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Time flies: time of day and social environment affect cuticular hydrocarbon sexual displays in *Drosophila serrata*

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Recent work on *Drosophila* cuticular hydrocarbons (CHCs) challenges a historical assumption that CHCs in flies are largely invariant. Here, we examine the effect of time of day and social environment on a suite of sexually selected CHCs in *Drosophila serrata*. We demonstrate that males become more attractive to females during the time of day that flies are most active and when most matings occur, but females become less attractive to males during the same time of day. These opposing temporal changes may reflect differences in selection among the sexes. To evaluate the effect of social environment on male CHC attractiveness, we manipulated male opportunity for mating: male flies were housed either alone, with five females, with five males or with five males and five females. We found that males had the most attractive CHCs when with females, and less attractive CHCs when with competitor males. Social environment mediated how male CHC attractiveness cycled: males housed with females and/or other males showed temporal changes in CHC attractiveness, whereas males housed alone did not. In total, our results demonstrate temporal patterning of male CHCs that is dependent on social environment, and suggest that such changes may be beneficial to males.

1. Introduction

Chemical communication is widespread among animals, with species-specific signals having been identified in 54 orders, including mammals, reptiles, amphibians, insects, diplopods arachnids, annelids, echinoderms, gastropods and nematodes [1–3]. In insects, chemical signals are especially pervasive and vary widely in form and function. Volatile chemicals are often used as long-distance signals, whereas non-volatile chemicals play a role in short-range communication. Functionally, insects rely on chemical communication for species recognition [4,5], mate recognition [6] and social organization [7,8]. There is substantial variation among species in the chemical composition of signals [3]. Chemical production within a species can also vary, and although such variation has received less attention, it is clear that in many species, females prefer males that display specific chemical combinations during mate choice [9].

Cuticular hydrocarbons (CHCs)—large, generally non-volatile, fatty-acid-derived hydrocarbons found on the cuticle of many insects [10]—are the most abundant and well-understood chemical signals produced by *Drosophila* flies. *Drosophila* CHCs are processed by oenocyte cells on the body and are perceived at short distances by the antennae or maxillary palps [11]. CHCs are used in sex [12,13] and species recognition [13–15], as sexual displays [15–19], and also provide a waxy layer of protection against desiccation [20,21]. The role of CHCs in mate choice has been extensively studied in the Australian fruit fly *Drosophila serrata*. In this species, males and females are sexually dimorphic in the relative concentrations of a homologous set of CHCs [22], and both sexes use these traits during mate choice [17,18,23]. CHC production is costly [24] and expression in males is condition-dependent [25]. CHCs and female preferences for them have also been shown to respond to altered selection [26–31].

Sexually selected chemical signals such as CHCs involve a suite of compounds and represent a fundamentally complex trait. Further, not all chemicals are used in communication: animals produce a wide range of compounds as by-products of physiological processes [32]. Consequently, the analysis of chemical signals depends on understanding the relationship between social and/or environmental context and the expression of different chemical combinations [12,16,33]. In *D. serrata*, binomial mate choice trials have been used to determine the multivariate combination of CHCs that is associated with higher mating success, both in males and females [17,18,34], and much of the work on the evolutionary genetics of CHCs in this species has focused on these particular trait combinations [23,31].

Chemical signals can also be affected by context. If environment, ontogeny or condition affects the expression of even one chemical in a complex profile, the multivariate signal will be altered. A detailed understanding of the behavioural ecology and evolutionary genetics of such traits therefore requires knowledge of the environmental factors that impact their expression. *Drosophila* were historically assumed to have relatively stable chemical profiles when compared with insects such as moths that display rapid daily cycling [11]. However, recent findings that both physical and social environment can affect CHC expression [34–38] have called this assumption into question. Moreover, because CHCs are often used in varying social contexts, the effect of social environment is of particular interest. Recent studies demonstrate that social context matters in *Drosophila*: Petfield *et al*. [39] showed that *D. serrata* males change their CHCs during sexual encounters in a way that is predictable by the genotype of the female with which they are interacting. In *Drosophila melanogaster*, a model organism for the study of circadian rhythms [40,41], an individual's social environment affects circadian patterns in CHC expression [34,42]. As a sexual display, CHC expression is therefore dynamic with respect to both time of day and social environment, although a detailed characterization of such variation within a single system is lacking.

Here, we seek to characterize temporal effects on sexual traits in *D. serrata*, including CHCs, and to explore effects of social environment on these traits. Because, in most cases, we characterize temporal variation over 24 h, and have neither studied their recurrence across multiple cycles nor in the absence of external cues, we refrain from interpreting these as circadian patterns in the formal sense. Our study has two parts. First, we present descriptive data on the effect of time of day on locomotion, mating activity, CHCs and the multivariate combination of CHCs associated with greatest mating success in each sex. Second, we manipulate male social environment to determine its effect on both average CHC-based attractiveness and temporal changes in this across 24 h.

2. Methods

All assays used flies from a previously described laboratory-adapted, outbred stock population of *D. serrata* that is maintained at large population size (16 half-pint bottles) via non-overlapping generations [43]. Experimental animals were maintained under constant conditions mirroring that of the stock, including temperature (28°C) and a photoperiod (12 L : 12 D), with the lights turning on at 07.00 and off at 19.00 daily.

(a) Locomotor activity

Virgin adults were collected at emergence using light CO_2 anaesthesia, separated by sex and housed in groups of either seven males or 10 females per vial. Four days after emergence, the locomotor activity of individual flies was measured using a DAM 2 activity monitor (Trikinetics, Waltham, MA). The monitor uses an infrared beam to measure the number of times that a single fly, housed in a 5 × 65 mm polycarbonate tube, crosses the midpoint of the tube. We programmed the DAMSystem Collection software (Trikinetics) to sum activity over 10 min periods. We simultaneously used three arrays to measure the separate activity of 43 females, 44 males and two empty tubes as negative controls, with the arrays set up as described in Charette *et al*. [44]. The tubes contained a non-nutritive 2% agarose medium for moisture. Males and females were visually separated from one another by cardboard dividers. Flies were lightly anaesthetized with CO_2, transferred into the activity monitor at 21.00 and remained in it for 48 h. Although the monitor collected all 48 h of data (see the electronic supplementary material, figure S1), statistical analyses were restricted to 24 h starting from 07.00 the morning after their introduction. This was done to allow the flies time to acclimatize to their new conditions and for their activity to settle after introduction, yet to avoid possible effects of desiccation stress (by 48 h, the medium had begun to dry and pull away from the sides of the tubes).

(b) Mating activity

This assay was designed to quantify sexual activity in 4 day old males and females. Males and females were collected at emergence using light CO_2 anaesthesia and housed in mixed-sex vials of approximately 12 flies per vial where they had the opportunity to gain mating experience. Three days later, six males and six females were transferred to a 35 × 10 mm Petri dish 'arena' containing a layer of non-nutritive 2% agarose medium on the bottom and sealed with parafilm to prevent water loss. The flies were allowed to acclimatize to each other and the arena for 24 h before image collection started.

