus far we have tested for temporal escape by asking whether Oahu males sing less at "dusk" and more during the "darkest" part of the night than Mangaia males, and whether this results in different allocation to singing at "dawn". We now test whether parameters ("phase markers") that formally characterize the circadian rhythms of each population

follow suit, in both entrained (LD) and free running conditions (DD). Specifically, we compared the onset, peak, and offset during entrained and free-running conditions (in ZT or CT, respectively) by modelling each phase marker as a response of photoschedule and population.

While the best fitting and most parsimonious model for onset included only "population" (ΔWAIC=0, Table 5), the HPD for the mean difference between populations contained zero and so onset is unlikely to significantly vary between populations. Indeed, onset occurred around ~ZT/CT14 for both populations, regardless of photoschedule (Table 6). Concerning peak, the best fitting and most parsimonious model contained both photoschedule and population (ΔWAIC=0, Table 5), though the HPD for the mean difference between populations contained zero, indicating that while population may explain minor variation in peak, this effect is largely driven by photoschedule. Specifically, while singing peaks at ~ZT19 when entrained, it becomes phase delayed until ~CT20 during free running conditions (Table 6). Finally, the model containing the photoschedule by population interaction was both the best fitting and most parsimonious model of offset (ΔWAIC=0, Table 5). Offset occurs ~2 hours apart between the populations (at approx. ZT22.3 and ZT24/0 for males from Mangaia and Oahu respectively) under entrained conditions, but becomes more similar in free running conditions, occurring around CT1.3. Specifically, offset is delayed by ~2h 45m for males from Mangaia under free-running conditions, but only by 1h 23m for males from Oahu (Table 6). Thus, while onset remains robust across both populations regardless of entrainment or free-running conditions, both peak and offset experience a delay in free-running conditions but only offset differs between the populations (delaying relatively more in DD for Mangaia versus Oahu males).

We also characterised variation around the circular mean of each phase marker. Whilst entrained, onset is equally variable amongst populations (V_m = 0.6 and 0.65 for Mangaia and Oahu, respectively; Table 6) though while free-running the onset for Mangaia is 3.3-

fold more variable than for Oahu. Peak is twice as variable for Mangaia whilst entrained and 5.3-fold as variable during free-running conditions (Table 6). Similarly, offset is 3.8- and 6.7-fold more variable for Mangaia than Oahu during entrained and free-running conditions, respectively (Table 6). Thus, excluding onset whilst entrained, Mangaia males exhibit greater variation for all phase markers throughout both photoschedules than Oahu males (Table 6).

Response~	Covariates	pWAIC	lppd	WAIC	ΔWAIC	WAIC *w*
Onset	***Population***	4.06	-299.92	607.97	0.00	0.41
	Photoschedule+population	6.23	-298.17	608.80	0.83	0.27
	Photoschedule*population	8.33	-296.63	609.91	1.93	0.15
	Null	2.02	-303.34	610.72	2.75	0.10
	Photoschedule	4.13	-301.62	611.50	3.53	0.07
Peak	***Photoschedule+population***	5.88	-258.65	529.05	0.00	0.82
	Photoschedule*population	7.94	-258.22	532.32	3.27	0.16
	Population	4.00	-264.21	536.42	7.37	0.02
	Photoschedule	3.87	-266.11	539.97	10.92	0.00
	Null	1.98	-271.31	546.58	17.53	0.00
Offset	***Photoschedule*population***	10.07	-238.21	496.57	0.00	0.76
	Photoschedule+population	7.02	-242.40	498.83	2.26	0.24
	Photoschedule	4.79	-259.30	528.17	31.60	0.00
	Population	4.52	-260.13	529.31	32.73	0.00
	Null	2.24	-274.44	553.36	56.79	0.00

Table 5. WAIC, estimated number of parameters (pWAIC), ΔWAIC ($WAIC_{model} - WAIC_{min\ model}$), log pointwise predictive density (lppd) and WAIC *w* (WAIC weight) for Bayesian projected normal models of the phase markers ("Response" = "onset", "peak", or "offset") ordered in descending fit (best-fitting model at the top and models competitive with the best are bolded and italicized). Covariates are "photoschedule" (12L:12D or 12D:12L) and "population" ("Mangaia" or "Oahu").

Photoschedule	Population	Onset	Peak	Offset	V_m Onset	V_m Peak	V_m Offset
12L:12D	Mangaia	14.28 ± 3.23	18.41±2.56	22.35 ± 3.8	0.65	0.51	0.57
	Oahu	13.43 ± 3.14	19.14 ± 2.1	0.6 ± 1.53	0.60	0.26	0.15
12D:12D	Mangaia	14.60 ± 3.55	20.00±3.42	1.20 ± 3.52	1.40	1.16	1.27
	Oahu	14.50 ± 2.33	20.1 ± 2.26	1.40 ± 224	0.43	0.22	0.19

Table 6. Phase markers (onset, peak, and offset; mean ZT ± S.D. rounded to the nearest minute) and angular variance (V_m onset, peak, and offset) for each population (Mangaia and Oahu) grouped by photoschedule. Times refer to Zeitgeber Time ("ZT") in LD and clock time ("CT") in DD.

4.5. Discussion

Here we ask whether a population of crickets (normal-wing *T. oceanicus* males from the Hawaiian Island of Oahu) has evolved altered timing of singing as a strategy for evading the parasitoid fly, *O. ochracea*. We predicted that having experienced strong selection to evade a lethal infection, males from Oahu are less likely to sing during the time-of-day the fly is most phonotactic (i.e., dusk; (Kolluru 1999; W. H. Cade, Ciceran, and Murray 1996) than males from an ancestral unparasitised population (Mangaia). Whilst some of our analyses may suffer from being underpowered due to using conservative metrics for quantifying singing and low sample sizes, the significant effects and borderline trends we uncover suggest that differences between the populations are consistent with temporal escape shaping the singing rhythm of Oahu males.

We first characterized the singing rhythms of both populations and found them to be strongly nocturnal with 85-90% of singing occurring during the dark phase of the circadian cycle (Fig. 6). Singing rhythms entrain following a phase shift (Fig. 3) and free-run in constant conditions with a period ~1h longer than in entrained conditions, indicative of

clock control (Fig. 4) consistent with Westwood *et al.* in prep (Chapter 3). Next, we compared how often males sing during different parts of the circadian cycle controlling for the approximately double singing minutes of Oahu compared to Mangaia males (Fig. 5). We find trends for the populations under ecologically realistic conditions (i.e., when entrained) to differ in manners consistent with temporal escape including: (i) during our proxy for dusk, Oahu males sing ~2.5-fold less often, which equates to ~5% less of their daily singing, than Mangaia males, and (ii) Oahu males undertake 7% more of their singing during the darkest part of night. However, these differences in timing appear too subtle to manifest in the parameters (onset, peak, offset) that characterize the waveforms of circadian rhythms. This may be explained by the temporal organisation of singing within the dark phase being less constrained than core properties of circadian rhythms. Additionally, the quantification of circadian parameters assumes a symmetrical wave form, which does not appear to be the case for *T. oceanicus* singing rhythms (Fig. 7) and is unlikely to be the case for many biological rhythms. Considering this, we argue our approach compartmentalizing and comparing different "bins" of time throughout the "night" is a more nuanced and accurate means of testing for temporal escape than by comparing phase markers derived from a symmetrical wave form.

Our results also hint at stricter scheduling of singing in Oahu males: (i) while the free-running period of >24h results in more singing by males in constant than entrained conditions, there is a trend for this to be exacerbated for males from Mangaia (Fig. 5); (ii) the allocation of singing to light and dark phases by Oahu males is similar to their allocation when free-running, unlike Mangaia males who sing 1.2-fold more in subjective light than Oahu males; (iii) all but one metric characterizing the wave form of the singing rhythm is more variable for Mangaia males; ~2-4-fold and ~3-7-fold higher for entrained and free-running rhythms, respectively. Further work is required to determine whether greater variation in the rhythms of males from Mangaia could reflect strong stabilizing

selection in response to fly driven selection and/or could simply due to a loss of genetic variation in the population bottleneck during colonization of Hawaii.

While our results suggest that Oahu males have shifted the temporal allocation of singing during the night, we did not find strong evidence of their singing window being condensed into the darkest part of the night. This may be due to multiple factors, including the timing of fly activity and whether the fly is phonotactic throughout the entirety of its active phase. For example, studies examining *O. ochracea* phonotaxis to host species across their geographic range have resulted in inconsistent findings over successive years. While Kolluru (1999) and Cade (1996) both found that *O. ochracea* phonotaxis is limited to dusk, with decreasing attraction approaching dawn and no attraction at all during the daytime, another study found phonotaxis to be greatest at dawn, though this was limited to one night's observations (William Cade 1979). Other findings focussed on the temporal organisation of male singing in parasitised and unparasitised populations suggest the fly is most active at both dusk and dawn (Zuk, Simmons, and Cupp 1993). Which factors drive the daily partitioning of fly activity and phonotaxis remains unclear (Cade 1975), though one possibility is it must temporally decouple rhythmic behaviours, such as foraging for food, seeking hosts, and finding mates. For example, adult Tachinid flies feed on the nectar of flowering plants, and many attractive qualities of flowering plants are circadian (Overland 1960; Matile 2006; Yon *et al.* 2016). If *O. ochracea* feed on a rhythmically available/attractive food source, gravid females may need to temporally de-couple foraging for hosts and foraging for food. While questions remain about whether an active fly is always phonotactic, the most robust prediction is that dusk and early part of the night poses the greatest risk of infection to male *T. oceanicus*, which may explain why we find the singing levels of both populations are most similar at dawn.

If temporal escape has selected for the differences between the singing rhythm of Oahu versus Mangaia males, the shifts are modest. Changes to the onset/peak/offset of the

singing window may be constrained because of the complexity and interconnectedness of the host circadian system. Specifically, because circadian rhythms govern so many behaviours and physiologies, a shift in one rhythmic behaviour may be hard to achieve without a concurrent shift in the timing of other rhythms. We found that onset was the most robust phase marker across populations and photoschedules. One reason for this may be that the onset of singing is tightly linked to other intrinsic or extrinsic rhythms. For instance, if spermatophore formation precedes the onset of singing by a fixed amount of time (such as in *T. commodus*; (Werner Loher 1974) a phase delay in the onset of singing may be difficult or impossible without an accompanied shift in the onset of spermatophore formation. Similarly, the activity rhythms of females (Loher and Orsak 1985) may constrain the singing schedule. If females are not receptive to male signals outside of the typical timeframe for their species, then condensing singing within a narrow window within the typical timeframe is a better strategy than shifting singing to a novel time of day. Our finding that peak and offset are relatively more flexible than onset may be a product of the advantage of being able to either increase or decrease levels of singing dependent upon variability in individual condition. For example, hungry/poor condition males may need to spend more time foraging for food and less time singing. Future directions for probing differences in clock control of singing between populations could include examining phase response curves (i.e., to elucidate which times of the circadian cycle singing is most sensitive to light) and/or to ramp light intensity up and down to mimic dusk and dawn to better reflect natural environmental lighting conditions, as well characterising rhythms in mate seeking by females and exploring whether sexual antagonism constrains the schedule of singing by males.

An unexpected finding was that Oahu males sing during approximately twice as many minutes throughout the circadian cycle as Mangaia males. However, as previously noted, this does not necessarily mean that Oahu males sang twice as much as Mangaia males. The “singing minute” metric we employ only informs whether a male sang at all during a

given minute. As such, singing may be equal between populations if Oahu males favour short singing bouts and Mangaia males favour relatively longer singing bouts. Recalibrating our methods to classify singing in shorter increments (e.g., whether or not a cricket sings in a 10s clip instead of a 1min clip) or re-training the model to differentiate between different lengths of singing in a given clip could help to elucidate population differences in singing effort. Previous work has shown that parasitised populations generally sing less than unparasitized populations, and that like female crickets, *O. ochracea* favours longer singing bouts (Zuk, Rotenberry, & Simmons 1998; Wagner 1996). Thus, if Oahu males favour shorter singing bouts they will be less attractive to female crickets but this cost may be offset by the overall reduction in the number of singing males in a parasitised population causing females to be less choosy. Furthermore, unless *O. ochracea* has other host species in the Hawaiian Islands, it faces selection to cope with the potentially altered schedule and duration of song bouts of its hosts, raising the possibility of coevolution of rhythms in host and parasitoid. A better understanding of the links between the characteristics of song bouts of Oahu males, what kind of song attracts female crickets and *O. ochracea,* and what time of day each are most receptive is now warranted.

Taken together, our results reveal differences between the temporal organisation of singing between the Oahu and Mangaia populations that are consistent with temporal escape. Whether these differences do constitute temporal escape requires a closer examination that considers whether differences in other aspects of habitat between the Hawaiian and Cook Islands and/or the process of colonisation (e.g. consequences of bottlenecks) of Hawaii could generate temporal shifts in the same directions. For example, extending this approach to include populations from other Hawaiian Islands with varying levels of parasite pressure could help elucidate if a gradient of selection has resulted in a gradient of temporal escape (and reveal how this is balanced against other external pressures, such as e.g. greater attraction of female crickets to males which sing earlier;

Orsak 1988). Accounting for variation in the chronology of *O. ochracea* introduction may also be informative because contrary to our findings, Kolluru (1999) found singing in male *T. oceanicus* peaked at dusk, which also coincided with peak fly phonotaxis, and Zuk (1993) suggests fly phonotaxis occurs at both dusk and dawn. Both experiments were performed on the Big Island of Hawaii over two decades ago, and a hallmark of this species is rapid evolution, particularly in response to parasitism by *O. ochracea* (Tinghitella 2008). Thus, our findings may indicate the evolution of a shift in circadian singing rhythms which has occurred only recently. Overall, we find that while most studies concerning the *T. oceanicus – O. ochracea* system in Hawaii have focussed on signal loss or variation due to changing morphology, our findings suggest evasion strategies may be more diverse.

5. Chapter 5: Testing possible causes of gametocyte reduction in temporally out-of-synch malaria infections

This work has been published as:

Westwood, Mary L., *et al.* "Testing possible causes of gametocyte reduction in temporally out-of-synch malaria infections." *Malaria journal* 19.1 (2020): 1-10.

5.1. Abstract

The intraerythrocytic development cycle (IDC) of the rodent malaria *Plasmodium chabaudi* is coordinated with host circadian rhythms. When this coordination is disrupted, parasites suffer a 50% reduction in both asexual stages and sexual stage gametocytes over the acute phase of infection. Reduced gametocyte density may not simply follow from a loss of asexuals because investment into gametocytes ("conversion rate") is a plastic trait; furthermore, the densities of both asexuals and gametocytes are highly dynamic during infection. Hence, the reasons for the reduction of gametocytes in infections that are out-of-synch with host circadian rhythms remain unclear. Here, two explanations are tested: first, whether out-of-synch parasites reduce their conversion rate to prioritise asexual replication via reproductive restraint; second, whether out-of-synch gametocytes experience elevated clearance by the host's circadian immune responses. First, conversion rate data were analysed from a previous experiment comparing infections of *P. chabaudi* that were in-synch or 12 hours out-of-synch with host circadian rhythms. Second, three new experiments examined whether the inflammatory cytokine TNF-α

varies in its gametocytocidal efficacy according to host time-of-day and gametocyte age. There was no evidence that parasites reduce conversion or that their gametocytes become more vulnerable to TNF-α when out-of-synch with host circadian rhythms. The factors causing the reduction of gametocytes in out-of-synch infections remain mysterious. Candidates for future investigation include alternative rhythmic factors involved in innate immune responses and the rhythmicity in essential resources required for gametocyte development. Explaining why it matters for gametocytes to be synchronised to host circadian rhythms might suggest novel approaches to blocking transmission.

5.2. Introduction

A hallmark of many species of malaria (*Plasmodium*) parasite is synchronous, rhythmic development during asexual replication cycles within host red blood cells. For *Plasmodium chabaudi*, each intraerythrocytic development cycle (IDC) spans 24 hours, at the end of which mature parasites burst to release their merozoite progeny. Each merozoite is committed to either asexual replication or to differentiating into a sexual stage (gametocyte) for transmission to mosquitoes. Like the human malaria *P. falciparum,* the rodent malaria *P. chabaudi*'s asexual development progresses through sequential stages within the IDC in synchrony with each other, transitioning between IDC stages at particular times-of-day (O'Donnell, Mideo, & Reece 2013; Mideo *et al.* 2013). Specifically, the IDC schedule is determined by the timing of the host's circadian feeding rhythm, with IDC

completion (schizogony) switching from the night (dark phase) to the daytime when hosts only have access to food in the daytime (light phase) (Prior *et al.* 2018; Hirako *et al.* 2018).

Maintaining coordination with host circadian rhythms appears important to parasites. If infections are initiated such that early IDC stages are inoculated into the host in the evening (12 hours out-of-synch with the host rhythm) rather than in the early morning (when they usually occur; in-synch), parasites suffer a 50% reduction of both asexually replicating stages and gametocytes across the acute phase (Donnell *et al.* 2011; O'Donnell, Mideo, & Reece 2013). Asexual stages become increasingly demanding of host resources as they progress through the IDC. If these resources appear in the blood in a circadian manner, asexual parasites that are out-of-synch with host rhythms may be unable to fulfil their needs and die, or have to pause development until resources are available (Babbitt *et al.* 2012). Either death or a delay to replication could explain why asexual density is lower in out-of-synch infections (Sarah E. Reece, Prior, and Mideo 2017). However, accounting for the reduction of gametocytes in out-of-synch infections is more complex. Intuitively, the reduction in the density of asexual stages might be expected to translate directly into an equal reduction in gametocyte density. However, investment in gametocytes (the proportion of asexuals in a given IDC cohort that produce gametocyte-committed progeny; "conversion rate") is a plastic trait that varies considerably during infections (Carter et al. 2013; P. Schneider, Greischar, et al. 2018). Furthermore, given the different developmental durations and lifespans of asexuals and gametocytes, and their

rapidly changing densities during infections, close correlation between asexual and gametocyte densities is unusual. Instead, the reduction in gametocyte density in infections that are out-of-synch with the host's circadian rhythms could be explained by either (or both) a "parasite strategy" to promote within-host survival, or increased host-mediated removal of out-of-synch gametocytes from circulation.

The "parasite strategy" scenario stems from a body of work revealing that malaria parasites adjust their conversion rate in response to changes in the within-host environment in ways that maximise their fitness (Carter *et al.* 2013; Birget *et al.* 2017; Dyer & Day 2003; Buckling *et al.* 1999; Drakeley *et al.* 1999). Specifically, under stressful conditions, parasites reduce conversion by adopting reproductive restraint and investing more in survival (P. Schneider, Greischar, et al. 2018). However, under extremely stressful conditions (when the infection is at risk of being cleared by the host immune system or drugs), parasites increase conversion, producing mostly transmission stages (gametocytes) and thus making a terminal investment (P. Schneider, Greischar, et al. 2018). Reproductive restraint enables more parasites to be allocated to asexual replication, which equips the parasite with "safety in numbers" to withstand within-host stressors. The loss of short-term transmission potential that results from reproductive restraint is compensated for by the improved prospects for within-host survival and future transmission opportunities (P. Schneider, Greischar, et al. 2018). Such reproductive restraint has been observed in both *P. chabaudi* and *P. falciparum* in response to

treatment with low doses of anti-malarial drugs and within-host competition (Sarah E. Reece et al. 2010; Mideo and Day 2008; P. Schneider, Greischar, et al. 2018; Pollitt et al. 2011; Sokhina, Trape, and Robert 2001; Peatey et al. 2009; Dyer and Day 2003). Thus, parasites may interpret the reduction in asexual density caused by being out-of-synch to the host's rhythm as a situation in which reproductive restraint is beneficial to them. Therefore, parasites in out-of-synch infections are expected to reduce conversion, at least during the first few IDCs when asexual densities are most affected by being out-of-synch.

Alternatively (or additionally), the host's circadian immune responses could be more effective at removing out-of-synch gametocytes from circulation. This "immune killing" hypothesis requires that: (i) the appearance of a gametocytocidal immune factor in the blood follows a circadian rhythm set by the host's circadian clock; (ii) the vulnerability of gametocytes varies throughout their development, such that during in-synch infections, gametocytes are at a less vulnerable age when the immune factor appears or peaks, and so, a more vulnerable age coincides with the immune factor in out-of-synch infections; and (iii) the gametocytocidal factor is part of the innate immune response because the costs of being out-of-synch occur in the first few days of infection when primarily innate responses are active. The only gametocytocidal factor reported to rapidly clear *P. chabaudi* gametocytes from the blood is the pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) (Long *et al.* 2008). When the host's TNF-α receptor is blocked, the gametocyte density of *P. chabaudi*-infected mice increases (on average by 44%),

regardless of parasite clone and asexual parasite density (Long *et al.* 2008). This increase occurs within 24 hours which is too soon for mature gametocytes produced via an increase in conversion rate to be detected, and the rate of gametocytogenesis was not affected by the TNF-α receptor blockade, implying that gametocyte survival was improved in the absence of TNF-α (Long *et al.* 2008). Asexual stages are also vulnerable to TNF-α which acts on, for example, *P. falciparum* via a calcium-cAMP downstream signalling, with PCNA1 (proliferating-cell nuclear antigen-1) as a possible target (Cruz *et al.* 2016). Whether a similar mechanism also operates in gametocytes and could mediate age-specific vulnerability to TNF-α is unknown. TNF-α expression is rhythmic in mice and generally peaks during the resting phase – i.e. during the day (Keller *et al.* 2009). However, standing rhythms in inflammation may be altered by infection: in *P. chabaudi* infected mice, rhythmicity in TNF-α is also linked to the time-of-day that schizogony occurs (Prior *et al.* 2018). Further complexity in TNF-α rhythms may arise from host rhythms of TNF-α production and decay, induction of TNF-α expression in response to schizogony, and possibly from time-of-day-dependent activities of the innate immune cells that TNF-α stimulates. Therefore, it is hard to predict the time-of-day (i.e. age) at which gametocytes are most vulnerable, or exposed, to TNF-α.

