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Within-population variability in a moth sex pheromone blend: genetic basis and behavioural consequences

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Evolutionary diversification of sexual communication systems in moths is perplexing because signal and response are under stabilizing selection in many species, and this is expected to constrain evolutionary change. In the moth *Heliothis virescens*, we consistently found high phenotypic variability in the female sex pheromone blend within each of four geographically distant populations. Here, we assess the heritability, genetic basis and behavioural consequences of this variation. Artificial selection with field-collected moths dramatically increased the relative amount of the saturated compound 16:Ald and decreased its unsaturated counterpart Z11-16:Ald, the major sex pheromone component (high line). In a cross between the high- and low-selected lines, one quantitative trait locus (QTL) explained 11–21% of the phenotypic variance in the 16:Ald/Z11-16:Ald ratio. Because changes in activity of desaturase enzymes could affect this ratio, we measured their expression levels in pheromone glands and mapped desaturase genes onto our linkage map. A delta-11-desaturase had lower expression in females producing less Z11-16:Ald; however, this gene mapped to a different chromosome than the QTL. A model in which the QTL is a trans-acting repressor of delta-11 desaturase expression explains many features of the data. Selection favouring heterozygotes which produce more unsaturated components could maintain a polymorphism at this locus.

1. Introduction

Sexual communication signals and responses can be influenced by different types of selection. When under sexual selection, mate choice often exerts directional selection on signal traits [1–5], which may cause substantial variation in the signals. When different phenotypes have similar mating success *within* one population, i.e. when there are alternative mating strategies, extremes may be selected for, causing discontinuous variation and multiple phenotypes. This has been mostly described in fish and lizard species where different colour morphs exist in the same population [6], and in birds and insects with different mating strategies (e.g. sneaker males) [7–9] or with differential attraction by predators [10]. The existence of multiple phenotypes within one population can be maintained through balancing selection, e.g. heterozygote advantage or frequency-dependent selection [11,12].

Sexual communication signals and responses can also be under stabilizing selection [2,13,14]. Preference for intermediate phenotypes is generally found in acoustic signals in frogs, crickets and grasshoppers [15–19] and in chemical signals in moths [20–26]. Stabilizing selection on sexual communication is likely

to evolve when sympatrically occurring, closely related species have very similar mating signals, and there is thus a risk of interspecific attraction; i.e. when the predominant role of sexual communication is species recognition [18,27–29]. Stabilizing selection depletes genetic variation through selection against extremes [13,30–32].

In most moth species, females attract males from a distance using volatile, species-specific sex pheromones that usually consist of two or more compounds [33]. Geographical variation has been found both in female signals [34–37] and male responses [22,23,38]. This suggests that moth sex pheromone signals and behavioural responses are affected by local environmental factors, although this variation may also be due to environmentally independent sexual selection. However, variability in chemical mating signals in moths *within* populations has been largely ignored, likely due to the presumption of stabilizing selection, because moth sexual communication has been found to be important for species recognition. Pheromone races such as described in *Ostrinia nubilalis* [39,40] are already reproductively isolated and hence are not considered to be within-population differences. To our knowledge, the only example of a polymorphism in volatile chemical signals occurring within as well as among insect populations exists in the bark beetle *Ips pini* [10,41]. Males of this species produce both enantiomers of ipsdienol, and extreme ratios attract more females than intermediate ratios: evidence of disruptive selection on the enantiomer ratio [10,41].

Here, we describe and analyse the phenomenon of within-population variation found in the sexual signal of the noctuid moth *Heliothis virescens*. The female sex pheromone of this species consists of (Z)-11-hexadecenal (Z11–16:Ald) as the critical major component and (Z)-9-tetradecenal (Z9–14:Ald) as the critical secondary component [42]. These two components are essential to attract *H. virescens* males [42–44]. Additional compounds in the pheromone gland are tetradecanal (14:Ald), hexadecanal (16:Ald), (Z)-7-hexadecenal (Z7–16:Ald), (Z)-9-hexadecenal (Z9–16:Ald) and (Z)-11-hexadecen-1-ol (Z11–16:OH) [42–44]. Previous studies all report Z11–16:Ald as the most abundant component, while the abundance of its saturated counterpart 16:Ald normally is only 8–46% of that of Z11–16:Ald on average (see the electronic supplementary materials and methods and table S1). None of these earlier studies reported within-population variation among females in the relative proportion of pheromone components. However, we found substantial within-population variation in these proportions, most notably in Z11–16:Ald and 16:Ald. In some females, we even found 16:Ald to be much more abundant than Z11–16:Ald. This variation was puzzling, because 16:Ald plays only a minor role in the attraction of *H. virescens* males [44–46]. To gain insight into the possible causes and consequences of this variation, we selected for two extreme phenotypes to assess heritability, we determined the genetic basis of the pheromone phenotypes and we assessed the attraction of males to the different female phenotypes in the field.

