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Genotype × environment interaction, environmental heterogeneity and the lek paradox

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Abstract

Substantial additive genetic variance (V_A) often exists for male signalling traits in spite of the directional selection that female choice imposes. One solution to this problem, a conundrum generally termed the 'lek paradox', is that genotype × environment interaction (GEI) occurs and generates a 'crossover' of reaction norms in which no one genotype performs in a superior manner in all environments. Theoretical work indicates that such crossover can sustain genetic variance provided that either (i) spatial heterogeneity in environmental conditions combined with limited migration among populations or (ii) temporal heterogeneity in environmental conditions combined with occasional generation overlap is present. Whereas some recent studies have revealed the intersection of reaction norms for sexually selected traits in laboratory and in natural populations, associated information on environmental heterogeneity, migration and generation overlap has not been investigated. We studied this question in an acoustic pyralid moth, *Achroia grisella*, in which previous work indicated GEI and crossover of reaction norms for several parameters of the male song evaluated by females. We measured reaction norms for male song as expressed when development was completed under different environmental conditions in four neighbouring, yet isolated, populations during 1 year and in one of these populations during consecutive years. Crossover occurred for the various song parameters in the several populations, but we did not observe a higher incidence of crossover between genotypes taken from two different populations than from the same population. However, for several key song parameters, crossover between genotypes taken from two different years was higher than that between genotypes from the same year. We suggest that temporal heterogeneity in the form of varying selection could potentially conserve V_A in *A. grisella*, but we also note other factors that might contribute.

Introduction

The presence of intraspecific variation in mating signals and courtship behaviour remains a major problem in reproductive biology in general and in sexual selection in particular (Kokko & Heubel, 2008; Higginson & Reader, 2009; Ingleby *et al.*, 2010). Observations and experimental studies of many species of invertebrates and vertebrates reveal substantial differences between individuals in the intensity, duration, rhythm and other characteristics of sexual advertisements. In males, these differences may significantly influence the signaller's mating and reproductive success (Andersson, 1994; Shuster & Wade, 2003), and in the case where signal variation reflects additive genetic variance (V_A), an evolutionary dilemma

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known as the 'paradox of the lek' (Taylor & Williams, 1982; Hunt *et al.*, 2004; Bussière *et al.*, 2008; Radwan, 2008) occurs: Given the directional selection pressure that female choice often imposes, what maintains the persistent genetic variation for signalling traits that is observed within a population? In other words, why do relatively unattractive, genetically determined male signal variants remain?

Applying the general mechanisms by which genetic variance can be maintained (Lewontin, 1974; Roff, 1997; Lynch & Walsh, 1998), biologists have proposed several resolutions to the above conundrum. These propositions include variation among females in preferences for and responses to males and male signals (e.g. Brooks & Endler, 2001; Miller & Moore, 2007; Foley *et al.*, 2010; Narraway *et al.*, 2010), mutation–selection balance (Rowe & Houle, 1996; Tomkins *et al.*, 2004; e.g. Kotiaho *et al.*, 2001), antagonistic pleiotropy or genetic trade-offs (Curtsinger *et al.*, 1994; Charlesworth & Hughes, 2000; e.g. Brooks, 2000; Hall *et al.*, 2009; Hine *et al.*, 2011), frequency-dependent selection (Kokko *et al.*, 2007; e.g. Fitzpatrick *et al.*, 2007) and GEI (Felsenstein, 1976; Slatkin, 1978; Charlesworth & Hughes, 2000; Charmantier & Garant, 2007; Kent *et al.*, 2008). Although these propositions are theoretically sound to varying degrees and under certain conditions (see Turelli & Barton, 2004 for an overview), relatively few empirical studies have addressed whether, and how, the various mechanisms might function in natural populations (e.g. Hine *et al.*, 2004) – or even in laboratory ones. Moreover, in those studies that have experimentally approached the problem of genetic variance for sexually selected traits, critical pieces of information are typically missing. For example, several population and quantitative genetic models indicate that GEI can maintain genetic variance for traits subject to selection provided that (i) ecological crossover (=intersection) of reaction norms occurs, (ii) the overall environment is spatially or temporally heterogeneous and (iii) some migration occurs between the different parts of a subdivided population or that generations occasionally overlap (Charlesworth, 1988; Gillespie & Turelli, 1989; Ellner & Hairston, 1994; Zhang, 2006; Bussière *et al.*, 2008; Kokko & Heubel, 2008; Cornwallis & Uller, 2010; Yeaman *et al.*, 2010; Rodríguez & Al-Wathiqui, 2011). Whereas a few studies report the requisite presence of ecological crossover within a local population such that no one genotype has 'superior' traits under all environmental conditions (Qvarnström, 1999; Jia *et al.*, 2000; Mills *et al.*, 2007), the level of environmental heterogeneity across space or time, the extent to which different 'reaction norm variants' exist in the various parts of a subdivided population and the amounts of migration and generational overlap across environments are largely unknown. That is, we do not know whether the ecological crossover, and hence V_A, currently observed within a local subpopulation might be sustained over the long term by virtue of migrants arriving from neighbouring subpopulations or one generation overlapping another. Here, we note that, given sufficient time, the individuals comprising a single panmictic population can be expected to evolve towards expressing a single reaction norm (Via & Lande, 1985). But in the stochastic and dynamic situation presented by a subdivided population, each subpopulation of which may be subject to different environmental conditions and selection pressures, certain regimes of relatively infrequent migration may preserve a diversity of reaction norms within a local subpopulation and render ecological crossover and the maintenance of V_A possible. Analogous effects may also occur where environmental conditions change across time and generations, but generations occasionally overlap. Thus, information on overall population structure, the distribution of reaction norm variants among the local subpopulations and the occurrence of migration between these local subpopulations would be critical for assessing the potential role of ecological crossover in regulating and maintaining V_A for all traits, including those subject to sexual selection.

