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Level up: the expression of male sexually selected cuticular hydrocarbons is mediated by sexual experience

Susan N. Gershman [a, *], Howard D. Rundle [b]

[a] Department of Evolution, Ecology and Organismal Biology, The Ohio State University at Marion, Marion, OH, U.S.A.
[b] Department of Biology, University of Ottawa, Ottawa, ON, Canada

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The use of cuticular hydrocarbons (CHCs) in species recognition, sex identification and sexual selection is widespread in insects. However, few studies have studied plasticity in CHCs. Here we examine the effect of age and social environment on a suite of sexually selected CHCs in *Drosophila serrata*. We demonstrate that the combination of CHCs that is associated with increased male mating success (CHCβ) changes as males age, and this effect is mediated by social environment. When single males were housed with multiple females, their expression of CHCβ increased across the first few days of their adult life, after which expression declined with increasing age. In contrast, sexually selected CHCs of males housed with other males, males housed with other males and females, and males housed alone all decreased across days. To determine the long-term consequences of mating on CHC expression, we allowed males a single mating opportunity and subsequently found some indication of a brief spike in CHCβ. Finally, to determine whether visual and olfactory contact with females, copulation, or intromission causes males to express high values of CHCβ, we manipulated male access and physical contact with females. We found that although prolonged copulation causes a slight increase in male CHCβ, only a successful copulation with sperm transfer induced males to develop CHCs associated with high mating success. Taken as a whole, our results demonstrate that the expression of sexually selected CHCs in males varies with both age and social context, and suggest that the latter is mediated at least in part by successful matings with females. More generally, contextual plasticity in CHCs is likely to affect both the experimental design of CHC-based experiments and the evolution of CHC signals as naturally and sexually selected traits.

Cuticular hydrocarbons (CHCs) are long-chain, largely nonvolatile waxes that are produced by oenocyte cells on the cuticle of most insects (Antony & Jallon, 1982; Wigglesworth, 1970). CHCs serve to reduce water loss through the cuticle and thus protect against desiccation (Foley & Telonis-Scott, 2011; Gibbs, Fukuzato, & Matzkin, 2003; Howard & Blomquist, 2005). In many species, CHCs and their derivatives containing various functional groups have been secondarily co-opted as a means of chemical communication and have been shown to function in species recognition (e.g. Alves et al., 2010; Buellesbach et al., 2013; Maroja et al., 2014), sex recognition (e.g. Antony & Jallon, 1982; Chenoweth & Blows, 2003, 2005), and as a means to discriminate among individuals within a population. With respect to the latter, CHC profiles are involved in recognizing previous mates (Weddle et al., 2013), in recognizing nestmates (Marten, Kaib, & Brandl, 2010; Ozaki, Kidokoro-Kobayashi, & Hiraguchi, 2012), and as a means of nest marking (Lorenzi, Cervo, & Bagneres, 2011). Specific combinations of CHCs have been associated with mate choice in both males and females (Chenoweth & Blows, 2005; Hunt, Snook, Mitchell, Crudgington, & Moore, 2012), and in some cases serve to mediate male–male competition (Thomas & Simmons, 2009).

Unlike the long-range mate attraction pheromones of moths and beetles that tend to have just one or a few dominant compounds that are perceived by the receiver, CHCs are often substantially more complex, being composed of multiple different alkanes, alkenes and methyl-branched hydrocarbons (Ferveur & Cobb, 2010). Furthermore, an individual's CHC profile can vary in response to factors such as diet (Fedina et al., 2012), desiccation stress (Stinziano, Sové, Rundle, & Sinclair, 2015) and age (Kuo et al., 2012). Social environment can also influence CHC expression in males and females (Farine, Ferveur, & Everaerts, 2012; Kent, Azanchi, Smith, Formosa, & Levine, 2008; Petfield, Chenoweth, Rundle, & Blows, 2005). These changes in CHC expression suggest

* Correspondence: S. N. Gershman, Department of Evolution, Ecology and Organismal Biology, 1465 Mount Vernon Ave, The Ohio State University at Marion, Marion, OH 43302, U.S.A.
E-mail address: gershman.6@osu.edu (S. N. Gershman).

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that male CHC expression may exhibit contextual plasticity, defined as 'variation in an individual's behaviour as a function of variation in the external stimuli (context) at the time the individual expresses that behaviour' (Ord, Stamps, & Losos, 2010, page 3135). Furthermore, these instances of contextual plasticity in CHC expression may potentially be beneficial to the male, although this has been addressed in surprisingly few studies (but see Thomas & Simmons, 2009, 2011).

In *Drosophila serrata*, an Australian species of fruit fly, CHCs have been studied extensively, with the majority of work (over 40 publications to date) focusing on their role as contact pheromones that are used in species recognition and during mate choice within populations (Blows & Allan, 1998; Chenoweth & Blows, 2003, 2005). Males and females express the same set of nine compounds, although their relative concentrations are sexually dimorphic. Both sexes also use these traits when choosing mates, although the preferred blend, and the form of sexual selection (e.g. directional, stabilizing), vary between males and females (Chenoweth & Blows, 2005; Rundle & Chenoweth, 2011). CHC appear costly to produce (Blows, 2002) and their expression in males is condition dependent (Delcourt & Rundle, 2011). Both CHCs and female preferences for them have a genetic basis and have been shown to respond to altered selection (Blows, 1998; Delcourt, Blows, & Rundle, 2010; Higgie, Chenoweth, & Blows, 2000; Hine, McGuigan, & Blows, 2011; Rundle, Chenoweth, & Blows, 2009; Rundle, Chenoweth, Doughty, & Blows, 2005). Consequently, *D. serrata* has also been used as a model system to examine hypotheses about the genetic architecture of multivariate, sexually selected traits.