After the acclimatization period, images were collected every 2 min for 24 h. Image capture was performed by a Canon Powershot G10 digital camera using RemoteCapture 2.7 software (Canon USA. Inc., Melville, NY) suspended above the arena on a fixed arm. Flies were not disturbed during the acclimatization or data collection periods. During the 12 h dark phase, all external light was blocked, and the arena was lit by 830 nm wavelength infrared lights. Previous research suggests that *Drosophila* are insensitive to light above approximately 650 nm [45–47]. Images were captured from only one arena at a time. Over a 51 day period, 51 cohorts of flies were reared to emerge on consecutive days, and 51 replicate arenas were observed. All individuals were 4 day old adults at the time of observation, and no individual was ever observed in more than one replicate arena.

All images were examined by a human observer (S.N.G.) to score all instances in which a fly was observed mounting another in a configuration consistent with copulation. If the same pair of flies remained in copula for at least two successive images, then this was scored as a mating, because previous studies indicate that a *D. serrata* male must remain mounted for at least 157 s, for successful sperm transfer [47,48]. Because individual flies could not be identified, the number of matings may underestimate the true number (i.e. if, between images, one pair stopped copulating and another started), although such an effect is probably small given the observed mating rate relative to the short time interval between images. Copulations that were observed in only a single image were classified as 'mounts'. We know from previous observations that *D. serrata* males will occasionally mount other males, although these are generally brief (less than 20 s; S. Gershman & H. D. Rundle 2005–2014, personal observation).

It was not possible to determine from the captured images whether the mounted fly was a male or a female, and mounts therefore include both unsuccessful male–female copulations as well as male–male mounts. Summing the number of matings and mounts provides a measure of total mating activity. Because mating rates were low, observations were grouped into 24 1 h intervals, comparable to the CHC data below, summing all occurrences within an arena in a given hour.

(c) Cuticular hydrocarbon experiment 1: temporal changes in male and female cuticular hydrocarbons

Virgin adults were collected at emergence using light CO_2 anaesthesia, separated by sex and housed in groups of eight individuals per vial. Starting at the beginning of the light cycle on the fourth day after emergence, CHCs were extracted hourly for 24 h. Each hour, extractions were performed on 16 males and 16 females, with four individuals randomly sampled from each of four housing vials for each sex (discarding the remaining individuals in these vials). CHCs were extracted as previously described [25]. To ensure that individuals sampled during the dark phase of the cycle were not exposed to light, at the beginning of the dark cycle, all housing vials were wrapped in aluminium foil and plugged with a dense cotton plug. To extract CHCs, the cotton plug was pierced with a wide-bore needle, and CO_2 was introduced into the vial at a high flow rate to rapidly anaesthetize the flies. Only after flies were unconscious were they removed from the dark vial, at which point their CHCs were extracted within seconds.

The resulting samples were analysed via gas chromatography as described in Sztepanacz & Rundle [49]. Individual CHC profiles were determined by integration of the area under nine peaks, corresponding to those used in past studies of this species, and identified in order of their retention times as: (Z,Z)-5,9-$C_{24:2}$; (Z,Z)-5,9-$C_{25:2}$; (Z)-9-$C_{25:1}$; (Z)-9-$C_{26:1}$; 2-Me-C_{26}; (Z,Z)-5,9-$C_{27:2}$; 2-Me-C_{28}; (Z,Z)-5,9-$C_{29:2}$ and 2-Me-C_{30} [50]. After integration, to correct for technical error associated with quantifying absolute abundances, relative abundances were calculated separately for each individual by dividing the area integrated for each of their CHCs by the total area for all nine CHCs. To break the unit-sum constraint inherent in such compositional data, proportions were transformed into eight logcontrast values [51], using Z,Z-5,9-$C_{24:2}$ as the common divisor, following past studies on this species [15,17,18,34,49]. We used the Mahalanobis distance technique in the multivariate analysis procedure of JMP v. 9.02 (SAS Institute, Cary, NC) to remove a small number of multivariate outliers (see below), probably representing integration errors or contaminated samples [52].

To determine multivariate CHC attractiveness, individuals were scored using the vector of sexual selection gradients ($\boldsymbol{\beta}$) calculated from an independent set of binomial choice mating trials. These mating trials were conducted separately for each sex (see the electronic supplementary material). This scoring generated individual values of the single trait (i.e. linear combination of logcontrast CHCs) that was most strongly associated with mating success in both males ($\mathrm{CHC}\beta_{\mathrm{males}}$) and females ($\mathrm{CHC}\beta_{\mathrm{females}}$), interpreted as their CHC-based attractiveness to the opposite sex.

(d) Cuticular hydrocarbon experiment 2: manipulating social environment

Virgin adults were collected at emergence using light CO_2 anaesthesia, separated by sex and then housed in vials in one of four social treatment groups: one male alone, six males together, one male with five females or six males with five females. Starting at the beginning of the light cycle on the fourth day after emergence, we extracted CHCs hourly from 10 males from each social environment each hour for a total of 24 h. For the two treatments with multiple males, CHCs were extracted from two males per vial, with the remaining individuals discarded. Extractions were performed, the resulting samples analysed and log contrast trait values calculated and scored as described above.

(e) Statistical analyses

To test for the presence of a 24 h temporal cycle without *a priori* assumption about its shape, we used an approach based on the vector sum (see the electronic supplementary material for a detailed description). In brief, if observations are expressed as a vector from the origin within a circular plot, with magnitude equal to their value and direction determined by their time of measurement [53], then in the presence of a temporal cycle, the sum of 24 vectors collected hourly will have a length that is significantly greater than zero. We tested this using a randomization approach that shuffled observations among times of the day to calculate a null distribution against which to compare our observed value (see the electronic supplementary material for details). We performed separate randomizations for average (across replicate tubes) male and female locomotor activity, average (across replicate arenas) hourly total mating activity and the actual number of matings, average (across replicate individuals) $\mathrm{CHC}\beta_{\mathrm{females}}$ (CHC experiment 1) and average (across replicate individuals) $\mathrm{CHC}\beta_{\mathrm{males}}$ (CHC experiments 1 and 2). To provide additional insights into whether the temporal cycle for $\mathrm{CHC}\beta$ was usually strong relative to other combinations of CHCs, we employed an additional randomization to generate a distribution of vector sums representing the strength of temporal patterning for 10 000 different linear combinations of CHCs in each sex (see the electronic supplementary material). The observed vector sum ($\mathrm{CHC}\beta_{\mathrm{males}}$ and $\mathrm{CHC}\beta_{\mathrm{females}}$) was compared with its respective distribution to assess whether it cycled significantly more than other combinations of CHCs in that sex.

