Evolution of sexual signals

*Within and between species variation in a dual function sex-pheromone component in two noctuid moths*

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High acetate levels in *H. subflexa* sex pheromone blend are associated with reduced fitness in a stressful environment

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Chapter 5

Abstract

Sexual communication allows individuals to find and choose a mate, but also to avoid hybridization with individuals from different species. Sexual signals can thus play an important role in speciation. Sexual signals also provide potential information about mate quality if signals are costly and vary due to trade-offs between investment in sexual traits and life-history traits. Importantly, whether sexual signal costs result in trade-offs may be condition dependent, meaning that costs or trade-offs associated with the signal will only be revealed under stressful conditions.

To determine if intra-specific variation in sexual signals can be driven by condition-dependent costs, we studied variation in the sex pheromone of the noctuid moth *Heliothis subflexa*. Females of this species produce acetate esters in their sex pheromone blend that attract males from the same species, while repelling a sympatrically occurring species, *Heliothis virescens*. As *H. subflexa* females produce high amounts of acetates when the interfering species is present but low amounts in its absence, we hypothesized that high-acetate sex pheromone signals are costly. To identify potential trade-offs between high acetate levels and female fitness, we manipulated the quantity of resources available. We showed that under food stress, females with high ratios of acetates in their sex pheromone had longer developmental times and lower fertility compared to females producing less acetates. These results support the hypothesis that a balance between costly acetates and the benefit of deterring heterospecific mates may underly intra-specific variation in the *H. subflexa* female sex pheromone blend.
Introduction

Finding a suitable mate is crucial for many species. Intra-specific variation in sexual signals can be used to infer the quality and mating status of a potential mate (Andersson and Simmons, 2006; Coyne and Orr, 2004; Ritchie, 2007; Schaefer and Ruxton, 2015; Wiens and Tuschhoff, 2020). When sexual signals are involved in species recognition, which prevents hybridization with members of other species, sexual communication also plays an important role in reproductive isolation and speciation (Coyne and Orr, 2004). Thus, the evolution of sexual communication is under a complex balance between natural and sexual selection (Blows, 2002). In order to comprehend how sexual signals may evolve, it is important to identify the role of selection in shaping intra-specific variation.

First, natural selection can drive both between- and within-species variation in sexual signals. For example, when sexual signals from closely related species overlap, communication interference may occur, leading to negative impacts on fitness due to sexual harassment or hybridization (Verrell 1994; Gröning and Hochkirch 2008). To avoid communication interference, sexual signals may diverge between closely related species that live in sympatry, which is known as reproductive character displacement (Brown et al., 1956; Crampton et al., 2011; Higgie et al., 2000; Kirschel et al., 2009; Lemmon, 2009). Consequently, intra-specific variation may appear between sympatric and allopatric populations (Hoskin et al., 2005).

Second, as organisms have limited resources available, trade-offs between sexually and naturally selected traits can generate intra-specific variation in sexual signals depending on how individuals allocate their resources (Selander, 1965). The expression of a sexual signal may bear a direct energetic cost, like acoustic calling in frogs or insects (Ryan, 1988) or bright coloration in birds (Hill, 1996). There can also be ecological trade-offs, such as an
increased risk of predation and parasitism for the signalers (Marshall et al., 2015; Sheldon et al., 1996; White et al., 2022).

Because of these direct or indirect cost, investment in sexual signals may be condition-dependent, and thus vary as a function of individual condition (Jennions et al., 2001). When only high-quality individuals can afford the cost of the signal, we expect to find a positive correlation between the signal and fitness (Agrawal et al., 2010). Signaling investment may differ depending on environmental conditions, thus the costs of sexual ornaments may be more easily detectable under stressful conditions than under ideal laboratory conditions (Cotton et al., 2004; Reznick, 1985). In stressful environments, allocation of more energy to survival is needed, making investment in sexual ornaments more costly. For example, Xu and Fincke (2022) showed that *Megaloprepus caerulatus* damselflies are smaller when food is restricted and that small males have smaller wing ornaments than larger ones even if they allocated a higher percentage of fat to this trait. Hence, when resources are scarce exaggerated ornaments are less affordable.