Here, we present the findings from a continuing study on the potential role of GEI in maintaining genetic variance for male signal traits in an acoustic insect species, *Achroia grisella* (Lepidoptera: Pyralidae), where ecological crossover of reaction norms has been well documented (Danielson-François *et al.*, 2006; Zhou *et al.*, 2008) but the other requisite elements of the proposed mechanism have not yet been confirmed. Male *A. grisella* broadcast an ultrasonic advertisement song (Spangler *et al.*, 1984) that attracts females. Laboratory tests using live insects (Jang & Greenfield, 1998), as well as playback experiments testing the broadcasts of synthetic signals (Jang & Greenfield, 1996), show that a significant proportion of a male's overall attractiveness can be explained by several song characters. Subsequent quantitative genetic experiments employing either half-sib/full-sib breeding designs or random inbred lines demonstrated (i) that these signal characters as well as overall attractiveness are repeatable (Jang *et al.*, 1997) and heritable/evolvable traits (Collins *et al.*, 1999; Brandt & Greenfield, 2004; see Hansen *et al.*, 2011 on this designation), (ii) that these song traits exhibit phenotypic plasticity along several environmental gradients that are likely to occur in natural populations (Jia & Greenfield, 1997) and (iii) that GEI and crossover of reaction norms exist for these song traits as expressed along the environmental gradients (Jia *et al.*, 2000). We have found that ecological crossover in *A. grisella* can reflect the co-occurrence of relatively canalized and plastic reaction norm variants within a population (Danielson-François *et al.*, 2006, 2009). We also have verified the presence of diverse reaction norm variants and crossover within natural populations (Zhou *et al.*, 2008) as well as laboratory ones. In a parallel study, we have also examined the antagonistic pleiotropy hypothesis and found no evidence for any trade-off, that is, negative