Here, both the "parasite strategy" (conversion rate modulation) and "immune killing" hypotheses were investigated using *P. chabaudi*. First, conversion was estimated for a previously collected dataset in which parasites were either in-synch or out-of-synch with

host rhythms. Conversion rates were estimated using a method for statistical inference which follows each time-series of within-host infection dynamics, including the densities of asexual parasites, RBCs, and the starting gametocyte density for each infection (Greischar et al. 2016; P. Schneider, Greischar, et al. 2018). Next, the immune killing hypothesis was examined using a series of three experiments. The first tested whether host circadian rhythms affect the clearance of TNF-α from the blood. The second and third experiments examined whether TNF-α differentially affects gametocyte survival at different times of day, using wild type (WT) and clock mutant mice, respectively. Unravelling the cause of gametocyte reduction in temporally desynchronised infections will further the understanding of the causes and consequences of rhythmicity in the IDC. This knowledge could guide the development of novel antimalarial treatments, and may inform predictions for the proximate and ultimate responses of parasites to temporal shifts in vector behaviour caused by the widespread usage of insecticide-treated bednets.

5.3. Methods

Testing the parasite strategy hypothesis

Do parasites in out-of-synch infections reduce investment into conversion?

A previously published data set (O'Donnell *et al.* 2011) was used to compare conversion rates at the start of infections for parasites that were in- and out-of-synch with host rhythms. Briefly, *P. chabaudi* AJ ring stage parasites (1 x 10^6 parasitised RBCs) were harvested from donor mice kept in standard (12-hour light : 12-hour dark) or reversed (12-hour dark : 12-hour light) lighting schedules and used to infect recipient mice (10- to

12-week-old male MF1) in the same lighting schedule as their donor mice, or into mice kept under the opposite (reversed) lighting schedule. Thus, parasites in recipient hosts kept in the same lighting schedule as the donor host remained in synchrony or "in-synch" with the host circadian rhythm and parasites moved between lighting schedules became 12-hours "out-of-synch". This produced four groups of infections: two in-synch and two out-of-synch. Data were collected for days 0-7 post-treatment; blood samples to quantify gametocyte densities (10 μL) were taken every day and total parasite densities (5 μL) were taken on days 1, 3, 5, and 7. DNA and RNA were extracted as described in Schneider *et al.* 2018 (P. Schneider, Greischar, et al. 2018). Total parasite densities were quantified by qPCR (quantitative polymerase chain reaction) and gametocytes by RT-qPCR (reverse-transcriptase qPCR), both targeting the CG2 gene (PCHAS_0620900, previously named PC302249.00.0 (Wargo *et al.* 2007). Asexual parasite density was calculated by subtracting gametocyte numbers from total parasite density. Red blood cell (RBC) densities were measured using flow cytometry (Beckman Coulter) every day.

Unlike previous methods which made unrealistic assumptions about infections (such as fixed conversion during maturation of sexual stages; equal death rates for asexuals and gametocytes; short survival of gametocytes and thus, non-overlapping cohorts of gametocytes), the method used here more realistically infers conversion in dynamic infections (Greischar *et al.* 2016). The method requires at least seven days of continuous data, including daily RBC, asexual, and gametocyte densities for each infection. Therefore,

to provide daily estimates of asexual density, missing values were interpolated between sequential data points by taking the mean of the preceding and subsequent day. These autocorrelated data resulted in an average estimate of constant conversion throughout each infection. These average conversion rates were compared between in-synch and out-of-synch parasites, for a data set including all infections, and a dataset comprising the subset of infections which met strict criteria for model fitting. Infections are excluded if the residuals showed a significant relationship to natural logged densities of gametocytes, and/or if less than four of the five candidate splines could be fitted (Greischar et al. 2016; P. Schneider, Greischar, et al. 2018). This resulted in the exclusion of 1 in-synch infection and 7 out-of-synch infections (out of 12 infections per each treatment; random subsets of 5 in-synch infections resulted in qualitatively similar analysis outcomes).

Testing the immune killing hypothesis

Three experiments were conducted to test: (i) whether changes in the concentration of TNF-α vary by time-of-day of injection and dose (which also informed the dose of TNF-α used in following experiments); (ii) whether gametocyte vulnerability to injected TNF-α varies according to host time-of-day; (iii) whether gametocyte age mediates vulnerability to injected TNF-α.

All mice were 6- to 8-week-old male and female WT C57/BL6 mice (in-house supplier, The University of Edinburgh). Mice were provided water containing 0.05% para-aminobenzoic

acid (PABA; to enhance parasite growth) and food *ad libitum* and kept under a standard 12-hour light : 12-hour dark schedule (except in experiment iii). All experimental mice that were infected received ring stage *P. chabaudi* clone ER parasitized red blood cells (1×10^6) via intraperitoneal (IP) injection.

(i) *Do changes in concentration of TNF-α vary by host time-of-day and dose?*

Uninfected mice received either 30 μg/kg or 60 μg/kg TNF-α in 100 μL PBS carrier at either lights on (ZT0, n=5 for each group) or lights off (ZT12, n=4 for each group) via IP injection. ZT refers to "Zeitgeber Time" which is defined as the number of hours elapsed since lights on. Two hours post-treatment with TNF-α, blood was obtained from all mice individually by cardiac puncture and centrifuged to collect plasma for a murine TNF-α-specific quantitative enzyme-linked immunosorbent assay (ELISA; eBioscience catalog number 88-7324 Mouse TNF alpha ELISA Ready-SET-Go!®).

(ii) *Is gametocyte vulnerability to TNF- dependent on host time-of-day?*

To coincide with peak gametocyte density in control infections (S. E. Reece et al. 2003; P. Schneider, Greischar, et al. 2018) mice were injected on day 14 PI with either 60 μg/kg TNF-α dissolved in 100 μL PBS carrier at ZT0 (n=23) or at ZT12 (n=17), or with 100 μL PBS carrier at ZT0 (n=4) or ZT12 (n=3) via IP injection. RBC densities and thin blood smears were taken from the tail vein of all mice 1-hour before TNF-α or control treatment (providing baseline parameter estimates) and also at, 2-, and 12-hours post-treatment.

The proportion of RBCs containing gametocytes (quantified via microscopy) was multiplied by RBC density to estimate gametocyte density.

(iii) *Is gametocyte vulnerability to TNF-α dependent on gametocyte age?*

Both host-time-of day and gametocyte age co-vary in experiment ii and it is possible these factors oppose the effects of TNF-α on gametocyte density. Therefore, to focus on gametocyte age without the confounding effect of host rhythms, C57/BL6 *Per1/2* null mice (11- to 22-week-old males and females, mouse line kindly donated by Michael Hastings (MRC Laboratory of Molecular Biology, Cambridge, UK), generated by David Weaver (UMass Medical School, Massachusetts, USA) were used. These clock mutant have an impaired TTFL clock and exhibit arrhythmic behaviour when placed in constant conditions such as constant darkness (Bae *et al.* 2001; Maywood *et al.* 2014). To produce synchronous infections in these arrhythmic mice, wild type C57/BL6 mice were used as parasite donors. Further, to create infections that could be treated with TNF-α simultaneously (i.e. at the same GMT), yet have focal cohorts of gametocytes at different ages in the experimental mice, staggered infections were set up in the donor mice. This was achieved by offsetting the 12-hour light : 12-hour dark schedule of each of three donor groups by six hours (i.e. lights-on, ZT0, at 07.00, 13.00, and 19.00 GMT; Figure 1).

Under a 12-hour light : 12-hour dark schedule, a new cohort of gametocytes is produced at each ~ZT17 (Reece *et al.* 2003; Gautret *et al.* 1996), translating to 00.00, 06.00, and

12.00 GMT for the three donor groups. Ring stage parasites from these groups were collected at ZT0 to infect experimental mice and produce gametocytes of different ages (31, 25, and 19 hours old) at the time (07.00 GMT) of treatment. For example, ring stage parasites collected at ZT0 from donors whose "lights on" is 19:00 GMT produce a cohort of focal gametocytes via schizogony at 12:00 GMT, making these gametocytes 19 hours old when treated at 07:00 GMT. Figure 1 illustrates the schedules of the donor mice and their parasites, and the development of the gametocytes produced in the three groups of experimental mice to produce gametocyte cohorts of different ages.

P. chabaudi gametocytes reach maturity between 24-36 hours after RBC invasion and become identifiable on blood smears between 18-24 hours old by their morphology and senesce rapidly post-maturation (Reece *et al.* 2003). Although present during treatment and throughout sampling (Figure 1), younger, non-focal gametocyte cohorts were assumed to make negligible contributions to the observed gametocytes because they are too immature to be detected via microscopy. Assuming a post-maturation half-life of approximately 14 hours (Reece *et al.* 2003), older, non-focal gametocyte cohorts should be rapidly lost after 38-50 hours.

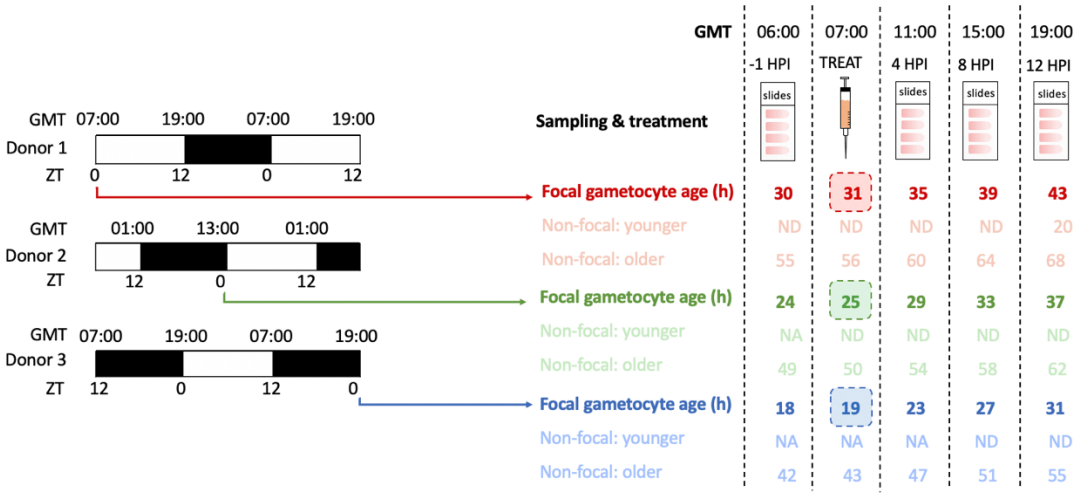


Figure 1. **Three photoschedules were used to generate temporally-staggered cohorts of gametocytes simultaneously perturbed at different ages.** Parasites were collected from donor mice at their ZT0, allowing infections in experimental mice to be staggered by 6-hours so that at the same times GMT, all infections could be sampled and treated with TNF-α or PBS yet, different ages of gametocytes (19-, 25-, and 31-hours-old) could be targeted. By using *Per1/2*(-/-) mice housed in constant darkness as the experimental hosts, the relevance of gametocyte age was decoupled from the canonical host circadian-clock-controlled rhythms. The ages of focal gametocyte cohorts (labelled "gametocyte age (h)", defined as hours post RBC invasion) at each sampling event and treatment (in GMT) are highlighted in bold and the ages of the previous ("younger") and subsequent ("older") cohorts are illustrated with faint text. Immature gametocytes not yet detectable via microscopy are denoted by "ND", and gametocytes not yet produced are denoted by "NA".

Donor mice were allowed to entrain to their schedules for one week prior to infection with *P. chabaudi*, then infections were run until day 7 PI and ring stages were collected to infect the experimental, arrhythmic *Per1/2*(-/-) mice. Parasites begin to lose synchrony in *Per1/2*(-/-) mice after five replication cycles (O'Donnell, Prior, and Reece 2019), and so, to capture gametocytes while still synchronous yet at quantifiable densities, experimental mice were treated with 125 mg/kg^{-1} phenylhydrazine dissolved in 100 μL PBS (PHZ, promotes gametocytogenesis via anaemia) (Birget *et al.* 2017) four days prior to infection. Experimental mice were randomly allocated to either the TNF-α or control group with respect to their response to PHZ, measured by RBC density. Then, on day 4 PI, experimental mice were treated with 60 μg/kg TNF-α dissolved in 100 μL PBS carrier (n=5 for each gametocyte age group, n=15 total) or 100 μL PBS carrier (n=4 for each gametocyte age group, n=12 total). Infections were sampled as per experiment ii to quantify gametocyte densities at -1 (baseline), 4-, 8-, and 12-hours post-injection (HPI) (Figure 1).

Data Analysis

All analyses were performed using R v. 3.5.1 (R Foundation for Statistical Computing, Vienna, Austria). To meet assumptions of homogeneity and variance, conversion rate estimates were log_{10}-transformed. Gametocyte densities for "testing the immune killing hypothesis" parts ii and iii were square root-transformed (in part ii only it was necessary to add half a measurement unit, i.e. 0.5 gametocytes/mL, to all counts to ensure no zero

counts). Five outliers were eliminated in "testing the immune killing hypothesis" part ii due to poor fit, and data were scaled in "testing the immune killing hypothesis" parts ii and iii to have a mean of 0 and a standard deviation of 1, post square root-transformation. Linear models were used to analyse conversion rate estimates (testing the conversion hypothesis) and the effects of host time-of-day on TNF concentration (testing the immune killing hypothesis part i). Linear mixed-effect models were used to analyse gametocyte density (testing the immune killing hypothesis parts ii and iii), using mouse ID as a random effect. To avoid overfitting due to small sample sizes, "Akaike information criterion - corrected " (AICc) values were calculated for each model, and a change in 2 AICc (ΔAICc=2) was chosen to select the most parsimonious model. Only models directly reflecting the hypotheses under test were fitted.

5.4. Results

Testing the conversion hypothesis

No evidence was found to support the hypothesis that parasites out-of-synch with host rhythms reduce conversion (Table 1; Figure 2). Conversion rates are best explained by the model containing only "donor" (donor mice housed in either the standard, or reversed, light : dark schedule) as a main effect (ΔAICc=0; Table 1). Specifically, the conversion rate of experimental mice infected with parasites from donors kept in the reversed light : dark schedule was on average 10.4% higher than compared to infections from donors kept in the standard light : dark schedule independent of the light schedule in which the receiving

mice were kept. Further, the inclusion of the interaction in the model does not improve model fit (ΔAICc=6.67, Table 1). Additionally, incorporating schedule (in-synch or out-of-synch) into the most parsimonious model did not improve model fit (ΔAICc=2.35, Table 1). Further, the model containing only "schedule" returned the least parsimonious fit (ΔAICc=7.11, Table 1), and has only a 2% chance of being the best approximating model in the given model set (AICc weight = 0.02, Table 1). Supporting a lack of effect of being out-of-synch on conversion rate, the same analysis performed on the full dataset including previously excluded infections also returned no evidence for a difference in conversion rates (Appendix Chapter 5).

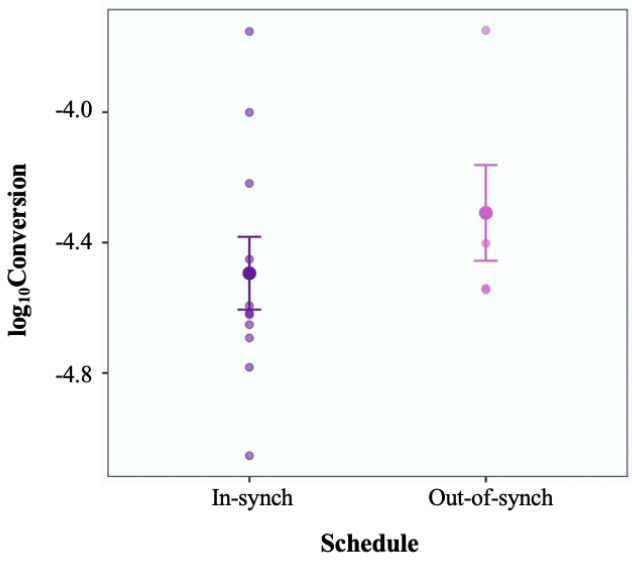


Figure 2. Conversion estimates, alongside mean ± S.E. (calculated post-transformation), for parasites in- and out-of-synch with host circadian rhythms. Points represent raw data,

log_{10}-transformed to approximate normality. Data from experiment in O'Donnell *et al.* 2011 (O'Donnell *et al.* 2011).

Model description: Log10(Conversion) ~	df	log(*L*)	AICc	ΔAICc	AICc *w*
donor	3	-1.704	11.41	0.000	0.688
donor + schedule	4	-1.061	13.76	2.351	0.212
null	2	-5.764	16.45	5.044	0.055
donor + schedule + donor*schedule	5	-1.037	18.07	6.667	0.025
schedule	3	-5.259	18.52	7.110	0.020

Table 1. Degrees of freedom (df), log-Likelihood (log(*L*)), AICc, ΔAICc ($AICc_{model} - AICc_{min\ model}$), and AICc *w* (AICc weight) for each linear model in the conversion analysis ordered in descending fit (best-fitting model at the top). The response variable for each model is the log_{10}-transformed conversion rate. "Schedule" refers to parasites either in-synch or out-of-synch with the host, and "donor" corresponds to parasites taken from donor mice kept in either the standard or reversed light : dark schedule.

II. Testing the immune killing hypothesis

(i) *Do changes in concentration of TNF-α vary by host time-of-day and dose?*

Dose ($P<0.0001$) and host time-of-day ($P<0.001$) contributed substantially to TNF-α concentration 2 HPI (Figure 3) but there was no interaction between them ($P=0.192$). The concentration of TNF-α in mice that were injected at ZT12 (i.e. lights-off, entering the

active phase) was 388.73 pg/mL (±112.88) lower at 2 HPI than in those mice injected at ZT0 (i.e. lights-on, entering the resting phase) (Figure 3).

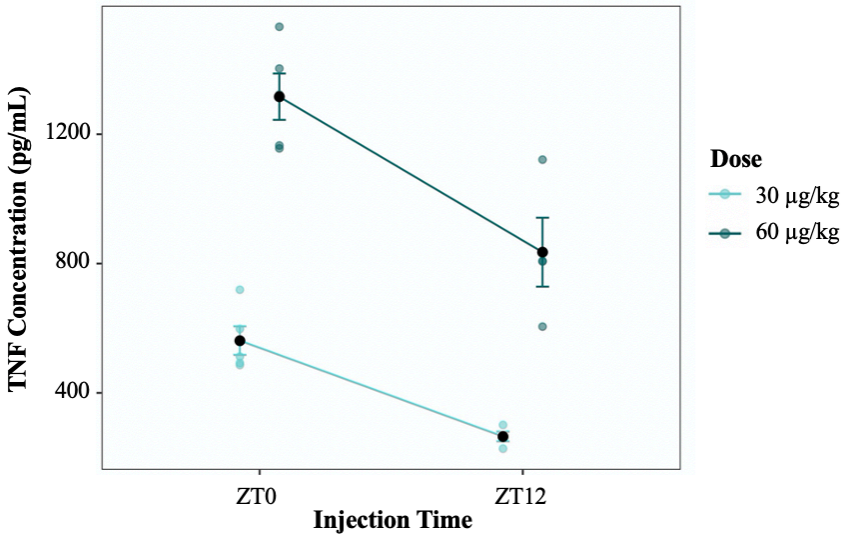


Figure 3. TNF-α concentrations (pg/ML) alongside mean ± S.E. at 2 HPI, after treatment at either ZT0 (lights-on) or ZT12 (lights-off) for two doses of TNF-alpha (30 µg/kg or 60 µg/kg). Points represent raw data.

(ii) *Is gametocyte vulnerability to TNF-α dependent on host time-of-day?*

No evidence was found to support the interaction between host time-of-day (i.e. injection time) and TNF-α on gametocyte density (Figure 4. A; Table 2). Indeed, the most parsimonious model included only host time-of-day (injection time ZT0 or ZT12) and sampling time (2- or 12-HPI) as main effects (ΔAICc=0; Table 2; Figure 4. B). The model incorporating only sampling time was within 2 ΔAICc (ΔAICc=1.588; Table 2) and is

therefore competitive with the most parsimonious model. Furthermore, the most parsimonious model returned only a ~48% chance of being the best approximating model in the given model set (AICc weight = 0.482, Table 2), indicating high model selection uncertainty. Notably, including treatment (TNF-α or PBS) reduced model fits (Table 2). Overall, this analysis finds no evidence to suggest that TNF-α or injection time (i.e. host time-of-day) effect gametocyte density.

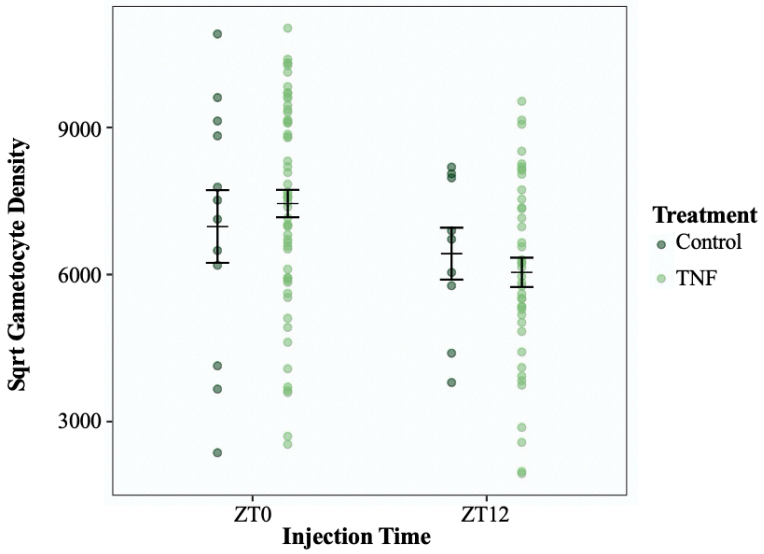


Figure 4. **A.** Gametocyte density (gametocytes/mL blood), alongside mean ± S.E. (calculated post-transformation), in mice injected with either TNF-α or carrier at either ZT0 or ZT12. **B.** Gametocyte density (gametocytes/mL blood), alongside mean ± S.E., for combined treatment groups (TNF-α and control) in mice injected at either ZT0 or ZT12 for each sampling time (-1 (baseline), 2-, and 12 HPI). In both plots, points represent raw data,

square-root-transformed to approximate normality, and scaled to have a mean of 0 and a standard deviation of 1.

