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Genetic variation and phenotypic plasticity in circadian rhythms in an armed beetle, *Gnatocerus cornutus* (Tenebrionidae)

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Circadian rhythms, their free-running periods and the power of the rhythms are often used as indicators of biological clocks, and there is evidence that the free-running periods of circadian rhythms are not affected by environmental factors, such as temperature. However, there are few studies of environmental effects on the power of the rhythms, and it is not clear whether temperature compensation is universal. Additionally, genetic variation and phenotypic plasticity in biological clocks are important for understanding the evolution of biological rhythms, but genetic and plastic effects are rarely investigated. Here, we used 18 isofemale lines (genotypes) of *Gnatocerus cornutus* to assess rhythms of locomotor activity, while also testing for temperature effects. We found that total activity and the power of the circadian rhythm were affected by interactions between sex and genotype or between sex, genotype and temperature. The males tended to be more active and showed greater increases in activity, but this effect varied across both genotypes and temperatures. The period of activity varied only by genotype and was thus independent of temperature. The complicated genotype–sex–environment interactions we recorded stress the importance of investigating circadian activity in more integrated ways.

ADDITIONAL KEYWORDS: circadian rhythm – *Gnatocerus cornutus* – isofemale line – power of circadian rhythm.

INTRODUCTION

Many species show a rhythmicity of activity, from the timing of flowering in plants to foraging behaviour in animals (Saunders, 2002). Examination of these rhythms in sexually dimorphic animals is interesting because males and females could have different life-history strategies and thus different daily patterns of activity (e.g. Anderson, 1994; Emlen, 2015). A rhythmicity approaching 24 h is called a circadian rhythm, and many studies have identified the genes underpinning circadian rhythms (Dunlap, 1996). Additionally, the time it takes for an organism to repeat endogenous rhythms in the absence of environmental cues is known as the free-running period and, amongst other things, this provides information on the accuracy of internal clocks (Klarsfeld *et al.*, 2003). The power of the circadian rhythm (i.e. the strength of the rhythm; hereafter, 'power') is another measure of activity cycles and indicates the inherent strength of the rhythm (Klarsfeld *et al.*, 2003). Although many previous studies have explored free-running periods, few have investigated the power of the circadian rhythm (but see Cavey *et al.*, 2016; Fujioka *et al.*, 2017; King *et al.*, 2017).

Free-running periods of circadian rhythms can vary with the light intensity (Aschoff, 1960, 1965), but rhythms can remain robust over a wide range of temperatures, a situation known as temperature compensation (e.g. Pittendrigh, 1954; Zimmerman *et al.*, 1968; Dunlap *et al.*, 2004). Be that as it may, there are a few published reports of temperature compensation in the power of circadian rhythms (e.g. Sorek & Levy, 2012).

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In addition to environmental effects, circadian rhythms can also differ between the sexes. For example, in the fly *Drosophila melanogaster*, the free-running period of males is shorter than that of females (Helfrich-Förster, 2000). This suggests that males have different periods of rhythm, possibly to facilitate searching for females (Helfrich-Förster, 2000). It is also possible that the power is different between males and females. To date, however, no investigation of the effect of sex on power has been reported, although in species with strong sexual selection (e.g. those with pronounced dimorphism in sexually selected morphology) there might well be dimorphism in the strength and period of biorhythms as a result of sex-specific selection.

Here, we investigated the effects of temperature on the free-running period, power and circadian rhythm of locomotor activity in the broad-horned flour beetle, *Gnatocerus cornutus* (Fabricius, 1798), while also testing for sex-specific effects. In *G. cornutus*, only males develop mandibles, and they often fight with rival males to obtain matings using these condition-dependent weapons (Okada *et al.*, 2006; House *et al.*, 2016). Furthermore, there is significant genetic variation in mandible size, and this affects fighting and mating outcomes (Harano *et al.*, 2010). Thus, this species is ideal to assess whether strong sexual selection has resulted in sex differences in activity rhythms, power and periods, because beetles generally show evidence of circadian activity (Harano & Miyatake, 2011). In our investigation, we used 18 iso-female lines (genotypes), making it possible also to test for genetic variation in circadian rhythms of locomotor activity, their free-running period and power. We also investigated the plasticity of the activity rhythm by measuring it at different temperatures.