In a recent paper we characterized short-term temporal changes of *D. serrata* CHC expression and plasticity of these changes in response to different social environments. We showed that when individuals were housed alongside others, the CHCs of both males and females varied across a 24 h period in an apparent circadian pattern. Focusing on the combination of CHCs targeted by sexual selection (termed CHCβ), males had higher values when housed under social conditions that provided greater access to females (and hence greater opportunity for mating), and their value peaked during the time of day when matings were most common. In both cases, these contextual changes in CHCs should enhance mating success (i.e. because males express higher values of the trait combination favoured by sexual selection), suggesting adaptive plasticity (Gershman, Toumishey, & Rundle, 2014b).

Building on this previous work, here we quantify longer-term changes (i.e. across days) in CHC expression over the early lives of males and the effects of social environment on CHC expression. Oenocyte cells mature over the first week after adult eclosion (Johnson & Butterworth, 1985), with both male and female *D. serrata* becoming sexually competent approximately 36 h after adult eclosion (S. N. Gershman & H. D. Rundle, personal observation). Our study has three parts. First, we characterized how the combination of CHCs targeted by sexual selection changes over the first week in adults, and we manipulated social environment to investigate its effects on this. In the second part, we tested the effect of mating on the expression of male sexually selected CHCs. Our interest here concerned whether and how a single mating may alter CHC expression across days. However, this assay also provided a test of a fundamental assumption of all sexual selection analyses that have been done using binomial mate choice trials in *D. serrata*: that the act of mating itself does not alter CHCs prior to their extraction (e.g. due to physical transfer or as a by-product of physiological changes induced by mating). Changes in CHCs induced by mating that occur prior to extraction would create a difference between chosen and rejected males that would confound any difference resulting from mate choice (i.e. sexual selection). Finally, in the third part, we investigated the specific cue that induces males to alter the expression of their sexually selected CHCs in the presence of females.

METHODS

In all assays, we used flies from a previously described laboratory-adapted, outbred stock population of *D. serrata* maintained at large population size (16 half-pint (237 ml) bottles/generation) via nonoverlapping generations (Chenoweth, Rundle, & Blows, 2008; Rundle, Chenoweth, & Blows, 2006). Experimental animals were maintained under constant conditions mirroring that of the stock, including temperature (25 °C) and photoperiod (12:12 h light:dark cycle), with the lights on at 0700 hours and off at 1900 hours daily.

Mating Trials and Identifying Sexually Selected CHCs

To test the effects of time and social environment on male CHC expression, we wanted to focus our analyses on the combination of CHCs that are most strongly associated with increased mating success, thereby reducing a high-dimensional data set to a single trait of biological interest. Our approach to doing this followed established methods that have previously been used in this species (e.g. Gershman, Delcourt, & Rundle, 2014a; Gershman et al., 2014b; Hine, McGuigan, & Blows, 2014; McGuigan, Petfield, & Blows, 2011; Sztepanacz & Rundle, 2012). In brief, we used a series of binomial choice mating trials to calculate the $\boldsymbol{\beta}$ vector of directional sexual selection gradients (i.e. partial regression coefficients; Lande & Arnold, 1983), equivalent to the linear combination of CHCs associated with highest mating success in males. We extracted and quantified CHCs of males from our experiments (i.e. males varied in age, social experience and mating experience) as described below. Then, we determined an individual's phenotypic score for the trait combination described by $\boldsymbol{\beta}$ in a manner identical to calculating an individual's score for a particular principal component, by using matrix algebra to multiply its measured values by the vector of loadings for that principal component. We calculated this trait, termed CHCβ, as $\mathrm{CHC}\beta = \boldsymbol{\beta}\,^{\mathrm{T}}\mathbf{Z}$, where $\mathbf{Z}$ is the vector of trait values measured on the individual (McGuigan et al., 2011).

As a trait, an individual's CHCβ score represents the individual's value for the linear combination of CHCs that is most strongly associated with increased male mating success. In previous studies in *D. serrata*, this score has been interpreted as CHC-based 'attractiveness' (Gershman et al., 2014a, 2014b; Hine, Lachish, Higgie, & Blows, 2002, 2011), under the assumption that, in the mating trials used to estimate β, variation in male mating success is caused by female mate choice alone. Although *D. serrata* females can choose which males to associate with, and can also dislodge unwanted copulations by shaking off the male (Hoikkala & Crossley, 2000), male–male interactions can also occur during choice trials and may contribute to the outcome. Although *D. serrata* males are not as visibly aggressive to one another as *Drosophila melanogaster* males, if given the opportunity, they will defend a territory and their success at doing so appears to affect their mating success (White & Rundle, 2014). However, CHCs do not appear to be associated with success in male territory defence (White & Rundle, 2014), and the arenas for mating trials used in estimating β were designed to minimize the opportunity for territorial defence (see Gershman et al., 2014b). Nevertheless, it is possible that subtle male–male interactions contribute to variation in male mating success and hence CHCβ values. Therefore, we refer to CHCβ as 'male sexually selected CHCs' and interpret this trait more broadly as the combination of CHCs associated with increased mating success.

