1 # Timing of host feeding drives rhythms in parasite replication

2 Kimberley F. Prior[1]*, Daan R. van der Veen[2], Aidan J. O'Donnell[1], Katherine Cumnock[4], David

3 Schneider[4], Arnab Pain[5], Amit Subudhi[5], Abhinay Ramaprasad[5], Samuel S.C. Rund[3], Nicholas J.

4 Savill[1,3] & Sarah E. Reece[1,3]

5

6

7

8 [1]Institutes of Evolution, Immunology and Infection Research, University of Edinburgh, Edinburgh, UK

9 [2]Faculty of Health and Medical Sciences, University of Surrey, Guildford, UK

10 [3]Centre for Immunity, Infection and Evolution, University of Edinburgh, Edinburgh, UK

11 [4]Department of Microbiology and Immunology, Stanford University, CA, USA

12 [5]King Abdullah University of Science and Technology, Saudi Arabia

13

14 *Corresponding author

15 E-mail: kimberley.prior@ed.ac.uk (KFP)

16

Abstract

Circadian rhythms enable organisms to synchronise the processes underpinning survival and reproduction to anticipate daily changes in the external environment. Recent work shows that daily (circadian) rhythms also enable parasites to maximise fitness in the context of ecological interactions with their hosts. Because parasite rhythms matter for their fitness, understanding how they are regulated could lead to innovative ways to reduce the severity and spread of diseases. Here, we examine how host circadian rhythms influence rhythms in the asexual replication of malaria parasites. Asexual replication is responsible for the severity of malaria and fuels transmission of the disease, yet, how parasite rhythms are driven remains a mystery. We perturbed feeding rhythms of hosts by 12 hours (i.e. diurnal feeding in nocturnal mice) to desynchronise the host's peripheral oscillators from the central, light-entrained oscillator in the brain and their rhythmic outputs. We demonstrate that the rhythms of rodent malaria parasites in day-fed hosts become inverted relative to the rhythms of parasites in night-fed hosts. Our results reveal that the host's peripheral rhythms (associated with the timing of feeding and metabolism), but not rhythms driven by the central, light-entrained circadian oscillator in the brain, determine the timing (phase) of parasite rhythms. Further investigation reveals that parasite rhythms correlate closely with blood glucose rhythms. In addition, we show that parasite rhythms resynchronise to the altered host feeding rhythms when food availability is shifted, which is not mediated through rhythms in the host immune system. Our observations suggest that parasites actively control their developmental rhythms. Finally, counter to expectation, the severity of disease symptoms expressed by hosts was not affected by desynchronisation of their central and peripheral rhythms. Our study at the intersection of disease ecology and chronobiology opens up a new arena for studying host-parasite-vector coevolution and has broad implications for applied bioscience.

Author summary

How cycles of asexual replication by malaria parasites are coordinated to occur in synchrony with the circadian rhythms of the host is a long-standing mystery. We reveal that rhythms associated with the time-of-day that hosts feed are responsible for the timing of rhythms in parasite development. Specifically, we altered host feeding time to phase-shift peripheral rhythms, whilst leaving rhythms driven by the central circadian oscillator in the brain unchanged. We found that parasite developmental rhythms remained synchronous but changed their phase, by 12 hours, to follow the timing of host feeding. Furthermore, our results suggest that parasites themselves schedule rhythms in their replication to coordinate with rhythms in glucose in the host's blood, rather than have rhythms imposed upon them by, for example, host immune responses. Our findings reveal a novel relationship between hosts and parasites that if disrupted, could reduce both the severity and transmission of malaria infection.

Introduction

The discovery of daily rhythms in parasites dates back to the Hippocratic era and a taxonomically diverse range of parasites (including fungi, helminths, Coccidia, nematodes, trypanosomes, and malaria parasites [1-6]) display rhythms in development and several behaviours. Yet, how rhythms in many parasite traits are established and maintained remains mysterious, despite their significance, as these traits underpin the replication and transmission of parasites [7]. For example, metabolic rhythms of *Trypanosoma brucei* have recently been demonstrated to be under the control of an oscillator belonging to the parasite, but the constituents of this oscillator are unknown [8]. In most organisms, endogenous circadian oscillators ("clocks") involve transcription-translation feedback loops whose timing is synchronised to external cues, such as light-dark and feeding-fasting cycles [9,10] but there is generally little homology across taxa in the genes underpinning oscillators. Multiple, convergent, evolutionary origins for circadian oscillators is thought to be explained by the fitness advantages of being able to anticipate and exploit predictable daily

69　changes in the external environment, as well as keeping internal processes optimally timed [11,12].

70　Indeed, the 2017 Nobel Prize in Physiology/Medicine recognises the importance of circadian

71　oscillators [13,14].

72　　　The environment that an endoparasite experiences inside its host is generated by many

73　rhythmic processes, including daily fluctuations in the availability of resources, and the nature and

74　strength of immune responses [15,16]. Coordinating development and behaviour with rhythms in the

75　host (or vector) matters for parasite fitness [17]. For example, disrupting synchrony between rhythms

76　in the host and rhythms in the development of malaria parasites during asexual replication reduces

77　parasite proliferation and transmission potential [18,19]. Malaria parasites develop synchronously

78　during cycles of asexual replication in the host's blood and each developmental stage occurs at a

79　particular time-of-day. The synchronous bursting of parasites at the end of their asexual cycle, when

80　they release their progeny to infect new red blood cells, causes fever with sufficient regularity (24,

81　48, or 72 hourly, depending on the species) to have been used as a diagnostic tool. Malaria parasites

82　are assumed to be intrinsically arrhythmic and mathematical modelling suggests that rhythms in host

83　immune effectors, particularly inflammatory responses, could generate rhythms in the development

84　of malaria parasites via time-of-day-specific killing of different parasite developmental stages [20,21].

85　However, the relevant processes operating within real infections remain unknown [22].

86　　　Our main aim is to use the rodent malaria parasite *Plasmodium chabaudi* to ask which

87　circadian rhythms of the host are involved in scheduling rhythms in parasite development. In the

88　blood, *P. chabaudi* develops synchronously and asexual cycles last 24 hours, bursting to release

89　progeny (schizogony) in the middle of the night when mice are awake and active. We perturbed host

90　feeding time (timing of food intake), which is known to desynchronise the phase of rhythms from the

91　host's central and peripheral oscillators, and we then examined the consequences for parasite

92　rhythms. In mammals, the central oscillator in the brain (suprachiasmatic nuclei of the hypothalamus,

93　SCN), is entrained by light [10,23]. The SCN is thought to shape rhythms in physiology and behaviour

94　(peripheral rhythms) by entraining peripheral oscillators via hormones such as glucocorticoids [24].

95　However, oscillators in peripheral tissues are self-sustained and can also be entrained by several
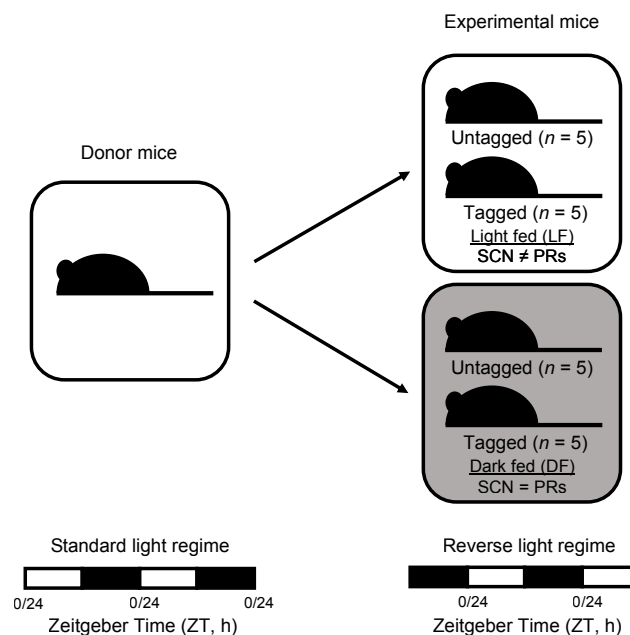
96   non-photic cues, such as the time-of-day at which feeding occurs [25,26]. Thus, eating at the wrong

97   time-of-day (e.g. diurnal feeding in nocturnal mice) leads to altered timing of oscillators, and their

98   associated rhythms in peripheral tissues. This phase-shift is particularly apparent in the liver where

99   an inversion in the peak phase of expression of the circadian oscillator genes *Per1* and *Per2* occurs

100 [26]. Importantly, eating at the wrong time-of-day does not alter rhythmic outputs from the central

101 oscillator [25].