Sexual communication through chemical signals such as sex pheromones has been shown in diverse taxa (Johansson and Jones, 2007; Wyatt, 2003). Even though chemical signals have been long assumed to be biosynthetically cheap (Greenfield, 1981; Johansson and Jones, 2007; Steiger and Stökl, 2014), recent empirical studies have suggested that sex pheromones can be costly. Empirical studies have shown that sex pheromone may vary in function of the nutritional state (Chemnitz et al., 2015; Foster, 2009; Rantala et al., 2003) and that body size can affect sex pheromone quantitively (Chemnitz et al., 2015; Harari et al., 2011) and qualitatively (Jaffe et al. 2007).

In moths, females usually emit species-specific multicomponent sex pheromone blends that attract males from long distances. To avoid cross-attraction, sex pheromone blends may contain antagonistic components that deter males of other species while increasing attraction of conspecific males (Fadamiro et al., 2015; Sheldon et al., 1996; White et al., 2022).
1999; Groot et al., 2006; Juárez et al., 2016; Löfstedt et al., 1991; Vickers and Baker, 1997). Since geographic variation in the relative percentage of an antagonist sex pheromone has been previously found (Groot et al., 2009b), we hypothesize that intraspecific variation could be explained by differential costs of pheromone signals depending on the composition of the signal. Following this hypothesis, investing in high concentrations of the antagonist components may be beneficial in sympatry, but not in allopatry which creates intra-specific variation.

In the sex pheromone of the noctuid moth Heliothis subflexa (renamed Chloridea, Pogue 2013), females produce three acetate esters (Z)-7-hexadecenyl acetate (Z7-16:OAc), (Z)-9-hexadecenyl acetate (Z9-16:OAc), and (Z)-11-hexadecenylacetate (Z11-16:OAc), hereafter referred to as acetates. The presence of acetates in the pheromone blend increases the attraction of conspecific males, while repelling males of a sympatrically occurring species, H. virescens (Groot et al., 2007, 2006; Vickers, 2002; Vickers and Baker, 1997), with which H. subflexa can hybridize in the laboratory but which results in sterile male offspring (Laster 1972). Intraspecific variation in acetate levels in this species has been found to correlate with the presence of H. virescens both geographically and seasonally (Groot et al., 2009b). In North Carolina (USA) where both species co-occur, H. subflexa females produced lower amounts of acetates in the years when fewer H. virescens were present. Moreover, in West Mexico where H. virescens is absent, H. subflexa females produce significantly less acetates than females in North Carolina, where H. virescens is abundantly present. As lower acetate levels are found when the chances of cross-attraction are reduced, one hypothesis for this pattern is that high acetate levels come at a cost. To test this hypothesis, we aimed to assess the costs associated with high acetate levels.

Previous work with H. subflexa in the laboratory showed that higher relative amounts of Z11-16:OAc were associated with an increased fecundity and fertility, but also with a reduced lifespan in
small females (Blankers et al., 2021). These results suggest that potential costs of high acetate levels may only become apparent under suboptimal conditions. In the laboratory insects are often kept in “ideal” conditions, (i.e in highly nutritious diets and in the absence of pathogens or parasites), which may mask costs or trade-offs experienced in the wild, where resources may be less abundant. In order to reveal any “hidden” condition-dependent costs, in this study we manipulated the resources available at the larval stage, which is known to affect adult morphology and fitness in holometabolous insects (Boggs and Freeman, 2005). Moreover, we used two experimental lines that had been selected for “High” and “Low” amounts of acetates. We hypothesized that high acetate levels are associated with reduced fitness in a stressful environment. Specifically, we expected that (i) when resources are limited, acetate levels are negatively correlated to developmental traits (i.e lower pupal mass or longer developmental time) and ii) when resources are limited, selection for higher acetate levels reduces the reproductive output.

Materials and methods

Creation and maintainance of the selection lines

To assess the potential cost of high acetate levels in H. subflexa sex pheromone blend, we used moths from the 13th-15th generations of ‘High’ and ‘Low’ selection lines (see supplementary table S1), as described in detail in Blankers et al. (2022) and summarized here. The two lines were created between 2018 and 2020 from a laboratory population of H. subflexa that has been reared at the University of Amsterdam since 2011. The lines were created by phenotyping mated females and using those with a relative
percentage of acetates above 22% or below 16% to found the High and Low lines, respectively. After generation one, the lines were kept separate and post-mating selection was performed for 10 generations to keep only those families where the mother’s sex pheromone blend had acetate levels that were below or above the set threshold levels.

