# Mating has opposite effects on male and female sexually selected cuticular hydrocarbons

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In *Drosophila serrata* flies, there is female choice for male cuticular hydrocarbon (CHC) profiles and male choice for female CHC profiles. Furthermore, both males and females can alter their CHCs: when there is more opportunity for mating, males express combinations of CHCs preferred by females; however, females appear to change CHC profiles to avoid male harassment. In this study, I investigate the effect of number of matings (0–4) on male and female sexually selected CHCs. Mating caused males to express CHCs associated with higher male mating success. Thus, successfully mating males are likely to have increased future mating success. Conversely, females that mated more times expressed CHC profiles that were associated with lower female mating success. Females maximized their offspring production by mating more than once, but additional matings did not provide additional benefits. Furthermore, number of matings did not affect female survival. In total, these results suggest that females alter CHC expression to discourage male courtship when additional matings are not beneficial. In conclusion, plasticity in male and female CHC expression can both increase variance in male mating success and decrease variance in female mating success, driving the evolution of sexually selected chemical signals.

*Keywords*:

CHC

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Cuticular hydrocarbons (CHCs) are waxy, heavy molecules produced by oenocyte cells in the cuticle of invertebrate animals. CHCs protect terrestrial invertebrates from desiccation (Gibbs et al., 2003; Howard & Blomquist, 2005) and serve as a means of chemical communication in many species. In some insects, individuals can use the chemical profile of CHCs to distinguish between species (Alves et al., 2010; Blows & Allan, 1998) and between sexes within a species (Antony & Jallon, 1982).

Although CHC profiles have a genetic basis (Gosden et al., 2018; Rundle et al., 2009; Rundle et al., 2005; Steiger et al., 2013; Thomas & Simmons, 2008), physical and social environment can alter an individual's CHC profile (Otte et al., 2018). In some species of insects including *Drosophila* flies and *Teleogryllus* field crickets, CHCs serve a role in sexual selection. In *Teleogryllus oceanicus* field crickets, males that lose fights avoid further aggression from competitors by altering their CHCs to resemble subordinate males (Thomas & Simmons, 2011). Furthermore, male crickets from an acoustic environment without the songs of conspecific competitors express CHCs that are more attractive to females than males exposed to the songs of conspecific competitors, suggesting that males produce more attractive CHCs when they perceive less competition for mates (Thomas et al., 2011).

In *Drosophila serrata*, an Australian fruit fly, females prefer a specific combination of nine CHCs in males (Chenoweth & Blows, 2003, 2005; Gosden et al., 2018; Rundle & Chenoweth, 2011), including methyl-branched CHCs (Chung et al., 2014). Recent studies have demonstrated that for male *D. serrata*, access to mating opportunities can influence the CHC-based mating success of males (Gershman & Rundle, 2016, 2017; Gershman et al., 2014). Males express CHCs associated with higher

mating success during times of day in which matings are most likely (Gershman et al., 2014). Furthermore, males housed with more females per male have CHC profiles associated with higher mating success, as compared to the CHC profiles of males housed in lower female-to-male ratios (Gershman & Rundle, 2017). These results suggest that males express CHCs associated with higher mating success when mating opportunities are more likely and fail to invest in successful CHCs when matings are less likely. In experiments to determine what experiences males use to detect the presence of potential mates and alter their CHCs, Gershman et al. (2016) found that male visual and olfactory contact with females, repeated mounting of females without copulation and even repeated copulation without sperm transfer were not sufficient to cause males to express CHC profiles associated with higher mating success, as compared to males housed with females (Gershman et al., 2016). Elimination of these other variables suggests the possibility that successful mating itself is the cue that *D. serrata* males use to assess the availability of potential mates and alter their sexually selected CHCs to females. In this paper, I directly test whether number and timing of matings affect male expression of sexually selected CHCs.

Although previous papers have examined plasticity in male CHC expression (e.g. Everaerts et al., 2010; Kent et al., 2007), there is little previous research on plasticity in female CHCs and plasticity in sexually selected female CHCs. In *D. serrata* flies, there is mutual mate choice: by measuring intersex genetic correlations, Chenoweth and Blows (2003) identified that male *D. serrata* prefer females with a different combination of the same nine CHCs that females prefer in males. Gershman et al. (2014) found that female CHC profiles cycle throughout the day, with females having CHCs associated with low

mating success during times of day when most matings occur and CHCs associated with higher mating success at night, when few matings occur (Gershman et al., 2014). If females alter their CHCs in an adaptive way, this result suggests that females either do not need to bear the cost of expressing CHCs that attract males during times of day that males are already actively attempting to find mates, or females avoid expressing CHCs that attract males during times of day that mating occurs to avoid the costs associated with male harassment. In this paper, I determine whether females are able to alter their CHCs to increase or decrease their chance of mating when it is most beneficial to do so.

In Part 1, I determine the effect of mating on male sexually selected CHCs. Based on the results of previous studies on the effect of sex ratio on male sexually selected CHCs (Gershman & Rundle, 2016; Gershman et al., 2014), I predicted that males that mated more times would have CHCs associated with higher mating success.