MATERIAL AND METHODS

Stock population and isolines

The *G. cornutus* beetle culture originated from adults collected in Miyazaki City (31°54′N, 131°25′E), Japan, in 1957 (see Okada *et al.*, 2006), and has been maintained in the laboratory of the National Food Research Institute and Okayama University for ~50 years on whole meal enriched with yeast as food. Beetles were reared on whole meal enriched with brewer's yeast and kept at 25–27 °C and 60% relative humidity under a photoperiod of 14 h light–10 h dark (House *et al.*, 2016). We established 18 iso-female lines (isolines, i.e. standardized genotypes) from the stock population. Initially, 18 males and 18 females were selected at random and paired. Subsequent full-sib matings within each family were used to propagate each line for 37 generations until the present study was conducted. Differences between lines are then largely genetic, and any within-line variance is largely environmental (David *et al.*, 2005). Lines can effectively be thought of as different genotypes (David *et al.*, 2005). To obtain adults for the present experiments, one final instar larva was placed in a separate well of a 24-well tissue culture plate, because pupation in *G. cornutus* is inhibited at a high larval density (Okada *et al.*, 2006).

Locomotor activity

To assess circadian rhythm, we maintained beetles in 14 h light–10 h dark conditions for > 20 days in an incubator kept at 25 °C before the measurement of locomotor activity, and we then measured the locomotor activity of *G. cornutus* for 10 days in darkness. A beetle from each isoline with enough food, as described above, was put in a clear plastic Petri dish (30 mm × 10 mm) in an incubator maintained at 25 or 29 °C. The beetles develop faster at 29 °C (unpublished observation, T.M.), and most investigation of the behaviour of this beetle has been conducted under 25 °C (Okada *et al.*, 2006; Okada & Miyatake, 2009, 2010); therefore, we chose these two temperatures, 25 and 29 °C, for this experiment. The locomotor activity of each individual was monitored using an infrared actograph. An infrared light beam was passed through a clear Petri dish, and the beam was projected onto a photomicrosensor (E3S-AT11; Omron, Kyoto, Japan) that detected all interruptions of the light beam. Signals of interruption of the infrared light beam were recorded every 6 min. The sample size of each isoline is shown in the Supporting Information (Table S1).

Statistical analysis

To determine the circadian rhythm, the locomotor activity data for 10 days (i.e. total activity) in the constant dark conditions was analysed. The free-running period of circadian rhythm was established using a χ^2 periodogram test (Sokolove & Bushell, 1978) for data on locomotor activity between 20 and 28 h (Halberg, 1969). Circadian rhythmicity was determined using χ^2 periodogram analysis, and we used 'power' as an index for the strength of the rhythms. The power of the circadian rhythm was defined as the maximal difference between the χ^2 value and the significance threshold line $P = 0.05$, i.e. the size of the peak above 5% threshold (see Klarsfeld *et al.*, 2003: fig. 1). The power is high when the rhythm is clear and strong, and a power of less than zero indicates a statistically arrhythmic state.

To analyse the effects of temperature, isoline and sex on the period and power of the circadian rhythm and on total activity, we used MANOVA and post-hoc ANOVA.

All statistics were based in R v.3.4.3 (R Development Core Team, 2017).

RESULTS

The MANOVA revealed significant main effects for all predictors and interactions on the multivariate combination of power, period and total activity. All variables were significant (see Supporting Information, Table S2). Post-hoc tests showed that power and total activity were the main drivers of these effects, with all predictors and interactions being significant for power, whereas sex, temperature and isoline plus the sex-by-isoline interaction were significant for total activity (Table 1).

Strictly speaking, it is the highest-order interactions that need to be interpreted, which for power was the three-way interaction between sex, temperature and isoline, indicating that there is a genotype-by-environment effect that varies by sex affecting the power of the rhythm (Fig. 1A; Table 1; Supporting Information, Fig. S1A). Males showed higher power than females at 25 °C, and the difference between sexes disappeared at 29 °C (Fig. 1A).