102   In murine hosts with an altered (diurnal) feeding schedule, the development rhythms of

103 parasites remained synchronous but became inverted relative to the rhythms of parasites in hosts

104 fed at night. Thus, feeding-related outputs from the hosts peripheral timing system, not the SCN, are

105 responsible for the timing (phase) of parasite rhythms. We also reveal that the inversion of parasite

106 rhythms corresponds to a phase-shift in blood glucose rhythms. That parasites remain synchronous

107 during the rescheduling of their rhythm coupled with evidence that immune responses do not set the

108 timing of parasite rhythms, suggests parasites are responsible for scheduling their developmental

109 rhythm, and may express their own circadian rhythms and/or oscillators. Furthermore, our perturbed

110 feeding regimes are comparable to shift work in humans. This lifestyle is well-known for increasing

111 the risk of non-communicable diseases (cancer, type 2 diabetes etc. [27]) but our data suggest the

112 severity of malaria infection (weight loss, anaemia) is not exacerbated by short-term

113 desynchronisation of the central and peripheral oscillators.

114

Results & Discussion

116   First, we examined the effects of changing the time of food intake on the phasing of circadian

117 rhythms in host body temperature and locomotor activity (Fig 1). Body temperature is a commonly

118 used phase marker of circadian timing because core body temperature increases during activity and

119 decreases during sleep [28,29]. Mice were given access to food for 12 hours in each circadian cycle,

120 either in the day (LF, light fed) or night (DF, dark fed). All food was available *ad libitum* and available

121 from ZT 0-12 (ZT refers to 'Zeitgeber Time'; ZT 0 is the time in hours since lights on) for LF mice,

122 and from ZT 12-24 for DF mice. All experimental mice were entrained to the same reversed

123 photoperiod, lights on: 7pm (ZT 0/24), lights off: 7am (ZT 12), for 2 weeks prior to starting the
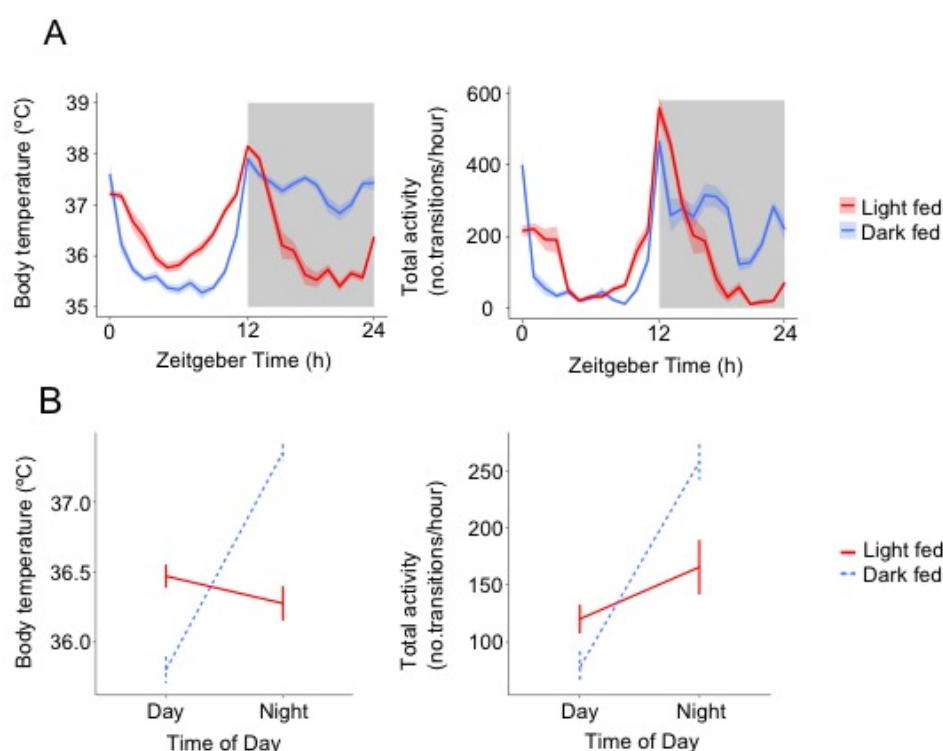
124 experiment (Fig 1).

125

Fig 1. Experimental design, feeding time. Infections were initiated with parasites raised in donor
mice entrained to a standard light regime [lights on: 7am (ZT 0/24) and lights off: 7pm (ZT 12)] and
used to create experimental infections in hosts entrained to a reverse light regime of 12-hours light:
12-hours dark [lights on: 7pm (ZT 0/24), lights off: 7am (ZT 12); ZT is Zeitgeber Time: hours after
lights on], leading to a 12-hour phase difference in SCN rhythms of donor and host, and
subsequently, parasite infections (see Materials and Methods for the rationale). Hosts were then
assigned to one of the two treatment groups. One group (N=10) were allowed access to food
between ZT0 and ZT12 ("light fed mice", LF, food access during the day) and the other group (N=10)
allowed access to food between ZT12 and ZT0 ("dark fed mice", DF, food access during the night).
Body temperature and locomotor activity were recorded from a subset of RFID "tagged" mice in each
group (N=5 per group). Changing feeding time (day time feeding of nocturnal mice) desynchronises
rhythmic outputs from the central (SCN) oscillator and the peripheral (peripheral rhythms, PRs)
oscillators ("SCN ≠ PRs"), whereas the SCN and peripheral rhythms remain synchronised in mice
fed at night ("SCN = PRs").

140 We found a significant interaction between feeding treatment (LF or DF) and the time-of-day

141 (day (ZT 0-12) or night (ZT 12-24)) that mice experience elevated body temperatures ($\chi^2_{(5,6)}$ = 75.89,

142 $p < 0.0001$) and increase their locomotor activity ($\chi^2_{(5,6)}$ = 39.57, $p < 0.0001$; S1 Table). Specifically,

143 DF mice have elevated body temperature and are mostly active during the night (as expected)

144 whereas LF mice show no such day-night difference in body temperature and locomotor activity, due

145 to a lack of night time elevation in both measures where food and light associated activity are

146 desynchronised (Fig 2). We also find the centres of gravity (CoG; a general phase marker of

6

147 circadian rhythms, estimated with CircWave), are slightly but significantly earlier in LF mice for both

148 body temperature (approximately 2 hours advanced: $\chi^2_{(3,4)}$ = 28.17, p < 0.0001) and locomotor

149 activity (approximately 4 hours advanced: $\chi^2_{(3,4)}$ = 27.32, p < 0.0001) (S1 Table). Therefore, the LF

150 mice experienced a significant change in the daily profile of activity, which is reflected in some phase

151 advance (but not inversion) relative to DF mice, and significant disruption to their body temperature

152 and locomotor activity rhythms, particularly during the night. Because an altered feeding schedule

153 does not affect the phase of the SCN [25], our data suggest that rhythms in body temperature and

154 locomotor activity in LF mice are shaped by both rhythms in feeding and the light-dark cycle [30].

155 Finally, the body weight of LF and DF mice did not differ significantly after 4 weeks ($\chi^2_{(3,4)}$ = 0.02, p

156 = 0.9) and both groups equally gained weight during the experiment (S1 Fig), corroborating that LF
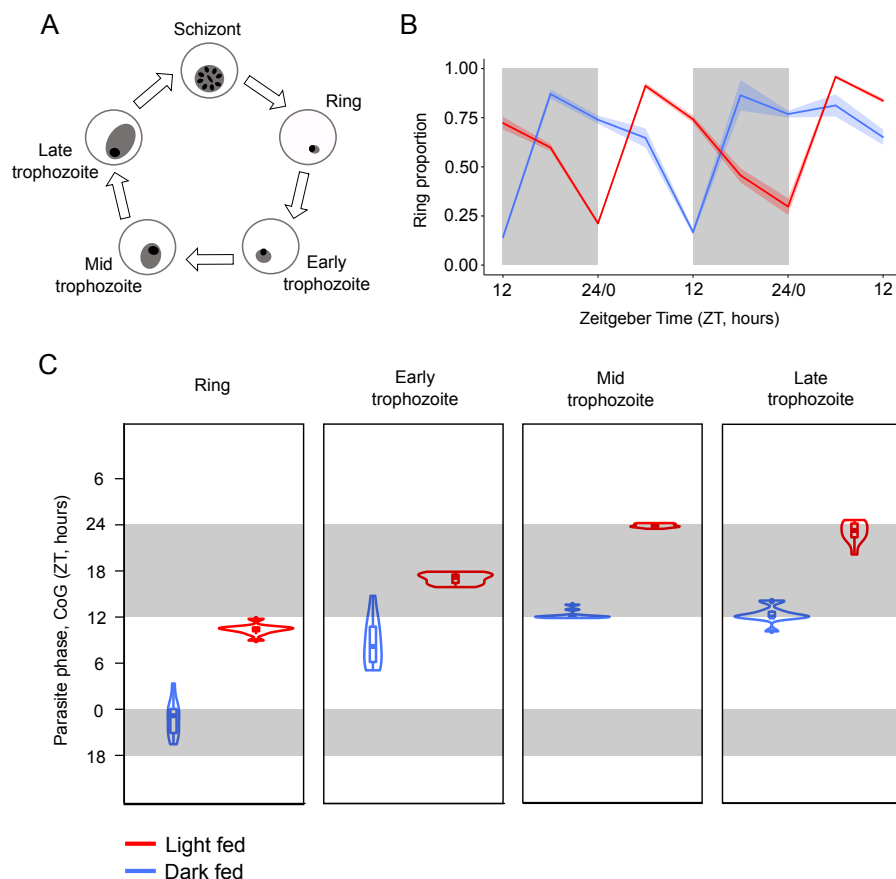
157 mice were not calorie restricted.