Model description: sqrt(Gametocyte Density) ~ (1|mouse)	df	log(*L*)	AICc	ΔAICc	AICc *w*
inj.time + samp.time	6	-137.7	288.1	0.000	0.482
samp.time	5	-139.6	289.7	1.588	0.218
inj.time + samp.time + treatment	7	-137.8	290.6	2.497	0.138
inj.time + samp.time + treatment+ inj.time*treatment	8	-137.1	291.4	3.318	0.092
samp.time + treatment	6	-139.7	292.1	3.953	0.067
inj.time	4	-145.3	299.0	10.872	0.002
null	3	-147.2	300.6	12.536	0.001
inj.time + treatment	5	-145.4	301.4	13.291	0.001
treatment	4	-147.3	302.9	14.825	0.000

Table 2. Degrees of freedom (df), log-Likelihood (log(*L*)), AICc, ΔAICc ($AICc_i - AICc_{min}$), and AIC *w* (AICc weight) for each linear model in the TNF ii analysis ordered in descending fit. The response variable for each model is the square root-transformed gametocyte density and the random effect is "mouse". "Samp.time" refers to sampling time (2- or 12-HPI), "inj.time" refers to injection time (ZT0 or ZT12 respectively), and treatment is either TNF-α or control.

(iii) *Is gametocyte vulnerability to TNF-α dependent on gametocyte age?*

The most parsimonious model included only gametocyte age (ΔAICc=0; Table 3), but evidence for this being the best fitting model is weak (AIC *w*=0.409; Table 3). The two next

most competitive models included age and treatment (ΔAICc= 0.850; Table 3) and age, treatment, and the treatment by age interaction (ΔAICc= 1.785; Table 3). Because the treatment by age interaction is only present in one of the competing models, and the AICc weight for this model is very low (AICc *w*=0.267; Table 3), it is unlikely that this parameter is important in explaining gametocyte density. Thus, in keeping with the results of experiment ii, there is no clear evidence to support a role for TNF-α in differentially affecting gametocytes of varying ages (Figure 5; Table 3).

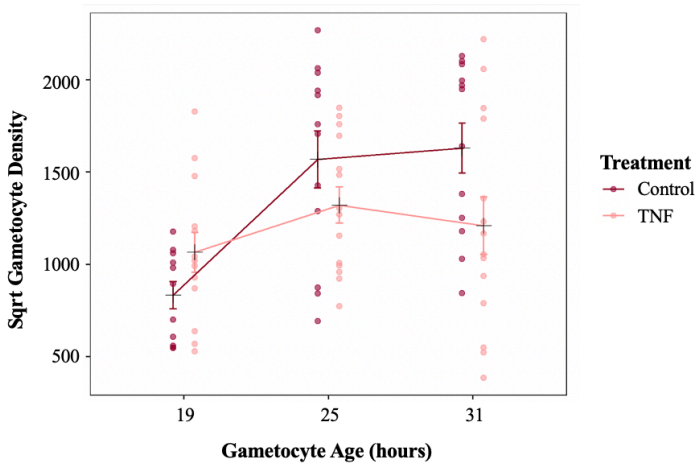


Figure 5. Gametocyte density (gametocytes/mL blood), alongside mean ± S.E. (calculated post-transformation), averaged across all sampling timepoints (4-, 8-, and 12-HPI) for gametocytes treated at ages 19-, 25-, and 31-hours old and according to whether they were treated with TNF-α or PBS carrier only. Points represent raw data, square root-transformed to approximate normality, and scaled to have a mean of 0 and a standard deviation of 1.

Model description: sqrt(Gametocyte Density) ~ + (1|mouse)	df	log(*L*)	AICc	ΔAICc	AICc *w*
age	5	-99.37	209.6	0.000	0.409
age + treatment + age*treatment	8	-96.15	210.4	0.850	0.267
age + treatment	6	-99.08	211.4	1.785	0.168
null	3	-103.5	213.3	3.759	0.062
age + samp.time	7	-99.52	214.7	5.088	0.032
treatment	4	-103.3	215.1	5.469	0.027
age + samp.time + treatment + age*treatment	10	-96.38	216.1	6.556	0.015
age + samp.time + treatment	8	-99.22	216.6	7.000	0.012
samp.time	5	-103.7	218.3	8.743	0.005
samp.time + treatment	6	-103.5	220.2	10.57	0.002

Table 3. Degrees of freedom (df), log-Likelihood (log(*L*)), AICc, ΔAICc ($AICc_{model} - AICc_{min\ model}$), and AICc *w* for each linear model in the analysis of experiment iii ordered in descending fit. The response variable for each model is the square root-transformed gametocyte density and the random effect is "mouse". "Samp.time" refers to sampling time (4-, 8- or 12-HPI), "age" refers to gametocyte age (19-, 25-, or 31-hours old) and treatment is either TNF-α or control.

5.5. Discussion

The experiments presented here suggest that neither the conversion nor the immune killing hypotheses can explain the reduction in gametocytes when parasites are out-of-synch with host circadian rhythms. First, parasites were predicted to reduce conversion in out-of-synch infections to reduce the impact of being out-of-synch on asexual densities. However, average conversion rates in the dataset that met strict model selection criteria

(P. Schneider, Greischar, et al. 2018) and also the full dataset of all infections (Appendix Chapter 5 Table 1) did not vary significantly between in- and out-of-synch infections (Table 1, Fig. 2, Appendix Chapter 5 Figure 1). Second, TNF-α (for both doses) introduced at the start of the resting phase (i.e. lights-on) was cleared at a slower rate compared to the start of the active phase (i.e. lights-off, experiment i). This time-of-day effect is likely explained by temporal variation in host metabolic rates: heightened metabolism during the active phase should clear incoming TNF-α more readily than the during the rest phase. Despite evidence for host circadian rhythms influencing TNF-α levels, which is consistent with other studies (Keller *et al.* 2009; Young *et al.* 1995; Bredow *et al.* 1997), two experiments (ii and iii; Fig. 4, Table 2; Fig. 5, Table 3) suggest that exposure to more TNF-α, or for longer, have no significant impact on the densities of gametocytes, even when exposed at different ages.

The conversion results suggest that either parasites do not adjust their conversion rate when out-of-synch with host rhythms, or that the methods utilised here were unable detect change. Conversion is a key determinant of transmission potential (thus, fitness) and a phenotypically plastic trait. Conversion is reduced by *P. chabaudi* in response to a loss of "state" (i.e. reduction in density or replication) (P. Schneider, Greischar, et al. 2018) so parasites would not need to detect that they are out-of-synch but simply respond to the impact of being out-of-synch upon state. It is possible that the modest drop in asexual density in the first couple of days post-infection (O'Donnell *et al.* 2011) is not sufficiently

stressful to elicit reproductive restraint (P. Schneider, Greischar, et al. 2018) – particularly because out-of-synch parasites still experience high replication rates despite being at a lower initial density. In addition, the data and approach used to test the conversion hypothesis were likely conservative. For example, there may be a minimal level of change in the conversion rate is required for Greischar *et al.*'s (Greischar *et al.* 2016) approach to reliably return different conversion estimates, and this level may not have been met. When out-of-synch with host rhythms, parasites begin the process of rescheduling to regain coordination between the IDC and host rhythms (Gautret *et al.* 1995). Whether rescheduling affects the gametocyte developmental schedule is unknown, but variation in how gametocytes accumulate over time could compromise the reliability of conversion estimates. It is therefore possible that when out-of-synch with host rhythms, parasites do reduce conversion accordingly, but more severe perturbations are required to detect this. For example, future work might compare conversion of in- and out-of-synch parasites when also exposed to in-host competition or resource limitation.

That TNF-α rhythms and the age of gametocytes had no significant effect on gametocyte density was unexpected. Schizogony (the production of a new cohort of asexuals and gametocytes) causes an elevation of TNF-α that may be capable of sterilising gametocytes (Prior *et al.* 2018). The concentration of TNF-α used in experiment ii is greater than schizogony-induced TNF-α levels, and should therefore represent a meaningful change to the within-host environment. However, time-of-day-dependent clearance of TNF-α may

have resulted in confounding host and parasite time-of-day in experiment ii. Experiment iii corrected for this by directly testing gametocyte age without host time-of-day as a confounding factor (via the use of circadian-knockout mice), although it was impossible to eliminate TNF-α that was produced as a result of schizogony. It remains uncertain how schizogony-induced TNF-α might have affected the focal gametocyte cohorts, but it may have acted either directly by attacking gametocytes, or indirectly by activating related immune cells or factors involved in clearing TNF-α. Further, gametocyte numbers are generally low, rendering them extremely difficult to detect, particularly where minor perturbations to density may have been confounded by other within-host rhythms. Why blocking the TNF-α receptor results in an increase in gametocyte density (Long *et al.* 2008), yet introducing TNF-α does not appear to decrease gametocyte density, remains mysterious. One possibility is that downstream immune cells activated by TNF-α are responsible for reducing gametocyte density, and in the absence of TNF-α these cells remain inactive. In this scenario, introducing more TNF-α (as done here) may have little to no additional impact at all on gametocyte density if the threshold for activation of these immune cells is low.

To address the potentially confounding effects of schizogony-induced TNF-α, future work could aim to block TNF-α (following (Long *et al.* 2008) in circadian-knockout mice at the onset of schizogony. The effect of rhythmic TNF-α on gametocyte survivability (replicated by artificial injection) could then be better understood without the presence of potentially

opposing rhythms. Additionally, other rhythmic host resources required for gametocyte development such as LysoPC (host-derived lipid) (Brancucci *et al.* 2017) may be limited in out-of-synch infections. Repeating a similar experiment to experiment iii but using resources essential to gametocyte development could elucidate whether the gametocyte density decrease in out-of-synch infections can be attributed to host offensive rhythms, or whether gametocyte development and survival is passively modulated through host circadian processes (e.g. the availability of nutrients). Further, out-of-synch gametocytes may alter their developmental rate to reschedule to match host circadian rhythms, accounting for some of the observed reduction in gametocyte density. Simultaneously considering the multitude of factors contributing to gametocyte reduction could provide an explanation for the substantial decrease in gametocytes in out-of-synch infections.

Understanding consequences of being out-of-synch with host circadian rhythms is important for unravelling the evolutionary drivers of rhythmic development in malaria parasites. Knowledge of the benefits, or costs, to parasites of being in-synch with host rhythms will allow them to be harnessed for the development of novel antimalarial treatments; for example, if gametocytes show time-of-day-specific vulnerabilities, drugs could take advantage of rhythmic weaknesses. Further, understanding how malaria parasites respond to temporal variation in the within-host environment is increasingly important as some mosquito populations are reported to have shifted the timing of blood-foraging rhythms in response to the widespread use of long-lasting insecticide treated

bed nets (Moiroux *et al.* 2012; Sougoufara *et al.* 2014). Since gametocytes are necessary for transmission, understanding what causes their reduction in out-of-sync infections could have implications for shifts in vector rhythms. If out-of-synch parasites are less fit, changes in vector behaviour could be beneficial in minimising malaria burden. However, without fully understanding the evolutionary drivers behind parasite rhythms it is difficult to predict how, or if, parasites might evolve to cope with these changes.

6. General Discussion

6.1. Introduction

The goal of this thesis was to uncover how circadian rhythms mediate interactions between hosts and parasites. Specifically, I focussed on how rhythms mediate activities which underpin sexual reproduction of hosts (the pacific field cricket *T. oceanicus*) and parasite (the rodent malaria *P. chabaudi*). First, I (i) introduced the value of integrating evolutionary ecology with circadian biology to understand infection biology, then I (ii) uncovered and characterised circadian singing rhythms in male *T. oceanicus* and (iii) applied this knowledge towards elucidating how parasitism may drive the evolution of male *T. oceanicus* circadian reproductive tactics, and (iiii) examined the role of host rhythms in shaping parasite reproductive activities that underpin transmission. In this chapter, I summarise these results whilst considering the limitations of my approaches, explain their significance in a broader context, and suggest future directions.

6.2. Integrating evolutionary ecology into chronobiology to study infections

Although circadian biology began in the mid-20th century as a field focussed on evolutionary and ecological questions, most work over the last 2 decades has focused on uncovering the molecular and mechanistic underpinnings of clocks. In Chapter 2, I made the case for returning chronobiology to its roots; in particular, I outlined the benefits to be gained from using a circadian framework to understand rhythms in host-parasite interactions. I focussed on hosts and parasites because infections are a common and fundamental ecological interaction between species (and so, lessons learned are broadly applicable) and because exploiting and/or disrupting rhythms during infections may be harnessed for clinical benefit. I examined this topic from three complementary angles: rhythms in host defence, rhythms in parasite offence, and parasite manipulation of host rhythms.

Concerning host defence, immune rhythms have received the most attention. One of the primary roles of the immune system is to ward off parasites, and mounting evidence suggests circadian clocks play a pivotal role in many immune functions (Scheiermann *et al.* 2018). This has led to the assumption that immune efficacy against infection peaks during the active phase and troughs during the resting phase. However, a key finding from Chapter 2 challenges this conventional wisdom; while host defence strategies against infection are often more effective during the active phase (and mortality and severity of infections are higher when infection occurs during the rest phase), this is not always the case (Westwood *et al.* 2019). In one notable example, the clock-controlled secretion of chemoattractants by host neutrophils and macrophages facilitates infection by *Leishmania* parasites, meaning that peak infection risk occurs concurrently with peak immune function during the active phase (Kiessling *et al.* 2017). Thus, to parse how immune rhythms relate to infection, studies which decouple the effects of immune rhythms on preventing and/or dealing with ongoing infection performed on a diversity of host-parasite systems are necessary, as well as consideration of whether immune responses simply represent a danger to parasites or can facilitate parasite activities. Beyond immune function, behavioural and morphological defences (such as those observed in the evolution of silent morphs in the *T. oceanicus – O. ochracea* host-parasite system) have received markedly less attention though I suggest they are often lower-cost defence strategies against parasitism.

6.3. Evolution of host rhythms for defence against infection

Chapters 3 and 4 focus on the reproductive circadian rhythms of *T. oceanicus*. The *T. oceanicus – O. ochracea* system in the Hawaiian Islands offers an exciting opportunity to uncover how circadian rhythms affect host-parasite coevolution in real-time following the groundwork laid in Chapters 3 and 4. In Chapter 3, I performed a robust characterisation of singing rhythms in normal-wing male *T. oceanicus*, revealing that singing fulfils all criteria necessary to be deemed circadian. Namely, that (1) singing persists in the absence

of external time cues with a periodicity slightly greater than 24h, (2) is temperature compensated, and (3) entrains to light. I also found that males sing overwhelmingly during the dark phase, and that singing rhythms vary across individuals, suggesting that the timing of singing may be a target of selection.

In this chapter I also combined machine learning, high performance computing, and bioacoustics to develop a pipeline capable of processing large, continuous audio data files for the purpose of circadian analysis. Other methods trialled to document singing rhythms are outlined in Appendix 8.2, but the selected method has numerous benefits, including (i) broad applicability to many sound and non-sound producing species, (ii) fine-scale temporal resolution of signal production, and (iii) continuous, longitudinal data collection. While much focus has been spent examining the rapid evolution of novel *T. oceanicus* wing morphs in response to lethal infection *O. ochracea*, relatively less attention has been paid to how normal-wing males have persisted in the Hawaiian Islands in spite of overwhelming natural selection. Phenotypic silencing may be a drastic means of avoiding *O. ochracea* but in Chapter 3 I lay the foundation to examine a much more nuanced strategy for parasite evasion – temporal escape.

In light of the method presented in Chapter 3 and the findings it contains, I was able to carry out Chapter 4 to examine whether Hawaiian *T. oceanicus* have evolved to reduce singing at the time-of-day when risk from *O. ochracea* is at its greatest. Few studies have formally characterised temporal variation of *O. ochracea* phonotaxis in the wild (or Tachinid flies generally), and even rarer are examples of *O. ochracea* phonotaxis to *T. oceanicus* in the Hawaiian Islands (to my knowledge there is one such study; (Kolluru 1999). However, evidence suggests that *O. ochracea* is most phonotactic around dusk, with decreasing phonotaxis as the night progresses, and no phonotaxis at all during the daytime (Kolluru 1999; W. H. Cade, Ciceran, and Murray 1996). I found that although Hawaiian *T. oceanicus* sing more overall than an unparasitised, ancestral population from

Mangaia, they appear to undertake a lower proportion of their overall singing during the hours approximating "dusk" and more during the "darkest part of night", but the same amount during "dawn". Although my findings are consistent with temporal escape from infection risk at dusk, further work is necessary to elucidate this with more certainty. For example, the inclusion of island replicates especially if their chronology of *O. ochracea* introduction varies, and more recent and robust temporal phonotactic data on *O. ochracea* would be helpful. These data are difficult to collect so repeating my study with the design altered to ramp the lighting up and down to mimic dusk and dawn, as well as increasing sample size (especially for the ancestral population because it is more variable) would be a good place to start.

More broadly, my research on *T. oceanicus* circadian singing rhythms highlights the need for a more comprehensive picture of how each player in this system partitions its time. For example, female rhythms (in e.g., locomotor activity/mate receptiveness and phonotaxis) may either constrain or facilitate the evolution of male rhythms. If female phonotaxis to male calling song is circadian controlled and time-of-day specific, the evolution of altered singing rhythms in response to parasite pressure is unlikely without a correlated response in females (and may account for the observed similarity in onset of singing between the Oahu and Mangaia populations in Chapter 4). Conversely, if females from parasitised populations with greater numbers of silent males are less choosy and more responsive to normal-wing song (Nathan W. Bailey and Zuk 2012), and females are able to reduce their own levels of parasitism by shifting phonotaxis to times of day when *O. ochracea* is least active, this may facilitate the evolution of temporal escape in singing males. Another big unknown is whether males have evolved other rhythmic defence strategies beyond a likely shift in singing. For example, if male crickets are capable of singing, and indeed even mating, for some time after being parasitised by *O. ochracea* then although infection is lethal, selection could favour immune defence strategies that prolong a male's ability to copulate even once infected. It is well documented that time-

of-day of infection can affect the severity of illness and outcome, and so, priming the immune system to peak when infection risk is highest may be one possible strategy to increase fitness. However, as outlined in Chapter 2, the immune system operates within a wide range of constraints, and crickets will need to balance the best defence against *O. ochracea* with defences against other parasites and pathogens they encounter. Understanding how crickets respond to *O. ochracea* is also necessary to determine whether time of day of infection impacts on *O. ochracea* activities as well impacting on the consequences for crickets.

6.4. Evolution of parasite rhythms for offence against host rhythms

When out-of-synch with host rhythms, the rodent malaria parasite *P. chabaudi* suffers a 50% reduction in both asexual and sexual stage parasites (gametocytes). Sexual reproduction is essential for parasite transmission so in my final data chapter (Chapter 5) tested why parasites incur a fitness cost when temporally misaligned to rhythms in its environment (i.e. within the host). Specifically, I examined two possible explanations: (1) reduced investment into the production of sexual stage gametocytes (i.e., conversion) to ameliorate the impact of mismatch on asexual replication, and (2) time of day specific elevated clearance of gametocytes by the host immune system. I did not find evidence supporting either hypothesis, even though conversion is a highly plastic trait and so it would be expected to vary in response to stressors that change aspects of the within-host environment, and the host immune factor I examined (TNF-α) is known to have a strong gametocytocidal effect.

While TNF-α is the only component of the innate immune response known to rapidly clear gametocytes from the circulation (Long *et al.* 2008), few rhythmic immune factors have been studied so it remains possible that immune rhythms are relevant. It is also possible that the sampling regime I followed may be at least in part possible for the observed reduction in gametocytes. For example, when mismatched to host time-of-day, sampling

may occur when only newly infective gametocytes are present, and when matched to host time-of-day, sampling may occur at a time-of-day that counts both young gametocytes only just reaching sexual maturity plus older senesced gametocytes that have not yet been cleared. Thus, in the former, gametocyte density may be lower, but infectivity may be higher, compared to the latter in which gametocyte density is higher but overall infectivity is relatively lower (Schneider *et al.* 2018c). Future studies which account for not only gametocyte density but maturity/infectivity, or sample more frequently during the circadian cycle, may parse the real effect of mismatch on transmission potential. Because sexual stage parasites are necessary for onwards transmission, uncovering why it is important for parasites to be in-synch with host rhythms may inform proximate and ultimate responses of parasites to temporal shifts in vector biting behaviour caused by the usage of insecticide-treated bed nets.

6.5. Conclusion

The work presented in this thesis reveals that circadian rhythms underpin traits relevant to host-parasite interactions as well to reproductive activities that underpin fitness. I show that knowledge gained from insights into the molecular underpinnings of clocks can be harnessed towards understanding the evolutionary ecology of circadian rhythms, particularly in the context of infections. Lessons from interrogating evolutionary and ecological questions from a chronobiological framework include both understanding the coevolution of hosts and parasites in the wild, as well as using this knowledge towards making medical interventions which capitalise on disrupting or harnessing rhythms robust to evolution. Thus, understanding how rhythms in parasite offense and host defence evolve provides both basic and applied knowledge, and as parasites are ubiquitous in nature, lessons learned from this research may be broadly applicable and could help elucidate open questions in evolutionary ecology.

7. Literature Cited

Abdelsalam, Salaheldin, Hiroyuki Uemura, Yujiro Umezaki, A. S. M. Saifullah, Miki Shimohigashi, and Kenji Tomioka. 2008. "Characterization of PDF-Immunoreactive Neurons in the Optic Lobe and Cerebral Lobe of the Cricket, Gryllus Bimaculatus." *Journal of Insect Physiology* 54 (7): 1205–12.

Abe, Yoshiteru, Hiroshi Ushirogawa, and Kenji Tomioka. 1997. "Circadian Locomotor Rhythms in the Cricket, Gryllodes Sigiilatus I. Localization of the Pacemaker and the Photoreceptor." *Zoological Science* 14 (5): 719–27.

Aide, T. Mitchell, Carlos Corrada-Bravo, Marconi Campos-Cerqueira, Carlos Milan, Giovany Vega, and Rafael Alvarez. 2013. "Real-Time Bioacoustics Monitoring and Automated Species Identification." *PeerJ* 1: e103.

Alexander, Richard D., and Daniel Otte. 2009. "Crickets." In *Encyclopedia of Insects*, 232–36. Elsevier.

Aschoff, Jürgen. 1965. "Circadian Rhythms in Man." *Science* 148 (3676): 1427–32.

Azevedo, Graciele Vivian de, Rubens Rodriguez, Sérgio Machado Porto, Carlos Graeff-Teixeira, and Fernando Fornari. 2011. "Elimination of Angiostrongylus Costaricensis Larvae in Feces from Experimentally Infected Swiss Mice: Circadian Rhythm and Correlation with Survival." *Parasitology Research* 108 (3): 537–40.

Babbitt, Shalon E., Lindsey Altenhofen, Simon A. Cobbold, Eva S. Istvan, Clare Fennell, Christian Doerig, Manuel Llinás, and Daniel E. Goldberg. 2012. "Plasmodium Falciparum Responds to Amino Acid Starvation by Entering into a Hibernatory State." *Proceedings of the National Academy of Sciences* 109 (47): E3278–87.