The binomial choice trials used in calculating β are described in detail in Gershman et al. (2014b). These trials used individuals from the same population of flies and were completed within the same year as the experiments described below. In brief, in each trial a single virgin female was placed together in a vial with two virgin males and the flies were observed continuously until a successful mating occurred, at which point the chosen and rejected males were anaesthetized and their CHCs were immediately extracted. Samples were analysed via gas chromatography and individual CHC profiles were determined by integration of the area under nine peaks, as described below. CHC relative concentrations were calculated and transformed to logcontrasts (see below), and then a standard first-order polynomial regression of mating success (mated versus not, coded as 1 versus 0) on these eight logcontrast CHCs was used to estimate the β vector of linear sexual selection (Lande & Arnold, 1983).

Part 1: The Effect of Male Age and Social Environment on Male Sexually Selected CHCs

To characterize how male sexually selected CHCs (i.e. CHCβ) varies across days, and the effects of social environment on this, males were housed under different social conditions and then their CHCs were collected at specific times. To begin the assay, virgin adults were collected at eclosion using light CO_2 anaesthesia, separated by sex, and then housed in 95 × 25 mm vials with ad libitum food (standard medium with live yeast sprinkled on top) under one of four social treatments: one male alone, six males together, one male with five females, or six males with five females. These are the same social treatments that caused differences in CHCs over a 24 h period in 4-day-old males (Gershman et al., 2014b).

CHCs were collected on days 2–7 after adult eclosion. Because CHC extraction is lethal, different males were used at each time point. For the treatments with multiple males, CHCs were extracted from two males/vial, with the remaining individuals discarded. Using two males per vial reduced the total time per day that was required to extract CHCs, which is important because previous studies demonstrate substantial time of day effects on CHCβ, but no effect of vial identity (Gershman et al., 2014b). All extractions were performed from 0900 to 1015 hours, the time of day that males express peak values of CHCβ (Gershman et al., 2014b). CHCs were collected from 24 males per social treatment per day (144 males in total for each social treatment).

Our statistical analysis used an ANCOVA-based approach that modelled variation in CHCβ as a function of the continuous effect of day and the fixed effects of the categorical variables of male social treatment (i.e. presence versus absence of five additional males) and female social treatment (i.e. presence versus absence of five females), as well as all of the two-way and three-way interactions among these. The interactions of the fixed social effects with day allow the slope describing changes in CHCβ across days to vary between different levels of the social treatments. A second-order effect of day (i.e. day^2) was also included, along with interactions of this with the fixed social effects, because a plot of CHCβ over time for the different treatments showed a curvilinear relationship (with a single point of inflection) that appeared to vary between social treatments (see Results).

Part 2: The Effect of Mating on Male Sexually Selected CHCs

To determine the effect of mating on male sexually selected CHCs (CHCβ), we tracked CHCs over time (days 3–7) by sampling males from each of two groups: one group of males was not exposed to females and thus remained virgin throughout the entire period ('virgin treatment'); males in the other group were allowed to mate once on the morning of day 5 but were otherwise held in the absence of females before and after this mating ('mated treatment'). All males were collected as virgins at eclosion using light CO_2 anaesthesia, and then housed individually in vials with ad libitum food. From day 3 to day 7, we extracted CHC at 0900 hours daily from males sampled from both groups. On the fifth day after eclosion, we paired males in the mated treatment with a female and observed them until they copulated, at which point we removed the female from the vial. We extracted CHCs from these males immediately after mating, and simultaneously extracted CHCs from males in the virgin treatment. Additional extractions were performed at 1600 hours on days 5 and 6 to determine whether CHCβ was affected by mating on a timescale shorter than 24 h. CHCs were extracted from 19–20 males per treatment per sampling time.

Because the mating treatment was applied part-way through the assay, our statistical analyses were split into two components. First, to quantify how male sexually selected CHCs changed through time, we used data from across all days but from only the virgin treatment to perform a second-order regression of CHCβ on day and day^2. The virgin mating treatment is equivalent to the 'one male alone' male treatment in Part 1, and if we observe the same pattern of changes in male CHCβ across these two experiments, conducted at different times, this strongly implies that the changes in CHCβ were due to male age, and not an artefact of some effect specific to one assay. Second, to test for effects of mating on changes in CHCβ across days, we used only data from after the application of the mating treatments (i.e. days 5–7). We used an ANCOVA-based approach to model variation in male CHCβ as a function of the continuous effect of day and day^2, and the interaction of each of these with the fixed effect of mating status (i.e. mated or not). We also used a series of two-sample *t* tests to compare mating treatments separately for the different time points (see Results).

Part 3: The Effect of Interactions with Females on Male Sexually Selected CHCs

To determine whether visual and/or olfactory contact with females is sufficient to elicit increased expression of CHCβ in males, we extracted CHCs from males that had been exposed to females across a permeable barrier that prevented physical contact. Virgin males were collected at eclosion using light CO_2 anaesthesia and then housed in one of three treatments: one male alone in a vial, one male with five females in a vial, or one male with five females in a vial with a cloth-mesh barrier that allowed the males and females to have visual and olfactory contact with one another, yet not mate. Based on previous results, males held with five females should increase their expression of CHCβ relative to males held singly (Gershman et al., 2014b), providing a positive and negative control against which the males from the mesh barrier could be compared. We extracted CHCs from 30 males per day on day 3, 4 and 5 after eclosion. Our statistical analysis used an ANCOVA in which variation in CHCβ was modelled as a function of the continuous effect of day and the fixed effect of a categorical variable denoting the social treatment (i.e. with females, without females, or exposure to females across a barrier). The second-order effect of day (i.e. day^2) and the interaction of this with social treatment were nonsignificant and were excluded from the model.