In Part 2, I investigate the effect of mating on female CHCs. I predicted that as females mated more times, mating would be less beneficial and that females would downregulate CHCs associated with mating success. I determined the effect of number of matings on female sexually selected CHCs. I also investigated the effect of number of matings on female fertility and longevity to determine the optimal number of matings for female fitness. If females alter their sexually selected CHCs in an adaptive way, I predicted that females would express CHCs associated with lower mating success when additional matings reduced female fitness.

**<H1>METHODS**

All experiments used laboratory-adapted, outbred stock populations of *D. serrata* maintained at large population sizes (15–16 standard stock bottles) in nonoverlapping generations (see Rundle et al., 2006, for a full description) for more than 10 years. All flies were maintained at 25 °C on 12:12 h light:dark cycle, with the lights turning on at 0700 hours and turning off at 1900 hours daily. All mating trials took place during the light period of the day.

*<H2>Identifying Sexually Selected CHCs*

I focused my CHC analyses on the combination of nine CHCs that have been most strongly associated with increased mating success in *D. serrata* in previous studies (Chenoweth & Blows, 2003, 2005; Gershman et al., 2014; Rundle et al., 2009; Sztepanacz & Rundle, 2012). Although there exist additional CHCs in *D. serrata*, previous unpublished studies have found that adding these CHCs does not alter the resulting selection gradients (S. N. Gershman, personal observation). Using the nine CHCs that are most strongly associated with increased mating success reduces a complex data set to a single trait of biological interest. This approach has been frequently used in *D. serrata* (e.g. Gershman et al., 2014; Hine et al., 2014; McGuigan et al., 2011; Sztepanacz & Rundle, 2012). I performed binomial choice mating trials (one trial for female mate choice and one trial for male mate choice) and calculated the $\beta$ vector of directional sexual selection gradients (i.e. partial regression coefficients) by regression of mating success against CHC values (Lande & Arnold, 1983). This analysis estimates the linear combination of CHCs most strongly associated with male and female mating

success (Lande & Arnold, 1983). In female choice trials, a virgin female was placed in a vial with two virgin males. In male choice trials, a virgin male was placed in a vial with two virgin females. The flies were continuously observed until a successful mating occurred. Immediately after a successful mating was observed, the chosen and the rejected individuals were anaesthetized and their CHCs were extracted. Samples were analysed by gas chromatography and individual CHC profiles were determined by integrating the area below nine peaks, as described below. In this paper, as in previous studies (e.g. Gershman et al., 2014; Hine et al., 2014; McGuigan et al., 2011; Sztepanacz & Rundle, 2012), rather than using total concentrations of CHCs, relative concentrations of CHCs were used. This measure is robust against deviations in the quantity of CHCs extracted from each individual due to incidental variation in how long each individual is soaked and agitated, as well as how much hexane is removed from the sample when each fly is extracted from the vial. The relative concentrations of CHCs were calculated and transformed to logcontrasts (see below) before estimating selection gradients. The female selection gradient was based on CHC samples extracted in 2015, at the same time as the Part 2 experiments (Appendix, Table A1). The male selection gradient was based on CHC samples extracted in 2012 (see supplemental materials in Gershman et al., 2014,), using the same population of flies, mating trial methods, CHC extraction methods and the same GC machine and method (see supplemental materials in Gershman et al., 2014).

*<H2>Using Sexually Selected CHCs to Calculate CHCβ*

CHCβs for individual males were calculated as CHCβ = $\boldsymbol{\beta}^{\mathrm{T}}\boldsymbol{Z}$, where $\boldsymbol{Z}$ is the

vector of the CHCs that were measured for each male. An individual male's CHCβ score represents his value for the linear combination of CHCs most strongly associated with male mating success. In many previous papers CHCβ is interpreted as male CHC-based attractiveness (Gershman et al., 2014; Hine et al., 2002; Hine et al., 2011). This interpretation assumes that female choice alone determines the outcome of the binomial trials used to estimate $\beta$, but the role of male–male interactions in female *D. serrata* mate choice is unclear. *Drosophila serrata* females can choose which males to approach and can also dislodge unwanted copulations (Hoikkala & Crossley, 2000; S. N. Gershman, personal observation). Unlike in *Drosophila melanogaster, D. serrata* males do not display an extensive repertoire of physically aggressive behaviours (Chen et al., 2002) and male *D. serrata* CHCs are not associated with successful territory defence (White & Rundle, 2014). Nevertheless, it is possible that subtle male–male interactions contribute to variation in male mating success and CHCβ values. Similarly, it is unclear whether female–female interactions have an effect on variation in female mating success and female CHCβ values. Consequently, I refer to CHCβ as 'sexually selected CHCs' and interpret this trait more broadly as the combination of CHCs associated with greater mating success. Full details of the male choice $\beta$s used in this study can be found in Gershman et al. (2014). Details of the female choice $\boldsymbol{\beta}$s are included in the Appendix below.