2. Material and methods

(a) Field sampling

Third to fifth instar larvae of *H. virescens* were collected annually from at least two of four different field sites in 2005, 2006, 2007 and 2008 (see table 1). All larvae were shipped to the laboratory

Table 1. Frequency of *H. virescens* females with ratio of 16:Ald to Z11–16:Ald ≥ 1 (unusual phenotype) in all field-collections. Fraction of females in each sample exhibiting a ratio of 16:Ald to Z11–16:Ald of more than or equal to 1 in extracts of the pheromone gland. In parenthesis, the number of females with a ratio of more than or equal to 1 divided by the total number of females phenotyped. Sampling sites are North Carolina: 35°39'58" N, 78°30'36" W; Mississippi: 33°25'04" N, 90°54'37" W; Texas: 30°38'22" N, 96°21'39" W; Eastern Mexico: 22°13'02" N, 97°50'40" W.

| | North Carolina | Mississippi | Texas | Eastern Mexico |
|------|----------------|-------------|-------------|----------------|
| 2005 | 0.11 (5/46) | 0.20 (4/20) | 0.16 (9/57) | 0.32 (14/44) |
| 2006 | | 0.06 (1/18) | 0.22 (5/23) | 0.12 (2/17) |
| 2007 | 0.11 (7/62) | 0.10 (2/20) | 0.14 (8/49) | |
| 2008 | 0.07 (2/29) | 0.09 (4/45) | | |

at NCSU and reared to adults on artificial Corn–Soy Blend diet. Pupae were sexed and daily checked for adult emergence. Two- to 5-day-old virgin females were used for pheromone analysis (described in the electronic supplementary material).

(b) Selection lines and heritability analysis

To assess the heritability of the female pheromone composition, we constructed 'high' and 'low' selection lines, where *high* refers to an unusually high ratio (greater than or equal to 1) of 16:Ald to Z11–16:Ald (the rare condition) and *low* refers to a low ratio (less than 1, the common condition). The starting population was from field-collected eggs in 2006 in North Carolina which were reared to adulthood in the laboratory (see [35]). In generation 0, 60 single-pair crosses of these individuals were sorted based on the mother's ratio of 16:Ald to Z11–16:Ald. Offspring of the six families whose mothers had the highest ratio constituted generation 1 of the high line. These were reared to adults and used to establish single-pair crosses, from which the six highest mothers were chosen and their offspring carried forward; this process was repeated for a total of eight generations of selection. The low line was developed in a similar fashion, starting with the six mothers with the lowest ratio and subsequently selecting the six lowest females of each generation (see the electronic supplementary material for a more detailed description).

Heritability of pheromone composition was estimated (i) as heritability based on the response to selection (realized heritability), and (ii) as heritability estimated from the resemblance between relatives, using an animal-model framework (see the electronic supplementary material for a detailed description). Least-squares estimates based on trait averages were used for realized heritability (see the electronic supplementary material). Additive genetic and environmental variance components for the pheromone data were estimated separately for the high and low line using an animal model based on the Bayesian implementation of generalized linear mixed-effects models provided by the R package MCMCglmm [47]. In contrast to the commonly employed least-squares estimates, this mixed-model analysis makes full use of the covariance structure not just between trait averages, but between all individuals measured during the course of the experiment, based on the available pedigree information. These animal-model-based estimates of components of variance generally represent the unselected base population, because the cross-generational relationship matrix fully accounts for effects of non-random mating, selection-induced changes in gametic-phase disequilibrium and allele-frequency changes by drift over the generations [48–50]. See the electronic supplementary material for a detailed description of this analysis.

(c) Quantitative trait locus analysis of pheromone variability in a backcross family

To determine the genetic basis of the intraspecific variability in the sex pheromone of *H. virescens*, we conducted quantitative trait locus (QTL) analysis using the high and low lines. After six generations of selection, we hybridized females from the low line with males of the high line, again in single-pair-matings. Females from the F₁ family with most offspring were mated with males of the high or low lines, in single-pair-matings. The offspring of two families from backcrosses to low were phenotyped by extracting the pheromone glands of 2–5-day-old females after injecting them with PBAN (see the electronic supplementary material). One of these families (HvNC-6YR) that generated the most female offspring (100) was phenotyped and genotyped for QTL analysis. This cross was (LH) × L (L = low, H = high; the first letter in each cross refers to the female), so that for each autosome, about 50% of these female offspring were LL and 50% were HL. Phenotypic (Pearson's) correlations between the different pheromone compounds among females of this backcross family are given in the electronic supplementary material, figure S6, along with phenotypic correlations among females in the selection lines for comparison.

A genetic map of *H. virescens* was created using 258 AFLP markers generated by using 35 primer combinations (see [51] for details). We used paired-end RADtags to homologize the linkage groups to our previously constructed interspecific map and the reference genome of *Bombyx mori* (see the electronic supplementary material and [52] for more details). As this was a female-informative cross and there is no crossing-over in female Lepidoptera, each linkage group corresponds to a separate chromosome [53]. To determine which of the linkage groups explained a significant portion of the variance in the pheromone compounds, QTL analysis was conducted on five arcsine transformed pheromone compound proportions (excluding the minor components Z7–16:Ald and Z9–16:Ald, which are difficult to separate in the chemical analysis and are present in minute amounts [54]), as well as on the log-ratio of 16:Ald/Z11–16:Ald, and the overall log-ratio of saturated (14:Ald, 16:Ald) to unsaturated (Z9–14:Ald, Z11–16:Ald, Z11–16:OH) compounds, using *t*-test based marker regression as implemented in R/qtl [55]. Evidence for a QTL is provided by a LOD score directly derived from the *t*-statistic [55]. We established significance thresholds for LOD scores empirically by permutation tests using 10 000 permutations.

(d) Candidate genes

As the difference in the 16:Ald/Z11–16:Ald ratio between the high- and low-line females could be explained by a difference in enzyme activity of desaturases, we conducted qRT-PCR experiments with five different desaturase genes that we identified and named based on their characteristic motifs following the nomenclature of Knipple *et al.* [56]: delta-11-desaturase (HvirLPAQ), two delta-9-desaturases (HvirNPVE and HvirKPSE) and two desaturases with as yet unknown function (HvirKSVE and HvirGATD). All desaturase sequences we detected in the pheromone gland and other tissues have been deposited in GenBank (accession numbers JX531653–JX531674). We constructed pools of 10 pheromone glands of high females and of low females, with four biological replicates of the high females (i.e. four pools of 10 glands each) and three biological replicates of the low females (i.e. three pools of 10 glands each). A detailed description of our qPCR experiments is given in the electronic supplementary material.