Bae, Kiho, Xiaowei Jin, Elizabeth S. Maywood, Michael H. Hastings, Steven M. Reppert, and David R. Weaver. 2001. "Differential Functions of MPer1, MPer2, and MPer3 in the SCN Circadian Clock." *Neuron* 30 (2): 525–36.

Bailey, N. W., and E. Macleod. 2014. "Socially Flexible Female Choice and Premating Isolation in Field Crickets (Teleogryllus Spp.)." *Journal of Evolutionary Biology* 27 (1): 170–80.

Bailey, Nathan W., Brian Gray, and Marlene Zuk. 2010. "Acoustic Experience Shapes Alternative Mating Tactics and Reproductive Investment in Male Field Crickets." *Current Biology: CB* 20 (9): 845–49.

Bailey, Nathan W., Janelle R. McNabb, and Marlene Zuk. 2007. "Preexisting Behavior Facilitated the Loss of a Sexual Signal in the Field Cricket Teleogryllus Oceanicus." *Behavioral Ecology: Official Journal of the International Society for Behavioral Ecology* 19 (1): 202–7.

Bailey, Nathan W., and Marlene Zuk. 2008. "Acoustic Experience Shapes Female Mate Choice in Field Crickets." *Proceedings. Biological Sciences / The Royal Society* 275 (1651): 2645–50.

———. 2012. “Socially Flexible Female Choice Differs among Populations of the Pacific Field Cricket: Geographical Variation in the Interaction Coefficient Psi (Ψ).” *Proceedings. Biological Sciences / The Royal Society* 279 (1742): 3589–96.

Balakrishnan, Rohini, and Gerald S. Pollack. 1996. “Recognition of Courtship Song in the Field Cricket, Teleogryllus Oceanicus.” *Animal Behaviour* 51 (2): 353–66.

Ballesta, Annabelle, Pasquale F. Innominato, Robert Dallmann, David A. Rand, and Francis A. Lévi. 2017. “Systems Chronotherapeutics.” *Pharmacological Reviews* 69 (2): 161–99.

Barbosa, Pedro, and Astrid Caldas. 2007. “Do Larvae of Species in Macrolepidopteran Assemblages Share Traits That Influence Susceptibility to Parasitism?” *Environmental Entomology* 36 (2): 329–36.

Batschelet, Edward. 1981. “Circular Statistics in Biology.” *ACADEMIC PRESS, 111 FIFTH AVE., NEW YORK, NY 10003, 1981, 388*.

Beaver, L. M., B. O. Gvakharia, T. S. Vollintine, D. M. Hege, R. Stanewsky, and J. M. Giebultowicz. 2002. “Loss of Circadian Clock Function Decreases Reproductive Fitness in Males of Drosophila Melanogaster.” *Proceedings of the National Academy of Sciences of the United States of America* 99 (4): 2134–39.

Bekker, Charissa de, Robin A. Ohm, Raquel G. Loreto, Aswathy Sebastian, Istvan Albert, Martha Merrow, Andreas Brachmann, and David P. Hughes. 2015. “Gene Expression during Zombie Ant Biting Behavior Reflects the Complexity Underlying Fungal Parasitic Behavioral Manipulation.” *BMC Genomics* 16 (1): 620.

Bekker, Charissa de, Ian Will, Biplabendu Das, and Rachelle M. M. Adams. 2018. “The Ants (Hymenoptera: Formicidae) and Their Parasites: Effects of Parasitic Manipulations and Host Responses on Ant Behavioral Ecology.” *Myrmecological News / Osterreichische Gesellschaft Fur Entomofaunistik* 28: 1–24.

Bellet, Marina M., Elisa Deriu, Janet Z. Liu, Benedetto Grimaldi, Christoph Blaschitz, Michael Zeller, Robert A. Edwards, et al. 2013. “Circadian Clock Regulates the Host Response to Salmonella.” *Proceedings of the National Academy of Sciences* 110 (24): 9897 LP – 9902.

Bell-Pedersen, Deborah, Vincent M. Cassone, David J. Earnest, Susan S. Golden, Paul E. Hardin, Terry L. Thomas, and Mark J. Zoran. 2005. “Circadian Rhythms from Multiple Oscillators: Lessons from Diverse Organisms.” *Nature Reviews. Genetics* 6 (7): 544–56.

Bhardwaj, Vaibhav, Stuart Meier, Lindsay N. Petersen, Robert A. Ingle, and Laura C. Roden. 2011. “Defence Responses of Arabidopsis Thaliana to Infection by Pseudomonas Syringae Are Regulated by the Circadian Clock.” *PloS One* 6 (10): e26968.

Birget, Philip L. G., Charlotte Repton, Aidan J. O’Donnell, Petra Schneider, and Sarah E. Reece. 2017. “Phenotypic Plasticity in Reproductive Effort: Malaria Parasites Respond to Resource Availability.” *Proceedings of the Royal Society B: Biological Sciences* 284 (1860): 20171229.

Biron, D. G., Fleur Ponton, L. Marché, N. Galeotti, L. Renault, E. Demey-Thomas, J. Poncet, S. P. Brown, P. Jouin, and F. Thomas. 2006. "'Suicide'of Crickets Harbouring Hairworms: A Proteomics Investigation." *Insect Molecular Biology* 15 (6): 731–42.

Brancucci, Nicolas M. B., Joseph P. Gerdt, Chengqi Wang, Mariana De Niz, Nisha Philip, Swamy R. Adapa, Min Zhang, Eva Hitz, Igor Niederwieser, and Sylwia D. Boltryk. 2017. "Lysophosphatidylcholine Regulates Sexual Stage Differentiation in the Human Malaria Parasite Plasmodium Falciparum." *Cell* 171 (7): 1532–44.

Bredow, Sebastian, Nandita Guha-Thakurta, Ping Taishi, Ferenc Obál Jr, and James M. Krueger. 1997. "Diurnal Variations of Tumor Necrosis Factor Alpha MRNA and Alpha-Tubulin MRNA in Rat Brain." *Neuroimmunomodulation* 4 (2): 84–90.

Breiman, Leo. 2001. "Random Forests." *Machine Learning* 45 (1): 5–32.

Buckling, A., L. C. Ranford-Cartwright, A. Miles, and Andrew Fraser Read. 1999. "Chloroquine Increases Plasmodium Falciparum Gametocytogenesis in Vitro." *Parasitology* 118 (4): 339–46.

Cade, W. 1975. "Acoustically Orienting Parasitoids: Fly Phonotaxis to Cricket Song." *Science* 190 (4221): 1312–13.

Cade, William. 1979. "The Evolution of Alternative Male Reproductive Strategies in Field Crickets." In *Sexual Selection and Reproductive Competition in Insects*, 343–79. Elsevier.

Cade, William H., Mark Ciceran, and Anne-Marie Murray. 1996. "Temporal Patterns of Parasitoid Fly (Ormia Ochracea) Attraction to Field Cricket Song (Gryllus Integer)." *Canadian Journal of Zoology* 74 (2): 393–95.

Carter, Lucy M., Björn F. C. Kafsack, Manuel Llinás, Nicole Mideo, Laura C. Pollitt, and Sarah E. Reece. 2013. "Stress and Sex in Malaria ParasitesWhy Does Commitment Vary?" *Evolution, Medicine, and Public Health* 2013 (1): 135–47.

Cavallari, Nicola, Elena Frigato, Daniela Vallone, Nadine Fröhlich, Jose Fernando Lopez-Olmeda, Augusto Foà, Roberto Berti, Francisco Javier Sánchez-Vázquez, Cristiano Bertolucci, and Nicholas S. Foulkes. 2011. "A Blind Circadian Clock in Cavefish Reveals That Opsins Mediate Peripheral Clock Photoreception." *PLoS Biology* 9 (9): e1001142.

Chen, Zheng, Elizabeth A. Odstrcil, Benjamin P. Tu, and Steven L. McKnight. 2007. "Restriction of DNA Replication to the Reductive Phase of the Metabolic Cycle Protects Genome Integrity." *Science* 316 (5833): 1916 LP – 1919.

Clark, Ian A., Alison C. Budd, and Lisa M. Alleva. 2008. "Sickness Behaviour Pushed Too Far–the Basis of the Syndrome Seen in Severe Protozoal, Bacterial and Viral Diseases and Post-Trauma." *Malaria Journal* 7 (1): 208.

Cotter, S. C., J. P. Myatt, C. M. H. Benskin, and Ken Wilson. 2008. "Selection for Cuticular Melanism Reveals Immune Function and Life-history Trade-offs in Spodoptera Littoralis." *Journal of Evolutionary Biology* 21 (6): 1744–54.

Cremers, Jolien, Kees Tim Mulder, and Irene Klugkist. 2018. “Circular Interpretation of Regression Coefficients.” *The British Journal of Mathematical and Statistical Psychology* 71 (1): 75–95.

Cruz, Laura N., Yang Wu, Henning Ulrich, Alister G. Craig, and Célia R. S. Garcia. 2016. “Tumor Necrosis Factor Reduces Plasmodium Falciparum Growth and Activates Calcium Signaling in Human Malaria Parasites.” *Biochimica et Biophysica Acta (BBA)-General Subjects* 1860 (7): 1489–97.

Curtis, Anne M., Marina M. Bellet, Paolo Sassone-Corsi, and Luke A. J. O’Neill. 2014. “Circadian Clock Proteins and Immunity.” *Immunity* 40 (2): 178–86.

Danbara, Yoshiki, Tomoaki Sakamoto, Outa Uryu, and Kenji Tomioka. 2010. “RNA Interference of Timeless Gene Does Not Disrupt Circadian Locomotor Rhythms in the Cricket Gryllus Bimaculatus.” *Journal of Insect Physiology* 56 (12): 1738–45.

Danley, Patrick D., Tagide N. DeCarvalho, Daniel J. Fergus, and Kerry L. Shaw. 2007. “Reproductive Asynchrony and the Divergence of Hawaiian Crickets.” *Ethology: Formerly Zeitschrift Fur Tierpsychologie* 113 (12): 1125–32.

Dantzer, Robert, Jason C. O’Connor, Gregory G. Freund, Rodney W. Johnson, and Keith W. Kelley. 2008. “From Inflammation to Sickness and Depression: When the Immune System Subjugates the Brain.” *Nature Reviews. Neuroscience* 9 (1): 46.

De Bekker, Charissa, Martha Merrow, and David P. Hughes. 2014. “From Behavior to Mechanisms: An Integrative Approach to the Manipulation by a Parasitic Fungus (Ophiocordyceps Unilateralis Sl) of Its Host Ants (Camponotus Spp.).” *Integrative and Comparative Biology* 54 (2): 166–76.

DeCoursey, P. J., J. K. Walker, and S. A. Smith. 2000. “A Circadian Pacemaker in Free-Living Chipmunks: Essential for Survival?” *Journal of Comparative Physiology A* 186 (2): 169–80.

DeCoursey, Patricia J., Jill R. Krulas, Gary Mele, and Daniel C. Holley. 1997. “Circadian Performance of Suprachiasmatic Nuclei (SCN)-Lesioned Antelope Ground Squirrels in a Desert Enclosure.” *Physiology & Behavior* 62 (5): 1099–1108.

Dodd, Antony N., Neeraj Salathia, Anthony Hall, Eva Kévei, Réka Tóth, Ferenc Nagy, Julian M. Hibberd, Andrew J. Millar, and Alex A. R. Webb. 2005. “Plant Circadian Clocks Increase Photosynthesis, Growth, Survival, and Competitive Advantage.” *Science* 309 (5734): 630 LP – 633.

Donnell, Aidan J. O., Petra Schneider, Harriet G. Mcwatters, and Sarah E. Reece. 2011. “Fitness Costs of Disrupting Circadian Rhythms in Malaria Parasites Fitness Costs of Disrupting Circadian Rhythms in Malaria Parasites.” *Proceedings of the Royal Society B: Biological Sciences*, no. January (January): 2429–36.

Drakeley, C. J., I. Secka, S. Correa, B. M. Greenwood, and G. A. T. Targett. 1999. “Host Haematological Factors Influencing the Transmission of Plasmodium Falciparum Gametocytes to Anopheles Gambiae Ss Mosquitoes.” *Tropical Medicine & International Health: TM & IH* 4 (2): 131–38.

Druzd, David, Olga Matveeva, Louise Ince, Ute Harrison, Wenyan He, Christoph Schmal, Hanspeter Herzel, Anthony H. Tsang, Naoto Kawakami, and Alexei Leliavski.

2017. “Lymphocyte Circadian Clocks Control Lymph Node Trafficking and Adaptive Immune Responses.” *Immunity* 46 (1): 120–32.

Du, Lucia Y., Hannah Darroch, Pramuk Keerthisinghe, Elina Ashimbayeva, Jonathan W. Astin, Kathryn E. Crosier, Philip S. Crosier, Guy Warman, James Cheeseman, and Christopher J. Hall. 2017. “The Innate Immune Cell Response to Bacterial Infection in Larval Zebrafish Is Light-Regulated.” *Scientific Reports* 7 (1): 12657.

Dyer, Mike, and Karen P. Day. 2003. “Regulation of the Rate of Asexual Growth and Commitment to Sexual Development by Diffusible Factors from in Vitro Cultures of Plasmodium Falciparum.” *The American Journal of Tropical Medicine and Hygiene* 68 (4): 403–9.

Earn, David J. D., Paul W. Andrews, and Benjamin M. Bolker. 2014. “Population-Level Effects of Suppressing Fever.” *Proc. R. Soc. B* 281 (1778): 20132570.

Edgar, Rachel S., Alessandra Stangherlin, Andras D. Nagy, Michael P. Nicoll, Stacey Efstathiou, John S. O’Neill, and Akhilesh B. Reddy. 2016. “Cell Autonomous Regulation of Herpes and Influenza Virus Infection by the Circadian Clock.” *Proceedings of the National Academy of Sciences* 113 (36): 10085–90.

Eldredge, Lucius G., and Neal L. Evenhuis. 2003. *Hawaii’s Biodiversity: A Detailed Assessment of the Numbers of Species in the Hawaiian Islands*. Bishop Museum Press.

Elliot, Simon L., and Adam G. Hart. 2010. “Density-dependent Prophylactic Immunity Reconsidered in the Light of Host Group Living and Social Behavior.” *Ecology* 91 (1): 65–72.

Emerson, Kevin J., Alathea D. Letaw, William E. Bradshaw, and Christina M. Holzapfel. 2008. “Extrinsic Light:Dark Cycles, Rather than Endogenous Circadian Cycles, Affect the Photoperiodic Counter in the Pitcher-Plant Mosquito, Wyeomyia Smithii.” *Journal of Comparative Physiology. A, Neuroethology, Sensory, Neural, and Behavioral Physiology* 194 (7): 611–15.

Evans, Sharon S., Elizabeth A. Repasky, and Daniel T. Fisher. 2015. “Fever and the Thermal Regulation of Immunity: The Immune System Feels the Heat.” *Nature Reviews. Immunology* 15 (6): 335.

Feeney, Kevin A., Louise L. Hansen, Marrit Putker, Consuelo Olivares-Yañez, Jason Day, Lorna J. Eades, Luis F. Larrondo, Nathaniel P. Hoyle, John S. O’Neill, and Gerben van Ooijen. 2016. “Daily Magnesium Fluxes Regulate Cellular Timekeeping and Energy Balance.” *Nature* 532 (7599): 375–79.

Fenske, Myles P., Leann P. Nguyen, Erin K. Horn, Jeffrey A. Riffell, and Takato Imaizumi. 2018. “Circadian Clocks of Both Plants and Pollinators Influence Flower Seeking Behavior of the Pollinator Hawkmoth Manduca Sexta.” *Scientific Reports* 8 (1): 2842.

Fergus, Daniel J., Tagide N. Decarvalho, and Kerry L. Shaw. 2011. “Genetically Regulated Temporal Variation of Novel Courtship Elements in the Hawaiian Cricket Genus Laupala.” *Behavior Genetics* 41 (4): 607–14.

Fortier, Erin E., Julie Rooney, Hugues Dardente, Marie-Pierre Hardy, Nathalie Labrecque, and Nicolas Cermakian. 2011. “Circadian Variation of the Response of T Cells to Antigen.” *Journal of Immunology* 187 (12): 6291–6300.

Gagnidze, Khatuna, Kaitlyn H. Hajdarovic, Marina Moskalenko, Ilia N. Karatsoreos, Bruce S. McEwen, and Karen Bulloch. 2016. “Nuclear Receptor REV-ERBα Mediates Circadian Sensitivity to Mortality in Murine Vesicular Stomatitis Virus-Induced Encephalitis.” *Proceedings of the National Academy of Sciences*, 201520489.

Gautret, P., Eric Deharo, R. Tahar, A. G. Chabaud, and I. Landau. 1995. “The Adjustment of the Schizogonic Cycle of Plasmodium Chabaudi Chabaudi in the Blood to the Circadian Rhythm of the Host.” *Parasite* 2 (1): 69–74.

Gautret, Philippe, François Miltgen, Jean-Charles Gantier, Alain Gabriel Chabaud, and Irène Landau. 1996. “Enhanced Gametocyte Formation by Plasmodium Chabaudi in Immature Erythrocytes: Pattern of Production, Sequestration, and Infectivity to Mosquitoes.” *The Journal of Parasitology* 82 (6): 900–906.

Geissmann, Quentin, Luis Garcia Rodriguez, Esteban J. Beckwith, and Giorgio F. Gilestro. 2019. “Rethomics: An R Framework to Analyse High-Throughput Behavioural Data.” *PloS One* 14 (1): e0209331.

Ghai, Ria R., Vincent Fugere, Colin A. Chapman, Tony L. Goldberg, and T. Jonathan Davies. 2015. “Sickness Behaviour Associated with Non-Lethal Infections in Wild Primates.” *Proc. R. Soc. B* 282 (1814): 20151436.

Gibbs, Julie E., John Blaikley, Stephen Beesley, Laura Matthews, Karen D. Simpson, Susan H. Boyce, Stuart N. Farrow, Kathryn J. Else, Dave Singh, and David W. Ray. 2012. “The Nuclear Receptor REV-ERBα Mediates Circadian Regulation of Innate Immunity through Selective Regulation of Inflammatory Cytokines.” *Proceedings of the National Academy of Sciences* 109 (2): 582–87.

Gibbs, Julie, Louise Ince, Laura Matthews, Junjie Mei, Thomas Bell, Nan Yang, Ben Saer, Nicola Begley, Toryn Poolman, and Marie Pariollaud. 2014. “An Epithelial Circadian Clock Controls Pulmonary Inflammation and Glucocorticoid Action.” *Nature Medicine* 20 (8): 919.

Goodspeed, Danielle, E. Wassim Chehab, Amelia Min-Venditti, Janet Braam, and Michael F. Covington. 2012. “Arabidopsis Synchronizes Jasmonate-Mediated Defense with Insect Circadian Behavior.” *Proceedings of the National Academy of Sciences of the United States of America* 109 (12): 4674–77.

Goulson, Dave. 1997. “Wipfelkrankheit: Modification of Host Behaviour during Baculoviral Infection.” *Oecologia* 109 (2): 219–28.

Graham, Andrea L., Judith E. Allen, and Andrew F. Read. 2005. “Evolutionary Causes and Consequences of Immunopathology.” *Annual Review of Ecology, Evolution, and Systematics* 36 (1): 373–97.

Green, Rachel M., Sonia Tingay, Zhi-Yong Wang, and Elaine M. Tobin. 2002. “Circadian Rhythms Confer a Higher Level of Fitness to Arabidopsis Plants.” *Plant Physiology* 129 (2): 576 LP – 584.

Greenfield, Michael D. 1988. “Interspecific Acoustic Interactions among Katydids Neoconocephalus: Inhibition-Induced Shifts in Diel Periodicity.” *Animal Behaviour* 36 (3): 684–95.

———. 1997. “Acoustic Communication in Orthoptera.” *The Bionomics of Grasshoppers, Katydids and Their Kin*, 197–230.

Greischar, Megan A., Nicole Mideo, Andrew F. Read, and Ottar N. Bjørnstad. 2016. “Quantifying Transmission Investment in Malaria Parasites.” *PLoS Computational Biology* 12 (2): e1004718.

Greives, Timothy J., Sjouke A. Kingma, Bart Kranstauber, Kim Mortega, Martin Wikelski, Kees van Oers, A. Christa Mateman, Glen A. Ferguson, Giulia Beltrami, and Michaela Hau. 2015. “Costs of Sleeping in: Circadian Rhythms Influence Cuckoldry Risk in a Songbird.” *Functional Ecology* 29 (10): 1300–1307.

Griebel, Thomas, and Jürgen Zeier. 2008. “Light Regulation and Daytime Dependency of Inducible Plant Defenses in Arabidopsis: Phytochrome Signaling Controls Systemic Acquired Resistance Rather than Local Defense.” *Plant Physiology* 147 (2): 790–801.

Hack, Mace A. 1998. “The Energetics of Male Mating Strategies in Field Crickets (Orthoptera: Gryllinae: Gryllidae).” *Journal of Insect Behavior* 11 (6): 853–67.

Häfker, N. Sören, Bettina Meyer, Kim S. Last, David W. Pond, Lukas Hüppe, and Mathias Teschke. 2017. “Circadian Clock Involvement in Zooplankton Diel Vertical Migration.” *Current Biology: CB* 27 (14): 2194-2201.e3.

Hart, Benjamin L. 1994. “Behavioural Defense against Parasites: Interaction with Parasite Invasiveness.” *Parasitology* 109 (S1): S139–51.

Hassaneen, Ehab, Alaa El-Din Sallam, Ahmad Abo-Ghalia, Yoshiyuki Moriyama, Svetlana G. Karpova, Salah Abdelsalam, Ayami Matsushima, Yasuyuki Shimohigashi, and Kenji Tomioka. 2011. “Pigment-Dispersing Factor Affects Nocturnal Activity Rhythms, Photic Entrainment, and the Free-Running Period of the Circadian Clock in the Cricket Gryllus Bimaculatus.” *Journal of Biological Rhythms* 26 (1): 3–13.

Hau, Michaela, Davide Dominoni, Stefania Casagrande, C. Loren Buck, Gabriela Wagner, David Hazlerigg, Timothy Greives, and Roelof A. Hut. 2017. “Timing as a Sexually Selected Trait: The Right Mate at the Right Moment.” *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* 372 (1734): 20160249.