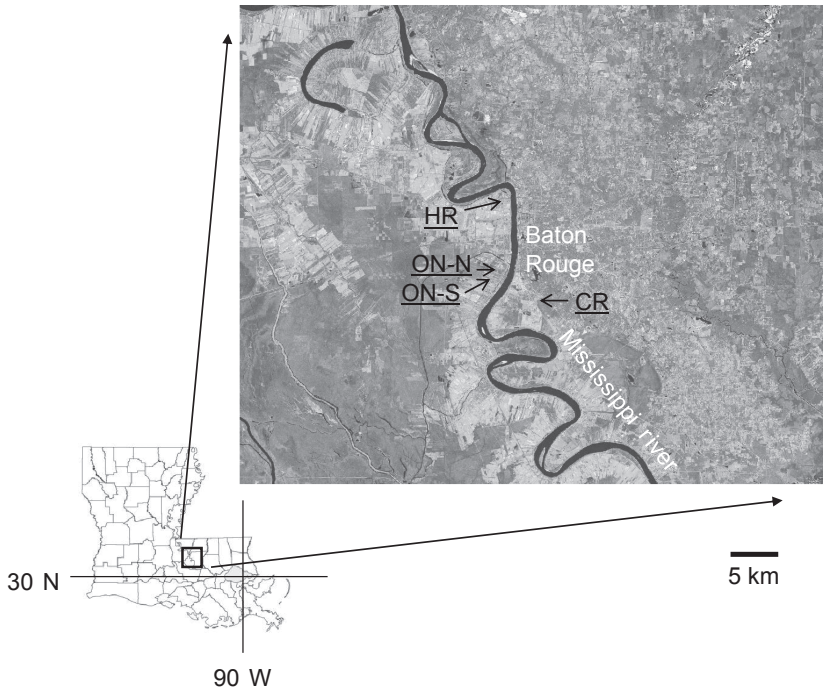

Fig. 1 Map showing locations of the four *Achroia grisella* populations (CR, HR, ON-N and ON-S) studied in the vicinity of Baton Rouge, Louisiana, during 2007.

genetic correlation, between adult longevity and male song attractiveness in laboratory populations (Brandt & Greenfield, 2004). Combined, these findings are consistent with and point towards the GEI hypothesis as responsible for the diversity of signal variants observed among males in *A. grisella* populations.

Our current study primarily addressed the degree to which neighbouring *A. grisella* populations exhibit different reaction norm variants and thereby present the opportunity for sustaining GEI. We also examined whether the reaction norm variants occurring in successive generations of the same population differ, which might preserve GEI if these generations overlap. *Achroia grisella* are symbionts of the western honeybee, *Apis mellifera*, at whose colonies the moth larvae feed on the organic detritus that accumulates as well as on some wax and stored pollen and honey. Feeding normally occurs at the periphery of the honeybee colony, away from the central core of worker bees, and colonies suffering a decline in worker population are favoured over those having high numbers of workers. The moths themselves are generally distributed among small, isolated populations, each representing an infested honeybee colony or group of colonies (bee yard) (Künike, 1930; Milum, 1940). *Achroia grisella* adults may migrate between these populations, either by means of their own flight or by human accident when honeybee colonies are moved. These features of population structure led us to predict that *A. grisella* could be exposed to the varying selection and migration demanded by the GEI hypothesis. We emphasize, however, that a complete analysis of this hypothesis would demand data on the actual incidence and regularity of interpopulation migration and also on the overlap of generations, data that are not yet available. Thus, our research on the variation of reaction norms between neighbouring populations and generations addresses only the potential for GEI to maintain V_A.

Materials and methods

Populations studied

We assessed the potential for spatial variation in reaction norm variants by studying four populations of *A. grisella* found at different bee yards in the vicinity of Baton Rouge, Louisiana, in late summer/early autumn 2007. Two of the populations (ON-S and ON-N) were separated by 2 km, whereas the remaining two (CR and HR) were at least 5 km distant from the other populations (Fig. 1). Honeybee colonies in this region of North America have a high incidence of infestation by *A. grisella*, particularly when they are not subject to regular apicultural management. We chose the four specific *A. grisella* populations used in our study because they were found at relatively unmanaged bee yards to which honeybee colonies had not been transferred from other sites for several years and because they were situated at least 1 km from the closest neighbouring honeybee colonies. *Achroia grisella* may be obligate symbionts of *A. mellifera* colonies, and we expected that the only gene flow into our four populations during 2007 and the immediately preceding years was that due to migrating adult moths.

Although adult *A. grisella* do have the capability to fly in search of resource sites, that is, *A. mellifera* colonies, and under natural conditions they would have exercised this ability when their natal site became depleted following the death of the honeybee colony, such circumstances and migration may be infrequent. These assumptions led us to consider the moths at each of the Louisiana sites as an independent population.

To effect a longitudinal analysis of reaction norm variants and thereby evaluate the potential contribution of temporal heterogeneity in conserving GEI, we selected one (ON-N) of the four populations examined in 2007 and studied it again during late summer 2008. Methods of collection, laboratory breeding and determination of reaction norms were identical for all four populations and both years.