Finally, to provide an alternative test for the presence of a 24 h cycle in CHC experiment 1, as well as differences between sexes (for locomotor activity) and social treatments (for CHC experiment 2), we employed a cubic polynomial regression of traits against time. The cubic model fit the data well (significantly better than a linear or quadratic; see Results), and the advantage of this approach is that it allows straightforward tests of fixed effects, such as between sexes and social treatments. This is done by including the fixed effect and its interactions with time within the relevant model. For example, the full model for CHC experiment 2 was

$$\mathrm{CHC}\beta_{\mathrm{males}} = \mathrm{time} + \mathrm{time}^2 + \mathrm{time}^3 + \mathit{treat} + \mathit{treat} \times \mathrm{time} + \mathit{treat} \times \mathrm{time}^2 + \mathit{treat} \times \mathrm{time}^3, \quad (2.1)$$

where *treat* denotes the fixed effect of social treatment. These models were fitted via maximum-likelihood using the mixed procedure in SAS v. 9.3 (SAS Institute). A likelihood ratio test (LRT) was used to compare the fit of the above model with one lacking all four terms that include *treat*, providing an overall test for differences between treatments. A subsequent LRT test of the main effect of treatment alone (i.e. comparing models with and without *treat* but including all the interactions) tests whether average $\mathrm{CHC}\beta_{\mathrm{males}}$ differs among treatments, and a comparison of models that include versus exclude the three *treat* × time interactions (with the main effect of *treat* present in both) tests for differences in the shape of the cycle. An analogous procedure was followed to test for differences between male and female locomotor activity, replacing *treat* with the fixed effect of sex. In this case, a repeated measures approach was used, because activity was measured on the same set of individuals throughout the 24 h period. Individual was therefore included as a random effect nested within sex, and a first-order autoregressive covariance structure was employed in which the correlation between

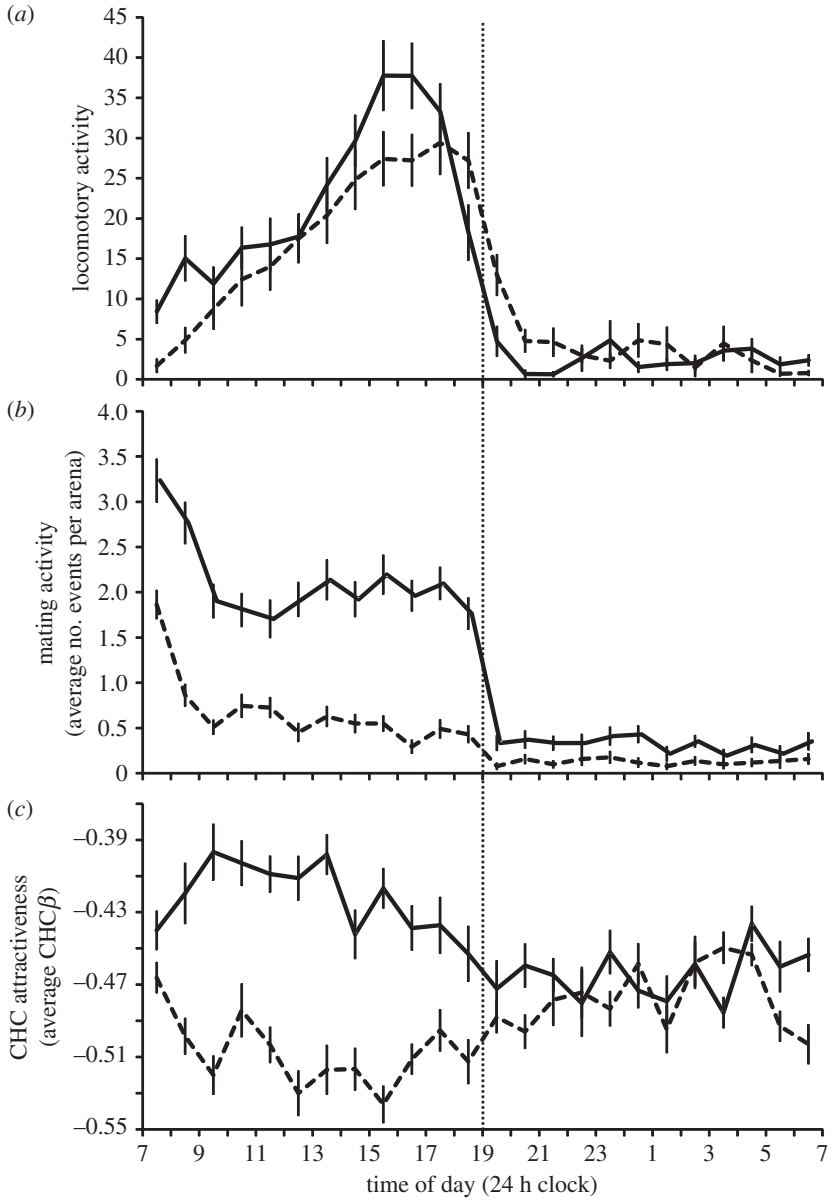

Figure 1. Daily cycles in *D. serrata*: (*a*) average hourly (±s.e.) locomotor activity of males (solid line) and females (dashed line), (*b*) average hourly (±s.e.) total mating activity (i.e. matings and mountings; solid line) and matings only (dashed line), and (*c*) average hourly (±s.e.) CHC-based attractiveness to the opposite sex for males (i.e. CHCβ_{males}; solid line) and females (i.e. CHC$\beta_{females}$; dashed line). Locomotor activity data were collected at 10 min intervals but for clarity are presented as sums over separate 1 h periods.

two measurements decreased exponentially as the length of time between them increased [54].

3. Results

(a) Locomotor activity

Locomotor activity in both sexes showed a strong temporal pattern over 24 h. Few movements were recorded throughout the 12 h dark phase, with activity rising steadily as soon as the light came on (07.00) and peaking in mid to late afternoon, followed by a rapid decline that began 1–2 h before the lights turned off and that continued into the early dark phase (figure 1*a*). This cycle repeated itself when observations were continued for a second 24 h (electronic supplementary material, figure S1). The observed vector sum of average activity over 10 min intervals was highly significant in a randomization test of the null hypothesis of no temporal patterning (observed value in males = 173.5, $p < 0.0001$; females = 150.9, $p < 0.0001$). Overall activity was very similar in the two sexes, although an LRT via a repeated measures cubic regression revealed a significantly better fit of a model that included the effect of sex along with its interactions with the time effects ($\chi^2_4 = 20.3$, $p < 0.001$), indicating differences between males and females. These differences appeared to arise both from a significantly higher average activity in males when compared with females (LRT of the main effect of sex, $\chi^2_1 = 5.1$, $p = 0.024$) and a difference in the shape of the temporal pattern (combined LRT of the sex × time, sex × time^2 and sex × time^3 interactions, $\chi^2_3 = 16.3$, $p = 0.001$), although significance of what appear to be fairly small differences probably reflects high statistical power given 12 528 observations (87 individuals × 6 observation periods per hour × 24 h).