Haus, Erhard, David J. Lakatua, Jacqueline Swoyer, and Linda Sackett-Lundeen. 2018. “Chronobiology in Hematology and Immunology.” *The American Journal of Anatomy* 168 (4): 467–517.

Haus, Erhard, and Michael H. Smolensky. 1999. “Biologic Rhythms in the Immune System.” *Chronobiology International* 16 (5): 581–622.

Hawking, Frank. 1967. “The 24-Hour Periodicity of Microfilariae: Biological Mechanisms Responsible for Its Production and Control.” *Proceedings of the Royal Society of*

London. Series B, Containing Papers of a Biological Character. Royal Society 169 (1014): 59–76.

Hays, G. C., H. Kennedy, and B. W. Frost. 2001. "Individual Variability in Diel Vertical Migration of a Marine Copepod: Why Some Individuals Remain at Depth When Others Migrate." *Limnology and Oceanography* 46 (8): 2050–54.

Heinen-Kay, Justa L., Daina B. Strub, Susan L. Balenger, and Marlene Zuk. 2019. "Direct and Indirect Effects of Sexual Signal Loss on Female Reproduction in the Pacific Field Cricket (Teleogryllus Oceanicus)." *Journal of Evolutionary Biology* 32 (12): 1382–90.

Heller, Klaus-Gerhard. 1986. "Warm-up and Stridulation in the Buschcricket, Hexacentrus Unicolor Serville (Orthoptera, Conocephalidae, Listroscelidinae)." *The Journal of Experimental Biology* 126 (1): 97–109.

Helm, Barbara, Marcel E. Visser, William Schwartz, Noga Kronfeld-Schor, Menno Gerkema, Theunis Piersma, and Guy Bloch. 2017. "Two Sides of a Coin: Ecological and Chronobiological Perspectives of Timing in the Wild." *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* 372 (1734). http://rstb.royalsocietypublishing.org/content/372/1734/20160246.abstract.

Herbison, Ryan, Clement Lagrue, and Robert Poulin. 2018. "The Missing Link in Parasite Manipulation of Host Behaviour." *Parasites & Vectors* 11 (1): 222.

Hevia, Montserrat A., Paulo Canessa, Hanna Müller-Esparza, and Luis F. Larrondo. 2015. "A Circadian Oscillator in the Fungus Botrytis Cinerea Regulates Virulence When Infecting Arabidopsis Thaliana." *Proceedings of the National Academy of Sciences* 112 (28): 8744–49.

Hirako, Isabella Cristina, Patrícia Aparecida Assis, Natália Satchiko Hojo-Souza, George Reed, Helder Nakaya, Douglas Taylor Golenbock, Roney Santos Coimbra, and Ricardo Tostes Gazzinelli. 2018. "Daily Rhythms of TNFα Expression and Food Intake Regulate Synchrony of Plasmodium Stages with the Host Circadian Cycle." *Cell Host & Microbe*.

Hoback, W. Wyatt, and W. E. J. R. Wagner. 1997. "The Energetic Cost of Calling in the Variable Field Cricket, Gryllus Lineaticeps." *Physiological Entomology* 22 (3): 286–90.

Holzer, Barbara, Alain Jacot, and Martin W. G. Brinkhof. 2003. "Condition-Dependent Signaling Affects Male Sexual Attractiveness in Field Crickets, Gryllus Campestris." *Behavioral Ecology: Official Journal of the International Society for Behavioral Ecology* 14 (3): 353–59.

Hoover, Kelli, Michael Grove, Matthew Gardner, David P. Hughes, James McNeil, and James Slavicek. 2011. "A Gene for an Extended Phenotype." *Science* 333 (6048): 1401.

Hopwood, Thomas W., Sarah Hall, Nicola Begley, Ruth Forman, Sheila Brown, Ryan Vonslow, Ben Saer, Matthew C. Little, Emma A. Murphy, and Rebecca J. Hurst.

2018. “The Circadian Regulator BMAL1 Programmes Responses to Parasitic Worm Infection via a Dendritic Cell Clock.” *Scientific Reports* 8 (1): 3782.
Horn, Melanie, Oliver Mitesser, Thomas Hovestadt, Taishi Yoshii, Dirk Rieger, and Charlotte Helfrich-Förster. 2019. “The Circadian Clock Improves Fitness in the Fruit Fly, Drosophila Melanogaster.” *Frontiers in Physiology* 10 (November): 1374.
Houslay, Thomas M., Kirsty F. Houslay, James Rapkin, John Hunt, and Luc F. Bussiere. 2017. “Mating Opportunities and Energetic Constraints Drive Variation in Age-dependent Sexual Signalling.” *Functional Ecology* 31 (3): 728–41.
Hozer, Clara, Martine Perret, Samuel Pavard, and Fabien Pifferi. 2020. “Survival Is Reduced When Endogenous Period Deviates from 24 h in a Non-Human Primate, Supporting the Circadian Resonance Theory.” *Scientific Reports* 10 (1): 1–9.
Hughes, Michael E., Katherine C. Abruzzi, Ravi Allada, Ron Anafi, Alaaddin Bulak Arpat, Gad Asher, Pierre Baldi, Charissa De Bekker, Deborah Bell-Pedersen, and Justin Blau. 2017. “Guidelines for Genome-Scale Analysis of Biological Rhythms.” *Journal of Biological Rhythms* 32 (5): 380–93.
Hunt, John, Robert Brooks, Michael D. Jennions, Michael J. Smith, Caroline L. Bentsen, and Luc F. Bussiere. 2004. “High-Quality Male Field Crickets Invest Heavily in Sexual Display but Die Young.” *Nature* 432 (7020): 1024–27.
Hurvich, Clifford M., and Chih-ling Tsai. 1993. “A Corrected Akaike Information Criterion for Vector Autoregressive Model Selection.” *Journal of Time Series Analysis / a Journal Sponsored by the Bernoulli Society for Mathematical Statistics and Probability* 14 (3): 271–79.
Hut, Roelof A., Violetta Pilorz, Ate S. Boerema, Arjen M. Strijkstra, and Serge Daan. 2011. “Working for Food Shifts Nocturnal Mouse Activity into the Day.” *PloS One* 6 (3): e17527.
Ingle, Robert A., Claire Stoker, Wendy Stone, Nicolette Adams, Rob Smith, Murray Grant, Isabelle Carré, Laura C. Roden, and Katherine J. Denby. 2015. “Jasmonate Signalling Drives Time-of-Day Differences in Susceptibility of Arabidopsis to the Fungal Pathogen Botrytis Cinerea.” *The Plant Journal: For Cell and Molecular Biology* 84 (5): 937–48.
Jabbur, Maria Luísa, Chi Zhao, and Carl Hirschie Johnson. 2021. “Insights into the Evolution of Circadian Clocks Gleaned from Bacteria.” In *Circadian Rhythms in Bacteria and Microbiomes*, edited by Carl Hirschie Johnson and Michael Joseph Rust, 111–35. Cham: Springer International Publishing.
Jammalamadaka, S. Rao, and Ambar Sengupta. 2001. *Topics in Circular Statistics*. Vol. 5. world scientific.
Johnson, Carl Hirschie, Chi Zhao, Yao Xu, and Tetsuya Mori. 2017. “Timing the Day: What Makes Bacterial Clocks Tick?” *Nature Reviews. Microbiology* 15 (4): 232.
Josephson, Robert K., and Roger C. Halverson. 1971. “High Frequency Muscles Used in Sound Production by a Katydid. I. Organization of the Motor System.” *The Biological Bulletin* 141 (3): 411–33.

Judge, Kevin A., Janice J. Ting, and Darryl T. Gwynne. 2008. “Condition Dependence of Male Life Span and Calling Effort in a Field Cricket.” *Evolution; International Journal of Organic Evolution* 62 (4): 868–78.

Keller, Maren, Jeannine Mazuch, Ute Abraham, Gina D. Eom, Erik D. Herzog, Hans-Dieter Volk, Achim Kramer, and Bert Maier. 2009. “A Circadian Clock in Macrophages Controls Inflammatory Immune Responses.” *Proceedings of the National Academy of Sciences* 106 (50): 21407 LP – 21412.

Kerr, Angela M., Susan N. Gershman, and Scott K. Sakaluk. 2010. “Experimentally Induced Spermatophore Production and Immune Responses Reveal a Trade-off in Crickets.” *Behavioral Ecology: Official Journal of the International Society for Behavioral Ecology* 21 (3): 647–54.

Kevan, D. K. 1990. “Introduced Grasshoppers and Crickets in Micronesia.” *Boletin de Sanidad Vegetal, Plagas*. https://agris.fao.org/agris-search/search.do?recordID=ES9100807.

Kharouba, Heather M., Johan Ehrlén, Andrew Gelman, Kjell Bolmgren, Jenica M. Allen, Steve E. Travers, and Elizabeth M. Wolkovich. 2018. “Global Shifts in the Phenological Synchrony of Species Interactions over Recent Decades.” *Proceedings of the National Academy of Sciences* 115 (20): 5211–16.

Kiessling, Silke, Geneviève Dubeau-Laramée, Hyejee Ohm, Nathalie Labrecque, Martin Olivier, and Nicolas Cermakian. 2017. “The Circadian Clock in Immune Cells Controls the Magnitude of Leishmania Parasite Infection.” *Scientific Reports* 7 (1): 10892.

Kluger, M. J. 1979. “Phylogeny of Fever.” In *Federation Proceedings*, 38:30–34.

Kluger, Matthew J., Daniel H. Ringler, and Miriam R. Anver. 1975. “Fever and Survival.” *Science* 188 (4184): 166–68.

Ko, Caroline H., and Joseph S. Takahashi. 2006. “Molecular Components of the Mammalian Circadian Clock.” *Human Molecular Genetics* 15 (suppl_2): R271–77.

Kolluru, Gita Raman. 1999. “Variation and Repeatability of Calling Behavior in Crickets Subject to a Phonotactic Parasitoid Fly.” *Journal of Insect Behavior* 12 (5): 611–26.

Koritala, Bala S. C., Craig Wager, Joshua C. Waters, Ryan Pachucki, Benedetto Piccoli, Yaping Feng, Laura B. Scheinfeldt, et al. 2020. “Habitat-Specific Clock Variation and Its Consequence on Reproductive Fitness.” *Journal of Biological Rhythms* 35 (2): 134–44.

Korneli, Christin, Selahattin Danisman, and Dorothee Staiger. 2014. “Differential Control of Pre-Invasive and Post-Invasive Antibacterial Defense by the Arabidopsis Circadian Clock.” *Plant & Cell Physiology* 55 (9): 1613–22.

Kruskal, William H., and W. Allen Wallis. 1952. “Use of Ranks in One-Criterion Variance Analysis.” *Journal of the American Statistical Association* 47 (260): 583–621.

Kuo, Tzu-Hsing, Douglas H. Pike, Zahra Beizaeipour, and Julie A. Williams. 2010. “Sleep Triggered by an Immune Response in Drosophila Is Regulated by the Circadian Clock and Requires the NFκB Relish.” *BMC Neuroscience* 11 (1): 17.

Kwiatkowski, D., and B. M. Greenwood. 1989. “Why Is Malaria Fever Periodic? A Hypothesis.” *Parasitology Today* 5 (8): 264–66.
Labrecque, Nathalie, and Nicolas Cermakian. 2015. “Circadian Clocks in the Immune System.” *Journal of Biological Rhythms* 30 (4): 277–90.
Lang, Veronika, Sebastian Ferencik, Bharath Ananthasubramaniam, Achim Kramer, and Bert Maier. 2021. “Susceptibility Rhythm to Bacterial Endotoxin in Myeloid Clock-Knockout Mice.” *ELife* 10 (October). https://doi.org/10.7554/eLife.62469.
Lazado, Carlo C., Peter Vilhelm Skov, and Per Bovbjerg Pedersen. 2016. “Innate Immune Defenses Exhibit Circadian Rhythmicity and Differential Temporal Sensitivity to a Bacterial Endotoxin in Nile Tilapia (Oreochromis Niloticus).” *Fish & Shellfish Immunology* 55: 613–22.
Lee, Jung-Eun, and Isaac Edery. 2008. “Circadian Regulation in the Ability of Drosophila to Combat Pathogenic Infections.” *Current Biology: CB* 18 (3): 195–99.
Lehmann, Gerlind U. C. 2003. “Review of Biogeography, Host Range and Evolution of Acoustic Hunting in Ormiini (Insecta, Diptera, Tachinidae), Parasitoids of Night-Calling Bushcrickets and Crickets (Insecta, Orthoptera, Ensifera).” *Zoologischer Anzeiger-A Journal of Comparative Zoology* 242 (2): 107–20.
Levi, Francis, and Ueli Schibler. 2007. “Circadian Rhythms: Mechanisms and Therapeutic Implications.” *Annual Review of Pharmacology and Toxicology* 47: 593–628.
Levri, Edward P. 1999. “Parasite-Induced Change in Host Behavior of a Freshwater Snail: Parasitic Manipulation or Byproduct of Infection?” *Behavioral Ecology: Official Journal of the International Society for Behavioral Ecology* 10 (3): 234–41.
Levri, Edward P., and Curtis M. Lively. 1996. “The Effects of Size, Reproductive Condition, and Parasitism on Foraging Behaviour in a Freshwater Snail, Potamopyrgus Antipodarum.” *Animal Behaviour* 51 (4): 891–901.
Liu, Elaine, Kevin Lewis, Hiba Al-Saffar, Catherine M. Krall, Anju Singh, Vladimir A. Kulchitsky, Joshua J. Corrigan, et al. 2012. “Naturally Occurring Hypothermia Is More Advantageous than Fever in Severe Forms of Lipopolysaccharide- and Escherichia Coli-Induced Systemic Inflammation.” *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology* 302 (12): R1372-83.
Liu, Yi, Martha Merrow, Jennifer J. Loros, and Jay C. Dunlap. 1998. “How Temperature Changes Reset a Circadian Oscillator.” *Science* 281 (5378): 825–29.
Lively, Curtis M. 1987. “Evidence from a New Zealand Snail for the Maintenance of Sex by Parasitism.” *Nature* 328 (6130): 519.
Loher, W., and K. Edson. 1973. “The Effect of Mating on Egg Production and Release in the Cricket Teleogryllus Commodus.” *Entomologia Experimentalis et Applicata* 16 (4): 483–90.
Loher, Werner. 1972. “Circadian Control of Stridulation in the CricketTeleogryllus Commodus Walker.” *Journal of Comparative Physiology. A, Neuroethology, Sensory, Neural, and Behavioral Physiology* 79 (2): 173–90.

———. 1974. “Circadian Control of Spermatophore Formation in the Cricket Teleogryllus Commodus Walker.” *Journal of Insect Physiology* 20 (7): 1155–72.
Loher, Werner, and Gottfried Weidenmann. 1981. “Temperature-dependent Changes in Circadian Patterns of Cricket Premating Behaviour.” *Physiological Entomology* 6 (1): 35–43.
Loher, Werrer, and Larry J. Orsak. 1985. “Circadian Patterns of Premating Behavior in Teleogryllus Oceanicus Le Guillou under Laboratory and Field Conditions.” *Behavioral Ecology and Sociobiology* 16 (3): 223–31.
Long G. H. Chan B. H. Allen J. E. Read A. F. & Graham, A. L. 2008. “Blockade of TNF Receptor 1 Reduces Disease Severity but Increases Parasite Transmission during Plasmodium Chabaudi Chabaudi Infection.” *International Journal for Parasitology* 38 (8–9): 1073–81.
Loudon, Andrew S. I. 2012. “Circadian Biology: A 2.5 Billion Year Old Clock.” *Current Biology: CB* 22 (14): R570–71.
Lupien, Mathieu, Stephanie Marshall, Winrich Leser, Gerald S. Pollack, and Hans Willi Honegger. 2003. “Antibodies against the PER Protein of Drosophila Label Neurons in the Optic Lobe, Central Brain, and Thoracic Ganglia of the Crickets Teleogryllus Commodus and Teleogryllus Oceanicus.” *Cell and Tissue Research* 312 (3): 377–91.
Mairan, D. E., and Jj. 1729. “Observation Botanique.” *Histoire de l’Academie Royale Des Sciences Paris*.
Marpegan, Luciano, María Juliana Leone, Marcelo E. Katz, Patricio M. Sobrero, Tristan A. Bekinstein, and Diego A. Golombek. 2009. “Diurnal Variation in Endotoxin-Induced Mortality in Mice: Correlation with Proinflammatory Factors.” *Chronobiology International* 26 (7): 1430–42.
Martinaud, Guillaume, M. Billaudelle, and Jérôme Moreau. 2009. “Circadian Variation in Shedding of the Oocysts of Isospora Turdi (Apicomplexa) in Blackbirds (Turdus Merula): An Adaptative Trait against Desiccation and Ultraviolet Radiation.” *International Journal for Parasitology* 39 (6): 735–39.
Martinez-Bakker, Micaela, and Barbara Helm. 2015. “The Influence of Biological Rhythms on Host–Parasite Interactions.” *Trends in Ecology & Evolution* 30 (6): 314–26.
Matile, Philippe. 2006. “Circadian Rhythmicity of Nectar Secretion in Hoya Carnosa.” *Botanica Helvetica* 116 (1): 1–7.
Matthews, James H., Egon Marte, and Franz Halberg. 1964. “A Circadian Susceptibility-Resistance Cycle to Fluothane in Male B 1 Mice.” *Canadian Anaesthetists’ Society Journal* 11 (3): 280.
Maywood, E. S., J. E. Chesham, N. J. Smyllie, and M. H. Hastings. 2014. “The Tau Mutation of Casein Kinase 1ε Sets the Period of the Mammalian Pacemaker via Regulation of Period1 or Period2 Clock Proteins.” *Journal of Biological Rhythms* 29 (2): 110–18.

McFarlane, J. E. 1968. “Diel Periodicity in Spermatophore Formation in the House Cricket, Acheta Domesticus (L.).” *Canadian Journal of Zoology* 46 (4): 695–98.
McMahon, J. E. 1976. “Circadian Rhythm in Schistosoma Haematobium Egg Excretion.” *International Journal for Parasitology* 6 (5): 373–77.
Michael, Todd P., Patrice A. Salomé, Hannah J. Yu, Taylor R. Spencer, Emily L. Sharp, Mark A. McPeek, José M. Alonso, Joseph R. Ecker, and C. Robertson McClung. 2003. “Enhanced Fitness Conferred by Naturally Occurring Variation in the Circadian Clock.” *Science* 302 (5647): 1049 LP – 1053.
Mideo, Nicole, and Troy Day. 2008. “On the Evolution of Reproductive Restraint in Malaria.” *Proceedings. Biological Sciences / The Royal Society* 275 (1639): 1217–24.
Mideo, Nicole, Sarah E. Reece, Adrian L. Smith, and C. Jessica E. Metcalf. 2013. “The Cinderella Syndrome: Why Do Malaria-Infected Cells Burst at Midnight?” *Trends in Parasitology* 29 (1): 10–16.
Moiroux, Nicolas, Marinely B. Gomez, Cédric Pennetier, Emmanuel Elanga, Armel Djènontin, Fabrice Chandre, Innocent Djègbé, Hélène Guis, and Vincent Corbel. 2012. “Changes in Anopheles Funestus Biting Behavior Following Universal Coverage of Long-Lasting Insecticidal Nets in Benin.” *The Journal of Infectious Diseases* 206 (10): 1622–29.
Moriyama, Yoshiyuki, Yuichi Kamae, Outa Uryu, and Kenji Tomioka. 2012. “Gb’clock Is Expressed in the Optic Lobe and Is Required for the Circadian Clock in the Cricket Gryllus Bimaculatus.” *Journal of Biological Rhythms* 27 (6): 467–77.
Moriyama, Yoshiyuki, Tomoaki Sakamoto, Svetlana G. Karpova, Akira Matsumoto, Sumihare Noji, and Kenji Tomioka. 2008. “RNA Interference of the Clock Gene Period Disrupts Circadian Rhythms in the Cricket Gryllus Bimaculatus.” *Journal of Biological Rhythms* 23 (4): 308–18.
Moriyama, Yoshiyuki, Tomoaki Sakamoto, Akira Matsumoto, Sumihare Noji, and Kenji Tomioka. 2009. “Functional Analysis of the Circadian Clock Gene Period by RNA Interference in Nymphal Crickets Gryllus Bimaculatus.” *Journal of Insect Physiology* 55 (2): 396–400.
Mouahid, Gabriel, Mohamed A. Idris, Olivier Verneau, André Théron, Mahmoud M. A. Shaban, and Hélène Moné. 2012. “A New Chronotype of Schistosoma Mansoni: Adaptive Significance.” *Tropical Medicine & International Health: TM & IH* 17 (6): 727–32.
Mrosovsky, N. 1999. “Masking: History, Definitions, and Measurement.” *Chronobiology International* 16 (4): 415–29.
Murdock, Courtney C., Lillian L. Moller-Jacobs, and Matthew B. Thomas. 2013. “Complex Environmental Drivers of Immunity and Resistance in Malaria Mosquitoes.” *Proc. R. Soc. B* 280 (1770): 20132030.
Mure, Ludovic S., Hiep D. Le, Giorgia Benegiamo, Max W. Chang, Luis Rios, Ngalla Jillani, Maina Ngotho, Thomas Kariuki, Ouria Dkhissi-Benyahya, and Howard M. Cooper.

2018. “Diurnal Transcriptome Atlas of a Primate across Major Neural and Peripheral Tissues.” *Science* 359 (6381).

Nelson B.V. & Vance, R. R. 1979. “Diel Foraging Patterns of the Sea Urchin Centrostephanus Coronatus as a Predator Avoidance Strategy.” *Marine Biology* 51 (3): 251–258.

Nguyen, Khoa D., Sarah J. Fentress, Yifu Qiu, Karen Yun, Jeffery S. Cox, and Ajay Chawla. 2013. “Circadian Gene Bmal1 Regulates Diurnal Oscillations of Ly6C^{hi} Inflammatory Monocytes.” *Science* 341 (6153): 1483 LP – 1488.

Nose, Motoki, Atsushi Tokuoka, Tetsuya Bando, and Kenji Tomioka. 2018. “Timeless2 Plays an Important Role in Reproduction and Circadian Rhythms in the Cricket Gryllus Bimaculatus.” *Journal of Insect Physiology* 105 (February): 9–17.

Nowosielski, J. W., and R. L. Patton. 1963. “Studies on Circadian Rhythm of the House Cricket, Gryllus Domesticus L.” *Journal of Insect Physiology* 9 (4): 401–10.