The three most likely candidate genes (based on previously determined function), i.e. delta-11-desaturase HvirLPAQ, delta-9-desaturase HvirNPVE and delta-9-desaturase HvirKPSE, were mapped onto our map. Homologues of these desaturases were identified in the genome sequence of *Bombyx mori* by

BLAST using SilkDB ([57], <http://silkworm.genomics.org.cn/>) and KAIKObase ([58], <http://sgp.dna.affrc.go.jp/KAIKObase/>), and by *tblastn* to the *Bombyx* scaffolds in the wgs dataset of NCBI (<http://www.ncbi.nlm.nih.gov/>). We used both the BGI protein predictions provided by SilkDB, and our own annotations when the BGI predictions were incomplete or missing (see the electronic supplementary material for more details on mapping these genes).

(e) Attraction of males by high- and low-line females in the field

To assess whether females with different ratios of 16:Ald to Z11–16:Ald were differentially attractive for males, we conducted field experiments in 2007 and 2008. We used live females from the fourth generation of high- and low-lines as trap lures (see also [59] and the electronic supplementary material for details on the experimental set-up).

3. Results

(a) Field samples

The ratio of 16:Ald to Z11–16:Ald exhibited continuous variation across all phenotyped females, but was centred at a median ratio of 0.40 with an interquartile range from 0.28 to 0.69 (see the electronic supplementary material, figure S1). Surprisingly, 13% of the females had an extreme phenotype with a ratio more than or equal to 1; a ratio this large had not been previously reported in the literature on this species. The same pattern was found for virgin and mated females (see the electronic supplementary material, figure S1). These extreme-phenotype females were present at 6–32% in all field populations that we measured over 4 consecutive years (table 1). Quantitative chemical analyses of all pheromone gland components revealed that in addition to Z11–16:Ald, the relative amounts of two other unsaturated compounds (Z11–16:OH and Z9–14:Ald) were lower, and the other saturated compound, 14:Ald, was higher in females with the extreme 16:Ald phenotype. Overall, the pheromone glands of these females contained more saturated aldehydes and less unsaturated aldehydes and alcohols.

(b) Selection lines and heritability analysis

The dynamics of response to selection appeared asymmetric (see the electronic supplementary material, figures S3–S5). Females in the high line showed a significant increase in the ratio of 16:Ald to Z11–16:Ald mostly in the second generation (see the electronic supplementary material, figures S3 and S4), with a moderate weighted least-squares estimate of realized heritability overall ($\hat{h}_r^2 = 0.20 \pm 0.07$, $t = 3.87$, $p = 0.006$; electronic supplementary material, figure S5A). Females in the low line responded to selection only in the first generation of selection (see the electronic supplementary material, figures S3 and S5) with no significant estimate of realized heritability overall ($\hat{h}_r^2 = 0.09 \pm 0.09$, $t = 1.14$, $p = 0.30$; electronic supplementary material, figure S5B). The slight asymmetry in the response to selection suggests that a lower limit of the ratio of 16:Ald to Z11–16:Ald was reached early.

In addition to the realized heritability from the selection experiments, we estimated the heritability of the ratio of 16:Ald to Z11–16:Ald and the relative amount of 16:Ald for the unselected base population over several generations

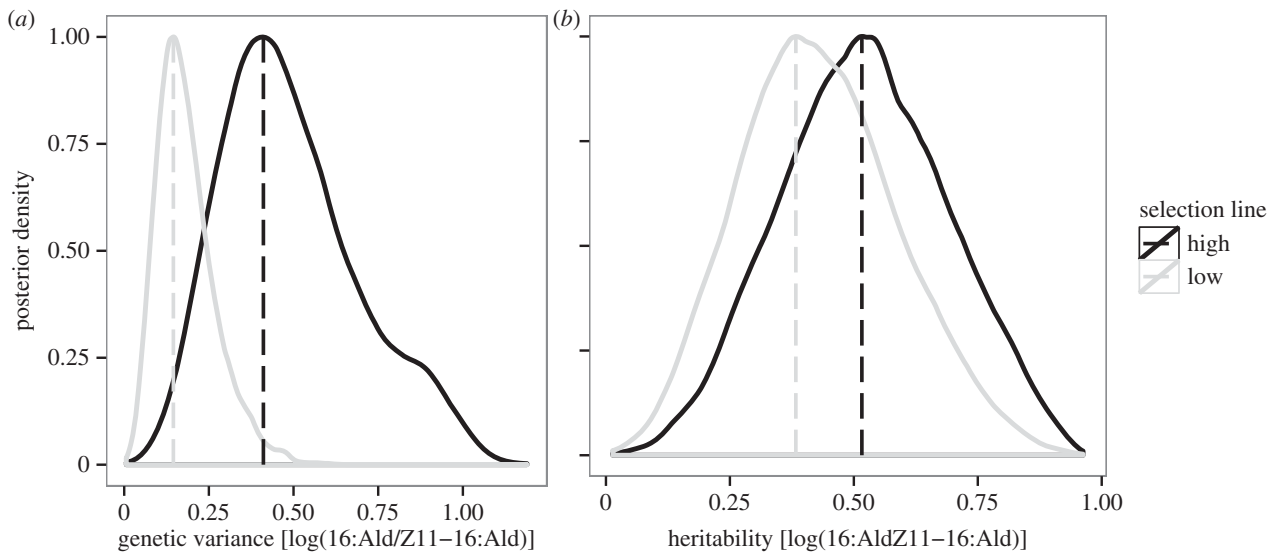


Figure 1. Marginal posterior distributions of additive (a) genetic variance and (b) heritability of the ratio of 16:Ald to Z11-16:Ald in *H. virescens*. The posterior distributions are based on a model including random generational effects. The dashed lines indicate the modes of the distributions.