*<H2>CHC Extraction and Analysis*

CHCs were extracted by aspirating each fly into 100 µl of hexane in a 400 µl

glass gas chromatography (GC) vial inserted within a 9 mm GC vial. Each fly was immersed in hexane for approximately 3 min. Vials were then agitated for 1 min with a vortex. Using forceps, flies were then immediately removed from vials and the vials were tightly sealed. GC vials were stored at –20 °C until analysis. Samples were analysed by gas chromatography (Sztepanacz & Rundle, 2012). I determined CHC profiles for individual flies by integrating the area under the nine peaks used in previous studies of *D. serrata*: (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 et al., 2003). Relative abundances of CHCs were calculated for each individual by dividing the area integrated under each of their CHC curves by the total area for all nine CHC curves. I used each individual's relative abundance of Z,Z-5,9-$C_{24:2}$ as the divisor for each of the individual's other eight relative CHC abundances, transforming the relative abundances of CHCs into eight logcontrast values (Aitchison, 1986). This transformation addresses the unit-sum constraint inherent in compositional data and has been used in past studies on this species (e.g. Blows & Allan, 1998; Chenoweth & Blows, 2003, 2005; Gershman et al., 2014; Rundle et al., 2009; Sztepanacz & Rundle, 2012). I used the Mahalanobis distance technique of JMP Pro 14 (SAS Institute, Cary, NC, U.S.A.; Sall et al., 2005) to remove a small number of multivariate outliers, most likely caused by integration errors or contaminated samples (see Appendix, Table A2, for numbers of outliers removed per treatment).

*<H2>Part 1: The Effect of Mating on Male CHCs*

Males were collected at eclosion using minimal $CO_2$ anaesthesia and housed individually in vials with yeast and yeast medium for food and moisture. At collection, males were randomly sorted into one of six treatment groups: zero matings, one mating, two matings (early), two matings (late), three matings or four matings (Table 1).

Starting 3 days after eclosion (when male flies are sexually mature and capable of reproduction), during 0900–1100 hours and 1300–1500 hours, males were transferred from their home vial to a fresh vial of unyeasted medium. Depending on the male's assigned treatment (Table 1), the fresh vial contained either a 3–5-day-old virgin female or no female. Males were continuously observed until they mated. Any copulation event with apparent intromission that lasted longer than 60 s was counted as a mating. If a mating did not occur within 30 min, the female was replaced with a different virgin female.

Mating opportunities were scheduled for twice a day, for a maximum of four matings over 3 days. Although males are capable of mating more often, this level of mating was chosen because it can be achieved by nearly all healthy males (Appendix, Table A2). Furthermore, because age affects male CHC profiles (Gershman & Rundle, 2016) and there is indirect evidence that time since last mating can affect CHCs (Gershman & Rundle, 2016), matings were clustered over a short period and timed so that the last mating occurred within 1 h of CHC extraction. To experimentally confirm that time since last mating can affect male sexually selected CHCs, in one treatment ('two matings late') males' last mating was 7 h before CHCs were extracted, while in another treatment ('two matings early') males' last mating was 31 h before CHC extraction. At 1600 hours on day 5, CHCs were extracted from all males, alternating

extractions among mating treatments to avoid any unintended effects of time of day or time since last mating on male CHCs. All CHC extractions were completed by 1730 hours.

The few males that failed to mate their assigned number of matings were not excluded from the statistical analyses (Appendix, Table A2). This prevented poor-quality males from being differentially removed from treatments with more matings. However, because injury and death can affect CHC profiles, individuals that died or had visible abdominal damage were removed from the study and replaced by other individuals. For each treatment level, males were assigned a number from 1 to 50 at eclosion. CHCs were extracted from the first 39–40 males within each treatment that were not dead or damaged. Based on a cutoff value of 4 Mahalanobis outlier distances, three individuals were dropped from the statistical analysis. Thus, 38–40 males from each treatment were retained in the statistical analyses (see Appendix, Table A2, for details).

*<H2>Part 2a: The Effect of Mating on Female CHCs*

Females were collected at eclosion using minimal $CO_2$ anaesthesia and housed individually in vials with yeast and yeast medium for food and moisture. At collection, females were randomly sorted into one of five treatment groups: zero matings, one mating, two matings, three matings or four matings (Table 1). Methods for female matings were parallel to the methods previously described for males in Part 1, however, the 'two matings early' treatment was not included in this experiment.

At eclosion, females were sorted into treatment groups. Females that failed to

mate their assigned number of matings were retained in the study. This was done to avoid having individuals self-select their treatments, which could cause poor-quality individuals to be differentially removed from the treatments with more matings. CHCs were analysed for 40 females in each treatment group. Based on a cutoff value of 4 Mahalanobis outlier distances, three individuals were dropped from the statistical analysis. Thus, 38–40 females from each treatment were retained in the statistical analyses (see Appendix, Table A2, for details).

*<H2>Part 2b: The Effect of Number of Matings on Female Fecundity and Longevity*

To assay female fecundity and longevity, a separate set of females experienced the same housing conditions and schedule of matings as in Part 2a (Table 1). Starting on the first day of scheduled matings, each female was individually housed in a vial that contained yeasted medium for eating and laying eggs. Every 3–4 days, each female was transferred into a fresh vial. All used vials were incubated until all viable pupae had eclosed, and all eclosing adults were counted. Two weeks after matings occurred, all females stopped laying eggs, potentially due to lack of stored sperm. After this time, each female was transferred to a vial of fresh food once per week. Throughout the study, each female was checked every 48 h to determine date of death. All females were monitored until 44 days after adult eclosion, at which time there is a dramatic drop in female survival (S. N. Gershman, unpublished data) and females are unlikely to be able to add to their lifetime reproductive success. Because both female fecundity and survival were non-normally distributed and could not be transformed to normality, Wilcoxon–Kruskal–

Wallis rank sums tests were used to determine the effect of number of matings on female fecundity and longevity, and pairwise comparisons were performed using the Wilcoxon method at a level of $\alpha < 0.05$. I also determined product-limit survival fit using the nonparametric Kaplan–Meier estimator to assess differences among treatments in survival time. No virgin females produced any larvae, pupae or eclosing adults. Consequently, the virgin female treatment was excluded from the statistical analysis of the effect of number of matings on female fecundity.