The period of activity was significantly affected only by isoline with a weak trend for a temperature–isoline effect, and all other effects non-significant (Fig. 1B; Table 1; Supporting Information, Fig. S1B). Examples of activity rhythms of males and females at the two temperatures are shown in the Supporting Information (Fig. S3).

For total activity, there was a sex-by-isoline interaction, suggesting genetic variation in activity that varied between the sexes (Fig. 1C; Table 1; Supporting Information, Fig. S1C). Males exhibited higher activity than females at both temperatures (Fig. 1C; Table 1; Supporting Information, Fig. S2).

DISCUSSION

In *G. cornutus*, only males fight with rival males to obtain matings using the developed mandibles (Okada *et al.*, 2006), which are condition dependent (House *et al.*, 2016), and the significant genetic variation in mandible size affects fighting and mating outcomes (Harano *et al.*, 2010). This could generate sexual differences in activity and circadian-related traits. Furthermore, a sexual difference in circadian characters might be especially prevalent across different environments because male sexual traits tend to be condition dependent; therefore, males may be susceptible to stressors such as elevated temperatures (Rashed & Polak, 2010). To test these ideas, we measured the locomotor activity of a number of *G. cornutus* genotypes (isolines) at 25 and 29 °C, assessing the free-running period and power of the circadian rhythm, in addition to total activity, across genotype, sex and temperature environments. Our findings suggested that males tended to be more active and showed greater increases in activity than females in this species. However, we found complicated interactions in addition to main effects (temperature, sex and genotype) for the multivariate combinations of these activity measures. There were very strong univariate effects that also included interactions between some of our predictors. The exception was the period of the activity rhythm, which was affected only by genotype (isoline).

Many previous studies have suggested that the free-running period of activity rhythm should show temperature compensation and remain robust over a wide range of temperatures (e.g. Pittendrigh, 1954; Zimmerman *et al.*, 1968; Dunlap *et al.*, 2004), and this is what we found. Temperature had no impact on the activity rhythm period (the rhythm was temperature compensated), which was impacted only by genotype, suggesting that period can evolve in spite of temperature compensation and that the environmental impacts on it are buffered across the range of temperatures we tested. Additionally, there were no sex effects or interactions affecting period, meaning that the genetic effects were the same across environments and were the same for males and females, but genotypes differed in their period of circadian rhythm. Given the strong sexual selection in this beetle (Okada & Miyatake, 2009, 2010; Harano *et al.*, 2010; Okada *et al.*, 2014), it was surprising that the sexes did not differ in their activity period, especially given that studies of other taxa have found sexual differences in this measure, even if they relate only to development and emergence times (e.g. Simmons *et al.*, 1994).

Many previous studies have investigated the effects of temperature on free-running periods, but few have focused on the power of circadian rhythms. We found that all main effects and their interactions affected power, generating a genotype-by-temperature-by-sex interaction for power, which measured how much activity increased during active periods. For example, the power of male circadian rhythms was much higher at 25 than at 29 °C, whereas females tended to be about the same at either temperature, but this effect differed across genotypes, such that for some genotypes the females were more active at the higher temperature, for example. The sexual element of this interaction affecting the power of the circadian rhythm response probably relates to reproductive behaviours, with males searching for mates and rivals, generating a much greater increase in male activity during the circadian cycle (e.g. Parker, 1978; Muniz *et al.*, 2018; Matsumura *et al.*, 2019). This effect seems to be very

Table 1. Post-hoc ANOVAs of the MANOVA outcomes testing effects on each activity trait (period and power of rhythm, and total activity) separately