Table 2. Sources of variation in the relative proportion of 16:Ald and the ratio of 16:Ald to Z11-16:Ald in *H. virescens*, estimated from the high- and low-selection lines. Variance components are presented as coefficients of variation ($CV = 100 \times \sigma/\mu$), where the mean proportion of 16:Ald and the ratio of 16:Ald to Z11-16:Ald were 0.43 and 1.51, respectively, for the high line, and 0.15 and 0.26 for the low line. Each parameter estimate is based on the mode of the respective marginal posterior distributions. h^2 is the narrow sense heritability, CV_A is the variation coefficient of the additive genetic variance and CV_G represents the between-generation coefficient of variation. The residual variation (CV_R) represents all remaining sources of individual variation (genetic and non-genetic). 95% confidence limits for each parameter are given in parentheses.

| selection line | trait | CV_A | CV_G | CV_R | h^2 |
|----------------|----------------------|------------------|------------------|------------------|------------------|
| high | proportion of 16:Ald | 32.4 (20.9–48.8) | 16.0 (8.38–40.4) | 25.9 (1.30–31.2) | 0.51 (0.22–0.85) |
| | 16:Ald/Z11-16:Ald | 41.7 (26.2–64.0) | 19.2 (9.74–49.0) | 35.2 (1.49–41.8) | 0.54 (0.21–0.84) |
| low | proportion of 16:Ald | 54.5 (32.8–86.8) | 8.71 (2.26–46.0) | 50.6 (2.56–60.3) | 0.51 (0.24–0.91) |
| | 16:Ald/Z11-16:Ald | 144.6 (77.2–233) | 28.0 (6.08–132) | 173.5 (117–210) | 0.35 (0.13–0.74) |

using mixed-model (animal model) analysis. For the proportion of 16:Ald, estimates of additive genetic variance (CV_A) were larger than the variance between generations (CV_G) and the residual variation (CV_R), which resulted in a high estimate of heritability ($h^2 \sim 0.50$) for both selection lines (table 2). The close agreement of heritability estimates between the high and the low line suggests that the animal model performed well in capturing the actual population heritabilities. Similar results were obtained for the ratio of 16:Ald to Z11-16:Ald, although the estimates for heritability differed slightly between selection lines (high line: $h^2 = 0.51$, low line: $h^2 = 0.35$; table 2 and figure 1). All heritability estimates based on mixed-effects model analysis were substantially higher than the realized heritabilities during the selection experiment. This difference is likely due to the lack of response to selection past generation 4 in the selection experiment (compare electronic supplementary material, figures S3 and S4). Pearson's correlations between the five most abundant pheromone components are shown in the electronic supplementary material, figure S6. Surprisingly, correlation patterns between the two unsaturated compounds Z11-16:Ald and Z9-14:Ald were reversed in the two selection lines with a negative correlation in the low line ($r = -0.292$) and a strong positive correlation in the high line ($r = 0.385$). However, in the absence of replicated lines, these results should be treated as preliminary.

(c) Quantitative trait locus analysis

Performing linkage analysis for the five components on backcrossing (LH) F_1 females to males of the low line (L) resulted in a significant QTL, chromosome 4 (chr.4), that explained 20.9% of the variance of arcsine transformed 16:Ald ($p < 0.001$) and 41.2% of the variance of arcsine transformed Z9-14:Ald ($p < 0.001$) (figure 2). The estimated effects for the QTL on chr.4 indicate that the H allele present in the heterozygous females results in a decrease in the saturated compound 16:Ald (back-transformed phenotypic means: 0.159 (LL); 0.118 (HL)) and an increase in the unsaturated compound Z9-14:Ald (back-transformed phenotypic means: 0.063 (LL); 0.116 (HL)). This pattern is in the opposite direction to that expected under a model with additive allele effects, because high 16:Ald females are defined as having a higher relative amount of the saturated compound and a lower relative amount of the unsaturated compound. Separate linkage analyses with respect to the ratio of 16:Ald to Z11-16:Ald, or the ratio of saturated to unsaturated compounds as the phenotypes, confirmed this pattern. Both ratios showed a significant QTL on chr.4 (figure 2). The QTL explained 11.3% of the phenotypic variance ($p = 0.046$) in the ratio 16:Ald/Z11-16:Ald (log-transformed) and 20.6% of the variance ($p < 0.001$) in the ratio of all saturated to unsaturated compounds (log-transformed). Again, effect sizes point in the opposite-to-expected direction with an average decrease