**<H1>RESULTS**

*<H2>Part 1: The Effect of Mating on Male CHCs*

Mating had an effect on male sexually selected CHCs, with males that mated more times having CHCβs associated with higher male mating success than males that mated fewer times (ANOVA: $F_{3,228} = 5.96$, $P < 0.0001$; Fig. 1).

Time since last mating had an effect on male sexually selected CHCs. Males that mated 7 h before CHCs were extracted had higher CHCβs than virgin males; males that mated 31 h before CHCs were extracted had CHCβs indistinguishable from virgin males' (Tukey HSD > 0.05). However, there was not a statistically significant difference in CHCβs among twice-mated males that mated 7 h before CHCs were extracted and twice-mated males that mated 31 h before CHCs were extracted (Tukey HSD > 0.05).

*<H2>Part 2a: The Effect of Mating on Female CHCs*

In females, mating had the opposite effect on sexually selected CHCs than with males: females that mated more times had CHCβs associated with lower mating success as compared to females that mated fewer times (ANOVA: $F_{4,192} = 5.79$, $P = 0.0002$; Fig. 2).

*<H2>Part 2b: The Effect of Number of Matings on Female Fecundity and Longevity*

Number of matings had an effect on the number of adult offspring that females produced (Wilcoxon–Kruskal–Wallis test: $\chi^2_3 = 18.3$, $P = 0.0004$), with females that mated more than once producing more offspring than females that mated only once (Wilcoxon, at a level of $\alpha < 0.05$; Fig. 3).

Number of matings had an effect of female longevity (Wilcoxon–Kruskal–Wallis test: $\chi^2_4 = 11.0$, $P = 0.027$; Wilcoxon product-limit survival fit: $\chi^2_4 = 10.1$, $P = 0.039$), with virgin females living longer than females that had mated (Wilcoxon, at a level of $\alpha < 0.05$; Fig. 4a). Furthermore, when virgin females were dropped from the analysis, there was not a statistically significant effect of number of matings on female longevity (Wilcoxon–Kruskal–Wallis test: $\chi^2_3 = 0.26$, $P = 0.97$; Wilcoxon product-limit survival fit $\chi^2_3 = 0.26$, $P = 0.97$; Fig. 4b), suggesting that mating, but not number of matings, has a negative effect on female longevity.

**<H1>DISCUSSION**

The results of this study demonstrate that mating causes male *D. serrata* to have CHC profiles that are associated with higher mating success. This result supports and extends previous experimental results that access to females causes male *D. serrata* to enhance their sexually selected CHCs (Gershman & Rundle, 2016, 2017). Gershman and Rundle (2016) compared the sexually selected CHC profiles of males housed with females to the CHCs of males with visual and olfactory but not tactile access to females, males with repeated opportunities to mount females but not copulate and males with repeated opportunities to mount and copulate with females but not transfer ejaculate. Female visual and olfactory cues, mounting and copulation treatments were not sufficient to cause males to alter their CHCs; only full access to cohabitating females over several days caused males to develop CHCs associated with higher mating success (Gershman et al., 2016). The current study completes the story, demonstrating that the cue that males use to alter their sexually selected CHCs is mating itself.

I investigated the effect of the timing of mating on male sexually selected CHCs by including a treatment that extended the time between a male's last mating and when CHCs were extracted. After 31 h since mating, a male's CHC profile appears to return to a profile that resembles a virgin male, suggesting that the effect of mating on male sexually selected CHCs decays over time. This effect is indirectly supported by previous studies: males housed in male-biased sex ratios had CHCs associated with lower mating success than males housed in female-biased sex ratios (Gershman & Rundle, 2017). If males housed in male-biased sex ratios experience both fewer matings and longer intervals between matings, this could cause males to express CHCs associated with lower mating success.

For male manipulation of sexually selected CHCs to be adaptive, males should become more attractive to females when there are fitness benefits for doing so. Previous studies have demonstrated that males housed alone have CHCs associated with lower mating success than males housed with access to females (Gershman & Rundle, 2017; Gershman et al., 2016), and during times of day when most matings occur, males had CHCs associated with higher mating success (Gershman et al., 2014). It is surprising that males are unable to use the physical presence of females to predict the opportunity for future mating opportunities. The results of this paper suggest that males require the experience of successfully transferring ejaculate to determine whether there will be opportunities to reproduce in the future. Male *Drosophila* routinely engage in same-sex sexual behaviour, potentially because males struggle to differentiate between sexes (Dukas et al., 2010; Macciano et al., 2018). The results of this study suggest the possibility that only by successfully transferring sperm can male *D. serrata* be assured that fertile females are present. Alternatively, there is evidence in *Caenorhabditis* species and *D. melanoga*ster that detecting the presence of females but being unable to mate may lower male fitness (García-Roa et al., 2018; Gendron et al., 2014; Harvanek et al., 2017). If this is the case in *D. serrata*, the inability of males to detect unavailable females may itself be adaptive.