Trait	Variable	d.f.	Sum of square	Mean square	*F*	*P*-value
Power of rhythm	Sex	1	13 358	13 838	8.9481	**0.0029**
	Temperature	1	5794	5794	3.8811	**0.0492**
	Isoline	17	283 308	16 665	11.1636	**< 0.0001**
	Sex*temperature	1	19 396	19 396	12.9927	**0.0003**
	Sex*isoline	17	82 189	4835	3.2386	**< 0.0001**
	Temperature*isoline	17	60 760	3574	2.3942	**0.0013**
	Sex*temperature*isoline	17	60 981	3587	2.4029	**0.0012**
	Residuals	793	1 183 807	1493		
Period of rhythm	Sex	1	15	15	0.0395	0.8425
	Temperature	1	316	316	0.8517	0.3564
	Isoline	17	14 474	851	2.2961	**0.0021**
	Sex*temperature	1	0	0	0.0001	0.9911
	Sex*isoline	17	4405	259	0.6988	0.806
	Temperature*isoline	17	9671	569	1.5341	0.0763
	Sex*temperature*isoline	17	4885	287	0.775	0.7234
	Residuals	793	294 043	371	–	–
Total activity	Sex	1	18 867 003	18 867 003	9.1322	**0.0026**
	Temperature	1	54 091 677	54 091 677	26.182	**< 0.0001**
	Isoline	17	4.97×10^8	29 211 404	14.1392	**< 0.0001**
	Sex*temperature	1	5 260 553	5 260 553	2.5463	0.1109
	Sex*isoline	17	1.35×10^8	7 952 461	3.8492	**< 0.0001**
	Temperature*isoline	17	24 922 413	1 466 024	0.7096	0.7949
	Sex*temperature*isoline	17	54 409 111	3 200 536	1.5492	0.0717
	Residuals	793	1.64×10^9	2 065 983	–	–

Bold means significance statistically.

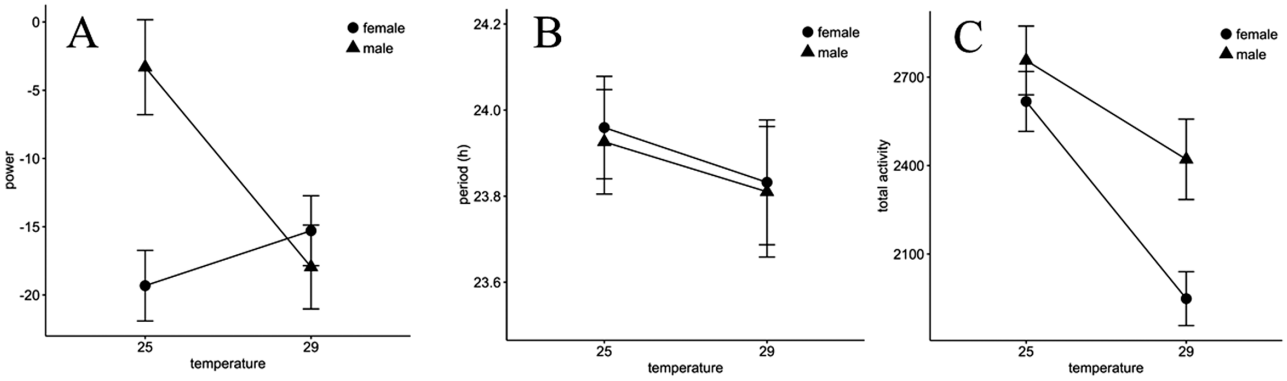

Figure 1. Power of activity rhythm (A), period of circadian rhythm (B) and total locomotor activity (C) by sex when measured at 25 and 29 °C, respectively. The error bars represent the SEM.

pronounced in some genotypes, which might relate to genetic variation in male aggression (Harano *et al.*, 2010; Okada & Miyatake, 2010).

It seems plausible that all this explains why circadian activity has greater power in males, but not why male power declines to female levels at 29 °C. Perhaps this decrease is stress related, because stress can shift resources from activity to maintenance, and the effects can be genotype specific (Mitton, 1997). Stress could be important here because beetles are normally reared at 25–27 °C, and because males have large condition-dependent mandibles (House *et al.*, 2016) they might be especially sensitive to stress (David *et al.*, 2000). We need to investigate further the effect of temperature on male–male competition and sexual selection more generally, but in any case, the present study seems to be the first report of a sex difference in the response of the power of circadian rhythm across temperature environments.