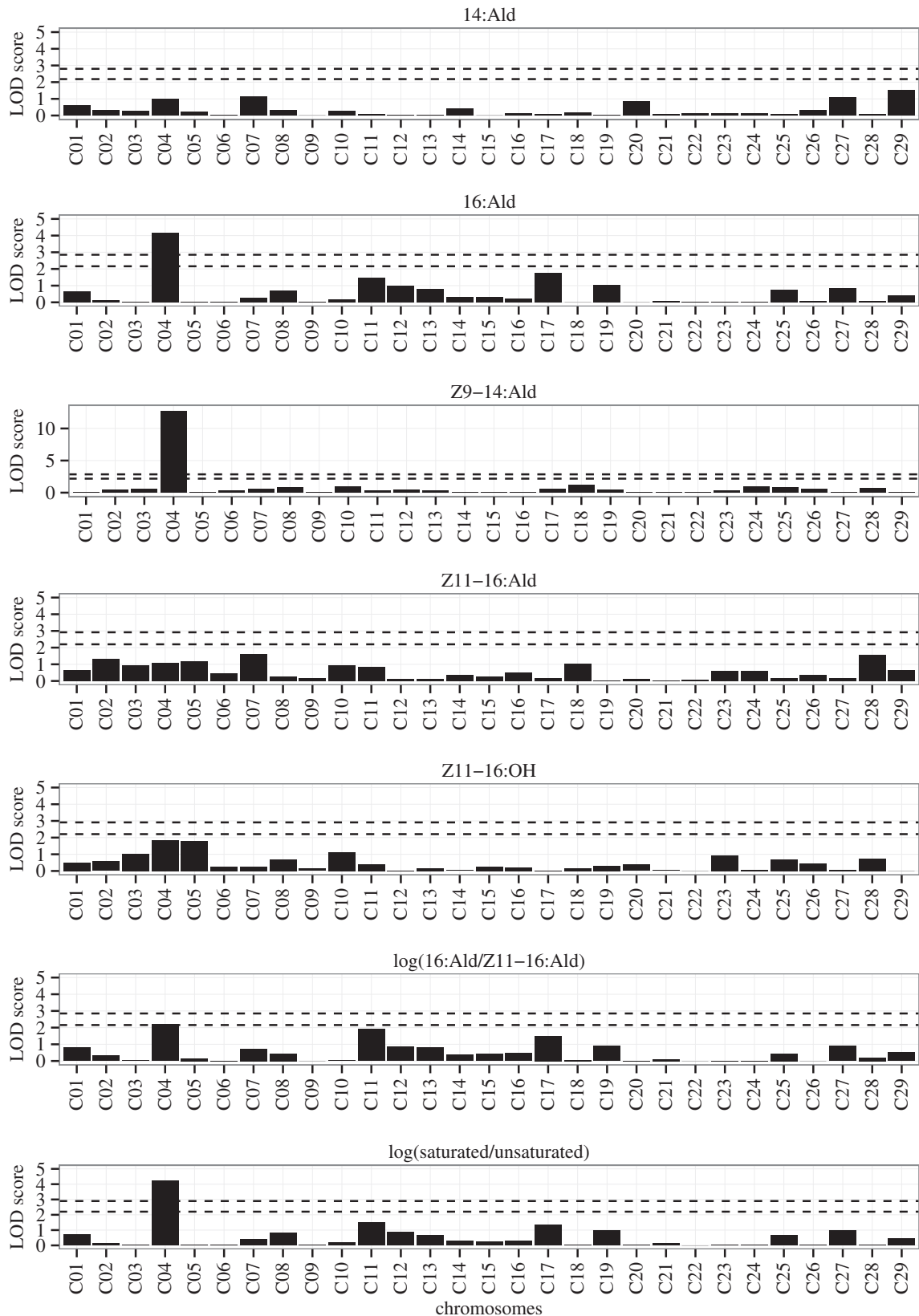


Figure 2. LOD scores for 29 chromosomes for all pheromone compounds (arcsine transformed), the log-ratio of 16:Ald to Z11-16:Ald and the log-ratio of saturated compounds (14:Ald and 16:Ald) to unsaturated compounds (Z9-14:Ald, Z11-16:Ald, Z11-16:OH). The dashed lines represent the 0.05 and 0.01 significance thresholds as empirically determined by 10 000 permutations.

of -0.058 in the ratio of 16:Ald/Z11-16:Ald and -0.063 for the ratio of saturated to unsaturated compounds in HL heterozygotes (values obtained after back-transformation of the phenotypic means). The relative amount of the different untransformed pheromone compounds is shown in the electronic supplementary material, figure S7. Thus, the

magnitude of the effect of the QTL on chr.4 is significantly non-zero, but the sign of its effect in this backcross is opposite to expectations under an additive model.

To determine whether this intraspecific QTL is homologous to our previously identified interspecific QTL affecting the amount of Z9-14:Ald in an interspecific cross between