To test whether mounting or attempted copulation with females (but without sperm/seminal fluid transfer) is required to elicit an increase in the expression of CHCβ, in a separate experiment we exposed males to treatments that varied the extent of their sexual contact with females. Virgin males were again collected at eclosion using light CO_2 anaesthesia and then exposed to one of four

treatments: one male alone in a vial, one male with five females in a vial, one male exposed to 'glued' females, which the male could mount but was incapable of copulating with, and one male repeatedly allowed to begin courtship and mount the female, but the mating was 'interrupted' before sperm could be transferred. We used a one-way ANOVA to test for treatment effects.

In our 'glued' treatment, males were collected within 24 h of eclosion and housed individually in vials. On days 3, 4 and 5, males were allowed the opportunity to interact with manipulated females. Females were collected within 12 h of eclosion and stored in vials in groups of five. On days 3, 4 and 5, females were lightly anaesthetized with CO_2 and their genitalia were painted with liquid bandage (Skin Shield, Del Laboratories, Uniondale, NY, U.S.A.). We gave females 30 min to recover after the application of the sealant, and then introduced two females into each male vial. Females painted with sealant were unable to mate, although males repeatedly mounted them and attempted to copulate. We removed females from the male vials after 2 h. Each female was only used once. Most females removed the skin sealant by grooming within a few hours after the trials ended and appeared to have unimpaired health (S. N. Gershman, personal observation). After exposure to males, females were housed for 12 days with oviposition medium to confirm that sperm transfer had not taken place.

In our 'interrupted' treatment, we collected males within 24 h of eclosion and housed them individually in vials. Males were exposed to females on days 3, 4 and 5 after eclosion. We collected females within 12 h of eclosion and stored them in vials in groups of five. On days 3, 4 and 5, we added two females to each male vial. We observed vials until the male mounted one of the females. Sixty seconds after mounting, we removed the male from the female using a puff of air from an aspirator. Males require more than 1 min to successfully transfer their sperm packet (Hoikkala, Crossley, & Castillo-Melendez, 2000). Both females were removed after the male attempted to mount one of them. Each female was only used once. After exposure to males, females were housed for 12 days with oviposition medium to confirm that sperm transfer had not taken place.

On the fifth day after male eclosion, CHCs were extracted from all males. For the males paired with glued females, CHCs were extracted 1–2 h after females were removed. For the interrupted males that could copulate but not transfer their sperm packet, CHCs were extracted 1–4 h after females were removed. For males housed continuously with females, males remained with females until CHCs were collected. Initially, we used 72 males per treatment. However, 26 males from the glued treatment mated with at least one female that eventually laid eggs, so we excluded these males from the analysis. No males from the interrupted treatment were excluded, as no females from these treatments laid any eggs.

CHC Extraction and Analysis

Samples were analysed via gas chromatography as described in Sztepanacz and Rundle (2012). 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} (Howard, Jackson, Banse, & Blows, 2003). After integration, to correct for error associated with quantifying absolute abundances via gas chromatography, we calculated relative abundances separately for each individual by dividing the area integrated for each individual's CHCs by the total area for all nine CHCs. The resulting proportions are a form of compositional data (parts of a whole) for which standard statistical methods are not appropriate (Aitchison, 1986; Egozcue & Pawlowsky-Glahn, 2011). To address this, we transformed relative concentrations into eight logcontrast values (Aitchison, 1986), using Z,Z-5,9-$C_{24:2}$ as the common divisor, following past studies on this species (e.g. Blows & Allan, 1998; Chenoweth & Blows, 2003, 2005; Gershman et al., 2014a, 2014b; Rundle, Chenoweth, & Blows, 2008; Sztepanacz & Rundle, 2012). We used the Mahalanobis distance technique in the multivariate analysis procedure of JMP v.9.02 (SAS Institute, Cary, NC, U.S.A.; Sall, Creighton, & Lehman, 2005) to remove a few multivariate outliers, probably representing integration errors or contaminated samples (0–3 individuals were removed from each data set).

RESULTS

Part 1: The Effect of Social Environment on the Development of Male CHCs

While the presence of females significantly increased male sexually selected CHCs (CHCβ) overall (main effect of female social treatment in Table 1), this arose primarily from changes that occurred in the absence of other competitor males (Fig. 1a), thereby generating a significant interaction between male and female social treatment (Table 1). Although in the absence of competitor males, CHCβ also increased in response to the presence of females, this change was much smaller in magnitude and was nonsignificant by post hoc comparison (Fig. 1a). In addition, while the addition of males decreased CHCβ overall, the interaction revealed that this effect was entirely due to the presence of females, and that in the absence of females, the addition of males caused, if anything, a small increase in CHCβ, although this difference was not significant in a post hoc comparison (Fig. 1a). Across days, CHCβ decreased, on average, although there was a significant effect of female social treatment on the quadratic (i.e. day^2) term, indicating a difference in the curvature of this relationship in the presence versus absence of females (Table 1). This difference in curvature appeared to arise from a convex relationship in the absence of females (i.e. the decrease in CHCβ slowed with time) that did not occur in the presence of females (Fig. 1b). Rather, in the presence of females the relationship was noticeably concave when competitor males were absent, with CHCβ actually increasing across the first few days, but not in the presence of additional males. This difference, however, was not sufficient to generate a male social treatment × female social treatment × day^2 interaction ($P = 0.096$; Table 1).