Moreover, the results of this study suggest a re-evaluation of the binomial female choice trials that are used to determine what combination of CHCs in virgin males is attractive to females: it is possible that in binomial trials, females prefer virgin males with CHCβs that best resemble those of mated males. Thus, the vector of $\beta$s generated by the trials may actually predict the combination of CHCs that most closely resembles a mated

male. It is possible that by choosing males that appear to have previously mated, females are copying the choices of other females. There is evidence that female *D. melanogaster* copy the mate choice of other females (Dagaeff et al., 2016; Mery et al., 2009). Female mate choice copying has not been previously found in *D. serrata* (Auld et al., 2009), however, mate choice copying is a plastic behaviour that may occur in some environments but not others (Dagaeff et al., 2016). Although there is no previous research on mate choice copying via CHC detection, in *Gryllodes sigillatus* crickets, there is evidence that females use CHCs on males to detect male mating history (Weddle et al., 2012). Thus, the results of this paper suggest a novel avenue for mate choice copying via chemical communication.

In contrast to males, female *D. serrata* that mated expressed CHCs associated with lower mating success than virgin females. This result is consistent with Gershman et al. (2014), in which female *D. serrata* had CHCs associated with the lowest mating success during times of day when most matings occurred and had CHCs associated with the highest mating success at night, when few matings occurred. If females suffer negative fitness effects from male harassment (Maklakov et al., 2013; Teseo et al., 2016), it could be beneficial for females to express less attractive CHCs when additional matings do not increase female fitness. In Gosden et al. (2018), when artificial selection on 'attractive' female *D. serrata* CHCβs was relaxed, females reverted to pre-selection CHCβs, suggesting a cost to maintaining these CHC profiles. It is possible that increased male harassment is this cost.

In follow-up experiments, I found that females that mated twice had more eclosing offspring than females that mated once; however, additional matings did not

increase female reproductive output. Mating decreased female survival versus no mating; however, females that mated one to four times did not differ in survival. In total, with the schedule of matings used in this experimental design, females gained maximum fitness benefits from mating twice. Although there was a tendency towards female sexually selected CHCs declining after two matings, this result was not statistically significant. Thus, although I did not find sufficient evidence to claim that females downregulate their sexually selected CHCs when they have mated the optimal number of times, it is possible that if my experimental design included more matings or matings over a longer span of time, a stronger pattern may have emerged.

In this study, the change in male CHCs after mating is unlikely to be due to physical transfer of CHCs from females. Several previous studies have documented CHC transfer. In crickets, female CHCs transferred to males during mating affect a male's future sexually selected CHCs (Weddle et al., 2012). In *Drosophila*, it is possible to intentionally transfer CHCs from donor flies to recipient flies, but this procedure requires an extended period of physical contact with many (25+) donor individuals trapped with one recipient within a confined space (Dyer et al., 2011). Conversely, there is evidence that mating alone is insufficient to transfer CHCs in *D. serrata*: when CHCs are extracted immediately after a single mating, mated males do not differ in their CHC profiles from virgin males, but several hours after mating, virgin and mated male CHCβs begin to diverge (Gershman & Rundle, 2016). Thus, in this study, any changes in male CHCs after mating are due to differences in male CHC expression, not transfer. There are no known experimental tests of CHC transfer from *D. serrata* males to females, so it is not possible to rule out the possibility that transfer has an influence on female CHC profiles. In *D.*

*melanogaster*, the transfer of 11-cis-vaccenyl acetate (Ejima et al., 2007) and oxygenated hydrocarbons (Yew et al., 2009) from males to females may inhibit male courtship with previously mated females. It is possible that transfer of these other molecules also influences the behaviour of male *D. serrata.* But regardless of the source of the CHCs, mating still has the potential to influence female CHC-based interactions with males.

Note, however, that the experiments in this paper were performed on outbred *D. serrata* that had lived for many generations under laboratory conditions. It is likely that these *D. serrata* represent only a subset of all possible genotypes and phenotypes that exist in nature. It would be valuable for future studies to determine how field-based mating rates and timings of matings affect male and female CHCβs. Furthermore, the experiments in this paper focus on a single species of *Drosophila*. However, CHCs have been identified in a multitude of arthropods (e.g. Grinsted et al., 2011; Otte et al., 2018; Zhang et al., 2011), and in a subset of these species, sex-specific and sexually selected CHCs have been identified (e.g. Berson & Simmons, 2019; Thomas et al., 2011). The finding that mating affects male and female sexually selected CHCs in *D. serrata* raises the possibility that mating may also affect CHC expression and behaviour in other sexually reproducing arthropods.

The results of this paper have implications for the evolution of CHCs by sexual selection. There is ample evidence that males that inherit attractive CHC profiles have higher mating success than other virgin males (e.g. Gershman et al., 2014; Hine et al., 2014; McGuigan et al., 2011; Sztepanacz & Rundle, 2012). As this paper demonstrates, males that are able to mate can increase their expression of CHCs associated with high mating success. Males that have the misfortune of inheriting CHC profiles associated

with low mating success are less likely to mate and will retain their unsuccessful CHCs. So, there is a synergistic effect of genetics and experience on male CHC-based mating success. Furthermore, because mating causes females to express less preferred CHC profiles, female mating success is self-limiting. This negative feedback loop may contribute to lower variance in mating success among females. Thus, the effect of mating experience on CHCs can contribute to more intense sexual selection.