158

159 **Fig 2. Feeding nocturnal mice in the day time disrupts rhythms in body temperature and**
160 **locomotor activity.** (A) Hourly mean ± SEM body temperature and locomotor activity (number of
161 transitions per hour is the average number of movements a mouse makes in an hour, between
162 antennae on the Home Cage Analysis system, see Materials and Methods) and (B) interaction
163 between time-of-day and treatment group on body temperature and locomotor activity (calculating
164 the mean temperature/activity across the day, ZT 0-12, and night, ZT 12-24, ± SEM) averaged from
165 48 hours of monitoring mice before infection. N=5 for each of the light fed (LF, red) and dark fed (DF,
166 blue) groups. Light and dark bars indicate lights on and lights off (lights on: ZT 0/24, lights off: ZT
167 12).

168   Having generated hosts in which the phase relationship between the light-entrained SCN and

169   food-entrained rhythms are altered (LF mice) or not (DF mice), we then infected all mice with the

170   rodent malaria parasite *Plasmodium chabaudi adami* genotype DK (Fig 1) from donor mice

171   experiencing a light-dark cycle 12 hours out of phase with the experimental host mice. After allowing

172   the parasite's developmental rhythms to become established (see Materials and Methods) we

173   compared the rhythms of parasites in LF and DF mice. We hypothesised that if parasite rhythms are

174   solely determined by rhythms driven by the host's SCN (which are inverted in the host mice

175   compared to the donor mice), parasite rhythms would equally shift and match in LF and DF mice

176   because both groups of hosts were entrained to the same light-dark conditions. Yet, if rhythms in

177   body temperature or locomotor activity directly or indirectly (via entraining other oscillators)

178   contribute to parasite rhythms, we expected that parasite rhythms would differ between LF and DF

179   hosts. Further, if feeding directly or indirectly (via food-entrained oscillators) drives parasite rhythms,

180   we predicted that parasite rhythms would become inverted (Fig 1).

181   In the blood, *P. chabaudi* parasites transition through five developmental stages during each

182   (~24hr) cycle of asexual replication (Fig 3A) [6,31]. We find that four of the five developmental stages

183   (rings, and early-, mid-, and late-trophozoites) display 24hr rhythms in both LF and DF mice (Fig 3B,

184   S2 Table, S2 Fig). The fifth stage - schizonts - appear arrhythmic but this stage sequesters in the

185   host's tissues [32,33] and so, are rarely collected in venous blood samples. Given that all other

186   stages are rhythmic, and that rhythms in ring stages likely require their parental schizonts to have

187   been rhythmic, we expect schizonts are rhythmic but that sequestration prevents a reliable

188   assessment of their rhythms.

189

190

191

192

193

194

195

Fig 3. Parasite rhythms are inverted in hosts fed during the day compared to the night. (A) The asexual cycle of malaria parasites is characterised by five morphologically distinct developmental stages (ring, early trophozoite, mid trophozoite, late trophozoite) differentiated by parasite size within the red blood cell, the size and number of nuclei, and the appearance of haemozoin [31]. (B) Mean ± SEM (N=10 per group) proportion of observed parasites in the blood at ring stage in light fed mice (red; allowed access to food during the day, between ZT 0 and ZT 12) and dark fed mice (blue; allowed access to food during the night, between ZT 12 and ZT 24). The proportion of parasites at ring stage in the peripheral blood is highest at night (ZT 22) in dark fed mice but in the day (ZT 10) for light fed mice, illustrating the patterns observed for all other (rhythmic) stages (see Fig S2). (C) CoG (estimate of phase) in ZT (h) for each rhythmic parasite stage in the blood. Each violin illustrates the median ± IQR overlaid with probability density (N=10 per group). The height of the violin illustrates the variation in the timing of the CoG between mice and the width illustrates the frequency of the CoGs at particular times within the distribution. Sampling occurred every 6 hours days 6-8 post infection. Light and dark bars indicate lights on and lights off (lights on: ZT 0, lights off: ZT 12).

The CoG estimates for ring, and early-, mid-, and late-trophozoite stages are approximately 10-12 hours out-of-phase between the LF and DF mice (Fig 3B,C, S2 Table). For example, rings peak at approximately ZT 10 in LF mice and peak close to ZT 23 in DF mice. The other stages peak in sequence. Schizogony (when parasites burst to release their progeny) occurs immediately prior to reinvasion, therefore we expect it occurs during the day for the LF mice and night for DF mice [7]. The almost complete inversion in parasite rhythms between LF and DF mice demonstrates that

9

218 feeding-related rhythms are responsible for the phase of parasite rhythms, with little to no apparent

219 contribution from the SCN and/or the light: dark cycle.
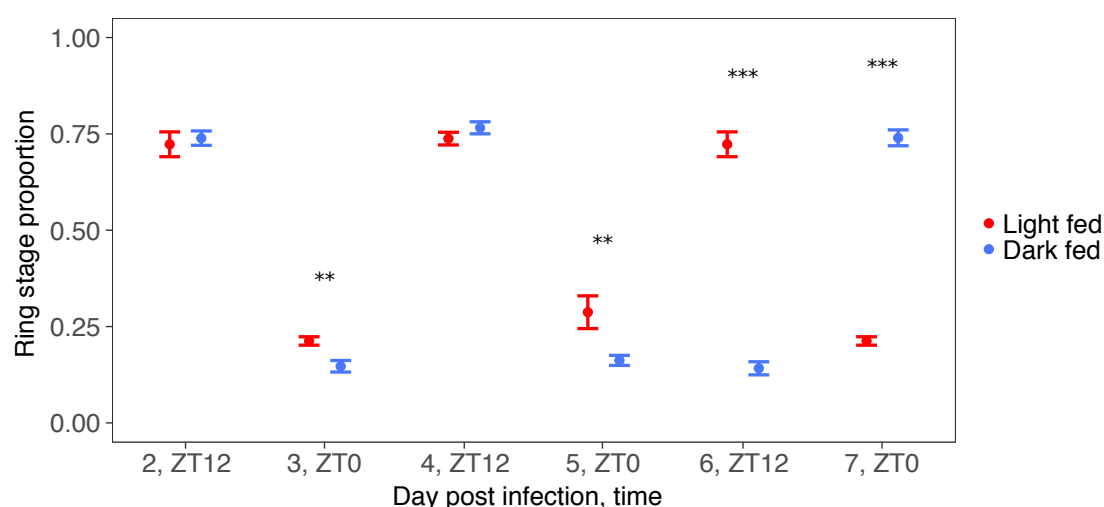
220 Changing the feeding time of nocturnal mice to the day time has similarities with shift work in

221 diurnal humans [34]. This lifestyle is associated with an increased risk of acquiring non-

222 communicable diseases (e.g. cancer, diabetes) [35] and has been recapitulated in mouse models

223 [e.g. 36,37,38]. In contrast, in response to perturbation of their feeding rhythm, infections are not

224 more severe in hosts whose circadian rhythms are desynchronised (i.e. LF hosts). Specifically, all

225 mice survived infection and virulence (measured as host anaemia; reduction in red blood cells) of

226 LF and DF infections is not significantly different (comparing minimum red blood cell density, $\chi^2_{(3,4)}$

227 $= 0.11$, $p = 0.74$; S3A Fig). As described above, changes in body mass were not significantly different

228 between treatments (S1 Fig). Using a longer-term model for shift work may reveal differences in

229 infection severity, especially when combined with the development of non-communicable disease.