Part 2: The Effect of Mating on Male CHCs

In a pattern matching the 'one male alone' treatment in Part 1, CHCβ decreased, on average, in virgins males across days 3–7 (day

Table 1
Statistical analysis of the effect of social environment on the expression of male sexually selected cuticular hydrocarbons across days

Source	Estimate	$F_{1,564}$	P
Day	**−0.023**	90.80	<0.0001
Males	**0.013**	4.38	0.037
Day*males	0.003	1.99	0.16
Females	**−0.047**	54.7	<0.0001
Day*females	−0.000	0.004	0.95
Males*females	**−0.028**	18.90	<0.0001
Day*males*females	−0.003	1.46	0.23
Day*day	0.001	0.21	0.65
Day*day*males	−0.001	0.55	0.4576
Day*day*females	**0.005**	8.06	0.0047
Day*day*males*females	0.003	2.78	0.0958

Results of an ANCOVA with unequal slopes that included day, day * day, females, males, and all possible two-way and three-way interactions as variables. Values shown in bold were statistically significant at $\alpha < 0.05$.

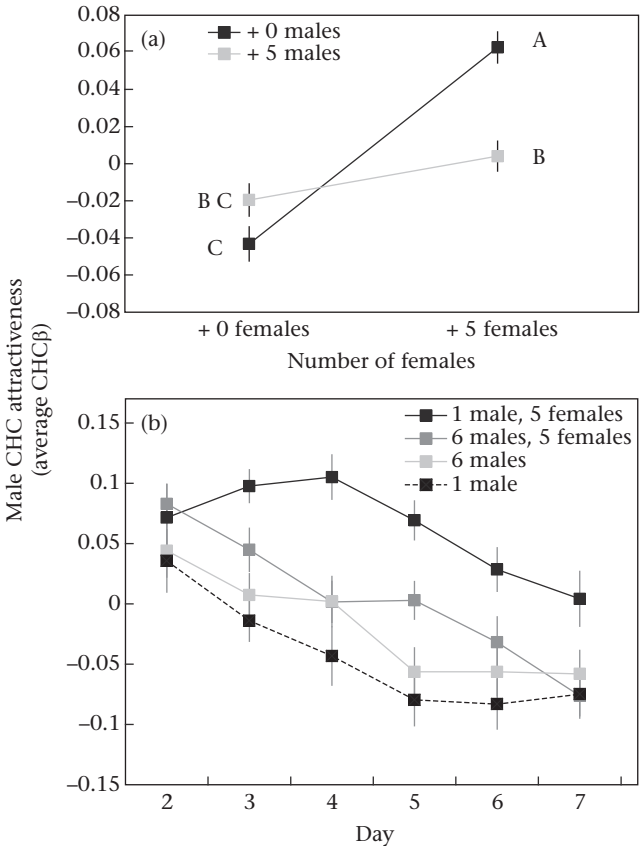

Figure 1. Effect of social environment on male sexually selected cuticular hydrocarbons (CHCβ). (a) Interaction plot of mean CHCβ across days for the factorial effects of the female and male social treatments. Mean ± SE for the CHCβs of a focal male in social environments with 0 vs 5 males and 0 vs 5 females added. Different uppercase letters indicate mean values that were significantly different at α <0.05 using Tukey HSD test of means. (b) Changes in male CHCβ across days with different social environments. Mean ± SE for replicate males collected on days 2–7 in each of the four combinations of male and female social environment: one male with five females, six males with five females, six males, and one male alone.

effect = −0.020, $F_{1,136} = 5.98$, $P = 0.0157$), but with a convex curvature such that it appeared to reach its lowest value on the morning of day 6 and then to subsequently increase, generating a significant quadratic term (day^2 effect = 0.026, $F_{1,136} = 18.15$, $P < 0.0001$; Fig. 2). Using only data collected after the application of the mating treatments (days 5–7), we detected no significant effect on CHCβ of mating status, day, day^2, or the interaction of these (Table 2). However, we also had an a priori interest in testing for potential short-term effects of mating on CHCs. In particular, in past binomial choice mating trials in *D. serrata*, CHCs were extracted immediately after mating. To determine whether mating itself alters male CHCs on this timescale, we conducted a planned comparison between virgin and mated males on the morning of day 5, immediately following the mating treatment. No significant difference in CHCβ was detected (two-sample t test: $t_{36} = -0.069$, $P = 0.945$).

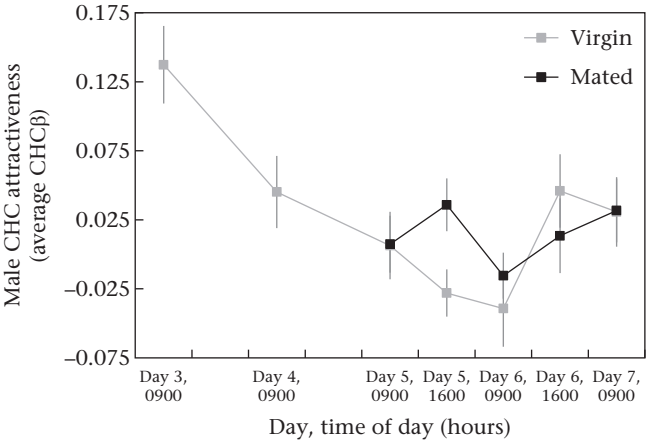

Figure 2. Effect of mating on male sexually selected cuticular hydrocarbons (CHCβ). Mean ± SE CHCβ on days 3–7, at 0900 or 1600 hours, for virgin and mated males.