Up until generation 10, between 189 and 295 females were phenotyped per line per generation to select families to include in the next generation. After generation 10, selection ceased but the lines were kept separate for five more generations. In generation 11-13, 20-40 females were phenotyped per line per generation. The last phenotyping was done in generation 13, the average relative amount of acetates was 17.2 % in the Low line and 32.4 % in the High line (as reported in Blankers et al. 2022). The rearing was kept at 25°C and 60 % relative humidity with 14L:10D light–dark cycle. Larvae were reared on a wheat germ/soy flour-based diet (BioServ Inc., Newark, DE, USA) in 37 ml individual cups. After emergence, single pair matings were set-up with individuals from distinct families to avoid inbreeding, with on average 200 matings per line set up per generation.

**Manipulation of larval food as a stressor**

As trade-offs are more easily detectable under stressful conditions (Reznick 1985; Cotton et al. 2004), we manipulated the larval diet (following Zweerus et al. 2021) to assess potential condition-dependent costs of producing high acetate levels. For both treatments, the same wheat germ/soy flour-based diet (BioServ Inc., Newark, DE, USA) was used. For the control treatment, we used the regular rearing protocol: 288 g of the flour was mixed with 38 g agar and 1750 ml demi-water. For the “reduced diet” treatment, the nutritional value was lowered to 25 % compared to the control by adding 25 % of wheat germ/soy flour-based diet to the regular amount of agar and water (Zweerus et al. 2021). Newly hatched
larvae were isolated to avoid cannibalism, in 37 ml cups filled with either control or reduced diet. In total, we set up 1,121 larvae on normal diet and 1,209 larvae on reduced diet. One day after pupation, individuals were weighed on a Sartorius analytical laboratory scale (model MC1 AC 210 S). After emergence, adults were sexed, fed with a cotton swab soaked in a 10 % sugar water solution, and their emergence date was recorded. Females were used either for pheromone extraction or were mated as described below.

**Pheromone extraction**

For all pheromone analyses in this study, we used 2-8 day old virgin female moths. Pheromone gland extractions were performed on calling females, 3-4 hours into scotophase. Some females were extracted outside of this time period and were first injected with pheromone biosynthesis activating neuropeptide (PBAN) 1–2 hours before gland extraction to stimulate pheromone production, following the protocol of (Groot et al., 2009a, 2005). After cutting the pheromone glands with microdissection scissors, the glands were soaked for 30 min in individual vials in 50 µL of hexane containing 200 ng of pentadecane as an internal standard. After 30-60 min, the glands were removed, and the extracts were stored at -20°C until GC analysis.

For the GC analysis, samples were reduced to approximately 2 µL under a gentle stream of nitrogen and 1 µL of octane was added to prevent evaporation of the sample. Each sample was then injected into a splitless inlet of a 7890A GC (Agilent Technologies, Santa Clara, CA, USA). The areas under the pheromone peaks of the 11 pheromone components present in the *H. subflexa* pheromone blend (i.e. tetradecanal, (Z)-9-tetradecanal, hexadecenal, (Z)-7-hexadecenal, (Z)-9-hexadecenal, (Z)-11-hexadecenal, (Z)-7-hexadecenyl acetate, (Z)-9-hexadecenyl acetate, (Z)-11-hexadecenyl acetate, (Z)-9-hexadecenol and (Z)-11-hexadecenol; Heath et al., 1990) were integrated using Agilent ChemStation.
High-acetate sex pheromone blends are costly

software (version B.04.03). Absolute amounts (in ng) of each component were calculated relative to the 200 ng pentadecane internal standard. The relative percentage of the acetates was calculated by summing the three acetate esters and dividing it by the total amount of pheromone produced.