O’donnell, Aidan J., Nicole Mideo, and Sarah E. Reece. 2013. “Disrupting Rhythms in Plasmodium Chabaudi: Costs Accrue Quickly and Independently of How Infections Are Initiated.” Vol. 12. https://doi.org/10.1186/1475-2875-12-372.

O’Donnell, Aidan J., Nicole Mideo, and Sarah E. Reece. 2013. “Disrupting Rhythms in Plasmodium Chabaudi: Costs Accrue Quickly and Independently of How Infections Are Initiated.” *Malaria Journal* 12 (1): 372.

O’Donnell, Aidan J., Kimberley F. Prior, and Sarah E. Reece. 2019. “Host Circadian Clocks Do Not Set the Schedule for the Within-Host Replication of Malaria Parasites.” *BioRxiv*, January, 777011.

O’Donnell, Aidan J., Kimberley F. Prior, and Sarah E. Reece. 2020. “Host Circadian Clocks Do Not Set the Schedule for the Within-Host Replication of Malaria Parasites.” *Proceedings. Biological Sciences / The Royal Society* 287 (1932): 20200347.

O’Donnell, Aidan J., and Sarah E. Reece. 2021. “Ecology of Asynchronous Asexual Replication: The Intraerythrocytic Development Cycle of Plasmodium Berghei Is Resistant to Host Rhythms.” *Malaria Journal* 20 (1): 105.

O’Donnell, Aidan J., Petra Schneider, Harriet G. McWatters, and Sarah E. Reece. 2011. “Fitness Costs of Disrupting Circadian Rhythms in Malaria Parasites.” *Proceedings. Biological Sciences / The Royal Society* 278 (1717): 2429–36.

O’Neill, John S., Gerben van Ooijen, Laura E. Dixon, Carl Troein, Florence Corellou, François-Yves Bouget, Akhilesh B. Reddy, and Andrew J. Millar. 2011. “Circadian Rhythms Persist without Transcription in a Eukaryote.” *Nature* 469 (7331): 554–58.

Orsak, Larry Joe. 1988. *Sexual Behavior in Teleogryllus Field Crickets (Orthoptera: Gryllidae): Elicitation in the Laboratory and in Nature*. University of California, Berkeley.

Otte, D. 1994. “The Crickets of Hawaii: Origin, Systematics, and Evolution. The Orthopterists’ Society.” In *Academy of Natural Sciences of Philadelphia, Philadelphia*. Vol. 396.

Ouyang, Yan, Carol R. Andersson, Takao Kondo, Susan S. Golden, and Carl Hirschie Johnson. 1998. “Resonating Circadian Clocks Enhance Fitness in Cyanobacteria.” *Proceedings of the National Academy of Sciences* 95 (15): 8660–64.

Overland, Lillian. 1960. “Endogenous Rhythm in Opening and Odor of Flowers of Cestrum Nocturnum.” *American Journal of Botany* 47 (5): 378–82.

Panda, Satchidananda, John B. Hogenesch, and Steve A. Kay. 2002. “Circadian Rhythms from Flies to Human.” *Nature* 417 (6886): 329–35.

Pascoal, Sonia, Timothee Cezard, Aasta Eik-Nes, Karim Gharbi, Jagoda Majewska, Elizabeth Payne, Michael G. Ritchie, Marlene Zuk, and Nathan W. Bailey. 2014. “Rapid Convergent Evolution in Wild Crickets.” *Current Biology: CB* 24 (12): 1369–74.

Peatey, Christopher L., Tina S. Skinner-Adams, Matthew W. A. Dixon, James S. McCarthy, Donald L. Gardiner, and Katharine R. Trenholme. 2009. “Effect of Antimalarial Drugs on Plasmodium Falciparum Gametocytes.” *The Journal of Infectious Diseases* 200 (10): 1518–21.

Pfau, H., and U. Koch. 1994. “The Functional Morphology of Singing in the Cricket.” *The Journal of Experimental Biology* 195 (1): 147–67.

Pigeault, Romain, Quentin Caudron, Antoine Nicot, Ana Rivero, and Sylvain Gandon. 2018. “Timing Malaria Transmission with Mosquito Fluctuations.” *Evolution Letters*.

Pittendrigh, C. S., and D. H. Minis. 1972. “Circadian Systems: Longevity as a Function of Circadian Resonance in Drosophila Melanogaster.” *Proceedings of the National Academy of Sciences of the United States of America* 69 (6): 1537–39.

Pittendrigh, Colin S. 1960. “Circadian Rhythms and the Circadian Organization of Living Systems.” In *Cold Spring Harbor Symposia on Quantitative Biology*, 25:159–84. Cold Spring Harbor Laboratory Press.

Pittendrigh, Colin S., and Serge Daan. 1976. “A Functional Analysis of Circadian Pacemakers in Nocturnal Rodents.” *Journal of Comparative Physiology. A, Neuroethology, Sensory, Neural, and Behavioral Physiology* 106 (3): 291–331.

PITTENDRIGH, and CS. 1981. “Circadian Organization and the Photoperiodic Phenomena.” *Biological Clocks in Seasonal Reproductive Cycles*, 1–35.

Pollitt, Laura C., Nicole Mideo, Damien R. Drew, Petra Schneider, Nick Colegrave, and Sarah E. Reece. 2011. “Competition and the Evolution of Reproductive Restraint in Malaria Parasites.” *The American Naturalist* 177 (3): 358–67.

Ponton, Fleur, Fernando Otálora-Luna, Thierry Lefevre, Patrick M. Guerin, Camille Lebarbenchon, David Duneau, David G. Biron, and Frédéric Thomas. 2011. “Water-Seeking Behavior in Worm-Infected Crickets and Reversibility of Parasitic Manipulation.” *Behavioral Ecology: Official Journal of the International Society for Behavioral Ecology* 22 (2): 392–400.

Poulin, R. 2007. “Evolutionary Ecology of Parasites Princeton University Press.” *Princeton, NJ. [Google Scholar]*.

Prendergast, Brian J., Erin J. Cable, Tyler J. Stevenson, Kenneth G. Onishi, Irving Zucker, and Leslie M. Kay. 2015. “Circadian Disruption Alters the Effects of Lipopolysaccharide Treatment on Circadian and Ultradian Locomotor Activity and Body Temperature Rhythms of Female Siberian Hamsters.” *Journal of Biological Rhythms* 30 (6): 543–56.

Prestwich, Kenneth N., and Thomas J. Walker. 1981. “Energetics of Singing in Crickets: Effect of Temperature in Three Trilling Species (Orthoptera: Gryllidae).” *Journal of Comparative Physiology* 143 (2): 199–212.

Prior, Kimberley F., Filipa Rijo-Ferreira, Patricia A. Assis, Isabella C. Hirako, David R. Weaver, Ricardo T. Gazzinelli, and Sarah E. Reece. 2020. “Periodic Parasites and Daily Host Rhythms.” *Cell Host & Microbe* 27 (2): 176–87.

Prior, Kimberley F., Daan R. van der Veen, Aidan J. O’Donnell, Katherine Cumnock, David Schneider, Arnab Pain, Amit Subudhi, Abhinay Ramaprasad, Samuel S. C. Rund, and Nicholas J. Savill. 2018. “Timing of Host Feeding Drives Rhythms in Parasite Replication.” *PLoS Pathogens* 14 (2): e1006900.

Rayner, Jack, Sarah Aldridge, Fernando Montealegre-Z, and Nathan William Bailey. 2019. “A Silent Orchestra: Convergent Song Loss in Hawaiian Crickets Is Repeated, Morphologically Varied, and Widespread.” *Ecology*.

Rayner, Jack G., Will T. Schneider, and Nathan W. Bailey. 2020. “Can Behaviour Impede Evolution? Persistence of Singing Effort after Morphological Song Loss in Crickets.” *Biology Letters* 16 (6): 20190931.

Reece, S. E., Alison B. Duncan, Stuart A. West, and Andrew F. Read. 2003. “Sex Ratios in the Rodent Malaria Parasite, Plasmodium Chabaudi.” *Parasitology* 127 (5): 419–25.

Reece, Sarah E., Eltayeb Ali, Petra Schneider, and Hamza A. Babiker. 2010. “Stress, Drugs and the Evolution of Reproductive Restraint in Malaria Parasites.” *Proceedings of the Royal Society B: Biological Sciences* 277 (1697): 3123–29.

Reece, Sarah E., and Kimberley F. Prior. 2018. “Malaria Makes the Most of Mealtimes.” *Cell Host & Microbe* 23 (6): 695–97.

Reece, Sarah E., Kimberley F. Prior, and Nicole Mideo. 2017. “The Life and Times of Parasites: Rhythms in Strategies for Within-Host Survival and Between-Host Transmission.” *Journal of Biological Rhythms* 32 (6): 516–33.

Refinetti, Roberto. 2010. “Entrainment of Circadian Rhythm by Ambient Temperature Cycles in Mice.” *Journal of Biological Rhythms* 25 (4): 247–56.

Rensing, Ludger, Ulf Meyer-Grahle, and Peter Ruoff. 2001. “Biological Timing and the Clock Metaphor: Oscillatory and Hourglass Mechanisms.” *Chronobiology International* 18 (3): 329–69.

Rijo-Ferreira, Filipa, Victoria A. Acosta-Rodriguez, John H. Abel, Izabela Kornblum, Ines Bento, Gokhul Kilaru, Elizabeth B. Klerman, Maria M. Mota, and Joseph S. Takahashi. 2020. “The Malaria Parasite Has an Intrinsic Clock.” *Science* 368 (6492): 746–53.

Rijo-Ferreira, Filipa, Tânia Carvalho, Cristina Afonso, Margarida Sanches-Vaz, Rui M. Costa, Luísa M. Figueiredo, and Joseph S. Takahashi. 2018. “Sleeping Sickness Is a Circadian Disorder.” *Nature Communications* 9 (1): 62.

Rijo-Ferreira, Filipa, Daniel Pinto-Neves, Nuno L. Barbosa-Morais, Joseph S. Takahashi, and Luisa M. Figueiredo. 2017. “Trypanosoma Brucei Metabolism Is under Circadian Control.” *Nature Microbiology* 2 (March): 17032.

Robert, Daniel, John Amoroso, and Ronald R. Hoy. 1992. “The Evolutionary Convergence of Hearing in a Parasitoid Fly and Its Cricket Host.” *Science* 258 (5085): 1135–37.

Roden, Laura C., and Robert A. Ingle. 2009. “Lights, Rhythms, Infection: The Role of Light and the Circadian Clock in Determining the Outcome of Plant–Pathogen Interactions.” *The Plant Cell* 21 (9): 2546 LP – 2552.

Romanovsky, A. A., and M. Székely. 1998. “Fever and Hypothermia: Two Adaptive Thermoregulatory Responses to Systemic Inflammation.” *Medical Hypotheses* 50 (3): 219–26.

Rubin, E. B., Y. Shemesh, M. Cohen, and S. Elgavish. 2006. “Molecular and Phylogenetic Analyses Reveal Mammalian-like Clockwork in the Honey Bee (Apis Mellifera) and Shed New Light on the Molecular Evolution of the ….” *Genome / National Research Council Canada = Genome / Conseil National de Recherches Canada*. https://genome.cshlp.org/content/16/11/1352.short.

Rubin, Matthew J., Marcus T. Brock, Amanda M. Davis, Zachary M. German, Mary Knapp, Stephen M. Welch, Stacey L. Harmer, Julin N. Maloof, Seth J. Davis, and Cynthia Weinig. 2017. “Circadian Rhythms Vary over the Growing Season and Correlate with Fitness Components.” *Molecular Ecology* 26 (20): 5528–40.

Rund, Samuel S. C., Aidan J. O’Donnell, James E. Gentile, and Sarah E. Reece. 2016. “Daily Rhythms in Mosquitoes and Their Consequences for Malaria Transmission.” *Insects* 7 (2): 14.

Rutila, J. E., V. Suri, M. Le, W. V. So, M. Rosbash, and J. C. Hall. 1998. “CYCLE Is a Second BHLH-PAS Clock Protein Essential for Circadian Rhythmicity and Transcription of Drosophila Period and Timeless.” *Cell* 93 (5): 805–14.

Sabrosky, Curtis W. 1953. “Taxonomy and Host Relations of the Tribe Ormiini in the Western Hemisphere (Diptera, Larvaevoridae). Taxonomía y Relaciones de Hospederos de La Tribu Ormiini En El Hemisferio Occidental (Diptera, Larvaevoridae).” *Proceedings of the Entomological Society of Washington* 55 (4): 167–83.

Scheiermann, Christoph, Julie Gibbs, Louise Ince, and Andrew Loudon. 2018. “Clocking in to Immunity.” *Nature Reviews. Immunology* 18 (7): 423–37.

Scheiermann, Christoph, Yuya Kunisaki, and Paul S. Frenette. 2013. “Circadian Control of the Immune System.” *Nature Reviews. Immunology* 13 (February): 190.

Schmid-Hempel, Paul. 2003. “Variation in Immune Defence as a Question of Evolutionary Ecology.” *Proceedings of the Royal Society of London. Series B: Biological Sciences* 270 (1513): 357–66.

Schneider, Petra, Megan A. Greischar, Philip L. G. Birget, Charlotte Repton, Nicole Mideo, and Sarah E. Reece. 2018. “Adaptive Plasticity in the Gametocyte Conversion Rate of Malaria Parasites.” *PLoS Pathogens* 14 (11): e1007371.

Schneider, Petra, and Sarah E. Reece. 2021. “The Private Life of Malaria Parasites: Strategies for Sexual Reproduction.” *Molecular and Biochemical Parasitology* 244 (July): 111375.

Schneider, Petra, Samuel S. C. Rund, Natasha L. Smith, Kimberley F. Prior, Aidan J. O’Donnell, and Sarah E. Reece. 2018. “Adaptive Periodicity in the Infectivity of Malaria Gametocytes to Mosquitoes.” *Proceedings of the Royal Society B*. https://doi.org/20181876.

Schneider, Will T., Christian Rutz, Berthold Hedwig, and Nathan W. Bailey. 2018. “Vestigial Singing Behaviour Persists after the Evolutionary Loss of Song in Crickets.” *Biology Letters* 14 (2): 20170654.

Schulman, Carl I., Nicholas Namias, James Doherty, Ronald J. Manning, Pamela Li, Ahmed Elhaddad, David Lasko, Jose Amortegui, Christopher J. Dy, and Lucie Dlugasch. 2005. “The Effect of Antipyretic Therapy upon Outcomes in Critically Ill Patients: A Randomized, Prospective Study.” *Surgical Infections* 6 (4): 369–75.

Sharma, Vijay Kumar. 2003. “Adaptive Significance of Circadian Clocks.” *Chronobiology International* 20 (6): 901–19.

Sheeba, V., Vijay Kumar Sharma, and Amitabh Joshi. 1999. “Adaptive Significance of Circadian Rhythms Biological Clocks and Darwinian Fitness in Cyanobacteria.” *Resonance* 4: 73–75.

Sidote, David, John Majercak, Vaishali Parikh, and Isaac Edery. 1998. “Differential Effects of Light and Heat on the Drosophila Circadian Clock Proteins PER and TIM.” *Molecular and Cellular Biology* 18 (4): 2004–13.

Sokhina, C. S., J-F Trape, and Vincent Robert. 2001. “Gametocytaemia in Senegalese Children with Uncomplicated Falciparum Malaria Treated with Chloroquine, Amodiaquine or Sulfadoxine+ Pyrimethamine.” *Parasite* 8 (3): 243–50.

Sokolove, P. G. 1975. “Locomotory and Stridulatory Circadian Rhythms in the Cricket, Teleogryllus Commodus.” *Journal of Insect Physiology* 21 (3): 537–58.

Sokolove, P. G., and W. Loher. 1975. “Rôle of Eyes, Optic Lobes, and Pars Intercerebralis in Locomotory and Stridulatory Circadian Rhythms of Teleogryllus Commodus.” *Journal of Insect Physiology* 21 (4): 785–99.

Sougoufara, Seynabou, Seynabou Mocote Diédhiou, Souleymane Doucouré, Nafissatou Diagne, Pape Mbacké Sembène, Myriam Harry, Jean-François Trape, Cheikh Sokhna, and Mamadou Ousmane Ndiath. 2014. “Biting by Anopheles Funestus in Broad Daylight after Use of Long-Lasting Insecticidal Nets: A New Challenge to Malaria Elimination.” *Malaria Journal* 13 (1): 125.

Spoelstra, Kamiel, Martin Wikelski, Serge Daan, Andrew S. I. Loudon, and Michaela Hau. 2016. “Natural Selection against a Circadian Clock Gene Mutation in Mice.” *Proceedings of the National Academy of Sciences* 113 (3): 686–91.

Stephan, Friedrich K. 2002. “The ‘Other’ Circadian System: Food as a Zeitgeber.” *Journal of Biological Rhythms* 17 (4): 284–92.

Stone, Elizabeth F., Ben O. Fulton, Janelle S. Ayres, Linh N. Pham, Junaid Ziauddin, and Mimi M. Shirasu-Hiza. 2012. “The Circadian Clock Protein Timeless Regulates Phagocytosis of Bacteria in Drosophila.” *PLoS Pathogens* 8 (1): e1002445.

Subudhi, Amit K., Aidan J. O’Donnell, Abhinay Ramaprasad, Hussein M. Abkallo, Abhinav Kaushik, Hifzur R. Ansari, Alyaa M. Abdel-Haleem, et al. 2020. “Malaria Parasites Regulate Intra-Erythrocytic Development Duration via Serpentine Receptor 10 to Coordinate with Host Rhythms.” *Nature Communications* 11 (1): 1–15.

Sueur, Jérôme. 2018. *Sound Analysis and Synthesis with R*. Springer.

Sueur, Jérôme, Thierry Aubin, and Caroline Simonis. 2008. “Sound Analysis and Synthesis with the Package Seewave.” *Bioacoustics* 18 (2): 213–26.

Tan, Ming Kai, and Tony Robillard. 2021. “Highly Diversified Circadian Rhythms in the Calling Activity of Eneopterine Crickets (Orthoptera: Grylloidea: Gryllidae) from Southeast Asia.” *Bioacoustics*, 1–20.

Tanner, Jessie C., Elizabeth Swanger, and Marlene Zuk. 2019. “Sexual Signal Loss in Field Crickets Maintained despite Strong Sexual Selection Favoring Singing Males.” *Evolution; International Journal of Organic Evolution* 73 (7): 1482–89.

Tei, H., H. Okamura, Y. Shigeyoshi, C. Fukuhara, R. Ozawa, M. Hirose, and Y. Sakaki. 1997. “Circadian Oscillation of a Mammalian Homologue of the Drosophila Period Gene.” *Nature* 389 (6650): 512–16.

Thomas, Édéric, Frédéric Thomas, Jean-Francois Guegan, François Renaud, and Jean-François Guegan. 2005. *Parasitism and Ecosystems*. OUP Oxford.

Thomas, F., A. Schmidt-Rhaesa, Guilhaume Martin, C. Manu, P. Durand, and F. Renaud. 2002. “Do Hairworms (Nematomorpha) Manipulate the Water Seeking Behaviour of Their Terrestrial Hosts?” *Journal of Evolutionary Biology* 15 (3): 356–61.

Thomas, Frédéric, Thierry Rigaud, and Jacques Brodeur. 2010. “Evolution of Parasite-Induced Behavioral Alterations.” Elsevier.

Tinbergen, Joost, and Serge Daan. 1979. “Young Guillemots (Uria Lomvia) Leaving Their Arctic Breeding Cliffs: A Daily Rhythm in Numbers and Risk.” https://agris.fao.org/agris-search/search.do?recordID=AV2012093362.

Tinghitella, R. M. 2008. “Rapid Evolutionary Change in a Sexual Signal: Genetic Control of the Mutation ‘flatwing’ That Renders Male Field Crickets (Teleogryllus Oceanicus) Mute.” *Heredity* 100 (3): 261–67.

Tinghitella, R. M., M. Zuk, M. Beveridge, and L. W. Simmons. 2011. “Island Hopping Introduces Polynesian Field Crickets to Novel Environments, Genetic Bottlenecks and Rapid Evolution.” *Journal of Evolutionary Biology* 24 (6): 1199–1211.

Tinghitella, Robin M., E. Dale Broder, James H. Gallagher, Aaron W. Wikle, and David M. Zonana. 2021. “Responses of Intended and Unintended Receivers to a Novel Sexual Signal Suggest Clandestine Communication.” *Nature Communications* 12 (1): 1–10.

Tinghitella, Robin M., E. Dale Broder, Gabrielle A. Gurule-Small, Claudia J. Hallagan, and Jacob D. Wilson. 2018. “Purring Crickets: The Evolution of a Novel Sexual Signal.” *The American Naturalist* 192 (6): 773–82.

Tinghitella, Robin M., and Marlene Zuk. 2009. “ASYMMETRIC MATING PREFERENCES ACCOMMODATED THE RAPID EVOLUTIONARY LOSS OF A SEXUAL SIGNAL.” *Evolution; International Journal of Organic Evolution* 63 (8): 2087–98.

Tomioka, K., M. Nakamichi, and M. Yukizane. 1994. “Optic Lobe Circadian Pacemaker Sends Its Information to the Contralateral Optic Lobe in the Cricket Gryllus Bimaculatus.” *Journal of Comparative Physiology. A, Neuroethology, Sensory, Neural, and Behavioral Physiology* 175 (4). https://doi.org/10.1007/bf00199246.

Tomioka, Kenji. 2014. “Chronobiology of Crickets: A Review.” *Zoological Science* 31 (10): 624–32.

Tomioka, Kenji, and Yoshihiko Chiba. 1986. “Circadian Rhythm in the Neurally Isolated Lamina-Medulla-Complex of the Cricket, Gryllus Bimaculatus.” *Journal of Insect Physiology* 32 (9): 747–55.

———. 1992. “Characterization of an Optic Lobe Circadian Pacemaker by in Situ and in Vitro Recording of Neural Activity in the Cricket, Gryllus Bimaculatus.” *Journal of Comparative Physiology. A, Neuroethology, Sensory, Neural, and Behavioral Physiology* 171 (1). https://doi.org/10.1007/bf00195955.

Uryu, Outa, Svetlana G. Karpova, and Kenji Tomioka. 2013. “The Clock Gene Cycle Plays an Important Role in the Circadian Clock of the Cricket Gryllus Bimaculatus.” *Journal of Insect Physiology* 59 (7): 697–704.