While the analysis above provided no statistical justification for pursuing additional pairwise comparisons between virgin and mated males, it is possible that the significance of a short-term effect of mating was lost due to the inclusion of subsequent time points for which a difference no longer existed. Therefore, for the sake of completeness and to inform future research, we compared CHCβ between mating treatments separately for the remaining sampling times, using a standard significance level of α <0.05. In the afternoon of day 5, approximately 6–7 h after mating, mated males had significantly higher values of CHCβ compared to virgin males ($t_{37} = -2.73$, $P = 0.010$). However, the difference in CHCβ between virgin and mated males was no longer significant by the morning of day 6 ($t_{37} = -0.560$, $P = 0.579$) and remained nonsignificant in the two subsequent time points ($P > 0.55$ in each).

Part 3: The Effect of Interactions with Females on Male CHCs

To test the effects of visual/olfactory contact, we compared sexually selected CHCs between males held in different social environments (i.e. alone, with females, and with females on the other side of a permeable barrier). Social environment had a significant effect overall ($F_{2,259} = 32.65$, $P < 0.0001$), with CHCβ being higher in males held with free access to females compared to solitary males and those in which females were behind a barrier (Fig. 3). Male sexually selected CHCs also changed across days, but this effect varied among social environments, generating a significant day * treatment interaction ($F_{2,259} = 7.13$, $P = 0.0010$). In the unrestricted presence of females, CHCβ increased across days 3–5, while it tended to decrease when females were absent or behind a barrier. When tested separately by day, the effect of social environment was significant for days 4 and 5 ($P < 0.0001$ in both cases) and Tukey post hoc comparisons revealed significant differences only between the males in the unrestricted presence of females compared to the other two treatments (i.e. not between the solitary males and those with females behind a barrier).

In a separate assay we tested the effects of mounting and copulation on sexually selected CHCs, comparing CHCs in males held without females, with females that mated, with females in which mating was interrupted, and with glued females with which

Table 2
Statistical analysis of the effect of mating on the expression of male sexually selected cuticular hydrocarbons across days 5–7

Source	Estimate	$F_{1,189}$	P
Mating status	0.010	0.79	0.37
Day	0.005	0.27	0.60
Day*day	0.021	1.57	0.21
Mating status*day	−0.010	0.95	0.33
Mating status*day*day	−0.005	0.07	0.79

Results of an ANCOVA with unequal slopes that included mating status, day, day * day, and all possible two-way and three-way interactions as variables. No values were statistically significant at α <0.05.

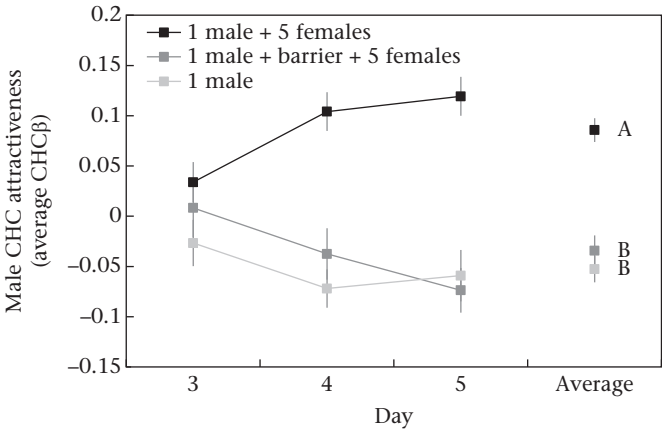

Figure 3. Effect of visual and olfactory contact with females on male sexually selected cuticular hydrocarbons (CHCβ). Mean ± SE for male CHCβ on days 3–5 in each of three social environments: one male with five females, one male housed with five females but separated by a barrier, and one male alone. Overall (i.e. across days) mean ± SE CHCβ for each social treatment are shown at the right. Different uppercase letters indicate mean values that were significantly different at α <0.05 using Tukey HSD test of means.

sperm transfer could not occur. These treatments had a significant effect on CHCβ overall ($F_{3,255} = 35.19$, $P < 0.0001$) and a Tukey's post hoc comparison revealed that males housed with females had significantly higher values than the other three social treatments (Fig. 4). Although none of the remaining three treatments differed significantly from one another in a post hoc comparison, males housed with glued females had intermediate values of CHCβ that differed significantly from the other treatments in pairwise *t* tests uncorrected for multiple comparisons ($P < 0.024$ in all cases).

DISCUSSION

In several insect species, cuticular hydrocarbons have been shown to be plastic in response to various internal and external stimuli, including aspects of an individual's social environment (Fedina et al., 2012; Kuo et al., 2012; Stinziano et al., 2015). Such contextual plasticity with respect to social environment in general, and during sexual interactions in particular (Kent et al., 2008; Petfield et al., 2005), suggests that CHCs may function as a means of chemical communication, consistent with their role as sexual displays that are the target of mate choice in some species (Chenoweth & Blows, 2005; Hunt et al., 2012). However, we lack a comprehensive understanding of the nature and extent of socially induced plasticity in any single species, and the impacts that this has on sexual interactions, including mating success, have received limited attention. Ultimately, if we want to understand the evolution of such plasticity, we will also need to know its consequences on fitness, including reproductive success.