Reproductive output measurements
To determine if reproductive output trades off with acetate levels under food stress, assortative single-pair matings were set-up using individuals from the same line (High or Low) and treatment (normal or reduced diet), but from different families. Each mating pair was placed in a transparent cup covered with gauze. To stimulate oviposition, a piece of Physalis fruit was placed on top of the gauze. Cotton soaked in a 10 % sugar water solution was provided and replenished every other day until female death.

Eggs laid on the gauze were collected daily, photographed, and placed in an empty petri dish at 25°C for two days. After this period, the number of fertile eggs was counted under the microscope. Non-fertile eggs usually shrink while fertile ones stay round and show two red dots or a black eyespot inside the eggshell. When fertile eggs were observed, the gauze was placed in a petri dish at 16°C to slow the hatching rate. The fertility was assessed again a day later to ensure all fertile eggs were detected.

To estimate the reproductive output, we measured mating failure, fecundity, fertility, and female lifespan, as described below. The percentage of mating failure was measured by dividing the number of matings that did not produce any eggs or only unfertile ones by the total number of matings. For the analysis of fecundity and fertility, the failed matings (i.e. when no or only unfertile eggs were laid) were excluded. The total fecundity was assessed by summing the total number of eggs laid by each female during her lifespan. The fecundity per night was calculated by dividing the total number of eggs laid by the number of nights during which the female
laid eggs. The total fertility and fertility per night were estimated in the same way, but now counting only the fertile eggs. Finally, female lifespan was measured as the number of days between adult emergence and death of the individual.

**Statistical analysis**

All statistical analyses were performed in R software version 4.1.2 (R Core Team, 2019). When we fitted generalized linear models, we used a stepwise model selection approach to identify significant explanatory variables and interactions. Model selection was done by first adding interactions to the explanatory variables and then removing predictors that did not significantly explain variation in the response. Comparison between the models was performed by ANOVA with a F test, and improvement of model fit was considered significant when \( P < 0.05 \). For each best fitted model, the \( R^2 \) was calculated with the function rsq for models with a binomial and quasi-binomial distribution, while for quasi-Poisson distributions the pseudo-\( R^2 \) was calculated as:

\[
\frac{\text{Residual Deviance}}{\text{Null Deviance}}
\]

**Expectation 1: When resources are limited, acetate levels are negatively correlated to developmental traits**

First, we evaluated if our diet treatment was effective at creating a stressful condition by comparing the development time, pupal mass and survival of larvae reared on control versus reduced diet. Pupae were not sexed prior to the analysis, so both males and females were included in this analysis and no sex-specific effects were measured. The difference in developmental time and pupal mass were tested with a Kruskal test, as the residuals were not normally distributed. The difference in survival rate until both the pupal and adult stage were performed with the function prop.test (R-package “rtatix”),
which evaluates the probabilities of success in several groups. To test whether acetate levels could be predicted by the diet treatment or developmental traits, we measured the sex pheromone composition of 40 and 39 females for the normal and reduced diet groups, respectively. We then fitted a generalized linear model with the relative percentage of acetates as response variable and a quasi-binomial distribution. As predictors we used the selection line, treatment, female pupal mass and developmental time. We also added whether females had been injected with PBAN prior to pheromone extraction as predictor to the model. Finally, to correct for the variation in the age of the females used for the pheromone extraction, we added age as a random effect.

**Expectation 2: When resources are limited, selection for higher acetate levels reduces the reproductive output**

Each reproductive output measurement was tested in a specific model with the same predictors. We were not able to measure the pheromone blend and reproductive output from the same female, because we performed pheromone extraction in an invasive way, (i.e. by cutting the pheromone gland), that led to the death of the female, as described above. However, 10 generations of divergent selection had resulted in lines that diverged in their acetate levels by about two standard deviations, while reared under otherwise identical conditions (Blankers et al., 2022). Therefore, we used the line (High or Low selection line) as a predictor, as well as the treatment (normal or reduced diet) and the female pupal mass. First, we tested the effect of selection line and treatment on mating failure (i.e percentage of matings that did not lay any fertile eggs) using a GLM with binomial distribution. Then, we did the same for fecundity and fertility using a GLM with quasi-Poisson distribution. Finally, the effect of selection line and treatment on variation in female lifespan was tested with a two-way ANOVA.
Figure 1: Effect of the reduced diet on *Heliothis subflexa* development