230 There are no significant differences between parasite densities in LF and DF hosts during

231 infections (LF versus DF on day 6 post infection, $\chi^2_{(3,5)} = 0.66$, $p = 0.42$, S3B Fig). This can be

232 explained by both groups being mismatched to the SCN of the host, which we have previously

233 demonstrated to have negative consequences for *P. chabaudi* [18]. Our previous work was carried

234 out using *P. chabaudi* genotype AJ so is not directly comparable to our results presented here,

235 because DK is a less virulent genotype [39]. Instead, a comparison of our results to data collected

236 previously for genotype DK, in an experiment where SCN rhythms of donor and host mice were

237 matched (see Materials and Methods; infections were initiated with the same strain, sex, and age of

238 mice, the same dose at ring stage) reveals a cost of mismatch of donor and host entrainment.

239 Specifically, parasite density on day 6 (when infections have established but before parasites start

240 being cleared by host immunity) is significantly lower in infections mismatched to the SCN (LF and

241 DF) compared to infections matched to the SCN ($\chi^2_{(3,5)} = 16.71$, $p = 0.0002$, difference = 2.21e+10

242 parasites per ml blood) (see S4A Fig). In keeping with a difference in parasite replication, hosts with

243 matched infections reach lower red blood cell densities ($\chi^2_{(3,5)} = 18.87$, $p < 0.0001$, mean difference

244 $= 5.29e+08$ red blood cells per ml blood).

245   The mismatched and matched infections compared above also differ in whether hosts had

246   food available throughout the 24-hour cycle or for 12 hours only (LF and DF). Restricting food to 12

247   hours per day does not affect host weight (S1 Fig) and mice still undergo their main activity bout at

248   lights off even when food is available all the time. Therefore, we propose that rather than feeding

249   duration, mismatch to the host SCN for as few as 5 cycles is costly to parasite replication and reduces

250   infection severity. Because peripheral and SCN driven rhythms are usually in synchrony, we suggest

251   parasites use information from food-entrained oscillators, or metabolic processes, to ensure their

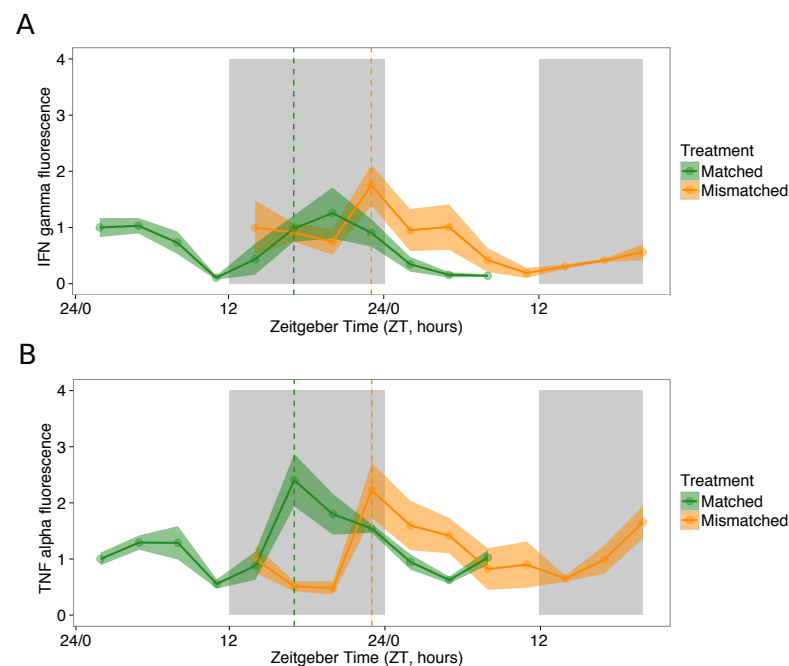252   development is timed to match the host's SCN rhythms.

253   Instead of organising their own rhythms (i.e. using an "oscillator" whose time is set by a

254   "Zeitgeber" or by responding directly to time-of-day cues), parasites may allow outputs of food-

255   entrained host oscillators to enforce developmental rhythms. Previous studies have focused on

256   rhythmic immune responses as the key mechanism that schedules parasite rhythms (via

257   developmental-stage and time-of-day specific killing [20,21]). Evidence that immune responses are

258   rhythmic in naïve as well as infected hosts is increasing [15,16], but the extent to which

259   peripheral/food-entrained oscillators and the SCN drive immune rhythms is unclear. Nonetheless,

260   we argue that rhythms in host immune responses do not play a significant role in scheduling

261   parasites for the following reasons: First, mismatch to the host's peripheral rhythms (which occurs

262   in DF mice but not LF mice as a feature of our experimental design) does not cause a significant

263   reduction in parasite number (S3B Fig), demonstrating that stage-specific killing cannot cause the

264   differently phased parasite rhythms in LF and DF mice. Second, while changing feeding time appears

265   to disrupt some rodent immune responses [40,41], effectors important in malaria infection, including

266   leukocytes in the blood, do not entrain to feeding rhythms [42,43]. Third, inflammatory responses

267   important for killing malaria parasites are upregulated within hours of blood stage infection [44] so

268   their footprint on parasite rhythms should be apparent from the first cycles of replication [19]. In

269   contrast, rhythms of parasites in LF and DF mice do not significantly diverge until 5-6 days post

270   infection, after 5 replication cycles (S3 Table, Fig 4). Fourth, an additional experiment (see Materials

271   and Methods) reveals that rhythms in the major inflammatory cytokines that mediate malaria infection

11

272   (e.g. IFN-gamma and TNF-alpha: [45,46,47,48]) follow the phase of parasite rhythms (Fig 5), with

273   other cytokines/chemokines also experiencing this phenomenon (S5 Fig). Specifically, mice infected

274   with *P. chabaudi* genotype AS undergoing schizogony at around midnight (ZT17), produce peaks in

275   the cytokines IFN-gamma and TNF-alpha at ZT21 and ZT19 respectively. Whereas mice infected

276   with mismatched parasites undergoing schizogony around ZT23 (6 hours later), experience 3-6 hour

277   delays in the peaks of IFN-gamma and TNF-alpha (IFN-gamma: ZT0, TNF-alpha: ZT1). Thus, even

278   if parasites at different development stages differ in their sensitivity to these cytokines, these immune

279   rhythms could only serve to increase synchrony in the parasite rhythm but not change its timing.

280

281   **Fig 4. Parasite rhythms in light and dark fed mice significantly diverge by day 5-6 post**
282   **infection.** The proportion of ring stage parasites across infections (light fed mice, red, and dark fed
283   mice, blue) as a phase marker reveals that rhythms of parasites in light fed mice (red) and dark fed
284   mice (blue) diverge. Mice were sampled at ZT 12 on days 2, 4 and 6 and at ZT 0 on days 3, 5 and 7
285   post infection (see Fig 3 and S2 Fig). Consistent significant differences (**, $p < 0.05$; ***, $p < 0.001$)
286   between feeding treatments begins on day 5. By days 6-7 post infection, rings in light fed mice are
287   present at ZT12 while rings in dark fed mice are present at ZT 0, indicating that parasites in dark fed
288   mice have rescheduled. Ring stages are presented as the phase marker because this is the most
289   accurately quantified stage but other stages follow a similar pattern (S3 Table). Mean ± SEM is
290   plotted and N=10 for each treatment group.

291

12

Fig 5. Rhythms in inflammatory cytokines follow rhythms in parasite development. Mean ± SEM (N=4 per time point) for cytokines (A) IFN-gamma and (B) TNF-alpha for parasites matched and mismatched to the SCN rhythms of the host (matched: green, mismatched: orange). Sampling occurred every 3 hours on days 4-5 post infection. Matched parasites undergo schizogony around ZT 17, (indicated by green dashed line) and mismatched parasites undergo schizogony 6 hours later, around ZT 23 (indicated by orange dashed line). IFN-gamma peaks at ZT 21.29 in matched infections (green) and at ZT 0 in mismatched infections (orange). TNF-alpha peaks at ZT 19.26 in matched infections (green) and at ZT 1.29 in mismatched infections (orange). Light and dark bars indicate lights on and lights off (lights on: ZT 0, lights off: ZT 12).