(b) Mating activity

Matings and general mating activity (matings + mounts) showed very similar temporal patterns over the 24 h, broadly

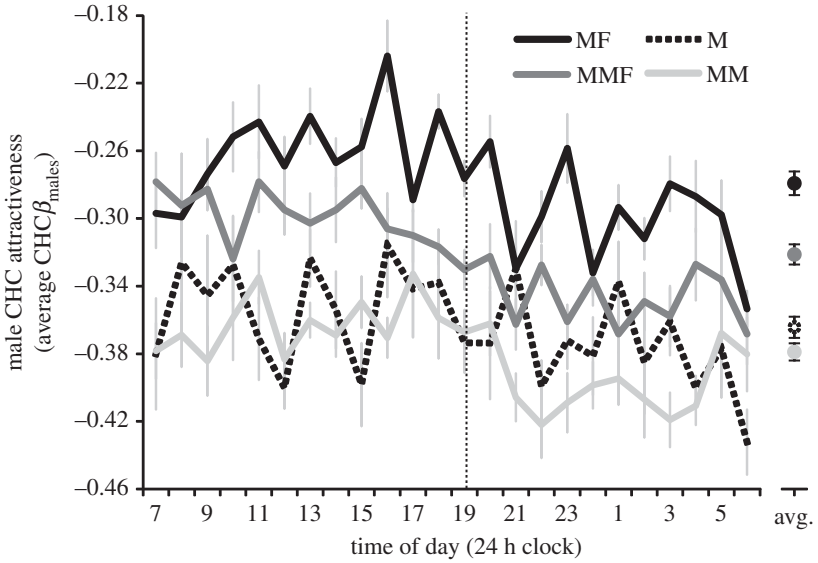

Figure 2. Average (±s.e.) hourly CHC-based attractiveness of males when held under one of four different social treatments (MF, one male + five females; MMF, six males + five females; M, single male; MM, six males). Average treatment values (±s.e.) across the 24 h are shown on the right. Capital letters indicate statistically significant pairwise comparisons ($\alpha < 0.05$).

mirroring that of locomotor activity in that both occurred almost exclusively during the light phase with little activity during in the dark. A burst of mating activity occurred immediately after the lights came on, followed by a decline over the next 1–2 h to an intermediate level of about half the peak value. This intermediate value held roughly constant throughout the day until the lights turned out, at which point mating activity immediately ceased (figure 1*b*). This pattern was highly significant in a randomization test of the null hypothesis of no temporal patterning, both for total mating activity (observed vector sum = 13.9, $p < 0.0001$) and actual matings (observed vector sum = 4.2, $p = 0.0003$).

(c) Cuticular hydrocarbons

In both sexes, a temporal pattern was evident over 24 h in the combination of CHCs most strongly associated with mating success (i.e. CHCβ; figure 1*c*). In males, CHCβ_{males} increased rapidly after the lights came on, held at a high value through to the early afternoon and then declined gradually through the late afternoon until the lights went out, holding at a low value throughout the dark phase. This pattern was highly significant in a randomization test of the null hypothesis of no temporal patterning (observed vector sum = 0.398, $p < 0.001$). A cubic regression of CHCβ_{males} against time also provided a good fit to the data (electronic supplementary material, figure S2) that was significantly better than a second-order (LRT, $\chi^2_1 = 6.2$, $p = 0.013$) model, which was itself a better fit compared with a first-order model (LRT, $\chi^2_1 = 58.0$, $p < 0.001$), providing additional evidence for temporal patterning. The linear effect of time was non-significant (LRT, $\chi^2_1 = 1.1$, $p = 0.294$), as might occur across one complete cycle of a circadian rhythm.

In females, CHCβ_{females} cycled in a pattern that was the mirror image of CHCβ_{males}, with trait values highest during the dark phase, declining rapidly when the lights came on, then holding at low values until the mid-afternoon, at which point they rose more gradually through the late day and into the early part of the dark phase. This pattern was also highly significant in a randomization test of the null hypothesis of no temporal patterning (observed vector sum = 0.319, $p < 0.001$). A cubic regression of CHCβ_{females} against time again provided a good fit to the data (electronic supplementary material, figure S3) that was significantly better than a second-order (LRT, $\chi^2_1 = 5.3$, $p = 0.021$) model, which was itself a better fit compared with a first-order model (LRT, $\chi^2_1 = 47.7$, $p < 0.001$). The linear effect of time was again non-significant (LRT, $\chi^2_1 = 1.9$, $p = 0.168$).

In males, CHCβ_{males} cycled more strongly than any of the individual log contrast CHCs, as revealed by a comparison of the vector sums of all of these traits (electronic supplementary material, table S1). In addition, when compared with 10 000 different traits representing different linear combinations of CHCs, the vector sum of CHCβ_{males} was also a borderline significant outlier ($p = 0.055$), indicating a temporal pattern in this trait that tended to be stronger than that for the majority of other possible CHC blends. In females, four individual log contrast CHCs had a stronger temporal pattern than CHCβ_{females} (electronic supplementary material, table S1), although when compared with 10 000 different traits against representing different linear combinations of CHCs, there was some evidence that CHCβ_{females} cycled to an unusual extent ($p = 0.089$), although it was not as strong an outlier as CHCβ_{males} in males.

When males were held under different social contexts that varied the presence/absence of other individuals of one or both sexes, CHCβ_{males} values varied depending on social treatment (LRT comparing cubic regression models that included versus excluded an effect of treatment and its interactions with the three time terms, $\chi^2_{12} = 316.7$, $p < 0.001$). This among-treatment variation arose in large part from differences in the average CHCβ_{males} value across the entire 24 h period (LRT of the main effect of treatment, $\chi^2_3 = 20.5$, $p < 0.001$), with the highest (i.e. most attractive) values expressed by males when they were held individually with five females ('MF' treatment in figure 2). Males were less attractive on average with the additional presence of five competing males (i.e. six males + five females; 'MMF' treatment in figure 2), and were least attractive in the absence of females, independent of the presence or absence of other males (i.e. single males or groups of six males; 'M' and 'MM' treatments, respectively, in figure 2).

In addition to these differences in average CHCβ_{males} values among social treatments, there was also some evidence for among-treatment variation in the presence and/or shape of the temporal patterning of this trait (figure 2), although this was marginally non-significant in a combined LRT of the interactions of treatment with the three time terms in a cubic regression ($\chi^2_9 = 15.3$, $p = 0.083$). Given a repeating circadian cycle, however, there may be no linear effect of time (because the trait returns to its original value as the pattern repeats every cycle) and differences in shape among treatments would therefore be captured by interactions with the second- and third-order time effects, not the treatment × time interaction. Consistent with this, the first-order time effect was non-significant in every model tested, including those lacking the higher-order time effects, and a test for a difference among treatments in the higher-order treatment × time interactions was significant (LRT of treatment × time^2 and treatment × time^3 interactions, $\chi^2_6 = 14.7$, $p = 0.023$), providing stronger evidence for variation among treatments in temporal patterning. This effect appeared to arise at least in part from the presence versus absence of a temporal pattern among treatments. In particular, when conducted separately by treatment, tests of the null hypothesis of no temporal patterning revealed a significant pattern in three of the treatments (MM: vector sum = 0.318, $p < 0.001$; MF: vector sum = 0.370, $p < 0.001$; MMF: vector sum = 0.392, $p < 0.001$), but not in the fourth (i.e. M, single male treatment: vector sum = 0.180, $p = 0.134$). Qualitatively identical results are obtained from an LRT of a cubic time model separately in each treatment (H. D. Rundle 2012, unpublished results).