Here we built on previous investigations in *D. serrata* to examine longer-term changes in CHCs and social plasticity in CHC expression, focusing on the combination of these traits that are important to male sexual fitness in this species. Our results provide direct evidence that the expression of sexually selected CHCs (i.e. CHCβ values) in males changes with age and that these changes are mediated by a male's social environment (Fig. 1). In particular, when single males were housed with multiple females, their expression of CHCβ increased across the first few days of their adult life, peaking at 4 days posteclosion, after which expression declined with increasing age. In contrast, sexually selected CHCs of males housed with other males, males housed with other males and females, and males housed alone all decreased across days, although in the absence of females the decline slowed and stopped in the last few days, generating significant convex curvature in these treatments. Overall, male CHCβ values were higher when males were housed with females than in any other social treatment, consistent with increased attractiveness of these males. However, there was an interaction between male and female social environment on average CHCβ: the effect of adding females was much greater when males were absent than when males were present. When females were present, additional males decreased male CHCβ, but when females were absent the presence of additional males had little effect.

Importantly, the changes in CHCs we observed across days, and the impacts of social environment on this, were repeatable. For example, the experiment in Part 2 included a treatment involving single, virgin males, replicating a treatment from Part 1. In these

Figure 4. Effect of mounting and copulation with females on male sexually selected cuticular hydrocarbons (CHCβ). Mean ± SE CHCβ of males exposed to each of four social treatments: one male housed with five females, one male repeatedly introduced to two glued females, one male repeatedly exposed to females but interrupted before successful intromission, and one male housed alone. Different uppercase letters indicate mean values that were significantly different at α <0.05 using Tukey HSD test of means.

males, CHCβ values decreased across the first few days, reaching a minimum early on day 6, and subsequently increased, generating a significant convex relationship very similar to that observed in Part 1 (see Figs 1, 2). Similarly, the first experiment in Part 3 included two social treatments from Part 1: single males and single males with unrestricted access to five females. Again, changes in CHC across days, and the effects on social environment on this, were very consistent in these two experiments (see Figs 1, 3). That the same patterns were observed in independent experiments, conducted at different times and using flies from different generations of the stock population, strongly infers that the changes were not the product of effects specific to an individual experiment (e.g. unidentified 'day' effects), but are rather general to the age of the males and the impact of these social environments.

Gershman et al. (2014b) characterized diurnal changes of CHCβ in 4-day-old males, revealing patterns consistent with the circadian cycling of sexually selected CHCs whenever other individuals were present (males and/or females). The effects of male and female social environment on average CHCβ across the 24 h of that study are entirely consistent with those of the current study. In particular, in both cases, males expressed the highest value of CHCβ when held singly with multiple females, and the lowest values when held in the absence of females, irrespective of the presence or absence of other males (see Figure 2 in Gershman et al., 2014; also see Fig. 1a). The combined results of both studies show temporal patterning of sexually selected CHCs both within and across days that varies with male and female social environment in a very similar way. In both studies, the social treatments altered the presence of females, and hence the potential for a male to encounter, court and potentially mate with them, and also varied the opportunity for males to compete with rival males. The precise cue or aspect of the social environment that produced these effects is therefore unclear and was the focus of our subsequent experiments.

In Part 2, we tested whether a single mating altered subsequent expression of sexually selected CHCs across days. Following a single mating, values of CHCβ in males appeared to increase within 6–7 h but then returned to values similar to that in the unmated (virgin) males by the following morning (Fig. 2). However, potentially due to the brief duration of this spike, this difference was not significant overall. This increase in CHCβ in the afternoon (1600 hours) following a morning (0900 hours) mating is nevertheless notable because Gershman et al. (2014b) found that under all social conditions except single males, CHCβ values were highest at 0900 hours and then decreased throughout the day. While this decrease in CHCβ values was associated with a decline in mating rate following the morning peak, mating still occurs throughout the afternoon (Gershman et al., 2014a, 2014b), providing the opportunity for males to benefit from an increase in their attractiveness. Our current results suggest that the effect of a recent (or potentially first) mating overrides the daily decline in CHCβ expression that would normally have occurred.

The analysis of the data from Part 2 also included a planned comparison of sexually selected CHCs between virgin and mated males when the latter were collected within minutes after mating. (CHCs were collected from the virgin males at the same time.) CHCβ values did not differ between these groups, confirming a previously untested assumption underlying the calculation of sexual selection gradients from mate choice trials in this species (i.e. Chenoweth & Blows, 2003, 2005; Hine et al., 2002; Rundle et al., 2008). If mating induced changes in CHCs on the timescale of minutes, for example due to their physical transfer between males and females (Yew, Dreisewerd, de Oliveira, & Etges, 2011), or rapid alterations by males themselves, such changes would confound the estimation of sexual selection gradients. Our results indicate that this is not a concern: not only was the difference nonsignificant, but the average CHCβ of the two groups of males was essentially identical (virgin: -0.017 ± 0.026; mated: -0.015 ± 0.019). The difference observed at 6–7 h does however suggest that extractions performed later after mating would be problematic, but how soon such changes arise following mating is not known.