Vaze, Koustubh M., and Vijay Kumar Sharma. 2013. “On the Adaptive Significance of Circadian Clocks for Their Owners.” *Chronobiology International* 30 (4): 413–33.

Vinne, Vincent van der, Sjaak J. Riede, Jenke A. Gorter, Willem G. Eijer, Michael T. Sellix, Michael Menaker, Serge Daan, Violetta Pilorz, and Roelof A. Hut. 2014. “Cold and Hunger Induce Diurnality in a Nocturnal Mammal.” *Proceedings of the National Academy of Sciences* 111 (42): 15256–60.

Wagner, William E. 1996. “Convergent Song Preferences between Female Field Crickets and Acoustically Orienting Parasitoid Flies.” *Behavioral Ecology: Official Journal of the International Society for Behavioral Ecology* 7 (3): 279–85.

Walker, T. J., and S. A. Wineriter. 1991. “Hosts of a Phonotactic Parasitoid and Levels of Parasitism (Diptera: Tachinidae: Ormia Ochracea).” *The Florida Entomologist* 74 (4): 554–59.

Walker, Thomas J. 1993. “Phonotaxis in FemaleOrmia Ochracea (Diptera: Tachinidae), a Parasitoid of Field Crickets.” *Journal of Insect Behavior* 6 (3): 389–410.

Wang, Wei, Jinyoung Yang Barnaby, Yasuomi Tada, Hairi Li, Mahmut Tör, Daniela Caldelari, Dae-Un Lee, Xiang-Dong Fu, and Xinnian Dong. 2011. “Timing of Plant Immune Responses by a Central Circadian Regulator.” *Nature* 470 (7332): 110.

Wargo, Andrew R., Jacobus C. De Roode, Silvie Huijben, Damien R. Drew, and Andrew F. Read. 2007. “Transmission Stage Investment of Malaria Parasites in Response to

In-Host Competition." *Proceedings of the Royal Society B: Biological Sciences* 274 (1625): 2629–38.
Watanabe, Sumio, and Manfred Opper. 2010. "Asymptotic Equivalence of Bayes Cross Validation and Widely Applicable Information Criterion in Singular Learning Theory." *Journal of Machine Learning Research: JMLR* 11 (12).
Westwood, Mary L., Aidan J. O'Donnell, Charissa de Bekker, Curtis M. Lively, Marlene Zuk, and Sarah E. Reece. 2019. "The Evolutionary Ecology of Circadian Rhythms in Infection." *Nature Ecology & Evolution* 3 (4): 552–60.
Wilson, Kenneth, and Sheena C. Cotter. 2013. "Host--Parasite Interactions and the Evolution of Immune Defense." *Advances in the Study of Behavior* 45: 81–174.
Woelfle, Mark A., Yan Ouyang, Kittiporn Phanvijhitsiri, and Carl Hirschie Johnson. 2004. "The Adaptive Value of Circadian Clocks: An Experimental Assessment in Cyanobacteria." *Current Biology: CB* 14 (16): 1481–86.
Yon, Felipe, Youngsung Joo, Lucas Cortés Llorca, Eva Rothe, Ian T. Baldwin, and Sang-Gyu Kim. 2016. "Silencing Nicotiana Attenuata LHY and ZTL Alters Circadian Rhythms in Flowers." *The New Phytologist* 209 (3): 1058–66.
Yon, Felipe, Danny Kessler, Youngsung Joo, Lucas Cortés Llorca, Sang-Gyu Kim, and Ian T. Baldwin. 2017. "Fitness Consequences of Altering Floral Circadian Oscillations for Nicotiana Attenuata." *Journal of Integrative Plant Biology* 59 (3): 180–89.
Young, M. Rita I., John P. Matthews, Eugene L. Kanabrocki, Robert B. Sothern, Beatrice Roitman-Johnson, and Lawrence E. Scheving. 1995. "Circadian Rhythmometry of Serum Interleukin-2, Interleukin-10, Tumor Necrosis Factor-α, and Granulocyte-Macrophage Colony-Stimulating Factor in Men." *Chronobiology International* 12 (1): 19–27.
Zar, J. H. 2010. "Biostatistical Analysis Fifth Edition." *Pearson Education Upper Saddie River, New Jersey, USA. Santos-Fita D, Naranjo EJ, Estrada E, Mariaca R, Bello E (2015) Symbolism and Ritual Practices Related to Hunting in Maya Communities from Central Quintana Roo, Mexico. Journal of Ethnobiology and Eth* 11: 71.
Zasłona, Zbigniew, Sarah Case, James O. Early, Stephen J. Lalor, Rachel M. McLoughlin, Anne M. Curtis, and Luke A. J. O'Neill. 2017. "The Circadian Protein BMAL1 in Myeloid Cells Is a Negative Regulator of Allergic Asthma." *American Journal of Physiology-Lung Cellular and Molecular Physiology* 312 (6): L855–60.
Zhang, Liang, Ibraheem Saleh, Sashi Thapaliya, Jonathan Louie, Jose Figueroa-Hernandez, and Hao Ji. 2017. "An Empirical Evaluation of Machine Learning Approaches for Species Identification through Bioacoustics." In *2017 International Conference on Computational Science and Computational Intelligence (CSCI)*, 489–94. IEEE.
Zhang, Ray, Nicholas F. Lahens, Heather I. Ballance, Michael E. Hughes, and John B. Hogenesch. 2014. "A Circadian Gene Expression Atlas in Mammals: Implications for Biology and Medicine." *Proceedings of the National Academy of Sciences* 111 (45): 16219–24.

Zhang, Xiao, Jack G. Rayner, Mark Blaxter, and Nathan W. Bailey. 2021. “Rapid Parallel Adaptation despite Gene Flow in Silent Crickets.” *Nature Communications* 12 (1): 50.

Zhu, Haisun, Ivo Sauman, Quan Yuan, Amy Casselman, Myai Emery-Le, Patrick Emery, and Steven M. Reppert. 2008. “Cryptochromes Define a Novel Circadian Clock Mechanism in Monarch Butterflies That May Underlie Sun Compass Navigation.” *PLoS Biology* 6 (1): e4.

Zuk, Marlene, Nathan W. Bailey, Brian Gray, and John T. Rotenberry. 2018. “Sexual Signal Loss: The Link between Behaviour and Rapid Evolutionary Dynamics in a Field Cricket.” *The Journal of Animal Ecology* 87 (3): 623–33.

Zuk, Marlene, and Gita R. Kolluru. 1998. “Exploitation of Sexual Signals by Predators and Parasitoids.” *The Quarterly Review of Biology* 73 (4): 415–38.

Zuk, Marlene, John T. Rotenberry, and Leigh W. Simmons. 1998. “CALLING SONGS OF FIELD CRICKETS (TELEOGRYLLUS OCEANICUS) WITH AND WITHOUT PHONOTACTIC PARASITOID INFECTION.” *Evolution; International Journal of Organic Evolution* 52 (1): 166–71.

Zuk, Marlene, John T. Rotenberry, and Robin M. Tinghitella. 2006. “Silent Night: Adaptive Disappearance of a Sexual Signal in a Parasitized Population of Field Crickets.” *Biology Letters* 2 (4): 521 LP – 524.

Zuk, Marlene, Leighw Simmons, and Luanne Cupp. 1993. “Calling Characteristics of Parasitized and Unparasitized Populations of the Field Cricket Teleogryllus Oceanicus.” *Behavioral Ecology and Sociobiology* 33 (5): 339–43.

8. Appendices

8.1. Appendix: Chapter 3

Parameter	Definition
freq.M	Median frequency
freq.IPR	Frequency interpercentile range
spec.mean	Mean of the frequency in the 3-6 kHz spectrum
spec.median	Median of the frequency in the 3-6 kHz spectrum
spec.mode	Mode of the frequency in the 3-6 kHz spectrum
freq.P2	Frequency terminal percentile
time.M	Time median
freq.P1	Frequency initial percentile
spec.sd	S.D. in the 3-6 kHz spectrum
spec.sem	S.E.M. in the 3-6 kHz spectrum
spec.Q25	First quartile in the 3-6 kHz spectrum
spec.Q75	Terminal quartile in the 3-6 kHz spectrum
spec.IQR	Interquartile range in the 3-6 kHz spectrum
spec.cent	Centroid in the 3-6 kHz spectrum
spec.skewness	Skewness in the 3-6 kHz spectrum
spec.kurtosis	Kurtosis in the 3-6 kHz spectrum
spec.sfm	Spectral flatness measure in the 3-6 kHz spectrum
spec.sh	Spectral entropy in the 3-6 kHz spectrum
spec.prec	Frequency precision of the 3-6 kHz spectrum

Table I. Parameters derived from audio clips using the Seewave R package. Parameters highlighted in yellow were chosen to train the models.

	Reference		
Prediction		n	y

	n	114	2
	y	0	22

Table II. Random forest model confusion matrix. Model "predictions" ("n" = no, "y" = yes, pertaining to whether a given clip contained chirping or not) on the left are compared against known "reference" clip values ("n" = no, "y" = yes along the top). The model predicted "no" correctly 114 times and incorrectly 2 times. The model predicted "yes" correctly 22 times, and "yes" incorrectly 0 times. Thus, the model (though overall very accurate, 98% as shown in Table III) is more likely to supply false negatives than false positives, rendering the model both highly accurate and conservative.

Accuracy	0.9855
95% CI	(0.9486, 0.9982)
No Information Rate	0.8261
P-Value	1.592e-09
Kappa	0.9478
Mcnemars Test P-value	0.4795
Sensitivity	1.0000
Specificity	0.9167
Pos Pred Value	0.9828
Neg Pred Value	1.0000
Prevalence	0.8261
Detection Rate	0.8261
Detection Prevalence	0.8406
Balance Accuracy	0.9583

Table III. Accuracy statistics associated with the random forest model.

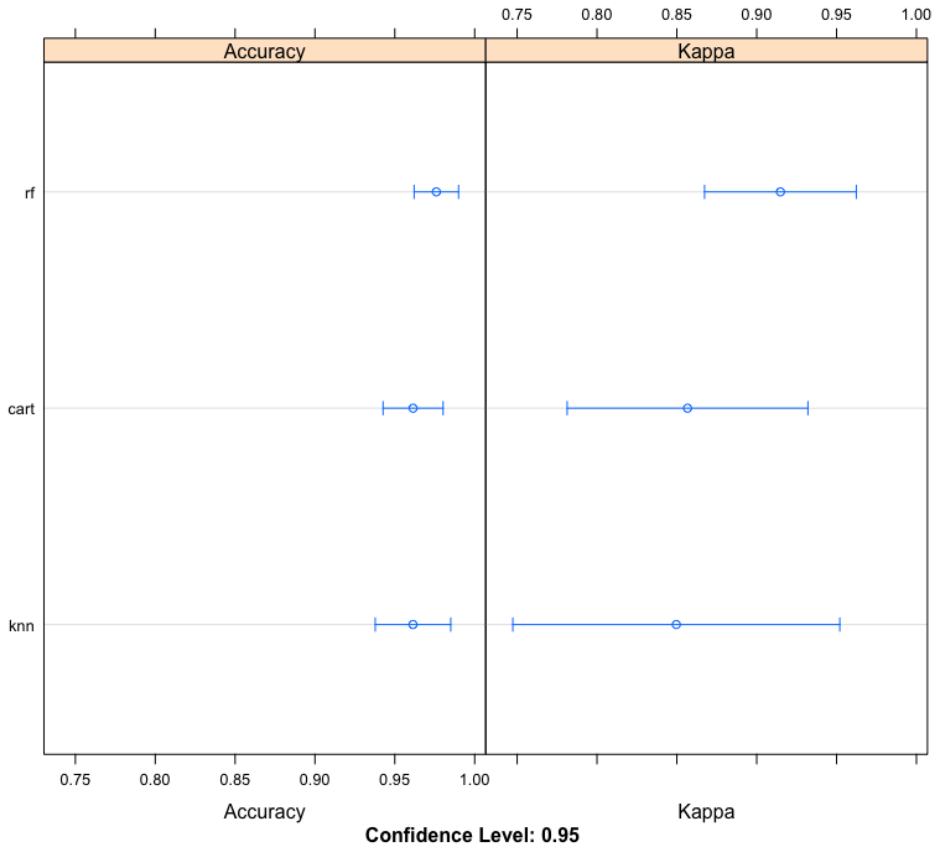


Figure I. Accuracy and kappa results from three algorithms (rf=random forest, cart=classification and regression tree, kNN=k-nearest neighbors) tested using k-fold cross validation.

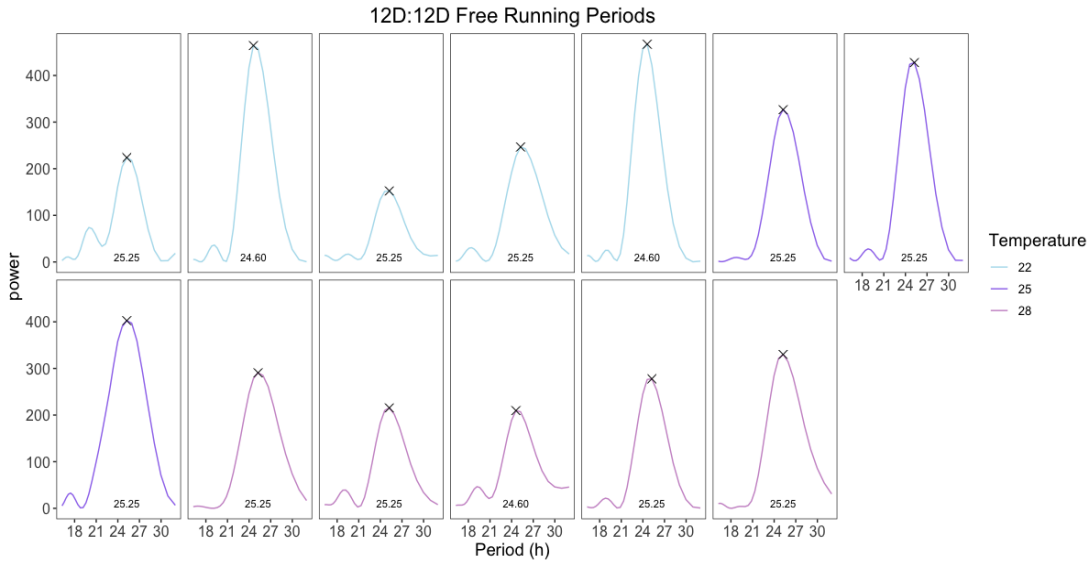


Figure II. Experiment 2: Individual Lomb-Scargle periodograms with most significant period estimate indicated by the black x on each plot. Temperature treatment groups are indicated by colours in the legend (22°C=blue, 25°C=purple, and 28°C=pink). Power of a given period estimate is located on the y-axis, and period (in hours) is located on the x-axis.

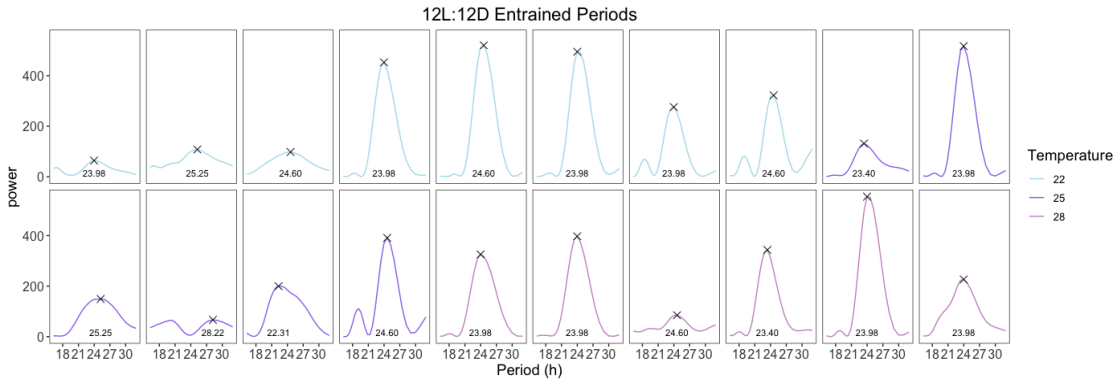


Figure III. Experiment 3: Individual Lomb-Scargle periodograms during the LD lighting regime with most significant period estimate indicated by the black x on each plot. Temperature treatment groups are indicated by colours in the legend (22°C=blue, 25°C=purple, and 28°C=pink). Power of a given period estimate is located on the y-axis, and period (in hours) is located on the x-axis.

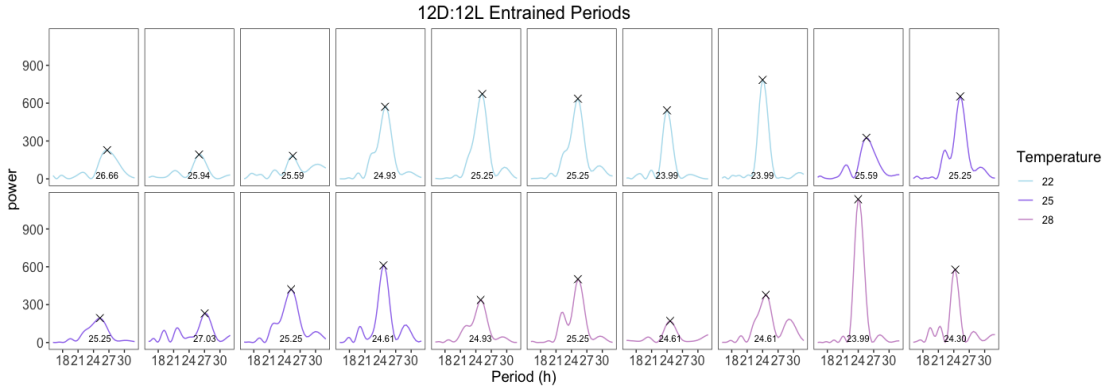


Figure IV. Experiment 3: Individual Lomb-Scargle periodograms during the DL lighting regime with most significant period estimate indicated by the black x on each plot. Temperature treatment groups are indicated by colours in the legend (22°C=blue, 25°C=purple, and 28°C=pink). Power of a given period estimate is located on the y-axis, and period (in hours) is located on the x-axis.

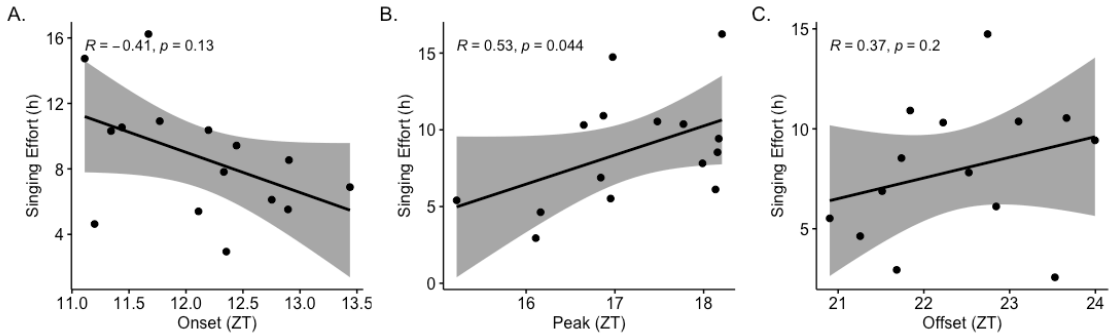


Figure V. Correlation plots for each phase marker (**A.** Onset, **B.** Peak, and **C.** Offset; x-axes) in ZT and "singing prevalence" in hours (y-axes). Spearman's correlation coefficient (*R*) and associated p-values are shown on each plot, along with points representing individuals, the regression line, and shaded areas are the confidence interval.

8.2. Appendix: Methods for detecting and quantifying singing by male crickets

Rationale & Background

Considerable time and effort were put towards developing and testing methods to collect high resolution data on the singing rhythms of male *T. oceanicus*. As my goal was to use this data towards characterising circadian singing rhythms, I had several general requirements: (1) song identification must be accurate and temporally precise, (2) data collection must be possible in both light and dark conditions, and (3) resulting high volumes of data spanning multiple circadian cycles must be able to be processed quickly. To that end, I tried three separate approaches towards collecting continuous singing data, the first of which involved video recordings while the second two relied on audio recordings.

Male *T. oceanicus* song is produced through stridulation, which involves the rhythmic opening and closing of the forewings, running two comb-like apparatuses (the scraper and the file) over one another (Pfau and Koch 1994). Initial attempts focussed on video recording because stridulation is visually distinct from other behaviours. If successful, using video recordings would have allowed the stridulatory rhythms of flat wing (as well as normal-wing) male morphs to be investigated because they stridulate in the same manner as normal wing males, but due to the feminisation of their wings, their stridulation does not produce typical calling song and they are largely silent (Zuk, Rotenberry, and Tinghitella 2006). Furthermore, it is possible to remove the scraper from normal wing males without interfering with their singing activity (J. G. Rayner, Schneider, and Bailey 2020). By rendering males silent, they do not acoustically interfere with the singing effort of their neighbours, which allows many males to be independently monitored in an array covered by a single video camera. Thus, using video to gather data offers the potential for good sample sizes of both normal and flatwing males simultaneously.

Unfortunately, video-based approaches proved insufficiently tractable, so I switched focus to audio recordings made by normal wing males who had to be acoustically isolated by using a separate incubator for each male. Here, I outline the methods trialled for the video recordings as well as the first attempt at processing the audio. I describe the shortcomings and pitfalls associated with both, ultimately leading to the development of the methods described in Chapters 3 and 4.

PLAN A: Video recording methods

I collaborated with Dr. Nathan Bailey, Dr. Will Schneider, and Dr. Jack Rayner to trial their method for collecting and extracting singing data from video recordings. Briefly, their method (see (J. G. Rayner, Schneider, and Bailey 2020) involves gluing a small reflective tag (3-4mg) to the distal tip of each male's right forewing using a small amount of superglue (Loctite, Germany). I first had to mute the normal wing males used for this trail (to prevent acoustic interference) by removing the scraper on their wings using scissors. Next, males were placed into an array (a craft supply box) with 20 small compartments of 55 × 43 × 35mm. I removed the front panel of the box and replaced it with a fine mesh covering to contain males. However, mesh proved unsuitable - the first cricket trialled using this covering made quick work of chewing through it and escaping (though it was later retrieved). I next modified the box to have a custom fitted Perspex covering which was resistant to cricket escape. Unfortunately, Perspex caused a high degree of reflection on the video recordings and the plastic panels of the array also obscured the recordings due to reflection. To solve the reflection problem, I sprayed the array with matte black spray paint and continued trials with different, clear anti-reflective Perspex.