4. Discussion

The evolutionary genetics of CHC-based sexual displays have been extensively studied in *D. serrata*, but the complex dynamics of these traits both temporally and in response to changes in social environment have not been well characterized. Here, we show that the combination of CHCs that engenders highest mating success in each sex varies temporally across 24 h. We also demonstrate that average male values, as well as the presence and shape of their temporal variation, are sensitive to different social conditions. These changes do not appear to be a simple physiological by-product of changes in other traits, nor are they the result of physical transfer among individuals (electronic supplementary material). Finally, we show broadly concordant temporal patterns in male and female locomotor and mating activity. Interestingly, the temporal variation in mating and locomotor activity we describe in *D. serrata* differs substantially from that seen in *D. melanogaster* in which both activities occur at appreciable frequencies during at least part of the dark phase [44,55]. If such divergence also characterized incipient species, it could contribute to an allochronic form of sexual isolation.

In males, significant directional sexual selection on CHCs was detected in female choice mating trials (electronic supplementary material), consistent with multiple past studies [17,18,34]. Using the resulting vectors of sexual selection gradients (β_{males}), we scored males for this linear combination of CHCs to generate their phenotypic value for this trait that best determines their mating success (termed CHCβ_{males}). While this is generally interpreted as male attractiveness in this species, CHCβ_{males} may also be influenced by male–male competition, although there is little indication that this occurs in these assays (electronic supplementary material). CHCs can be costly in *D. serrata* [24,31,56] and their expression depends on male condition [25,57]. Therefore, a potential explanation for the observed temporal changes is that males use this sexual signal economically, increasing their expression when mating is most likely. Broadly consistent with this, our results show that mating occurred almost exclusively during the day. Although mating activity and CHCβ_{male} values peak at slightly different times during the light phase (figure 1), this could represent temporal changes in the availability of receptive females that alter the costs and/or benefits of male signalling. Confirmation that temporal cycling is adaptive would require evidence that altered expression of the CHC blend observed in the dark increases male fitness, possibly through reduced cost of CHC synthesis and/or improved desiccation resistance.

Sexual selection on the homologous set of CHCs in females, as estimated via male choice mating trials, differed significantly from that on CHCs in males ($p < 0.001$; electronic supplementary material, table S1). Although linear selection was not significant when tested in females alone, we proceeded to examine this trait combination, because selection approached significance overall ($p = 0.075$), and the vector of selection gradients (i.e. $\beta_{females}$) was very similar (vector correlation = 0.925) to that found to be statistically significant in a previous study in this species [17]. When sampled hourly over 24 h, CHC$\beta_{females}$ values cycled in a pattern that was essentially the mirror image of those in males (figure 1*c*). In particular, females expressed higher values of this trait (i.e. were most attractive to males) during the dark phase when mating activity was low, and then lower values during the light phase when mating was more common.

Sexual conflict has been extensively studied in *D. melanogaster* [49,58,59] and *D. serrata* males are likewise also known to be harmful to females [43]. Reduced attractiveness of females when mating activity is highest may therefore be an adaptive response in females to ongoing interlocus conflict, allowing them to reduce costly male harassment and/or to avoid possibly harmful matings. Alternatively, as both males and females are more active and more likely to mate during the day, it is possible that females increase CHC-based attractiveness at night because it is more difficult to secure matings at that time.

In *D. melanogaster*, circadian rhythms may be maintained via social effects on CHC expression [35–38]. In *D. serrata*, our results show that average CHC-based male attractiveness, and daily temporal variation in this, can be altered under different social conditions that vary the opportunity for mating and the potential for male–male competition. With respect to average attractiveness across 24 h, we found that males expressed the highest value when individually confined with five females. Average attractiveness was observed to decrease with the additional presence of five competing males, and was lowest when males were held in the absence of any females, whether other males were present or not. This suggests that males only invest in expressing costly and attractive CHC profiles when in the presence of females (i.e. when mating is possible), and that males do not use this particular CHC blend in direct male–male interactions, at least in the absence of females. In the presence of females, decreased reproductive investment of

males in response to the presence of competitor males might represent an adaptive response to a diminishing return on their investment [60].

Finally, we found that social environment affected the presence and shape of temporal variation in CHCβ_{males}. Although males housed singly showed no temporal pattern, in the treatments in which a male was held with several other males, several females or both, there was evidence of significant temporal patterning. In the presence of only other males, males varied their attractiveness throughout the 24 h, but their average attractiveness remained low, only increasing with the addition of females. This suggests that average CHC attractiveness is sensitive to the presence of females, whereas cyclical patterns in CHCs are more broadly sensitive to social interactions involving either sex.

In total, our results demonstrate that males alter their CHCs in response to social environment in potentially adaptive ways. Similar results have been found in some species of crickets that, such as *Drosophila*, have sexually dimorphic CHCs that can be detected in close-range communication [61,62]. In *Teleogryllus oceanicus*, females use CHCs to evaluate mate quality [63], generating sexual selection on males [63]. Further, male *T. oceanicus* are able to rapidly alter their CHC profiles to minimize aggression from rival males [64].

Drosophila CHCs were once considered to be fixed within an individual at the time of emergence. However, it is now clear that these traits are plastic within an individual and serve as a dynamic mode of chemical communication. Accommodating such variation will be important in future studies, both from a practical perspective (e.g. the daily range of variation of in CHCβ_{males} in CHC experiment 1 was 2.7 times the average difference between chosen and rejected males in our mating trials) and because it may affect the evolutionary dynamics of these traits and our understanding of their genetic basis.

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Data accessibility. All data are accessible at The Knowledge Bank at Ohio State University: http://hdl.handle.net/1811/61482.

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Mating trials

For both CHC assays, we wanted to focus our analyses on the combination of CHCs associated with highest mating success, both to reduce dimensionality and because this is the trait of greatest biological interest with respect to sexual selection. We therefore conducted a series of replicate female and male binomial mating trials from which we could calculate directional (i.e. linear) sexual selection gradients on these traits in each sex. Such gradients can be used to score individuals, generating their values for the trait that is most strongly associated with mating success (termed CHCβ below).

In theory, both intersexual (i.e. mate choice) and intrasexual selection (e.g., male-male competition) may contribute to outcome of binomial trials, and this may be of particular concern with respect to female choice trials because males of many species compete, either directly or indirectly, for access to females and this may affect their mating success. In *D. serrata*, however, there is clear experimental evidence of female mate choice and little indication of male-male competition, at least during trials of this design. In particular, female mate choice was demonstrated in a quantitative genetic study in which single daughters were placed with five random stock males [1]. Sire-level genetic variance in male mating success was detected, indicating the presence of female mate preferences for male CHCs. With respect to male-male competition, during mating trials *D. serrata* males rarely make any physical contact with one another, or otherwise interact in any detectable way (S. Gershman and H.D. Rundle, pers. obs.). *D. serrata* females also have the capacity to dislodge males who attempt to force copulation, allowing females an additional means of exerting choice. Finally, while territorial behavior has been shown in other *Drosophila* species in which males will aggressively defend a limited food resource consisting of a single spot of live yeast [2], presumably to gain access to females coming to feed, the design of our trials minimizes the opportunity for territoriality by introducing two males that have not previously interacted into a vial containing abundant live yeast immediately prior to a mating trial. Mating generally occurs quickly under these conditions, often within minutes, providing little opportunity for males to establish a dominance hierarchy. Given the above, we interpret our results as reflective of mate preferences in one sex for particular combinations of CHCs in the other, while acknowledging that we cannot rule out some additional contribution on intrasexual selection to the resulting gradients.