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## Appendix

*<H2>Methods for Mating Trials to Estimate the Male Selection Gradient*

Mating trials followed the design of previous studies (Gershman et al., 2014). At eclosion, males were collected using light $CO_2$ anesthesia and housed in vials of eight males. Females were collected at eclosion and housed in vials of 12 females.

In each trial, two 4-day-old virgin females from different housing vials were simultaneously added to a vial containing a single virgin 4-day-old male. Vials were observed continuously until a successful mating occurred, at which point the chosen and rejected females were anaesthetized and CHCs were immediately extracted. Mating trials were conducted from 0900 to 1030 hours. I performed 449 trials to assess male choice of female partners.

**Table A1**

The male selection gradient

| CHC | $\beta$ | $P$ |
|---|---|---|
| (Z,Z)-5,9-$C_{25:2}$ | 0.436513427 | 0.6278 |
| (Z)-9-$C_{25:1}$ | 0.098394419 | 0.7839 |
| (Z)-9-$C_{26:1}$ | -0.474421185 | 0.2465 |
| 2-Me-$C_{26}$ | -0.328388388 | 0.4729 |
| (Z,Z)-5,9-$C_{27:2}$ | -0.943440127 | 0.0053 |
| 2-Me-$C_{28}$ | 0.57267544 | 0.4716 |
| (Z,Z)-5,9-$C_{29:2}$ | 0.724126896 | 0.0080 |
| 2-Me-$C_{30}$ | -0.279284842 | 0.4871 |

The male selection gradient was estimated using standard least squares. However, statistical significance was determined using logistic multiple regression because mating success was binomially distributed. This was done using a generalized linear model with a logistic link function, fitted via maximum likelihood, as implemented in JMP Pro 14 (SAS Institute, Cary, NC). Male directional sexual selection was statistically significant ($\chi^2_8 = 18.9$, $P$=0.0153, $R^2_{adj} = 0.023$, $N = 449$).

**Table A2**

Sample sizes and causes of exclusion for flies used in CHC analyses

| Sex | Number of matings | Number of flies that did not mate the assigned number of times (Number of times flies actually mated) | Initial sample size | Number of flies excluded due to death | Number of flies excluded due to morphological damage | Number of GC samples | Number of flies excluded after outlier analysis | Final sample size |
|---|---|---|---|---|---|---|---|---|
| M | 0 | — | 41 | 0 | 2 | 39 | 0 | 39 |
| M | 1 | 1 (0) | 39 | 0 | 0 | 39 | 0 | 39 |
| M | 2 late | 1 (1) | 42 | 1 | 2 | 39 | 1 | 38 |
| M | 2 early | 2 (1) | 40 | 0 | 0 | 40 | 1 | 39 |
| M | 3 | 1 (2) | 41 | 0 | 1 | 40 | 1 | 39 |
| M | 4 | 2 (3) | 42 | 1 | 1 | 40 | 0 | 40 |
| F | 0 | — | 40 | 0 | 0 | 40 | 2 | 38 |
| F | 1 | 0 | 40 | 0 | 0 | 40 | 0 | 40 |
| F | 2 | 0 | 40 | 0 | 0 | 40 | 0 | 40 |
| F | 3 | 0 | 40 | 0 | 0 | 40 | 1 | 39 |
| F | 4 | 0 | 40 | 0 | 0 | 40 | 0 | 40 |

**Table 1**

Timing of mating treatments ('mating') and CHC extractions ('CHCs') for Part 1 and Part 2a experiments

| | Sex | Number of matings | Day/time of day (hours) | | | | |
|---|---|---|---|---|---|---|---|
| | | | Day 3 1300 | Day 4 0900 | Day 4 1300 | Day 5 0900 | Day 5 1600 |
| Part 1 experimental design | Males | 0 | | | | | CHCs |
| | Males | 1 | | | | Mating | CHCs |
| | Males | 2 early | Mating | Mating | | | CHCs |
| | Males | 2 late | | | Mating | Mating | CHCs |
| | Males | 3 | | Mating | Mating | Mating | CHCs |
| | Males | 4 | Mating | Mating | Mating | Mating | CHCs |
| Part 2a experimental design | Females | 0 | | | | | CHCs |
| | Females | 1 | | | | Mating | CHCs |
| | Females | 2 | | | Mating | Mating | CHCs |
| | Females | 3 | | Mating | Mating | Mating | CHCs |
| | Females | 4 | Mating | Mating | Mating | Mating | CHCs |

**Figure 1.** The effect of male mating on male sexually selected CHCs (mean ± 1 SE). Letters indicate which pairwise comparisons are statistically significantly different using Tukey HSD.

**Figure 2.** The effect of female mating on female sexually selected CHCs (mean ± 1 SE). Letters indicate which pairwise comparisons are statistically significantly different using Tukey HSD.