**A)** Percentage of individuals that reached the pupal and adult stage for both treatments. **B)** Comparison of developmental time per treatment. The developmental time was estimated by counting the number of days from which the newly hatched larva was isolated in a cup with diet until adult emergence. **C)** Comparison of pupal mass per treatment. Grey: individuals reared on normal diet, Yellow: individuals reared on reduced diet. *: $P < 0.0001$
Results

**Expectation 1:** When resources are limited, acetate levels are negatively correlated with developmental traits.

Reduced diet induces stress

Resource availability during the larval stage had a strong effect on development. Compared to the control treatment, individuals reared on reduced diet had a significantly lower survival rate until pupation (respectively 60.5 % and 28 % individuals pupated, prop.test P < 0.0001) and lower pupal emergence rates (respectively 42.4 % and 21 % emerged, prop.test P < 0.0001). Moreover, individuals reared on reduced diet had a significantly longer developmental time than the control (number of days until adult emergence; control: median = 37 days; reduced diet: median = 39 days, Kruskal test P < 0.0001) and were lighter (control: median = 0.246 g; reduced diet: median = 0.167 g, Kruskal test P < 0.0001) (Figure 1).

In stressful conditions, acetate levels are positively correlated with developmental time

Most of the variance in acetates was explained by the line (High or Low selection lines, partial $R^2 = 0.46$), but some variance was also explained by the interaction between developmental time and treatment (partial $R^2 = 0.13$). On control diet, larval developmental time was not correlated with the relative amount of acetates in adult females (Figure 2.A, grey regression line: $r = -0.02$, $P = 0.29$). In contrast, in the reduced diet treatment, females with a short
developmental time had blends with lower amounts of acetates, while females with a longer developmental time contained higher acetate levels (Figure 2, yellow regression line: $r = 0.04, P = 0.047$). Even though pupal mass was negatively affected by the reduced diet treatment (Figure 1.C), the pupal mass did not have a significant effect on the relative percentage of acetates (Figure 2.B). Finally, PBAN had no significant effect on the acetate levels in the control diet (See supplementary results for more details).

**Figure 2: Correlation between acetate levels and developmental traits per treatment**

**A)** Correlation between acetate levels and the developmental time per treatment. The developmental time was estimated by counting the number of days from which the newly hatched larva was isolated in a cup with diet until adult emergence.

**B)** Correlation between acetate levels and the pupal mass per treatment. The Pupal mass was estimated to the nearest 0.01 grams.

Each dot represents one individual, Grey: individuals reared on normal diet (n= 40), Yellow: individuals reared on reduced diet (n=39). The regression lines with their correlation coefficient are in the corresponding color.
High-acetate sex pheromone blends are costly

**Expectation 2: When resources are limited, selection for higher acetate levels reduces reproductive output**

When we compared the pheromone blend between selection lines we found that High line females contained on average twice as much acetates as Low line females, respectively 30.5 % and 14.9 % (Figure 3) which matches with the relative percentage of acetate levels found in the previous generations of the selection lines (Blankers et al., 2022). Furthermore, ~ 90 % of High line females contained more than 20% of acetates in their pheromone blend, while only ~ 15% of Low line females contained that much. The best fitted model for each adult reproductive output measurement is described below.

![Figure 3: Relative percentage of acetates in the two selection lines.](image)

*Individuals from High line are shown in red, the ones from Low line are represented in blue. The horizontal barres indicate the median and each black dot correspond to an individual. *: P < 0.05*
Figure 4: Matings per line and treatment
Matings were classified according to the absence of any eggs (pink dotted), only non-fertile eggs present (red dotted) or fertile eggs present (green). Non-fertile eggs were those that did not show any eye pigmentation within three days after oviposition.