Mating trials followed the design of past studies in this species [e.g., 3,4]. In each trial, two four-day-old virgin males or females from different housing vials were simultaneously added to a vial containing a single virgin individual of the opposite sex (four days post-emergence in age). Vials were observed continuously until a successful mating occurred, at which point the chosen and rejected individuals were anaesthetized and CHCs were immediately extracted as described above. Mating trials were conducted from 10-11:30 am. Two hundred and seventy nine trials were performed to assess female choice of male partners, and 187 to assess male choice of female partners.

CHCs were quantified as described for the CHC assays and logcontrast values calculated. A standard first-order polynomial regression of mating success (mated vs. not, coded as 1 vs. 0) on these eight logcontrast CHCs was used to estimate the $\boldsymbol{\beta}$ vector of linear sexual selection gradients (i.e., partial regression coefficients; [see 5]) separately for both males and females. Traits were not standardized prior to analysis so that the resulting gradients could be used to score individuals from CHC Experiments 1 and 2 directly (i.e. so that the traits are all on the same scale). Although the selection gradients were estimated using standard least squares, significance was determined using logistic multiple regression because mating success is binomially distributed [6]. This was done using a generalized linear model with a logistic link function, fit via maximum likelihood, as implemented in JMP v. 9.02 (SAS Institute, Cary, NC). Significance of sexual selection overall was tested using a likelihood ratio test (LRT) comparing the full model to one lacking the eight logcontrast CHCs. Differences in sexual selection on these traits between the sexes (i.e. arising from female choice of male CHCs vs. male choice of female CHCs) was tested by adding to this model a fixed effect of sex along with the interaction terms of this effect with each of the eight traits. A LRT was then used to compare the fit of this model to one lacking these interaction terms [7]. To calculate CHCβ_{males}, the vector of linear sexual selection gradient on males ($\boldsymbol{\beta}_{\text{male}}$) was standardized to unit length and then used to score males from both CHC Experiments 1 and 2 [see 4,8]. The same procedure was used with females from CHC Experiment 1, with CHCβ_{females} calculated by scoring these females for $\boldsymbol{\beta}_{\text{female}}$ (standardized to unit length).

Directional sexual selection on the eight logcontrast CHCs differed significantly between the sexes (LRT, $\chi^2 = 40.1$, d.f. = 8, $P < 0.001$). Analyzing separately by sex, sexual selection was significant overall in males (LRT, $\chi^2 = 42.4$, d.f. = 8, $P < 0.0001$, $r^2_{\text{adj}} = 0.091$) and approached significance in females (LRT, $\chi^2 = 14.3$, d.f. = 8, $P = 0.075$, $r^2_{\text{adj}} = 0.033$). With respect to the individual traits, selection was significant on four of

the logcontrast CHCs in males and one in females (Table 1). Although selection was not significant in females alone, we scored females for this trait combination and proceeded with its analysis because selection approached significance overall (P = 0.075) and the sexual selection vector (i.e. $\boldsymbol{\beta}_{\text{females}}$) was very similar (vector correlation = 0.925) to that found to be statistically significant in a previous study in this species [see 3]. Individuals from CHC Experiments 1 and 2 were scored for their respective $\boldsymbol{\beta}$ vector, generating the single trait (i.e. linear combination of logcontrast CHCs) most strongly associated with mating success for both males (CHCβ_{males}) and females (CHCβ_{females}).

Vector Sum and randomization procedures

Presence of a temporal cycle.— The vector sum provides a general test for the presence of a 24 h temporal cycle that makes no *a priori* assumptions about the shape of the pattern. Converting the time of day (using a 24 h clock) to an angular direction, with 00:00 h corresponding to 0˚ and each hour to an additional 15˚ (i.e. 12:00 is 180˚), an observation at any given time can be expressed within a circle projection as a vector from the origin with its angular direction determined by its time and its magnitude by its observed value at that time (e.g., activity level, number of matings, CHCβ value; [see 9]). If samples are taken at regular intervals throughout the entire cycle (e.g., hourly over 24 h), as in our case, then a circadian pattern (i.e. observed values that are higher at some time of day and lower at another) will cause the sum of these equally spaced vectors to be displaced from the origin (i.e. to be a vector with a length significantly greater than zero). In contrast, the vector sum for a trait that varies randomly throughout the day, or that remains unchanged, will have a length that does not differ significantly from zero. The observed vector sum can be compared to a distribution of sums generated under the null hypothesis of no association between observations and the time of day at which they were collected. This null distribution was created by shuffling observations among times via 10,000 iterations of a randomization procedure.

Strength of the CHCβ temporal cycles.— To test whether CHCβ_{males} cycled to an unusual degree compared to other CHC blends, we employed a randomization approach, following Delcourt and Rundle [10], in which different linear combinations of CHCs were generated by randomly shuffling individuals among two equal sized groups. Multiple linear regression of each individual's eight logcontrast CHCs values against their group membership (coded as 0 vs. 1) was used to obtain a vector of partial regression coefficients representing an arbitrary axis of CHC variation (i.e. CHC blend). This approach

preserved the phenotypic covariance structure inherent in this CHC dataset, identifying linear combinations of CHCs that exist phenotypically within the population and that may (or may not) cycle temporally. We repeated this procedure 10,000 times, resulting in resulting in 10,000 different linear combinations of CHCs. Each vector of partial regression coefficients was standardized to a unit length and then used to score all individuals in the same dataset, generating their phenotypic values for the given trait combination. The length of the resulting vector sum for this trait was then calculated as a measure of the extent to which it cycles. The observed length of the vector sum for CHCβ_{males} was then compared to this distribution of lengths to assess whether it cycled significantly more than other combinations of CHCs. This same procedure was performed separately for females, comparing the extent to which CHCβ_{females} cycles relative to 10,000 other linear combinations of CHCs in females.

In theory, a temporal pattern in the linear combination of CHCs that best predicts male mating success CHCβ_{male} could arise simply as a physiological by-product of circadian changes in other traits such as activity. However, CHCβ_{male} had a stronger temporal pattern than the majority of other CHCs blends and was a borderline significant outlier in this respect (P = 0.055). Furthermore, CHCβ_{males} values peaked earlier in the light phase than did male locomotor activity, also suggesting that changes in it are not a simple byproduct of metabolic activity. Finally, when males were housed singly, there was no evidence of any temporal pattern, again implying that daily variation in this trait is not a necessary physiological side effect of other circadian rhythms in other traits. As in males, this pattern of temporal change in CHCβ_{females} does not align well with locomotor activity, suggesting that it is not a simple physiological byproduct of metabolic changes. There is also some evidence that the temporal pattern for this trait is unusually strong relative to other CHC blends (P = 0.089), although it is less of an outlier than CHCβ_{males}.