More in-depth analysis of LF and DF infections provides further support that parasites actively organise their developmental rhythms. We examined whether parasites in DF mice maintain synchrony and duration of different developmental stages during rescheduling to the host's SCN rhythms. Desynchronisation of oscillators manifests as a reduction in amplitude in rhythms that are driven by more than one oscillator (e.g. parasite and host oscillator). No loss in amplitude suggests that parasites shift their timing as a cohort without losing synchrony. Parasite rhythms in LF and DF mice did not differ significantly in amplitude ($\chi^2_{(6,7)}$ = 1.53, p = 0.22, S4A Table) and CoGs for sequential stages are equally spaced ($\chi^2_{(10,18)}$ = 11.75, p = 0.16, S2 Table) demonstrating that parasite stages develop at similar rates in both groups. The rhythms of parasites in LF and DF mice were not intensively sampled until days 6-8 PI, raising the possibility that parasites lost and regained synchrony before this. Previously collected data for *P. chabaudi* genotype AS infections mismatched

313   to the host SCN by 12 hours that have achieved a 6-hour shift by day 4 PI also exhibit synchronous

314   development (S4B Table and S6 Fig), suggesting that parasites reschedule in synch.

315   That parasite rhythms do not differ significantly between LF and DF mice until day 5-6 post

316   infection (Fig 4) could be explained by the parasites experiencing a phenomenon akin to jet lag. Jet

317   lag results from the fundamental, tissue-specific robustness of circadian oscillators to perturbation,

318   which slows down the phase shift of individual oscillators to match a change in 'time-zone' [10]. We

319   propose that the most likely explanation for the data gathered from our main experiment for genotype

320   DK, and that collected previously for AJ and AS, is that parasites possess intrinsic oscillators that

321   shift collectively, in a synchronous manner, by a few hours each day, until they re-entrain to the new

322   'time-zone'. Because there is no loss of amplitude of parasite rhythms, it is less likely that individual

323   parasites possess intrinsic oscillators that re-entrain at different rates to the new 'time-zone'. The

324   recently demonstrated ability of parasites to communicate decisions about asexual to sexual

325   developmental switches [49] could also be involved in organising asexual development.

326   If parasites have evolved a mechanism to keep time and schedule their rhythms, what

327   external information might they synchronise to? Despite melatonin peaks in lab mice being brief and

328   of low concentration [50,51], the host's pineal melatonin rhythms have been suggested as a parasite

329   time cue [52]. However, we can likely rule pineal melatonin, and other glucocorticoids, out because

330   they are largely driven by rhythms of the SCN, which follow the light-dark cycle and have not been

331   shown to phase shift by 12 hours as a result of perturbing feeding timing [25]; some glucocorticoid

332   rhythms appear resistant to changing feeding time [53]. Whether extra-pineal melatonin, produced

333   by the gut for example [54], could influence the rhythms of parasites residing in the blood merits

334   further investigation. Body temperature rhythms have recently been demonstrated as a Zeitgeber for

335   an endogenous oscillator in trypanosomes [8]. Malaria parasites are able to detect and respond to

336   changes in environmental temperature to make developmental transitions in the mosquito phase of

337   their lifecycle [55,56], and may deploy the same mechanisms to organise developmental transitions

338   in the host. Body temperature rhythms did not fully invert in LF mice but they did exhibit unusually

339   low (i.e. day time) temperatures at night. Thus, for body temperature to be a time-of-day cue or

14

340   Zeitgeber it requires that parasites at early developmental stages (e.g. rings or early trophozoites)

341   are responsible for time-keeping because they normally experience low temperatures during the day

342   when the host is resting. The same logic applies to rhythms in locomotor activity because it is very

343   tightly correlated to body temperature (Pearson's correlation R=0.85, 95% CI: 0.82-0.88). Locomotor

344   activity affects other rhythms, such as physiological oxygen levels (daily rhythms in blood and tissue

345   oxygen levels), which can reset circadian oscillators [57] and have been suggested as a time cue for

346   filarial nematodes [4].

347   Feeding rhythms were inverted in LF and DF mice and so, the most parsimonious explanation

348   is that parasites are sensitive to rhythms related to host metabolism and/or food-entrained oscillators.

349   Malaria parasites have the capacity to actively alter their replication rate in response to changes in

350   host nutritional status [58]. Thus, we propose that parasites also possess a mechanism to coordinate

351   their development with rhythms in the availability of nutritional resources in the blood. Rhythms in

352   blood glucose are a well-documented consequence of rhythms in feeding timing [59] and glucose is

353   an important resource for parasites [60]. We performed an additional experiment to quantify blood

354   glucose rhythms in (uninfected) LF and DF mice (Fig 6A,B). Despite the homeostatic regulation of

355   blood glucose, we find its concentration varies across the circadian cycle, and is borderline

356   significantly rhythmic in DF mice (p = 0.07, peak time = ZT17.84, estimated with CircWave) and

357   follows a significantly 24-hour pattern in LF mice (p < 0.0001, peak time = ZT8.78). Glucose

358   rhythms/patterns are shaped by feeding regime (time-of-day: feeding treatment $\chi^2_{(18,32)}$ = 45.49, p <

359   0.0001). Specifically, during the night, DF mice have significantly higher blood glucose than LF mice

360   (t = 3.41, p = 0.01, difference 20.6mg/dl±7.32) and there is a trend for LF mice to have higher blood

361   glucose than DF mice during the day (t = -0.94, p = 0.78, difference 7.9mg/dl±9.86).
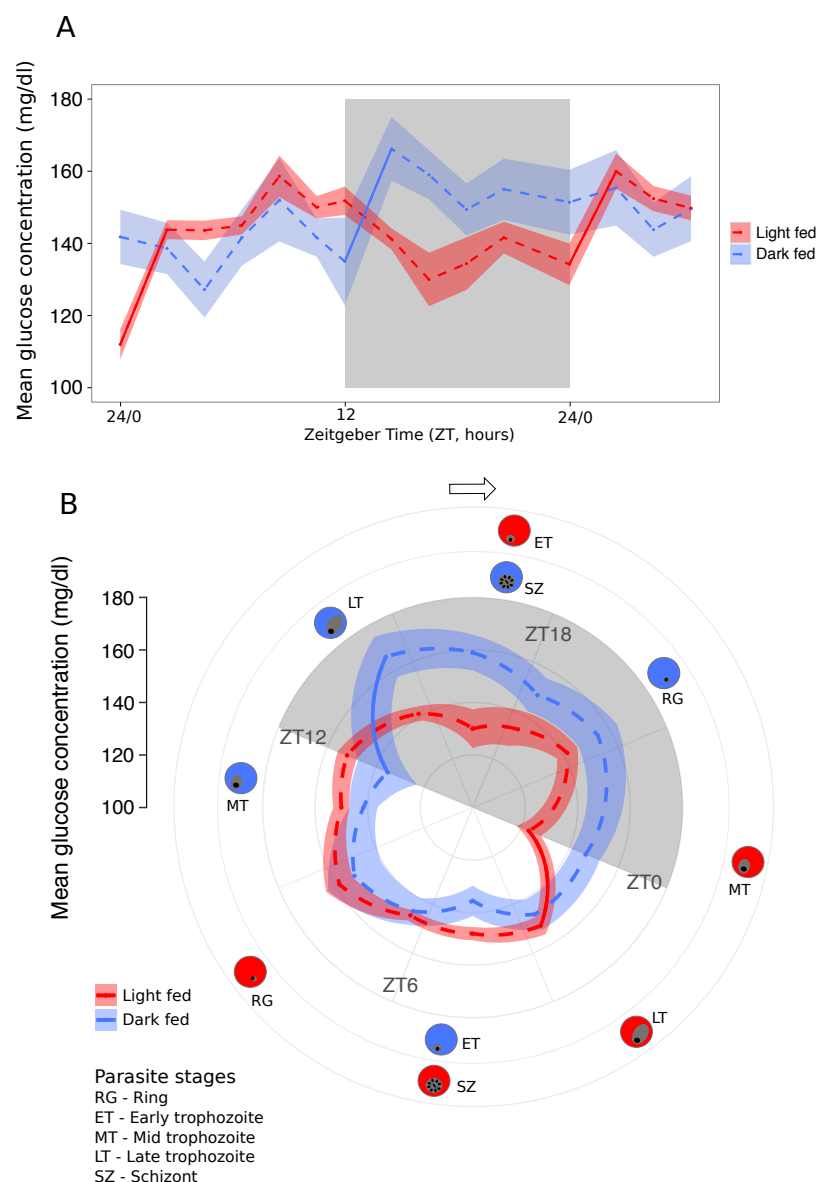
15

Fig 6. Feeding mice in the day time affects blood glucose regulation. A) Mean ± SEM (N=5 per group) for light fed mice (LF, white bars; allowed access to food from ZT 0-ZT 12) and dark fed mice (DF, grey bars; allowed access to food from ZT 12-ZT 0). Blood glucose concentration was measured every ~2 hours for 30 hours from ZT 0. Steep increases in blood glucose concentration occur as a result of the main bout of feeding in each group (i.e. just after lights on in LF mice and lights off in DF mice, illustrated by the regions with solid lines connecting before and after the main bout, see S5 Table), and suggests glucose concentration is inverted during the night. Light and dark bars indicate lights on and lights off (lights on: ZT 0, lights off: ZT 12). B) as for A, but plotted as a polar graph with corresponding developmental stages for each treatment group (red, LF; blue DF) on the perimeter.