**Figure 3.** The effect of female mating on number of eclosing offspring (mean ± 1 SE). Letters indicate which pairwise comparisons are statistically significantly different using the nonparametric Wilcoxon method at a level of $\alpha<0.05$).

**Figure 4.** (a) The effect of number of matings on female survival over 44 days. (b) Average number of days surviving (mean ± 1 SE). Letters indicate which pairwise comparisons are statistically significantly different using the nonparametric Wilcoxon method at a level of $\alpha<0.05$).

Figure 1.

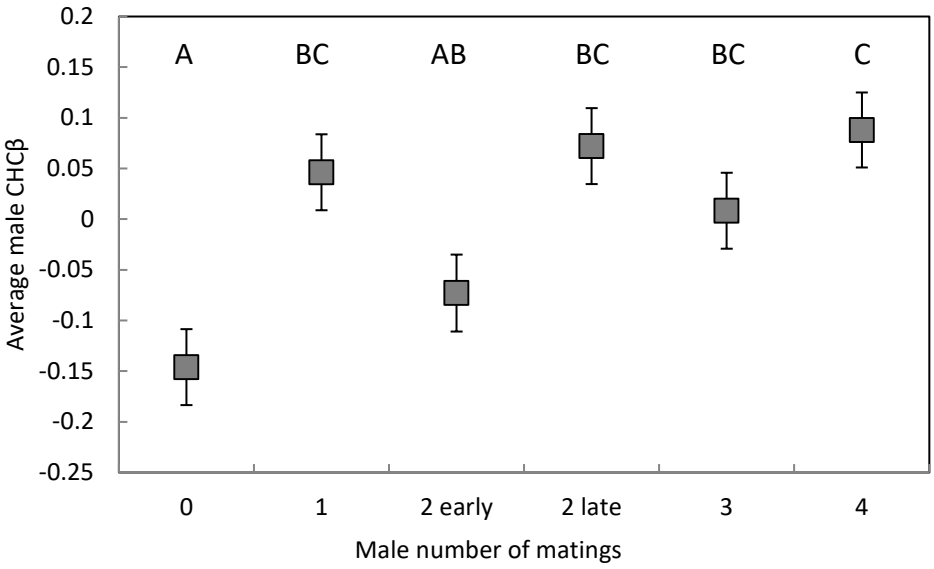

0.2
0.15
0.1
0.05
0
-0.05
-0.1
-0.15
-0.2
-0.25
Average male CHCβ
A
BC
AB
BC
BC
C
0
1
2 early
2 late
3
4
Male number of matings

Figure 2.

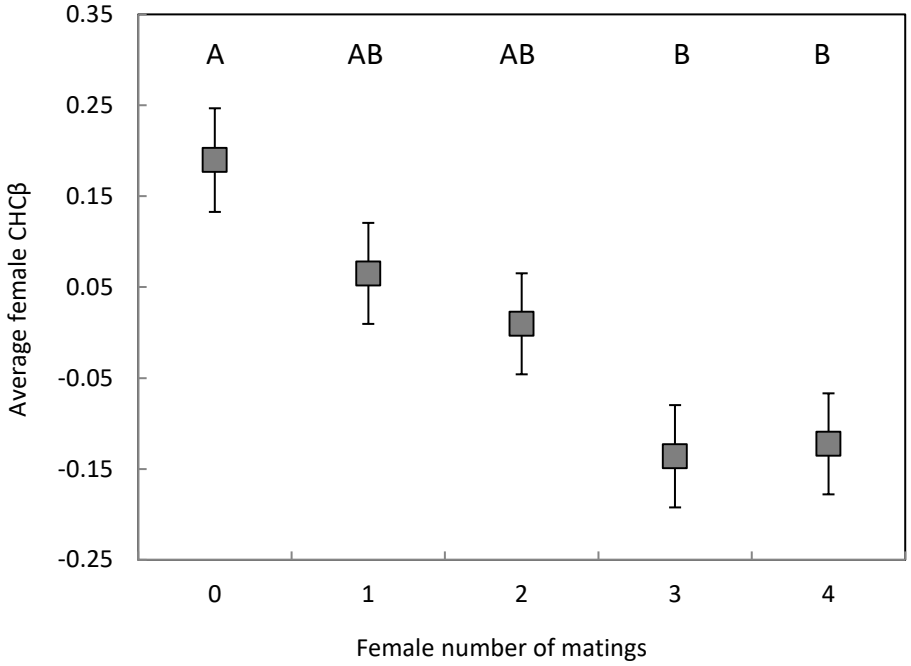

0.35
0.25
0.15
0.05
-0.05
-0.15
-0.25
Average female CHCβ
A
AB
AB
B
B
0
1
2
3
4
Female number of matings

Figure 3.