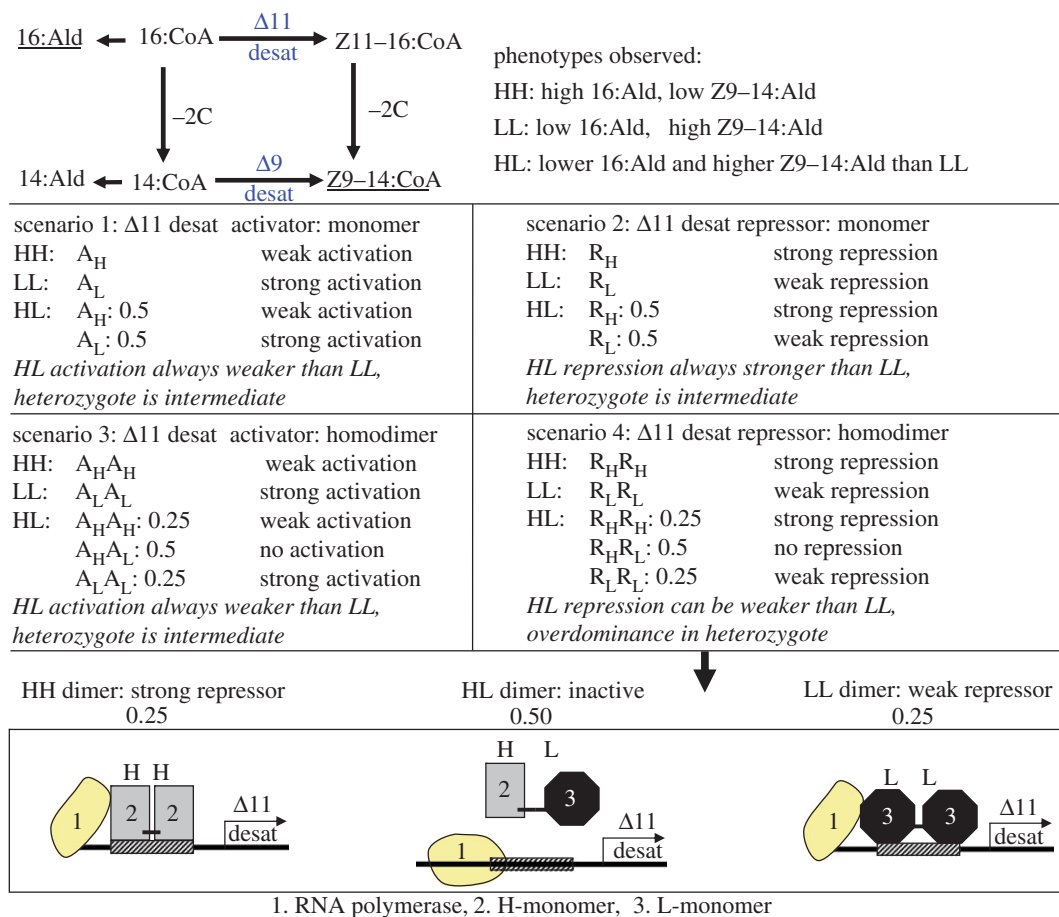


Figure 3. Four different scenarios to explain the opposite-to-expected effect of lower saturated compounds (16:Ald) and higher unsaturated compounds (Z9–14:Ald) in HL compared with LL females. Only scenario 4 can explain this opposite-to-expected effect. The illustration below the scenarios shows how only homodimers bind to the promoter-region to repress transcription of delta-11-desaturase, while the H- and L-monomers form dimers as well, but the mismatched dimer is non-functional as a repressor.

H. virescens and *Heliothis subflexa*, we used RADtag analysis, described in detail in [52] (see also the electronic supplementary material). With the paired-end RADtags, we found intraspecific chr.4 to be homologous to interspecific chr.27 and *B. mori* chr.12 (see the electronic supplementary material). Interestingly, interspecific chr.27 was found to be the major QTL for the interspecific variation of Z9–14:Ald between *H. virescens* and *H. subflexa* (F. Gould and J. Emerson 2013, unpublished results). This overlapping QTL between the species and within *H. virescens* indicates a common genetic basis for the production of Z9–14:Ald, which is the major secondary sex pheromone component of *H. virescens* [42,43,60].

(d) Candidate genes

The higher amounts of Z9–14:Ald and Z11–16:Ald versus 14:Ald and 16:Ald in females of the low- versus the high-selected lines could be caused by differences in the relative rates of the biosynthetic steps involved [51]. Direct reduction of 16:CoA produces the unsaturated 16:Ald. Differences in desaturase activities relative to this would affect the relative amounts of the two desaturated compounds. Increased activity of delta-11-desaturase (16:CoA \rightarrow Z11–16:CoA) would produce more Z11–16:Ald, and if followed by chain-shortening (Z11–16:CoA \rightarrow Z9–14:CoA), more Z9–14:Ald. Direct reduction of 14:CoA produces the unsaturated 14:Ald. Increased activity of delta-9-desaturase (14:CoA \rightarrow Z9–14:CoA) would result in more Z9–14:Ald. In females of the low line, we would thus expect the expression of one or both desaturases

to be enhanced, whereas in females of the high line this expression would be reduced (see figure 3).

qRT-PCR experiments with specific primers for five identified desaturases showed that as expected, delta-11-desaturase HvirLPAQ was expressed at significantly higher levels in the pools of pheromone glands from females of the low line (higher relative amounts of unsaturated components) compared with the pools of pheromone glands from females of the high line (higher relative amounts of saturated compounds; figure 4). One of the delta-9-desaturases, HvirKPSE, also showed higher expression in the low line, but the difference was not significant. The other three desaturases, two with functions as yet unknown (HvirKSVE and HvirGATD), showed no significant expression differences. The orthologues of these desaturases are described in the electronic supplementary material and depicted on a neighbour-joining tree (see the electronic supplementary material, figure S8).

Mapping of these desaturases onto our linkage map showed the following unexpected result: delta-11-desaturase HvirLPAQ did not map to QTL-chr.4, but to chr.21, which corresponds to *B. mori* Chr23 (see [52]). This is consistent with the location of *desat1*, the likely *Bombyx* orthologue of HvirLPAQ on BmChr23 (see the electronic supplementary material, figure S8). Thus, the significant differences in delta-11-desaturase expression cannot be explained by variation in the coding sequence or a *cis*-acting regulatory element, but could be explained by a *trans*-acting regulatory element mapping to QTL-chr.4. However, both delta-9-desaturases (HvirKPSE and HvirNPVE) did map to QTL-chr.4, consistent