Figure 5: Correlation between fecundity and fertility per day and pupal mass per selection line.
A) The fecundity per day was estimated as the average number of eggs produced per night during the laying period. B) The fertility per day was estimated as the average number of fertile eggs produced per night during the laying period. Individuals from High line are shown by red circles and the ones from Low line are represented by blue triangles. The regression lines and the Pearson correlation coefficients are in the corresponding color.
Selection for high acetate levels is associated with an increased mating failure

Between the two treatments or selection lines we found no significant differences in reproductive output, even though between 63.2 % and 84.2 % of the matings produced eggs (Figure 4). However, the percentage of failed matings (i.e. matings in which females did not lay fertile eggs) was significantly higher for individuals reared on the control diet (74.2 %, n = 83) compared to reduced diet (39.4 %, n = 40); the line also had a significant effect: Low line matings had a lower failure rate (42.5%, n = 64) than High line matings (67 %, n = 59).

Reproductive output is resource limited in the high line but not the low line

Focusing on the matings that successfully produced fertile eggs, the fecundity per day, fertility per day, total fecundity and total fertility, were all positively correlated to the pupal mass (see Table S1). Heavier females laid more eggs in total and had an increased fertility compared to lighter females, but neither the total fecundity or total fertility depended on diet treatment or on the interaction between the selection line and pupal mass. Nevertheless, while in the High line heavy females laid significantly more eggs per day than lighter females, (Spearman correlation test: ρ = 0.70, P = 0.002; Fig 5), we did not find such a correlation for the Low line (Pearson correlation test: r = 0.14, P = 0.48; Fig 5). Similarly, the number of fertile eggs laid per day increased with female pupal mass in the High line females (Spearman correlation test ρ = 0.53, P = 0.03; Fig 5) but not in the Low line females (Pearson correlation test r = 0.11, P = 0.56; Fig 5). Diet treatment had a marginally significant effect on the fertility per day (0.644, P = 0.09, partial R² = 0.05).
Discussion

Even though trade-offs are theoretically common, their detection in empirical studies has been difficult, particularly under laboratory conditions (Agrawal et al., 2010; Kotiaho, 2001). Here, we used a design to uncover condition-dependent costs for a sexual signal. We found that under reduced food availability, females with sex pheromone blends with a higher relative percentage of acetates had longer developmental times and lower fertility compared to females with blends lower in acetates. Our findings confirm our expectations that (i) when resources are limited, acetate levels are negatively correlated with developmental traits (i.e., lower pupal mass or longer developmental time), and (ii) when resources are limited, selection for higher acetate levels reduces reproductive output.

As hypothesized, the costs of higher rates of acetate are condition-dependent which may create intra-specific variation. Since acetates play a dual function by attracting conspecific males while deterring heterospecific H. virescens (Groot et al., 2007, 2006; Vickers, 2002; Vickers and Baker, 1997), intra-specific variation in acetate levels in the H. subflexa female sex pheromone blend may result from a balance between costly acetates and the benefit of deterring heterospecific mates. Below we elaborate on the possible causes of condition-dependent costs of acetate expression, and on the implications for the maintenance of variation in sexual signals more broadly.

Females with high acetate levels need a longer developmental time in stressful condition

In this study, we showed that food reduction at the larval stage resulted in a longer developmental time, which has been found before in different insects (Dmitriew and Rowe, 2007; Florez-
Cuadros et al., 2019; Poças et al., 2022). Moreover, we found that when resources were limited, individuals with longer developmental times had higher rates of acetates: on average for every extra percentage point of acetates, females reared on reduced diet needed an extra day to develop. In Drosophila, longer developmental time under food stress allows accumulation of greater mass from larval feeding (Chippendale et al. 1996; Harshman et al. 1999). Hence, if blends with high acetate levels require more resources, when the nutritional value of the diet is low (as is the case in the reduced diet treatment) a longer developmental time is needed, while this is not the case when the diet is of high nutritional value (control diet treatment).

However, longer developmental times increase the risk of predation before producing any offspring (Abrams and Rowe, 1996; Taylor and Gabriel, 1992; Urban, 2007). Although this may not be the case for H. subflexa larvae, as they develop only on Physalis plants (Laster, 1972) whose calyx covering the fruit creates what is known as an “enemy-free space” (Oppenheim and Gould, 2002; Puente et al., 2008; Sisterson and Gould, 1999). While H. subflexa with a longer developmental time may not suffer costs of increased predation, other factors likely constrain how long a developmental period can be. For example, pupae enter into diapause to overwinter, but late emerging females that reproduce at the end of the season may produce offspring that do not develop into pupae in time to overwinter. Hence, increased developmental time may generate fitness cost over multiple generations.