Physical transfer of CHCs

Flies in our experiments (and mating trials above) were held in groups prior to, and in some cases during, the assays, providing the opportunity for physical transfer of CHCs among individuals. Such transferred (i.e. 'rubbing') is known to occur to some extent in *Drosophila* and past studies taken advantage of this to perfume individuals in desired ways (e.g., [11]). However, such perfuming is achieved by confining a focal individual with many (i.e. 25+) donor individuals within a highly restricted space (e.g., a vial with the stopper pushed down to within 1 cm of the food) for an extended period of time. The densities used in our experiments are unlikely to produce such an effect and, more

importantly, such transfer would tend to produce homogeneity among individuals within a group that would tend to hamper the ability to associate differences in mating success with differences in CHC profiles among-individual (i.e., as we did in our mating trials above). Such transfer among individuals also provides no mechanism to explain the observed temporal patterns in relative concentrations that we observed in CHC Experiment 1.

Notwithstanding effects outside the context of reproduction, physical transfer during mating is of concern as it is known to occur in *D. melanogaster* [12]. In CHC Experiment 2 this could, in theory, have produced the higher average attractiveness of males in the two treatments in which females were present. However, in a direct test no detectable transfer of CHCβ_{males} was observed in *D. serrata* (S. Gershman and H.D. Rundle, unpublished data). In addition, the blend of CHCs that differs between the sexes (calculated using unmated females from the mating assay above) is oriented in multivariate trait-space at 127 degrees from CHCβ_{males}. Therefore, any transfer of female CHCs to males during mating in CHC Experiment 2 should not produce males with higher values of CHCβ_{males}.

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Figure legends

Fig. S1. Average (± SE) hourly locomotor activity of male and female *D. serrata*. Data were collected on 10 min intervals but for clarity are presented as sums over 1 h periods. Higher activity in the first hour is likely the result of the disturbance involved in introducing the flies to the activity monitor.

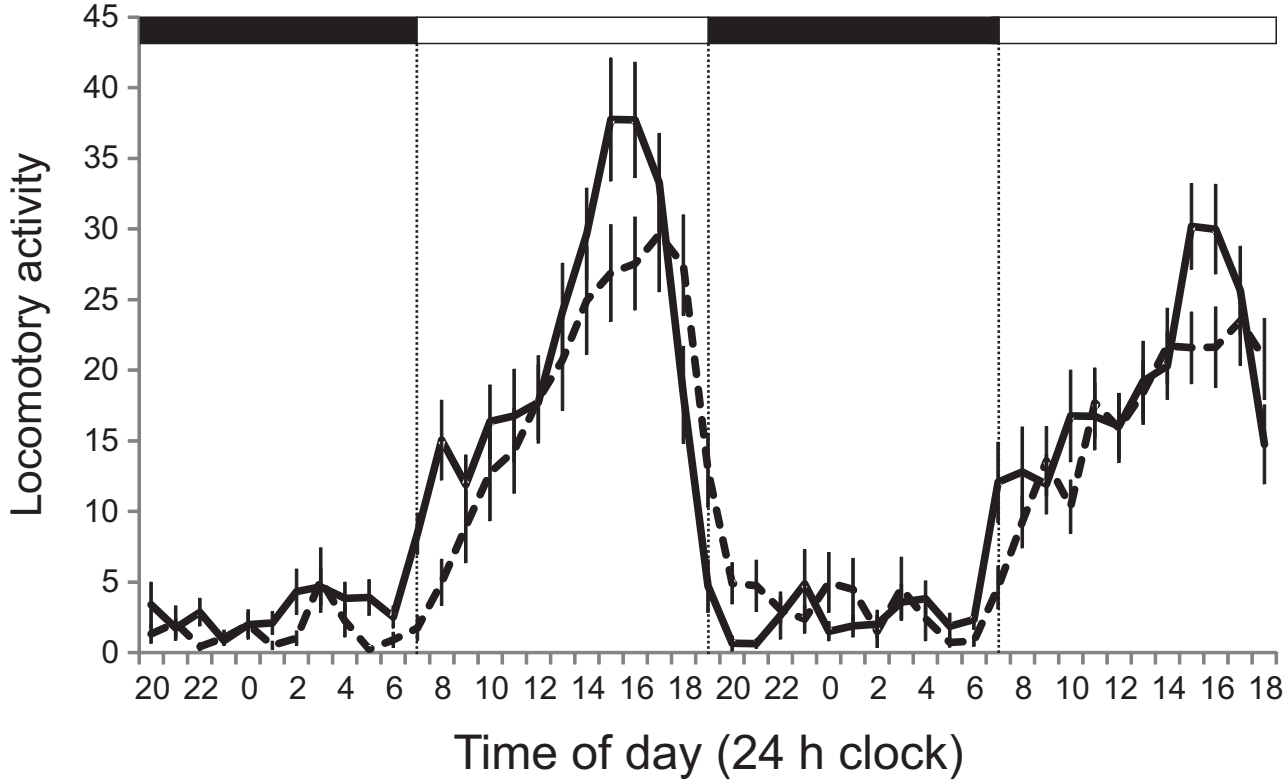
Fig. S2. Individual replicate CHCβ_{males} trait values over 24 h for males from CHC Experiment 1, showing (A) the best fit cubic regression against time and (B) the residuals plotted against predict trait values.

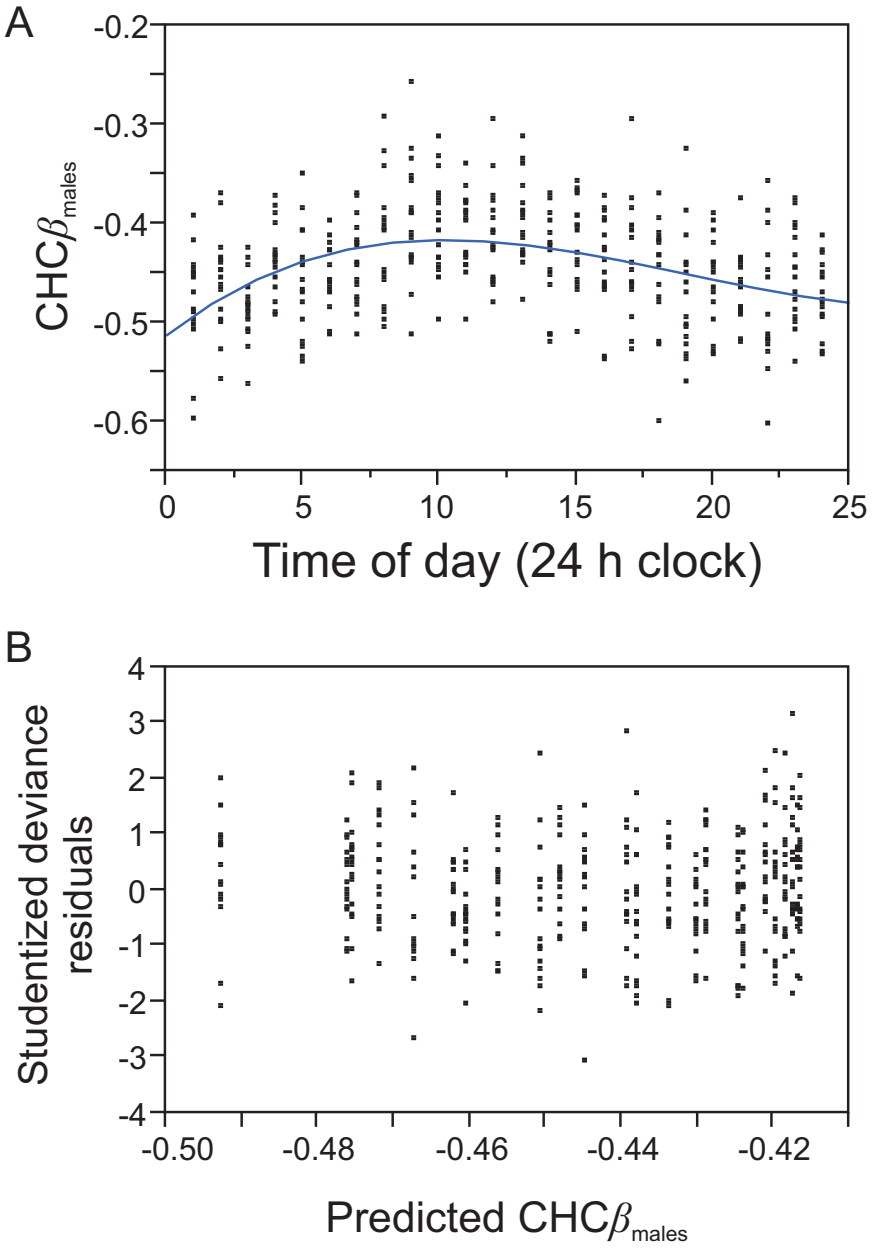
Fig. S3. Individual replicate CHCβ_{females} trait values over 24 h for females from CHC Experiment 1, showing (A) the best fit cubic regression against time and (B) the residuals plotted against predict trait values.