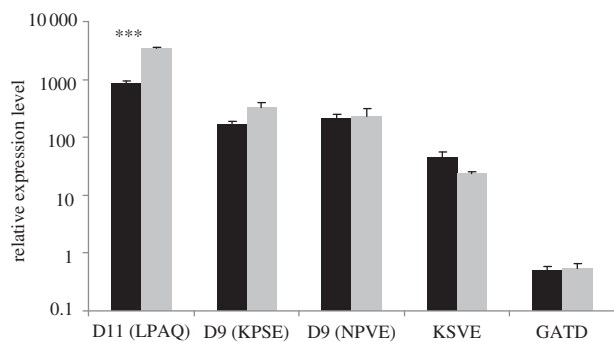


Figure 4. Gene expression levels of five different desaturases of *H. virescens* in pheromone glands of the high black bar and low grey bar lines. D11 LPAQ indicates the delta-11 desaturase and D9 KPSE and D9 NPVE the two delta-9 desaturases. The functions of the other two desaturases KSVE and GATD remain unknown. RPS8 was used as the reference housekeeping gene; results are expressed as copy number of desaturase gene per copy of RPS8; error bars indicate the standard error of the mean. Four biological replicates were used for the high line and three biological replicates were used for the low line. *** $p < 0.0001$

with the location of their *Bombyx* orthologues on BmChr12. Thus, variation in the coding sequences and/or *cis*-acting regulatory elements in HvirKPSE could account for the (non-significant) difference in its expression between HL and LL females.

We have noted that the sign of the effect of QTL-chr.4 in the backcross was observed to be opposite to expectations under a model of additive effects. This observation would predict that in HL heterozygous females, the expression of one or both desaturases would then be enhanced, whereas in LL homozygous females this expression would be reduced. Unfortunately, we could not test this prediction by qPCR because mRNA was not isolated from female pheromone glands in the backcross experiment.

(e) Attraction of males to high and low females in the field

In 2007, 41 of the 125 tested females attracted one or more *H. virescens* males, as assayed by trap catch. In 2008, 29 out of 56 females attracted one or more *H. virescens* males. All females that did not catch any males were excluded from the analysis, because we cannot distinguish whether these females did not call or were not attractive. Combining all trap catches in both years, there was a significant negative correlation between the (square-root transformed) number of *H. virescens* males caught and the log-ratio of 16:Ald/Z11-16:Ald in female pheromone glands (Pearson's correlation coefficient $r = -0.24$, $p = 0.021$; figure 5).

Note that inclusion of a curvilinear term did not improve the model fit significantly (likelihood ratio test, $\chi^2_1 = 0.013$, $p = 0.91$).

4. Discussion

The sex pheromone variation that we observed within all populations of *H. virescens* sampled is in large part based on reciprocal changes in the relative amounts of the major pheromone component Z11-16:Ald and its saturated counterpart 16:Ald. Moreover, concomitant reciprocal changes are evident between all the unsaturated and saturated 16- and 14-carbon

pheromone gland compounds. This variation has a genetic basis, as shown by the high heritability of this trait and by its response to selection. Our finding that one QTL accounts for 11–21% of the variance between the HL and LL genotypes indicates that this variation is at least partly due to one or a few linked genes in one genomic location. By homologizing this QTL by BLAST queries of the paired-end RADtags to the *B. mori* genome sequence, we identified this QTL to correspond to *B. mori* chr.12 (see http://sgp.dna.affrc.go.jp/KAIKObase/PGmap/PGmap.php?chr_id=12).

Interestingly, the intraspecific QTL between the high and low lines of *H. virescens* females is on the same chromosome as the QTL for the amount of Z9-14:Ald in an interspecific backcross between *H. virescens* and *H. subflexa* (F. Gould and J. Emerson 2013, unpublished data). The unsaturated compounds Z9-14:Ald and Z11-16:Ald are the most critical pheromone components: the presence of both is required for attracting *H. virescens* males, and both can be produced by the action of delta-11 desaturase (with subsequent chain-shortening required for Z9-14:Ald [51,59]). Our finding of an overlapping QTL within and between species suggests that the same gene(s), or maybe even the same allele, may be involved in the variation within and between species—an exciting result that we will explore further. However, we will first need to identify the gene(s) underlying both QTLs.

Z9-14:Ald can also be produced by delta-9 desaturase, acting directly on the 14:CoA produced by chain-shortening of 16:CoA. Part of the increase in Z9-14:Ald in females of the low-selected line might thus be due to their higher (but not statistically significant) expression level of the KPSE delta-9 desaturase compared with the high line (see figure 4). The gene encoding the KPSE delta-9 desaturase maps to the same chromosome as the QTL. However, variation in the coding sequence or *cis*-acting regulatory regions of KPSE alone cannot account for the sign of the QTL effect being opposite to expectations in the backcross. If the two alleles of KPSE are independently transcribed in heterozygous females, Z9-14:Ald levels would be intermediate to the two homozygotes, which was not observed (see the electronic supplementary material, figure S7).

As previously described, an increase in delta-11 desaturase activity could increase the production of both Z9-14:Ald and Z11-16:Ald relative to the unsaturated 16:Ald. Although the coding sequence or *cis*-active regulatory elements may differ between the high and low lines, any such effects on delta-11 desaturase expression which would be linked to chr.21 are insignificant compared with the QTL on chr.4. Delta-11 desaturase activity could, however, be affected by a *trans*-acting factor on chr.4. This could be a protein that modulates the activity of the enzyme by binding to its active site; such activity modulators have not yet been described for desaturases. Alternatively, a gene on chr.4 could encode a transcription factor that affects the rate of production of desaturase mRNA, either as a transcriptional activator or transcriptional repressor.