The genotype effect indicates genetic variance in the power of the circadian rhythm. Similar findings have been reported in other studies, including recent work investigating output pathways of the circadian clock in *Drosophila* (Cavey *et al.*, 2016; King *et al.*, 2017). That work revealed molecular genetic components that affect the power of the circadian rhythm and perhaps our findings likewise involve output pathways. Furthermore, if the output pathways were affected in our beetles, it would appear that the power, but not the period, of the circadian rhythm was impacted. More work on environmental effects needs to be undertaken to test for genotype–environment interactions, matching calls made in other fields of study (Hunt & Hosken, 2014). Additionally, although the power of the circadian rhythm has been investigated in several previous studies (Malpel *et al.*, 2004; Meshi & Bloch, 2007; Fujioka *et al.*, 2017), there are few studies that have investigated the fitness impacts of a variation in power. It would seem prudent to conduct such investigations across environments.

We found no significant effect of temperature on the free-running period. This is consistent with previous studies, which have reported temperature compensation in the free-running period of the circadian rhythm in other species (e.g. Pittendrigh, 1954; Zimmerman *et al.*, 1968; Dunlap *et al.*, 2004). We also found no sex differences. This contrasts with *D. melanogaster*, in which males had a shorter period of the circadian rhythm than females (Helfrich-Förster, 2000). This probably relates to male *Drosophila* searching for females (Helfrich-Förster, 2000), whereas in *G. cornutus*, the period of mate searching might be less beneficial because of male–male fighting and strong territoriality (Yamane *et al.*, 2010; Okada *et al.*, 2014). Additionally, males that lose fights do not engage in fighting behaviour for 4 days (Okada & Miyatake, 2010), and losers transfer more sperm to females during copulation (Yamane *et al.*, 2010). Therefore, males might invest more in fighting than in searching for females in *G. cornutus*. However, the males did seem generally to increase power more than females (see above). Rather than increasing the duration of activity, they instead increased how active they were during periods of activity. This suggests that finding females at the right times, when they are receptive, is important. In many insects, the males cannot force copulation after adulthood cuticles have hardened (Markow, 2000; Eberhard, 2002). In any case, the males seemed to increase the amount of activity within a period, rather than increasing the duration of activity, and we found significant genetic variation in the free-running period of the circadian rhythm; this was the only significant main effect. This again implies that period can evolve, which is, arguably, as expected for a behavioural phenotype (Hosken *et al.*, 2019) and all the more so given that there is variation in animal activity periods across taxa (e.g. diurnal vs. nocturnal).

The total locomotor activity over 10 days was affected by the interaction between genotype (isoline) and sex, with males tending to be more active than females at both temperatures, although this effect varied with genotype. Again, this interaction could be understood in terms of stress and the condition dependence of male sexual traits and how both vary across genotypes. There was also an independent (of the interaction) effect of temperature, such that activity was higher at 25 than at 29 °C, which again supports the notion of the higher temperature being more stressful. And again, the genotype (isoline) effect on activity shows there is genetic variation for this trait. We need to undertake additional work to uncover the details of all these effects to gain a greater understanding of some of our findings, especially as they relate to mechanism and fitness.

To conclude, we found strong effects of sex, genotype and environment (temperature) on a range of measures of circadian activity. These effects interacted and did not act consistently on all the activity elements we measured. Thus, it appears that activity is like many other behaviours, being affected by genes and environment and their interaction, and elements of activity seem to be somewhat independent of each other. However, additional work is needed both to clarify the detail of our findings and to test their generality across taxa.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. A, the three-way interaction between temperature, sex and genotype (isoline) affecting activity rhythm power. This shows that the effects of temperature depend on sex and on genotype; for example, for some genotypes the males are more active at 29 °C and for others the females are (Supporting Information, Fig. S2 shows the sex-by-temperature element of this interaction). B, the period of activity for different genotypes (isolines). Genotype was the only variable that explained significant variation in activity period. C, the interaction between sex and genotype (isoline) affecting total activity. On average, males tend to be more active but this varies across genotypes; in isoline 10, for example, females are more active. In the all graphs, the error bars represent the SEM.
Figure S2. The three-way interaction between temperature, sex and genotype (isoline) affecting total activity. This shows that the effects of temperature depend on sex and on genotype. The error bars represent SEM.
Figure S3. Examples of activity rhythm traces of males and females at two temperatures, 25 and 29 °C.
Table S1. Sample sizes for each isoline of *Gnatocerus cornutus*.
Table S2. MANOVA with period and power of circadian rhythm, and total activity.