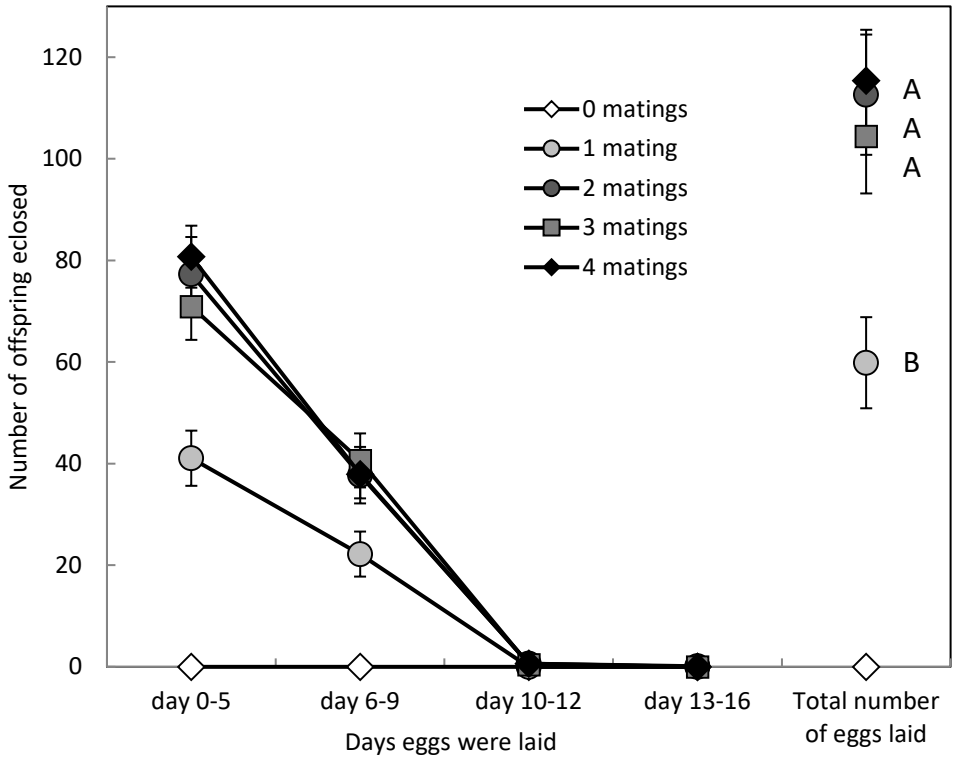
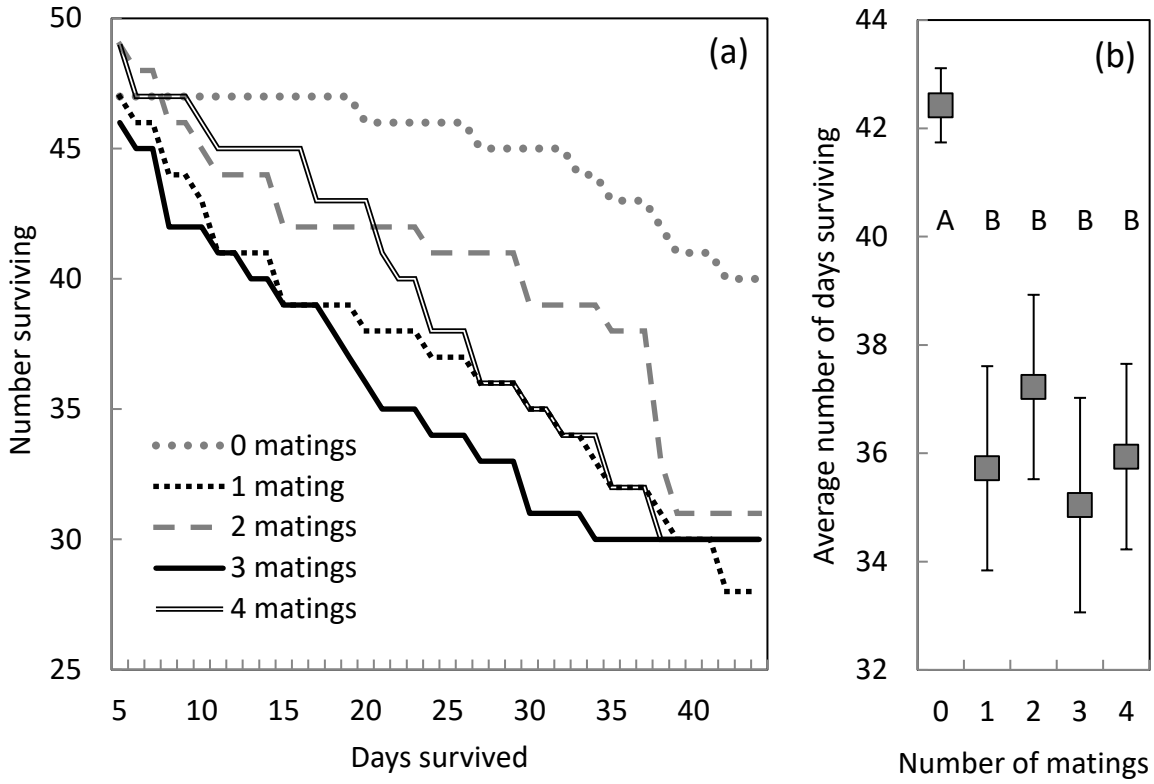

120
100
80
60
40
20
0
Number of offspring eclosed
0 matings
1 mating
2 matings
3 matings
4 matings
A
A
A
B
day 0-5
day 6-9
day 10-12
day 13-16
Total number of eggs laid
Days eggs were laid

Figure 4.


(a)
Number surviving
Days survived
0 matings
1 mating
2 matings
3 matings
4 matings
(b)
Average number of days surviving
Number of matings
A B B B B