Titrating whether glucose availability is high or low would only provide parasites with information on whether it is likely to be day or night, and a 12-hour window in which to make developmental transitions should erode synchrony, especially as glucose rhythms are weak in DF mice. Instead, parasites may use the sharp rise in blood glucose that occurs in both LF and DF mice after their main bout of feeding as a cue for dusk (S5 Table; regions with solid lines connecting before

16

377 and after feeding in Fig 6). In line with the effects of feeding timing we observe in mice, a recent

378 study of humans reveals that changing feeding time can induce a phase-shift in glucose rhythms,

379 but not insulin rhythms [43]. Alternatively, parasites may be sensitive to fluctuations in other factors

380 due to rhythms in food intake, such as amino acids [61] or other rhythmic metabolites that appear

381 briefly in the blood after feeding, changes in oxygen consumption, blood pressure or blood pH

382 [62,63].

383 In summary, we show that peripheral, food-entrained host rhythms, but not central, light-

384 entrained host rhythms are responsible for the timing of developmental transitions during the asexual

385 replication cycles of malaria parasites. Taken together, our observations suggest that parasites have

386 evolved a time-keeping mechanism that uses daily fluctuations in resource availability (e.g. glucose)

387 as a time-of-day cue or Zeitgeber to match the phase of asexual development to the host's SCN

388 rhythms. Why coordination with the SCN is important remains mysterious. Uncovering how parasites

389 tell the time could enable an intervention (ecological trap) to "trick" parasites into adopting suboptimal

390 rhythms for their fitness.

Materials and Methods

392 We conducted an experiment to investigate whether host peripheral rhythms or those driven by the

393 SCN affect rhythms in the asexual development of malaria parasites. Our findings stimulated the

394 analysis of four further data sets stemming from three independent experiments. Here, we detail the

395 approach used for our main experiment "Effect of feeding time on parasite rhythms" before briefly

396 outlining the approaches used in the analyses of additional data "Costs of mismatch to host SCN

397 rhythms", "Rhythms in cytokines during malaria infection", "Synchrony during rescheduling" and

398 "Effect of feeding time on blood glucose rhythms".

Effect of feeding time on parasite rhythms

400 Experimental design

401   Both LF ("light-fed mice", access to food during the day, ZT 0-12) and DF ("dark-fed mice", access

402   to food during the night, ZT 12-0) mice were kept in the same light-dark cycle to ensure the phase

403   of their central oscillators did not differ (because the SCN is primarily entrained by light [23]) (Fig 1).

404   Changing host feeding time in LF mice created an in-host environment where peripheral rhythms

405   associated with feeding are out of phase with the SCN, but in phase in DF mice. Every 12 hours,

406   food was added/removed from cages and the cages thoroughly checked for evidence of hoarding,

407   which was never observed. All experimental infections were initiated with parasites from donor mice

408   in light-dark cycles that were out of phase with the experimental host's light-dark cycles by 12 hours,

409   leading to a 12-hour phase difference in SCN entrainment of donor and host. Specifically, infections

410   were initiated with ring stage parasites (which appear in the early morning) collected from donor mice

411   and injected immediately into host mice which experiencing their evening. Parasites that are

412   mismatched by 12 hours to mice with synchronised SCN and peripheral rhythms (i.e. DF mice) take

413   around one week to reschedule [64,65,18]. Therefore, if peripheral rhythms but not SCN rhythms,

414   affect parasite rhythms, by starting infections with mismatched parasites we expected that parasites

415   in DF mice would reschedule within 7 days whereas rhythms in the LF mice would not change (or

416   change less). Because rhythms generally return to their original state after perturbation faster than

417   they can be shifted from homeostasis [66], studying the change in rhythms of mismatched parasites

418   ensured we could observe any divergence between parasite rhythms in LF and DF mice before host

419   immune responses and anaemia clear infections.

420   Parasites and hosts

421   We used 20 eight-week-old male mice, strain MF1 (in house supplier, University of Edinburgh),

422   entrained to a reverse lighting schedule for 2 weeks before starting the experiment. After

423   entrainment, mice were randomly allocated to one of two feeding treatments for the entire experiment

424   (Fig 1). After 2 weeks on the assigned feeding treatment we recorded body temperature and

425   locomotor activity for 48 hours. We used BioThermo13 RFID (radio frequency identification) tags

426   (Biomark, Idaho, USA) in conjunction with a Home Cage Analysis system (Actual HCA, Actual

427   Analytics Ltd, Edinburgh, Scotland), which enables body temperature and locomotor activity

428 readings to be taken every 0.05 seconds without disturbing the animals (using a network of antennae

429 spaced approximately 10.9 cm apart). Next, all mice were intravenously infected with 1 x 10^7

430 *Plasmodium chabaudi adami* (avirulent genotype, DK) parasitised red blood cells (at ring stage). We

431 used DK to minimise disruption to host feeding compared to infection with more virulent genotypes

432 that cause more severe sickness [39]. All mice were blood sampled from the tail vein twice daily

433 (ZT0 and ZT12) on days 0-5 and every 6 hours from days 6-8 post infection (PI). The densities and

434 developmental stages of parasites in experimental infections were determined from thin blood

435 smears (day 2 PI onwards, when parasites become visible in the blood) and red blood cell (RBC)

436 densities by flow cytometry (Beckman Coulter).

Costs of mismatch to host SCN rhythms

438 We compared the performance of parasites in our main experiment (in which infections were initiated

439 with parasites from donor mice that were mismatched to the host's SCN rhythms by 12 hours), to

440 the severity of infections when infections are initiated with parasites from donor mice that are

441 matched to the host's SCN rhythms. Twelve infections were established in the manner used in our

442 main experiment (eight-week-old male mice, strain MF1, intravenously infected with 1 x 10^7 *P.*

443 *chabaudi* DK parasitised RBC), except that donor SCN rhythms were matched to the experimental

444 host's SCN rhythm and hosts had access to food day and night. Densities of parasites were

445 quantified from blood smears and RBC density by flow cytometry on day 6 and 9 PI, respectively.

446 We chose to compare parasite density in matched infections to LF and DF infections on day 6 PI

447 because parasites are approaching peak numbers in the blood (before host immunity starts to clear

448 infections) and their high density facilitates accurate quantification when using microscopy.

Rhythms in cytokines during malaria infection

450 This experiment probes whether host immune responses mounted during the early phase of malaria

451 infection could impose development rhythms upon parasites. We entrained N=86 eight-week-old

452 female mice, strain MF1, to either a reverse lighting schedule (lights on 7pm, lights off 7am, N=43)

453 or a standard lighting schedule (lights on 7am, lights off 7pm, N=43). Donor mice, infected with *P.*

454 *chabaudi* genotype AS, were entrained to a standard lighting schedule to generate infections

455 matched and 12 hours mismatched relative to the SCN in the experimental mice. Mice were

456 intravenously injected with 1 x 10^7 parasitised RBC at ring stage. Genotype AS has intermediate

457 virulence [39] and was used to ensure immune responses were elicited by day 4 PI. We terminally

458 sampled 4 mice every 3 hours over 30 hours starting on day 4 PI, taking blood smears, red blood

459 cell counts and collecting plasma for Luminex cytokine assays.

460 Cytokines were assayed by the Human Immune Monitoring Centre at Stanford University using

461 mouse 38-plex kits (eBiosciences/Affymetrix) and used according to the manufacturer's

462 recommendations with modifications as described below. Briefly, beads were added to a 96-well

463 plate and washed in a Biotek ELx405 washer. 60uL of plasma per sample was submitted for

464 processing. Samples were added to the plate containing the mixed antibody-linked beads and

465 incubated at room temperature for one hour followed by overnight incubation at 4°C with shaking.

466 Cold and room temperature incubation steps were performed on an orbital shaker at 500-600 rpm.

467 Following the overnight incubation, plates were washed as above and then a biotinylated detection

468 antibody was added for 75 minutes at room temperature with shaking. Plates were washed as above

469 and streptavidin-PE was added. After incubation for 30 minutes at room temperature a wash was

470 performed as above and reading buffer was added to the wells. Each sample was measured as

471 singletons. Plates were read using a Luminex 200 instrument with a lower bound of 50 beads per

472 sample per cytokine. Custom assay control beads by Radix Biosolutions were added to each well.