Once contained within an array, males were filmed using a Nikon D3300 (720p at 60fps) (J. G. Rayner, Schneider, and Bailey 2020). We recorded one cricket per compartment, with 16 crickets visible in each field of view. Once recordings had finished, the videos were processed via a bespoke MATLAB script which identified individual males and quantified

the duration of wing movement bouts each made. To that end, video was adjusted for brightness and contrast such that the resulting image frames contained only the reflective tags. The location of the tag was then pinpointed on a coordinate plane by the script so that between frame movement could be calculated. I followed requirements for wing movement to be considered singing (as opposed to other wing movements, e.g. aggression displays) outlined in (J. G. Rayner, Schneider, and Bailey 2020)

The method developed by (J. G. Rayner, Schneider, and Bailey 2020) was designed to provide video recordings that seldom lasted more than a couple of hours. To quantify circadian rhythms, my experiments required males to be monitored for weeks. This caused several problems. After just a few hours, many of the males were able to remove the reflective wing tags or the tags would fall off due to movement. Because of this, I also trialled dabbing a bit of Tippex on the tip of the wings, but this did not provide a clear enough image on the recordings in dim red light (which must be used to record singing activity during the night) to accurately retrieve singing information. Thus, using the reflective tags and performing daily checks to replace lost tags appeared the best solution. However, dealing with continuous video recordings proved an unsurmountable obstacle, making the tag problem irrelevant.

Collecting and processing the large quantity of data produced via continuous video recordings presents challenges for data storage and the time required to run the bespoke MATLAB script.

I first aimed to minimise these issues by optimising the trade-off between video quality and processing time; to use video recordings of sufficient quality to accurately pinpoint singing, but not so high as to preclude timely processing. Using the initial Nikon D3300 produced significantly larger files than a microcomputer or webcam, so to minimise file size and so, facilitate timely processing, I trialled alternative camera set ups. In my first attempt, I used a low power Raspberry Pi microcomputer (Raspberry Pi 4 Model B) fitted

with a camera module (Raspberry Pi Camera Module V2). Recordings from this setup were not of sufficient quality to accurately detect the reflective wing tags using the MATLAB script. I next trialled a Logitech C922 Pro HD Stream Webcam, which similarly did not produce video data of sufficient quality to accurately detect the reflective wing tags. These trials demonstrated that high quality video recordings were essential and so I next used a Nikon D3500 Digital SLR Camera with 18-55mm AF-P Non-VR Lens and a tripod (Manfrotto MT055XPRO3). With this setup, I acquired video recordings of sufficient quality to detect wing tag movement using the MATLAB script. However, processing 1h of video took ~24h, and because my recordings were to be for weeks at a time, processing this vast quantity of recordings would not be possible using this script without access to a computing cluster.

Outcome

Because I had invested a considerable amount of time (~12 months; alongside Chapters 2 and 5) into trialling the video methods, and because there was no guarantee that spending further months engaging with a computing cluster was a solution, I pivoted to developing a method to use audio recording data instead. While basing my data on audio recordings meant that I could not include flatwing males as subjects in my experiments and constrains sample size, it does have the benefit of avoiding any negative consequences of the procedure require to mute normal wing males.

PLAN B: Audio recording methods

T. oceanicus song is readily distinguished from other sounds and characterised by a long chirp duration followed by a short chip duration (Figure A1) with a peak frequency of ~4.8kHz (Figure A2). To prevent singing by males within an experiment from stimulating or inhibiting other replicate males, each had to recorded in its own incubator. This required suitable housing for both crickets and their recording devices, and a method to differentiate song from the background noise of incubator mechanics. Individual crickets

were housed in 9L plastic containers with lids, with food and water available *ad libitum*, and egg carton for shelter with audio recorders fixed to the side of the box. I used a Sony™ ICD-UX560 Digital Voice Recorders equipped with Integral™ Micro Secure Digital eXtended Capacity cards) set to a sampling rate of 44.1 kHz and a 16-bit resolution (stereo MP3 file format) and connected to an external power supply cord for all trials.

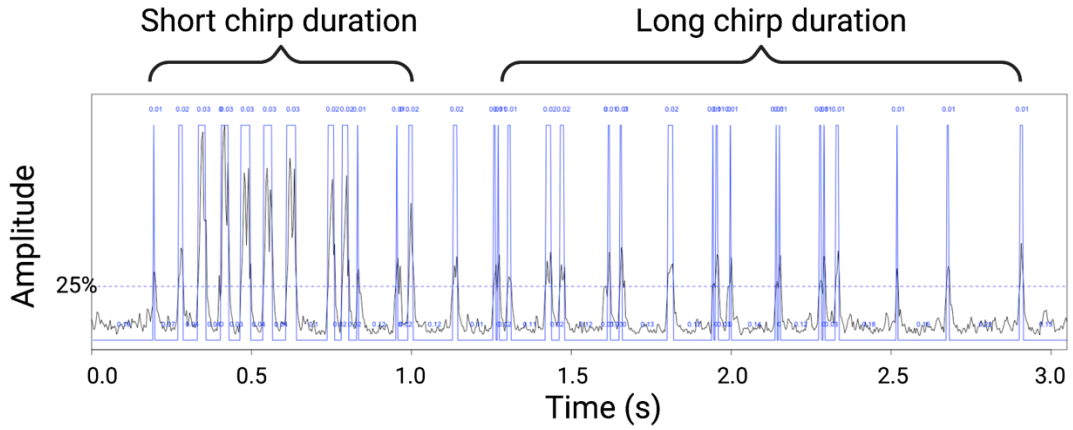


Fig. 1. *T. oceanicus* song is characterised by a short chirp duration (0-~1s; x-axis) followed by a long chirp duration (1.5-3.0s; x-axis). Amplitude is shown on the y-axis, and a 25% amplitude threshold is set (horizontal blue dashed line) such that any sample above this threshold is marked as signal (i.e. singing), and anything below the threshold is marked as pause (i.e. background noise). Signal events are also outlined in blue, and the length of time for each signal and pause event are indicated in small blue text on the figure. The sum of all signal and pause event times equals the total duration of the file.

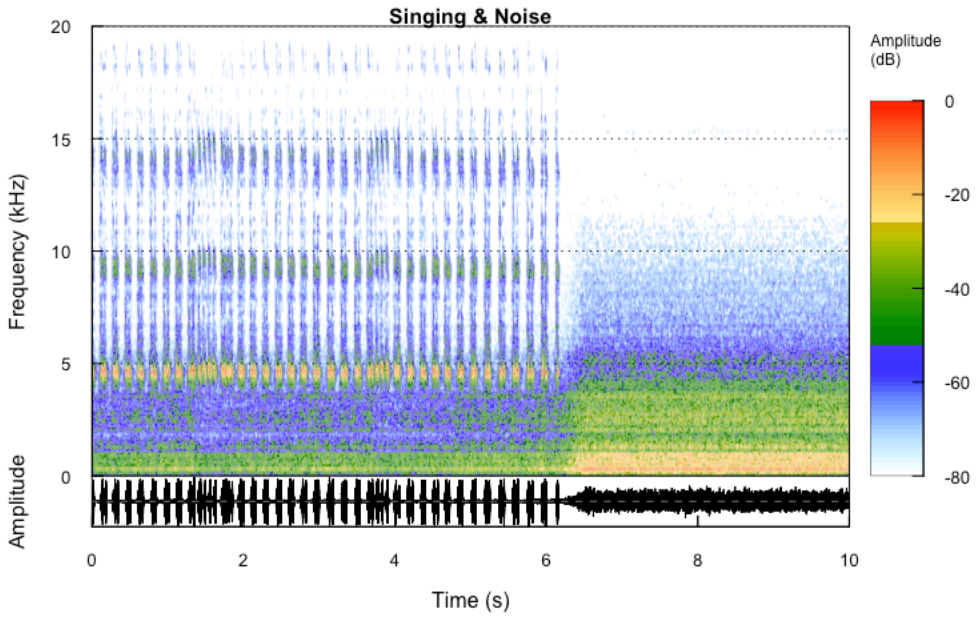


Fig. 2. Spectrogram (top) and associated oscillogram (bottom) for a normal-wing male calling song (from 0-6s) followed by background (i.e. incubator) noise (6-10s). Frequency is shown on the y-axis of the spectrogram and relative amplitude is shown on the y-axis of the oscillogram. Time in seconds is along the x-axis. Colours in the spectrogram indicate amplitude (dB, as shown in the legend).

I based my first audio analysis methods on those described in "Sound Analysis and Synthesis with R" (Sueur 2018). This involved breaking each continuous audio file (regardless of total duration) into 60s "clips", which were then passed through an amplitude threshold. The amplitude threshold marks any sample above the limit as signal and anything below as pause and records those event durations in seconds. It is then possible to extract the length (in seconds) of each signal and pause event in a given clip and determine their ratio (see Fig. A3 for 3 examples of this ratio). By setting an appropriate amplitude threshold, singing can be differentiated from background noise with a high degree of accuracy.

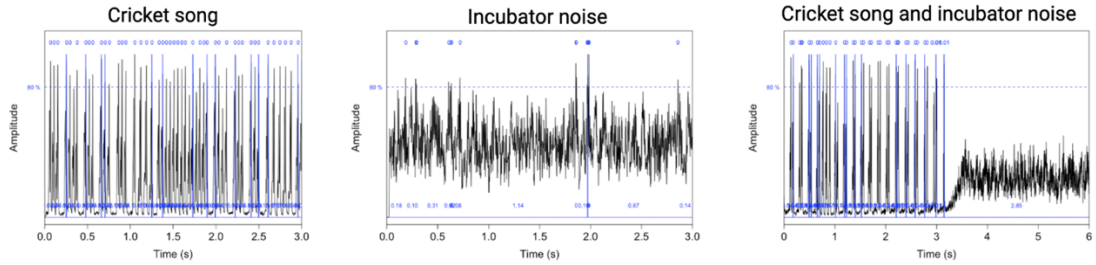


Fig. 3. Oscillograms of cricket song, incubator noise, and cricket song followed by incubator noise (y-axes indicate relative amplitude and x-axes indicate time in seconds). The horizontal blue dashed line across each plot indicates an 80% amplitude threshold. Each signal event is also outlined in blue, and the length of signal events are written in small blue text on the figure. In the left-most figure showing only cricket song , 46 signal events were detected (i.e., the number of times the amplitude threshold was crossed), and signal:pause = 0.048. In the middle figure showing only incubator noise, 14 signal events were detected and signal:pause = 0.011. In the right-most figure, for the first 3s the cricket is singing, and for the final 3s the cricket stops and so only incubator noise is recorded. In this figure, 37 signal events are detected and the signal:pause = 0.021.

While the signal:pause is a valid method to identify and quantify a sound of interest (Sueur 2018), several issues arose, making it a poor approach for my experiments. First, the parameters set for detecting signal need to be constant across audio files from different males and incubators (Sueur 2018). Because of this, a relatively high amplitude threshold must be set to minimise capturing rare instances of loud incubator background noise as signal (Fig. A3). Even with a threshold of 80%, some background noise is still captured (Fig. A3). However, such a high amplitude threshold also markedly reduces power to detect singing. For example, in Fig. A3i, the male sang consistently for the entire duration of the audio clip, though, the signal:pause is in a comparably low range (0.048; Fig. A3i) to the clip in which the male did not sing at all (0.011; Fig. A3ii). Yet, the signal:pause ratio reports erroneously there is ~4x less singing in the clip in Fig.A3ii when there is no male present to sing.

I tried to remedy this by placing a frequency filter on the audio files to home in on the frequency range of the calling song (~4.0-5.5 kHZ). However, this was accompanied by an overall decrease in the amplitude of files which do not contain any singing which forces an error in the R package I use. Specifically, files in which no signal is detected return an "error" message from the R package "Seewave" (the package which accompanies "Sound Analysis and Synthesis with R"; (Sueur 2018), which makes it impossible to differentiate between a true error or a file in which no signal was detected. Because male crickets do not sing for most of the circadian cycle, this uncertainty was unacceptable for our expectations of accuracy.

Outcome

For the reasons discussed, neither the video nor audio methods described in this appendix were selected to be used to detect singing in my experiments. Instead, I ultimately chose to pursue a machine learning approach in which I trained a Random Forest model to detect male song within a 60 second audio clip with >98% accuracy. Details for this method can be found in Chapters 3 and 4. My endeavours taught me a great deal about video processing and bioacoustic analysis (not to mention perseverance) and the bioacoustics methods provided the foundation for the machine learning algorithm, because the audio parameters describing *T. oceanicus* song were derived from my trial recordings and used to train the models. Finally, familiarising myself with video tracking methods will possibly prove useful for future work (e.g., for use whilst recording flatwing males).

8.3. Appendix: Chapter 4

Subjective "lights off" = $\left(\frac{DL_{lights-off} * \tau_{DD}}{24}\right) + (Day - 1) * \left(\frac{DL_{lights-off} * \tau_{DD}}{24} - 6\right)$

Subjective "dusk" begins = $\left[\left(\frac{DL_{lights-off} * \tau_{DD}}{24}\right) + (Day - 1) * \left(\frac{DL_{lights-off} * \tau_{DD}}{24} - 6\right)\right] - 1$

Subjective "lights on" = $\left[\left(\frac{DL_{lights-on} * \tau_{DD}}{24}\right) + (Day - 1) * \left(\frac{DL_{lights-on} * \tau_{DD}}{24} - 12\right)\right] + (.5 * \tau_{DD})$

Subjective "dawn" begins = $\left[\left(\frac{DL_{lights-on} * \tau_{DD}}{24}\right) + (Day - 1) * \left(\frac{DL_{lights-on} * \tau_{DD}}{24} - 12\right)\right] + (.5 * \tau_{DD}) + 1$

Equations I. Whilst free-running, subjective "lights on and off" do not simply follow from the previously experienced DL photoschedule; rather, it is based on their own unique FRP. Thus, we derived equations based on each individual's FRP to determine their subjective "lights on" and "lights off" whilst free-running. Subjective "lights off" and "lights on", and associated subjected "dusk" and "dawn", where $DL_{lights-off}$ is equal to lights-off from the 12D:12L photoschedule, $DL_{lights-on}$ is equal to the lights-on from the 12D:12L photoschedule, and τ_{DD} is equal to the free-running period for a given individual.

Parameter	Definition
freq.M	Median frequency
freq.IPR	Frequency interpercentile range
spec.mean	Mean of the frequency in the 3-6 kHz spectrum
spec.median	Median of the frequency in the 3-6 kHz spectrum
spec.mode	Mode of the frequency in the 3-6 kHz spectrum
freq.P2	Frequency terminal percentile
time.M	Time median
freq.P1	Frequency initial percentile
spec.sd	S.D. in the 3-6 kHz spectrum
spec.sem	S.E.M. in the 3-6 kHz spectrum
spec.Q25	First quartile in the 3-6 kHz spectrum
spec.Q75	Terminal quartile in the 3-6 kHz spectrum
spec.IQR	Interquartile range in the 3-6 kHz spectrum
spec.cent	Centroid in the 3-6 kHz spectrum

spec.skewness	Skewness in the 3-6 kHz spectrum
spec.kurtosis	Kurtosis in the 3-6 kHz spectrum
spec.sfm	Spectral flatness measure in the 3-6 kHz spectrum
spec.sh	Spectral entropy in the 3-6 kHz spectrum
spec.prec	Frequency precision of the 3-6 kHz spectrum

Table I. Parameters derived from audio clips using the Seewave R package. Parameters highlighted in yellow were chosen to train the models.

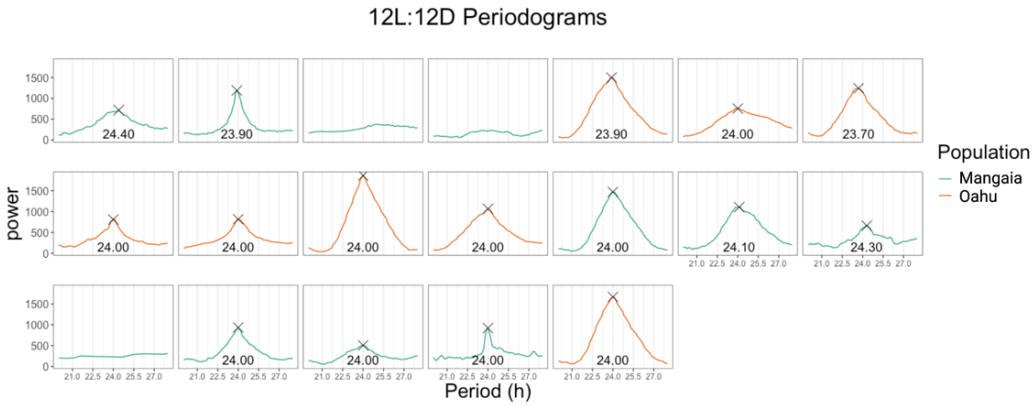


Figure I. Chi-square periodograms for individual males under the entraining LD photoschedule. Period estimate ("period" in hours) and power are shown on the x- and y-axes, respectively. Populations are indicated by colours in the legend (blue-green = "Mangaia Islands" and orange= "Oahu"). The most significant period estimate is marked with a black "X" on the line plot and written as text on the plot. Two individuals from the Mangaia Island population did not return significant period estimates for this photoschedule (the 3rd and 4th plots from the left along the top row).

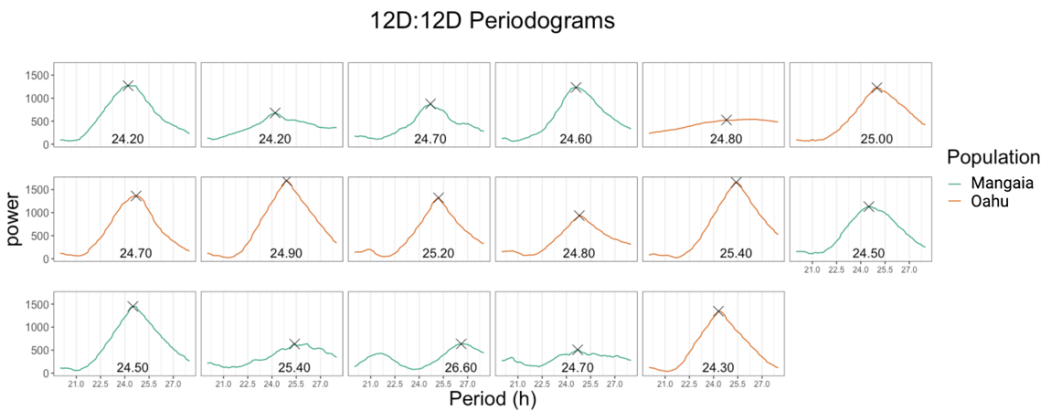


Figure II. Chi-square periodograms for individual males under the free running DD photoschedule. Period estimate ("period" in hours) and power are shown on the x- and y-axes, respectively. Populations are indicated by colours in the legend (blue-green = "Mangaia Islands" and orange= "Oahu"). The most significant period estimate is marked with a black "X" on the line plot and written as text on the plot. One individual from the Mangaia Island population did not return a significant period estimate for this photoschedule (the 2nd plot from the left along the middle row).

8.4. Appendix: Chapter 5

Model description: Log10(Conversion) ~	df	log(*L*)	AICc	ΔAICc	AICc *w*
donor	3	-13.80	34.80	0.000	0.648
donor + schedule	4	-13.77	37.64	2.842	0.156
null	2	-16.74	38.05	3.252	0.127
schedule	3	-16.72	40.63	5.831	0.035
donor + schedule + donor*schedule	5	-13.71	40.75	5.950	0.033

Table 1. Degrees of freedom (df), log-Likelihood (log(*L*)), AICc, ΔAICc ($AICc_i - AICc_{min}$), and AICc *w* (AICc weight) for each linear model in the conversion analysis using the full dataset ordered in descending fit (best-fitting model at the top). The response variable for each model is the log_{10}-transformed conversion rate. "Schedule" refers to parasites either in-synch or out-of-synch with the host, and "donor" corresponds to parasites taken from donor mice kept in either the standard or reversed light : dark schedule.

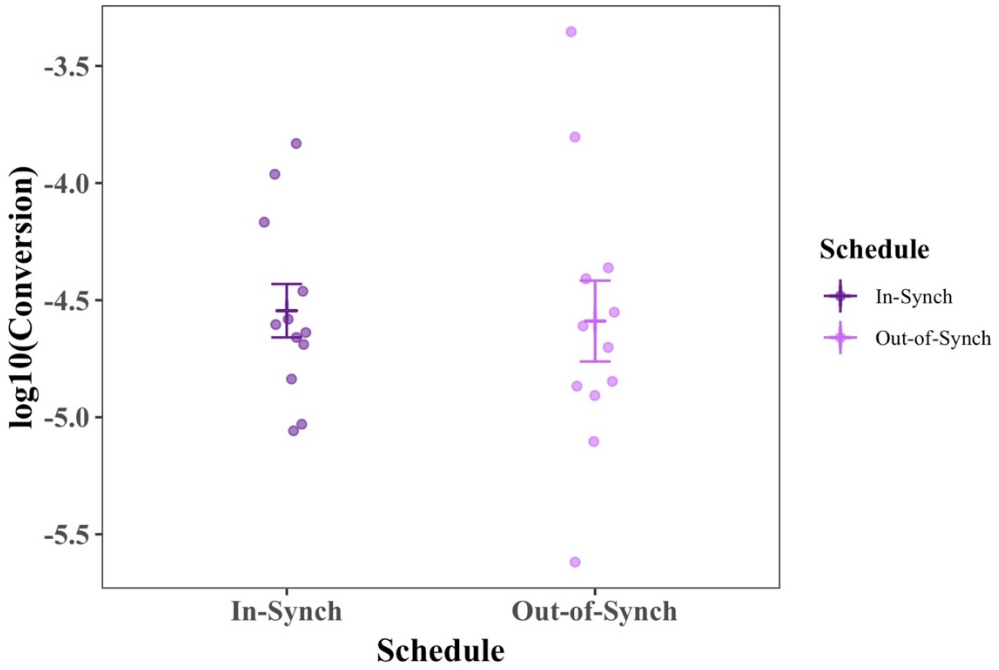


Figure 1. Conversion estimates, alongside mean ± S.E. (calculated post-transformation), for parasites in- and out-of-synch with host circadian rhythms using the full dataset. Points represent raw data, $\log_{10}$-transformed to approximate normality. Data from experiment in O'Donnell *et al.* 2011.