While Part 2 suggested that a single mating may be sufficient to induce at least short-term changes in sexually selected CHCs, in Part 3 we attempted to isolate the specific cue that stimulates the male response to varying social environment. The combined results of the two experiments indicated that visual, olfactory and physical contact, including the actual mounting of females by males, were all insufficient to produce an increase in CHCβ expression, even with multiple days of exposure to these treatments. Instead, only males that were able to successfully transfer sperm increased their CHCβ values. This implies that males use successful mating as the cue to increase investment in a sexual signal that should enhance subsequent mating success. Results from Part 1 are consistent with this because males housed alone with five females would have mated more often than males housed with females and other competitor males, and the former expressed higher CHCβ values than the latter. In addition, males housed in the absence of females would never mate, irrespective of the presence or absence of other males, and CHCβ values were lowest and largely indistinguishable between these two groups of males (Fig. 1). Results in Part 2 also suggested an increase in male sexually selected CHCs following a single successful mating.

The expression of CHCβ is condition dependent (Delcourt & Rundle, 2011) and appears to be costly in that further exaggeration of sexually selected CHCs is opposed by natural selection (Hine et al., 2011). Therefore, when the opportunity for mating is low, context dependence of CHCβ expression may help to reduce these costs to males. However, it is surprising that CHCs expressed after a successful mating would be preferred by females, as it would seem advantageous for a male to express higher CHCβ values whenever potentially receptive females are around, whether or not he had managed to previously mate. It is possible that males are poor at distinguishing males from females, or distinguishing nonreceptive females from receptive females. If males commonly encounter nonreceptive females, for example, then a successful mating may provide the male with a more reliable indicator of the presence of receptive females. *Drosophila serrata* males exhibit a territory defence mating system in which males compete over, and aggressively defend, an area containing both food and egg-laying substrate for females (White & Rundle, 2014). Nonreceptive females may therefore often be encountered in nature if they come to lay eggs fertilized by a previous mating. However, why the presence of one receptive female might signal increased potential of encountering additional ones is unclear.

Alternatively, males may increase their CHCβ after mating (possibly as a physiological consequence of mating itself) as a signal that they have successfully mated, and females have evolved a preference for this combination of CHCs as a form of mate choice copying. That is, a preference for CHCβ may provide females with information about the quality of potential mates as revealed by their past mating success. In *D. melanogaster*, Mery et al. (2009) found that females preferred to mate with males that visually resembled those previously observed mating, although an independent test of mate choice copying in this species failed to find any effect (Auld, Punzalan, Godin, & Rundle, 2009). In *D. serrata*, binomial choice trials most commonly use virgin males (i.e. Chenoweth & Blows, 2003, 2005; Hine et al., 2002; Rundle et al., 2008). In these

trials, females may have been demonstrating preference for virgin males with CHCs that most closely resembled those of mated males (or males that were best able to mimic the CHCs of mated males).

Conversely, mated males may gain an advantage from mimicking the CHC profile of young virgin males. Our results from Parts 1 and 2 suggest that young, virgin males have CHCs associated with highest mating success. Although some studies have found that females prefer experienced males (McNamara, McKenzie, Elgar, & Jones, 2012; Milonas, Farrell, & Andow, 2011; Saleem, Ruggles, Abbott, & Carney, 2014), there are examples in the fly literature of female preference for virgin males. In Markow, Quaid, and Kerr (1978), *D. melanogaster* females were more likely to mate with virgin males than nonvirgin males in no-choice trials. Sivinski (1984) also found that Caribbean fruit fly *Anastrepha suspense* females prefer virgin males to mated males in competitive trials. Female flies that mate with virgin males may gain benefits from doing so. Whittier and Kaneshiro (1991) found that females that mated with virgin males produced more offspring than females that mated with nonvirgin males. In *D. serrata*, it is possible that females prefer virgin males, because of a fertility benefit from mating with them. However, the effect of *D. serrata* male mating status on female fertility has not been determined, and changes in CHC-based attractiveness with age in wild populations is currently unknown. It is also possible that female preference for virgin males in laboratory-reared flies may be a result of our stock maintenance routine, with adults only living for a short period (2–4 days) each generation before being discarded. The mating success of young males may therefore be paramount to their lifetime fitness, generating strong sexual selection to express highly attractive profiles as soon as possible after eclosion.

Our results have practical implications for the study of CHCs. At a minimum, when quantifying CHCs, the age and mating status of all individuals, as well as the time of day they are sampled, need to be controlled experimentally or included as covariates. In *D. serrata* males, the range of variation in CHCβ associated with the time of day was more than twice the average difference between chosen and rejected males detected in binomial mating trials (Gershman et al., 2014b). Temporal changes across days and the influence of social environment detected in the current study are of similarly large relative magnitude. Mating also induces changes in CHCs in at least males, and although extractions performed immediately following mating avoid such effects, later CHC extractions may not.

In conclusion, male expression of sexually selected CHCs is dynamic with respect to age and social context, these effects are repeatable, and the longer-term effects of social environment generally mirror their influence on shorter-term (i.e. diurnal) changes described in a previous study (Gershman et al., 2014b). Successful mating appears to be the cue males use in responding to the presence of females, although their response is also mediated by unknown cues indicating the presence of other males. At least some of the among-individual variation in CHCs may therefore be the result of potentially adaptive plasticity related to time and age and to differences in social environment, although a definitive test will require the fitness consequences of these changes to be determined.

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