Models in which high and low alleles (of either activators or repressors) act independently predict heterozygote gene expression to be intermediate between the two homozygotes. However, if a repressor is only active as a homodimer, any sequence difference between high and low alleles that prevents function of the high-low dimer would result in the heterozygote exhibiting less repression than either homozygote (see electronic supplementary material, figure S8). Examples of homodimeric transcriptional repressors are known in other

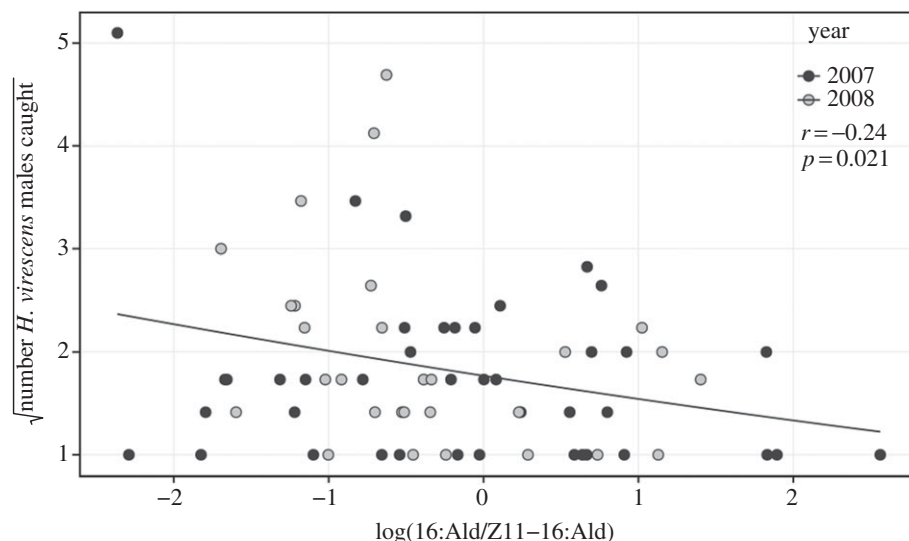


Figure 5. Field experiments with live *H. virescens* females in 2007 and 2008. Black dots refer to trap catches in 2007, grey dots are the trap catches in 2008. Correlation between trap catch (square-root transformed) and ratio 16:Ald/Z11-16:Ald (log-transformed) was conducted in R, using *lm*.

systems [61,62], although none have yet been described for desaturases. This could explain the finding that the HL heterozygote in the backcross unexpectedly produces more unsaturated compounds than does the LL homozygote: HL desaturase activity would be higher than LL because its desaturase expression is repressed to a lesser degree. Thus, the H and L alleles do not act additively in the heterozygote; and the phenotype of desaturase activity is overdominant.

Females producing a higher relative proportion of 16:Ald are less attractive to males (figure 5). Sexual selection for higher production of the bioactive components Z9-14:Ald and Z11-16:Ald [45] would favour heterozygous females and could maintain a polymorphism at the repressor locus, providing for the maintenance of standing genetic variation in pheromone composition in the population. We found *H. virescens* females expressing the unusual phenotype (high relative amounts of 16:Ald and lower amounts of Z11-16:Ald) in all populations, across regions and years, albeit at low frequency (see table 1). Plotting the change in proportion from t to $t + 1$ against proportion at time t reveals a strong negative relationship ($R^2 = 0.67$; see the electronic supplementary material, figure S9). This is a classic signature of stabilizing selection or balanced polymorphism, which further supports our hypothesis for selection for heterozygous females maintaining the polymorphism. This is in contrast to the situation in *I. pini*, in which the pheromone polymorphism is maintained by disruptive selection [10,41].

When selection has brought the population towards a balanced polymorphism, the additive genetic variance for the fraction of unsaturated compounds is near zero. Even if there is a uniform preference by males for a higher fraction of unsaturated compounds, the population average cannot increase beyond a certain limit defined by the genotype frequencies and their phenotypes. Heterozygous females are selected for by males, but matings always generate some female homozygous offspring, which pull down the population average. This can explain the asymmetric dynamics of the selection response we observed in the high and low lines (see the electronic supplementary material, figures S1-S3). Selection for the low line cannot increase the value of the trait (unsaturated compounds) very much

because the population is already near the stable equilibrium; little response was seen after generation 1. Selection for the high line, however, is similar to disruptive selection in that it selects against the heterozygote, and for the homozygote that produces the lowest fraction of unsaturated compounds. The additive genetic variance remains positive and a selection response will be observed until the population is fixed for that homozygote, which in the high line apparently occurred by generation 4.

In summary, we found a heritable intra-population variation in a moth sex pheromone, a large part of which is likely due to one or a few closely linked genes. An intra-population variation in moth sex pheromones has not been described before. A polymorphism at a *trans*-acting repressor locus for delta-11-desaturase can explain many features of the data. Such a polymorphism may be maintained through balancing selection, in this case specifically through heterozygote advantage, as heterozygote females produce significantly more of the critical sex pheromone component through which males are attracted. Thus, selection need not act solely in a purifying role, eliminating genetic variation in signal-response systems as is generally assumed, but can also act to maintain genetic variation in them. This is to our knowledge the first study that identifies a possible mechanism for the maintenance of such genetic variation in moth sexual communication.

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Data accessibility. Data deposited in Dryad: All raw data on selection lines, backcross map and phenotype, and field experiments are in this Excel file, containing eight sheets: doi:10.5061/dryad.694rn/1. The alignment on which electronic supplementary material, figure S8 is based is in doi:10.5061/dryad.nm5k1/1.

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