**More failed matings in the High selection line**

We found that a higher percentage of High line matings failed to produce any fertile eggs compared to Low line matings. This suggests that high levels of acetate have a cost on individual reproductive output and fitness. However, we also found that our
matings had a low success rate overall. Since we always rear the moths in single pair matings, one explanation may be that single pair matings reduce the opportunity for sexual selection to purge genetic load (Agrawal, 2001; Siller, 2001). As females did not have the opportunity to choose which male to mate with and there was no competition between males, this may have given the opportunity to low-quality individuals to reproduce.

However, genetic load can be reduced in harsh environments by the death of low-quality individuals (Agrawal and Whitlock, 2012). This may explain the fact that the control diet group had a high mating failure rate, while this rate was decreased by almost twofold in the reduced diet group. In other words, low-quality individuals may have died before reaching the adult phase, while good-quality individuals survived, so that adults from the reduced diet group had fewer matings with low-quality individuals. This could explain why more matings laid fertile eggs in the reduced diet group compared to the control diet group.

In stressful conditions, high acetate levels are associated with lower reproductive output

Female weight significantly affected reproductive output differently in the two acetate lines. Light females from the High acetate line laid on average fewer eggs per night compared to heavy females from the same line and compared to any females of the Low acetate line. A similar pattern was observed for the fertility per night. Reduced reproductive output due to lower pupal mass can be partly explained by the effect of diet on weight. This matches the findings of a study on the closely related *H. virescens*, where individuals reared on diet of lower nutritional quality had lower pupal mass (Zweerus et al., 2021). More generally in insects, reduction of food at the larval stage can negatively affect development and adult fitness (Boggs and
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Freeman, 2005). Since we found that pupal mass particularly affects fecundity per day in the High acetate line, acetate rates seem to trade off with fecundity in stressful conditions.

Since we showed that High line females had a longer lifespan on reduced diet, even though they laid fewer eggs per night, their longer lifespan could compensate for the reduced egg production per night and explain why no differences were observed in the total fecundity. Under natural conditions, females may die earlier from predation which increases the cost of laying fewer eggs per night found for High line females. We could thus expect that under less idealized conditions, we may see differences in the total fecundity of the High and Low lines that correlate with the acetate levels. Moreover, we previously found that light *H. subflexa* females with high relative acetate levels laid fewer eggs in total than females with lower acetate levels (Blankers et al. 2021). Both studies suggest a condition-dependent trade-off between fecundity and acetates levels.

**Intra-specific variation in acetate levels is shaped by different selective pressures**

Our results support the hypothesis that the expression of sexual signals may generate different costs and benefits in different conditions, creating intra-specific variation. In this study, we found that high acetate levels are associated with a longer developmental time and a reduced fertility per night under poor nutritional conditions. In combination, we conclude that high acetate levels in the sex pheromone blend are costly, and that this cost is condition dependent. Since acetate levels trade off with important life-history traits, females with lower levels of acetates will potentially have an advantage when *H. virescens* is absent. In this scenario, the cost/benefit balance of the expression of high acetate levels depends on the presence of heterospecifics and the resources available.
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This explanation is concordant with the variation in acetate levels found in wild populations of *H. subflexa*: in West Mexico where *H. virescens* is almost absent, *H. subflexa* females have significantly lower levels of acetates than females in North Carolina where both species co-occur (Groot et al., 2009b). Moreover, *H. subflexa* females from North Carolina have lower levels of acetates in the years when fewer *H. virescens* were present.

Even though chemical signals have long been assumed to be cheap (Greenfield, 1981; Johansson and Jones, 2007; Steiger and Stökl, 2014), our results together with other empirical studies show that the pheromone composition depends on the availability and allocation of resources (Blankers et al., 2021; Chemnitz et al., 2015; Foster and Johnson, 2011; Harari et al., 2011; Jaffe et al., 2007). Since the cost of sexual signals may vary under different conditions, potential costs may remain hidden under ideal laboratory conditions and may thus be underestimated in the literature. Hence, condition-dependent costs may be more common than previously thought and could be an important factor maintaining intra-specific variation in sexual signals, even those that appear invariant with life history traits under laboratory conditions.
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High-acetate sex pheromone blends are costly.