473 **Synchrony during rescheduling**

474 We staged the parasites from the blood smears collected from the infections used to assay cytokines

475 (above) to investigate their synchrony during rescheduling. The infections from mismatched donor

476 mice began 12 hours out of phase with the host SCN rhythms and the CoG for ring stage parasites

477 reveals they had become rescheduled by 6 hours on day 4 PI. We focus on the ring stage as a phase

478 marker – for the analysis of synchrony in these data and the divergence between LF and DF

479 parasites – because rings are the most morphologically distinct, and so, accurately quantified, stage.

20

Blood glucose concentration

In a third additional experiment, we entrained 10 eight-week-old male mice, strain MF1, to a standard lighting schedule for 2 weeks before randomly allocating them to one of two feeding treatments. One group (N=5) were allowed access to food between ZT 0 and ZT 12 (equivalent to the LF group in the main experiment) and the other group (N=5) allowed access to food between ZT 12 and ZT 0 (equivalent to the DF group). After 10 days of food restriction we recorded blood glucose concentration every 2 hours for 30 hours, using an Accu-Chek Performa glucometer.

Data analysis

We used CircWave (version 1.4, developed by R.A. Hut; available from http://www.euclock.org/) to characterise host and parasite rhythms, and R v. 3.1.3 (The R Foundation for Statistical Computing, Vienna, Austria) for analysis of summary metrics and non-circadian dynamics of infection. Specifically, testing for rhythmicity, estimating CoG (a reference point to compare circadian rhythms) for host (body temperature, locomotor activity, blood glucose concentration) and parasite rhythms, and amplitude for parasite stage proportions, was carried out with CircWave for each individual infection. However, the cytokine data display high variation between mice (due to a single sample from each mouse) so we calculated a more robust estimate of phase than CoG by fitting a sine curve with a 24h period (using CircWave) and finding the maxima. Linear regression models and simultaneous inference of group means (using the multcomp R package) were run with R to compare summary measures that characterise rhythms, parasite performance, glucose concentration and disease severity. R was also used to construct and compared linear mixed effects models using which included mouse ID as a random effect (to account for repeated measures from each infection) to compare dynamics of parasite and RBC density throughout infections, and glucose concentration throughout the day.

Ethics Statement

504 All procedures were carried out in accordance with the UK Home Office regulations (Animals

505 Scientific Procedures Act 1986; project licence number 70/8546) and approved by the University of

506 Edinburgh.

Acknowledgements

508 We thank Philip Birget, Nicole Mideo, Petra Schneider, Bert Maier, Phil Spence and Barbara Helm

509 for discussion, Christopher Hutton and Giles K.P. Barra for assistance, and three anonymous

510 reviewers for their helpful and insightful comments.

References

512 1. Hevia MA, Canessa P, Müller-Esparza H, Larrondo LF. A circadian oscillator in the fungus

513   *Botrytis cinerea* regulates virulence when infecting *Arabidopsis thaliana*. PNAS. 2015;112:

514   8744-8749.

515 2. N'Goran E, Brémond P, Sellin E, Sellin B, Théron A. Intraspecific diversity of *Schistosoma*

516   *haematobium* in West Africa: chronobiology of cercarial emergence. Acta Trop. 1997;66: 35-

517   44.

518 3. Dolnik OV, Metzger BJ, Loonen MJJE. Keeping the clock set under the midnight sun: diurnal

519   periodicity and synchrony of avian *Isospora* parasites cycle in the High Arctic. Parasitol.

520   2011;138: 1077-1081.

521 4. Hawking F. The 24-hour periodicity of microfilariae: biological mechanisms responsible for

522   its production and control. Proc Roy Soc B. 1967;169: 59-76.

523 5. Hawking F. Circadian rhythms of *Trypanosoma congolense* in laboratory rodents. Trans R

524   Soc Trop Med Hyg. 1978;72: 592-595.

525 6. Hawking F. The asexual and sexual circadian rhythms of *Plasmodium vinckei*, of *P. berghei*

526   and of *P. gallinaceum*. Parasitol. 1972;65: 189-201.

527 7. Mideo N, Reece SE, Smith AL, Metcalf CJE. The Cinderella syndrome: why do malaria-

infected cells burst at midnight? Trends Parasitol. 2013;29: 10-16.

8. Rijo-Ferreira F, Pinto-Neves D, Barbosa-Morais NL, Takahashi JS, Figueiredo LM. *Trypanosoma brucei* metabolism is under circadian control. Nat Microbiol. 2017;2: 17032.

9. Albrecht U. Timing to perfection: the biology of central and peripheral circadian clocks. Neuron. 2012;74:246-60.

10. Roenneberg T, Daan S, Merrow M. The art of entrainment. J Biol Rhythms. 2003;18: 183-94.

11. Sharma VK. On the significance of circadian clocks for insects. J Indian Inst Sci. 2003;83: 3-26.

12. van der Veen DR, Riede SJ, Heideman PD, Hau M, van der Vinne V, Hut RA. Flexible clock systems: adjusting the temporal programme. Philos Trans R Soc Lond B Biol Sci. 2017;19: 372(1734)

13. Hardin PE, Hall JC, Rosbash M. Feedback of the *Drosophila* period gene product on circadian cycling of its messenger RNA levels. Nature. 1990;343: 536-540.

14. Bargiello TA, Jackson FR, Young MW. Restoration of circadian behavioural rhythms by gene transfer in *Drosophila*. Nature. 1984;312: 752-754.

15. Scheiermann C, Kunisaki Y, Frenette PS. Circadian control of the immune system. Nat Rev Immunol. 2013;13: 190-198.

16. Curtis AM, Bellet MM, Sassone-Corsi P, O'Neill LAJ. Circadian clock proteins and immunity. Immunity. 2014;40: 178-186.

17. Martinez-Bakker M, Helm B. The influence of biological rhythms on host-parasite interactions. TREE. 2015; 30: 314-326.

18. O'Donnell AJ, Schneider P, McWatters HG, Reece SE. Fitness costs of disrupting circadian rhythms in malaria parasites. Proc R Soc B Biol Sci. 2011;278: 2429-36.

19. O'Donnell AJ, Mideo N, Reece SE. Disrupting rhythms in *Plasmodium chabaudi*: costs accrue quickly and independently of how infections are initiated. Malar J. 2013;12: 372.

20. Rouzine IM, McKenzie FE. Link between immune response and parasite synchronization in malaria. PNAS. 2003;100: 3473-3478.

21. Kwiatkowski D, Greenwood BM. Why is malaria fever periodic? A hypothesis. Parasitol Today. 1989;5: 264-266.

22. Reece SE, Prior KP, Mideo N. The life and times of parasites: rhythms in strategies for within-host survival and between-host transmission. J Biol Rhythms. 2017;doi: 10.1177/0748730417718904.

23. Mohawk JA, Green CB, Takahashi JS. Central and peripheral circadian clocks in mammals. Annu Rev Neurosci. 2012;35: 445-462.

24. Cuesta M, Cermakian N, Boivin DB. Glucocorticoids entrain molecular clock components in human peripheral cells. FASEB J. 2014;29: 1360-1370

25. Stokkan K, Yamazaki S, Tei H, Sakaki Y, Menaker M. Entrainment of the circadian clock in the liver by feeding. Science. 2001;291: 490-493.

26. Damiola F, Le Minli N, Preitner N, Kornmann B, Fleury-Olela F, Schibler U. Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. Genes Dev. 2000;14: 2950-61.

27. Rajaratnam SMW, Howard ME, Grunstein RR. Sleep loss and circadian disruption in shift work: health burden and management. MJA. 2013;199: S11-S15.

28. Brown EN, Czeisler CA. The statistical analysis of circadian phase and amplitude in constant-routine core-temperature data. J Biol Rhythms. 1992;7: 177-202.

29. Benloucif S, Guico MJ, Reid KJ, Wolfe LF, L'hermite-Balériaux M, Zee PC. Stability of melatonin and temperature as circadian phase markers and their relation to sleep times in humans. J Biol Rhythms. 2005;20: 178-188.

24

577   30. van der Veen DR, Saaltink D-J, Gerkema MP. Behavioral responses to combinations of timed
578   light, food availability, and ultradian rhythms in the common vole (*Microtus arvalis*).
579   Chronobiol Int. 2011;28: 563-571.