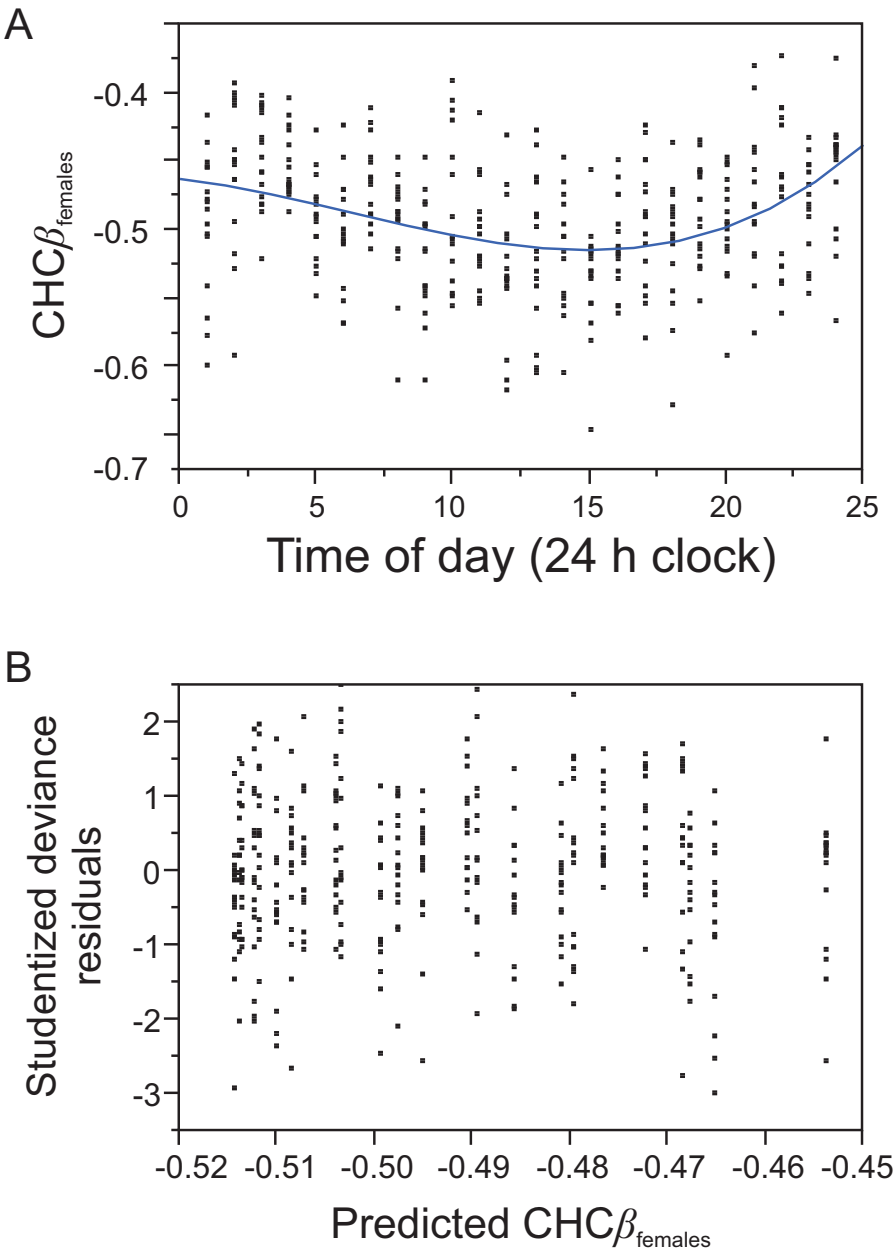
Table S1. Directional sexual selection (β) and circadian cycles for eight logcontrast CHCs in males and females.

	Females			Males		
Logcontrast CHC	β	**P**	**Vector sum**[a]	β_{males}	**P**	**Vector sum**[a]
(Z,Z)-5,9-$C_{25:2}$	0.430	0.789	0.067	-1.129	0.395	0.052
(Z)-9-$C_{25:1}$	0.454	0.435	0.143	**-1.471**	0.003	0.007
(Z)-9-$C_{26:1}$	0.757	0.133	0.201	-0.687	0.173	0.139
2-Me-C_{26}	0.947	0.150	0.449	-0.961	0.074	0.092
(Z,Z)-5,9-$C_{27:2}$	-0.113	0.718	0.258	-0.189	0.676	0.036
2-Me-C_{28}	**-2.832**	0.031	0.531	**2.040**	0.010	0.117
(Z,Z)-5,9-$C_{29:2}$	-0.191	0.419	1.041	**1.020**	<0.001	0.111
2-Me-C_{30}	0.987	0.090	0.707	**-1.130**	<0.001	0.148

[a]Magnitude of the vector sum for the given logcontrast CHC from CHC Experiment 1, indicative of the strength of the 24 h circadian cycle of that trait.


Locomotory activity
45
40
35
30
25
20
15
10
5
0
20 22 0 2 4 6 8 10 12 14 16 18 20 22 0 2 4 6 8 10 12 14 16 18
Time of day (24 h clock)


A
-0.2
-0.3
-0.4
-0.5
-0.6
CHCβ_{males}
0
5
10
15
20
25
Time of day (24 h clock)
B
4
3
2
1
0
-1
-2
-3
-4
Studentized deviance residuals
-0.50
-0.48
-0.46
-0.44
-0.42
Predicted CHCβ_{males}


A
-0.4
-0.5
-0.6
-0.7
CHC$\beta_{females}$
0
5
10
15
20
25
Time of day (24 h clock)
B
2
1
0
-1
-2
-3
Studentized deviance residuals
-0.52
-0.51
-0.50
-0.49
-0.48
-0.47
-0.46
-0.45
Predicted CHC$\beta_{females}$