Appendix

a. PBAN effect on acetate levels

Because of time constraints, some females were extracted during the pick calling time of *H. subflexa*, three to four hours into scotophase, without PBAN injections while females extracted outside this period were first injected with PBAN. Based on previous findings, PBAN was expected to have no effect on the relative amounts of pheromone components, including acetates. To deal with the unbalanced nature of these data, we used a non-parametric approach to test the effect of PBAN on acetate ratios in the High and Low line and for reduced and normal diet separately. In line with our expectations, none of these four groups showed significant differences in acetates between the females injected with PBAN and those not injected (High-Control: N= 68; W = 446; P = 0.158, High-Reduced: N= 25; W= 79; P = 0.051, Low-Control: N= 42; W= 158; P = 0.498, and Low-Reduced: N= 14; W= 25 ;P = 0.225).

b. Lifespan

The lifespan of High line females increased on reduced diet compared to the control treatment while for Low line, female lifespan was similar on both treatments (Supplementary Figure 1; line-type:treatment, F = 4.52, P = 0.036).
Supplementary Figure 1: Female lifespan per line and treatment
Individuals from High line are shown in the red boxplots while the ones from the Low line are in bleu. The black dots represent each female lifespan and the horizontal bar indicate the mean.

<table>
<thead>
<tr>
<th>Generation</th>
<th>Low line</th>
<th>High line</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>190</td>
<td>69</td>
</tr>
<tr>
<td>14</td>
<td>210</td>
<td>111</td>
</tr>
<tr>
<td>15</td>
<td>0</td>
<td>461</td>
</tr>
</tbody>
</table>

Supplementary Table 1
Number of individuals that reached the pupal stage and on which life-history traits were collected for this experiment per selection lines from generation 13-15.

<table>
<thead>
<tr>
<th></th>
<th>Mating failure</th>
<th>Total fecundity</th>
<th>Fecundity per day</th>
<th>Total fertility</th>
<th>Fertility per day</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>123</td>
<td>46</td>
<td>46</td>
<td>46</td>
<td>46</td>
</tr>
<tr>
<td>Distribution</td>
<td>Binomial</td>
<td>Quasi-Poisson</td>
<td>Quasi-Poisson</td>
<td>Quasi-Poisson</td>
<td>Quasi-Poisson</td>
</tr>
<tr>
<td>R2/pseudo-R2</td>
<td>-1.5689</td>
<td>4.9566</td>
<td>2.2266</td>
<td>4.627</td>
<td>0.5123</td>
</tr>
<tr>
<td>(z = 4.225, p = 2.39e-05)</td>
<td>(t = 13.806, p = &lt;2e-16)</td>
<td>(t = 4.319, p = 9.37e-05)</td>
<td>(t = 10.052, p = 5.7e-13)</td>
<td>(t = 0.470, p = 0.64)</td>
<td></td>
</tr>
<tr>
<td>Line</td>
<td>0.9204</td>
<td>2.2266</td>
<td>1.4087</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(z = 2.210, p = 0.027)</td>
<td>(t = 2.027, p = 0.049)</td>
<td>(t = 1.610, p = 0.12)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Treatment</td>
<td>1.5593</td>
<td>4.0134</td>
<td>12.0178</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(z = 3.649, p = 0.0003)</td>
<td>(t = 2.980, p = 0.0048)</td>
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<td></td>
</tr>
<tr>
<td>Pupal mass</td>
<td>3.899</td>
<td>7.0384</td>
<td>4.0134</td>
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<tr>
<td>(t = 2.589, p = 0.013)</td>
<td>(t = 3.327, p = 0.0018)</td>
<td>(t = 2.086, p = 0.043)</td>
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<td></td>
</tr>
<tr>
<td>Line:Treatment</td>
<td>-5.7611</td>
<td>-6.5067</td>
<td></td>
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<tr>
<td>(t = -2.170, p = 0.036)</td>
<td>(t = -1.785, p = 0.082)</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Supplementary Table 2: Detailed results of the glm models