580   31. Cambie G, Caillard V, Beaute-Lafitte A, Ginsburg H, Chabaud A, Landau I. Chronotherapy
581   of malaria: identification of drug-sensitive stage of parasite and timing of drug delivery for
582   improved therapy. Ann Parasitol Hum Comp. 1991;66: 14-21.

583   32. Brugat T, Cunningham D, Sodenkamp J, Coomes S, Wilson M, Spence PJ, et al.
584   Sequestration and histopathology in *Plasmodium chabaudi* malaria are influenced by the
585   immune response in an organ-specific manner. Cell Microbiol. 2014;16: 687-700.

586   33. David PH, Hommel M, Miller LH, Udeinya IJ, Oligino LD. Parasite sequestration in
587   *Plasmodium falciparum* malaria: spleen and antibody modulation of cytoadherence of
588   infected erythrocytes. Proc Natl Acad Sci U S A. 1983;80: 5075-9.

589   34. Salgado-Delgado RC, Saderi N, Basualdo MDC, Guerrero-Vargas NN, Escobar C, Buijs RM.
590   Shift work or food intake during the rest phase promotes metabolic disruption and
591   desynchrony of liver genes in male rats. PLoS One. 2013;8: e60052.

592   35. Rajaratnam SMW, Howard ME, Grunstein RR. Sleep loss and circadian disruption in shift
593   work: health burden and management. Med J Aust. 2013;199: 11-5.

594   36. Cheon DJ, Orsulic S. Mouse models of cancer. Annu Rev Pathol. 2011;6: 95-119.

595   37. Glastras SJ, Chen H, Teh R, McGrath RT, Chen J, Pollock CA, et al. Mouse models of
596   diabetes, obesity and related kidney disease. PLoS One. 2016;11: e0162131.

597   38. Wright JL, Cosio M, Churg A. Animal models of chronic obstructive pulmonary disease. Am
598   J Physiol Lung Cell Mol Physiol. 2008;295: L1-L15.

599   39. Bell AS, de Roode JC, Sim D, Read AF. Within-host competition in genetically diverse
600   malaria infections: parasite virulence and competitive success. Evolution. 2006:60: 1358-
601   1371.

40. Laermans J, Broers C, Beckers K, Vancleef L, Steensels S, Thijs T, et al. Shifting the circadian rhythm of feeding in mice induces gastrointestinal, metabolic and immune alterations which are influenced by ghrelin and the core clock gene Bmal1. PLoS One. 2014;9: 1-12.

41. Luna-Moreno D, Aguilar-Roblero R, Díaz-Muñoz M. Restricted feeding entrains rhythms of inflammation-related factors without promoting an acute-phase response. Chronobiol Int. 2009;26: 1409-29.

42. Nguyen KD, Fentress SJ, Qiu Y, Yun K, Cox JS, Chawla A. Circadian gene Bmal1 regulates diurnal oscillations of Ly6Chi inflammatory monocytes. Science. 2013;341: 1483-8.

43. Wehrens SMT, Christou S, Isherwood C, Middleton B, Gibbs MA, Archer SN, Skene DJ, Johnston JD. Meal timing regulates the human circadian system. Curr Biol. 2017;27: 1768-1775.

44. Artavanis-Tsakonas K, Riley EM. Innate immune response to malaria: rapid induction of IFN-γ from human NK cells by live *Plasmodium falciparum*-infected erythrocytes. J Immunol. 2002;169: 2956-2963.

45. Orengo JM, Evans JE, Bettiol E, Leliwa-Sytek A, Day K, Rodriguez A. *Plasmodium*-induced inflammation by uric acid. PLoS Pathog. 2008;4: e1000013.

46. Stevenson MM, Tam MF, Wolf SF, Sher A. IL-12-induced protection against blood-stage *Plasmodium chabaudi* AS requires IFN-gamma and TNF-alpha and occurs via a nitric oxide-dependent mechanism. J Immunol. 1995;155: 2545-2556.

47. Kumaratilake LM, Ferrante A, Rzepczyk C. The role of T lymphocytes in immunity to *Plasmodium falciparum*. Enhancement of neutrophil-mediated parasite killing by lymphotoxin and IFN-gamma: comparisons with tumor necrosis factor effects. J Immunol. 1991;146: 762-767.

48. Jacobs P, Radzioch D, Stevenson MM. In vivo regulation of nitric oxide production by tumor

627   necrosis factor alpha and gamma interferon, but not by interleukin-4, during blood stage
628   malaria in mice. Infect Immun. 1996;64: 44-49.

629   49. Regev-Rudzki N, Wilson DW, Carvalho TG, Sisquella X, Coleman BM, Rug M, et al. Cell-cell
630   communication between malaria-infected red blood cells via exosome-like vesicles. Cell.
631   2013;153: 1120-33.

632   50. Goto M, Oshima I, Tomita T, Ebihara S. Melatonin content of the pineal gland in different
633   mouse strains. J Pineal Res. 1989;7: 195-204.

634   51. Kennaway DJ, Voultsios A, Varcoe TJ, Moyer RW. Melatonin in mice: rhythms, response to
635   light, adrenergic stimulation, and metabolism. Am J Physiol Regulatory Integrative Comp
636   Physiol. 2002;282: R358-65.

637   52. Hotta CT, Gazarini ML, Beraldo FH, Varotti FP, Lopes C, Markus RP, et al. Calcium
638   dependent modulation by melatonin of the circadian rhythm in malaria parasites. Nat Cell
639   Biol. 2000;2: 466-8.

640   53. Yasumoto Y, Hashimoto C, Nakao R, Yamazaki H, Hiroyama H, Nemoto T, et al. Short-term
641   feeding at the wrong time is sufficient to desynchronize peripheral clocks and induce obesity
642   with hyperphagia, physical inactivity and metabolic disorders in mice. Metabolism. 2016;65:
643   714-27.

644   54. Acuña-Castroviejo D, Escames G, Venegas C, Díaz-Casado ME, Lima-Cabello E, López LC,
645   Rosales-Corral S, Tan DX, Reiter RJ. Extrapineal melatonin: sources, regulation, and
646   potential functions. Cell Mol Life Sci. 2014;71: 2997-3025.

647   55. Blanford JI, Blanford S, Crane RG, Mann ME, Paaijmans KP, Schreiber K V., et al.
648   Implications of temperature variation for malaria parasite development across Africa. Sci
649   Rep. 2013;3: 1-11.

650   56. Chao J, Ball G. The effect of low temperature on *Plasmodium relictum* in *Culex tarsalis*. J
651   Parasitol. 1962;48: 252-4.

57. Adamovich Y, Ladeuix B, Golik M, Koeners MP, Asher G, Adamovich Y, et al. Rhythmic oxygen levels reset circadian clocks through HIF1α. Cell Metab. 2016; 319-30.

58. Mancio-Silva L, Slavic K, Ruivo MTG, Grosso AR, Modrzynska KK, Vera IM, et al. Nutrient sensing modulates malaria parasite virulence. Nature. 2017; 547: 213-216.

59. McGinnis GR, Young ME. Circadian regulation of metabolic homeostasis: causes and consequences. Nat Sci Sleep. 2016;8: 163-80.

60. MacRae JI, Dixon MW, Dearnley MK, Chua HH, Chambers JM, Kenny S, et al. Mitochondrial metabolism of sexual and asexual blood stages of the malaria parasite *Plasmodium falciparum*. BMC Biol. 2013;11: 67.

61. Nasset ES, Heald FP, Calloway DH, Margen S, Schneeman P. Amino acids in human blood plasma after single meals of meat, oil, sucrose and whiskey. J Nutr. 1979;109: 621-30.

62. Christopherson RJ, Webster AJ. Changes during eating in oxygen consumption, cardiac function and body fluids of sheep. J Physiol. 1972;221: 441-57.

63. Matsukawa K, Ninomiya I. Changes in renal sympathetic nerve activity, heart rate and arterial blood pressure associated with eating in cats. J Physiol. 1987;390: 229-242.

64. Boyd GH. Induced variations in the asexual cycle of *Plasmodium cathemerium*. J Exp Zool. 1929;9: 111-26.

65. Gautret P, Deharo E, Tahar R, Chabaud AG, Landau I. The adjustment of the schizogonic cycle of *Plasmodium chabaudi chabaudi* in the blood to the circadian-rhythm of the host. Parasite-Journal La Soc Fr Parasitol. 1995;2: 69-74.

66. Le Minh N, Damiola F, Tronche F, Schütz G, Schibler U. Glucocorticoid hormones inhibit food-induced phase-shifting of peripheral circadian oscillators. EMBO J. 2002;20: 7128-36.