Collection, breeding design and rearing

At each site during both 2007 and 2008, we collected between 20 and 50 adult *A. grisella* of both sexes and enclosed these moths in a plastic box supplied with 100 g of a synthetic diet adapted for this species (Jang & Greenfield, 1996). Female *A. grisella* oviposited in the boxes, and their larvae developed on the synthetic diet after hatching. We maintained these rearing boxes in environmental chambers at the University of Kansas under 'standard conditions', 25 °C and a 12 : 12 L/D photoperiod. We later divided the developing larvae among several boxes to maintain a moderate density, approximately 200 insects per 100 g of diet. As these insects enclosed, we chose 20–22 males from each population to serve as sires for the subsequent, experimental generation. Each sire was mated with three dams from its population, and the resulting offspring, representing 60–66 full-sib families, were initially kept in boxes with diet as mentioned above. We transferred these offspring during the 2nd larval instar to 28-mL plastic cups, three insects per cup on 2 g of diet, and continued maintaining them under the standard 12 : 12 L/D photoperiod. These levels of larval density and food availability were the standard conditions in a previous study (Zhou *et al.*, 2008) of phenotypic plasticity and reaction norms in *A. grisella*. As in that previous study, we emphasize that we established our experimental generation and conducted our tests of reaction norms as soon as possible following collection of insects in the field, given the constraint of initiating our breeding design in the laboratory. Thus, our findings on reaction norm diversity may approximate the actual diversity existing within the populations in the field. We reared the insects of the experimental generation on the synthetic diet, as opposed to natural food from the honeybee colonies where the moth populations had been collected, to maintain a standard nutritional condition for each population. We did not transfer larvae prior to the 2nd instar because handling smaller insects could not be carried out reliably without injury.

Because previous studies of *A. grisella* populations, including one from the Baton Rouge, Louisiana region (Zhou *et al.*, 2008), indicated substantial phenotypic plasticity in male song traits as expressed along a gradient of rearing temperatures, we specifically examined the thermal reaction norm in our current study. Applying the basic methodology of ecological genetics, we split the offspring of each full-sib family in the experimental generation into two groups, one in which the initial boxes and 28-mL rearing cups were kept at 22 °C and one at 29 °C. For each full-sib family and temperature, we transferred 30–35 2nd-instar larvae to 28-mL rearing cups. To reduce bias towards insects expressing fast or slow development, we included a range of sizes in each group of transferred larvae. Among all four populations and both rearing temperatures, approximately 65% of the insects completed development to the adult stage. The cool and warm rearing temperatures represented conditions that would be experienced by developing larvae in natural *A. grisella* populations in Louisiana over the course of their season each year or at different locations in the vicinity of a honeybee colony on a given day (Zhou *et al.*, 2008).

We noted the developmental period, measured from oviposition to adult eclosion, for each male and female and weighed each individual to the nearest 0.01 mg on the day of eclosion. We then recorded the calling song of males later on that same day.

Acoustic recording and analysis

Each eclosing male was recorded during the initial 6 h of the night, the interval during which *A. grisella* sing under natural conditions. Males were placed individually in cylindrical (1.5-cm diam. × 2 cm height) screen cages and then were moved to an acoustically insulated chamber kept at 25 °C and illuminated with a 25-W incandescent red bulb. We began recording 30 min after transfer to allow for adjustment to the chamber.

Recordings were 1 s in length and were made with a 6.25-mm instrumentation condenser microphone (model 7016; ACO Pacific, Belmont, CA, USA frequency response: ±2 dB from 10 to 100 000 Hz, ±6 dB from 10 to 160 000 Hz) whose output was amplified 40 dB (model 4012 preamplifier, model PS9200 amplifier, ACO Pacific), filtered (model 3202 variable filter, Krohn-Hite), digitized at 500 000 samples s^{-1} and 16 bits (National Instruments DAQCard 6062E) and saved to a computer file using BatSound 4.0 software (Petterson Elektronik AB, Uppsala, Sweden) for later analysis. A previous study (Jang *et al.*, 1997) indicated that *A. grisella* male song is sufficiently repeatable during a given night that a 1-s recording is representative of an individual's signalling at that time. That study also showed that the screen used in the male cages did not affect the acoustic parameters of

the song as it passed to the outside of the cage. We arranged the male cages such that neighbours were separated by a minimum 30 cm and partially shielded from each other by acoustic insulation. Thus, a given male's singing was not unduly influenced by his neighbours' songs. All recordings were made with the microphone positioned 10 cm from the male and oriented directly towards him. These specifications allowed us to estimate relative amplitude of male song.

From the 1-s recording taken of each male, we measured three acoustic parameters known to influence female attraction in various *A. grisella* populations: pulse rate, mean peak amplitude and mean asynchrony interval (Jang & Greenfield, 1996, 1998; Zhou *et al.*, 2008). Measurements were taken with a custom script adapted to a commercially available signal processing program (Spike2, Cambridge Electronic Design; Cambridge, UK; see Brandt & Greenfield, 2004 for complete procedure) that automatically analysed multiple files arranged in a folder. These measurements were confirmed manually where necessary. Based on a previous study showing that *A. grisella* females may evaluate males, in part, by the acoustic power (=pulse rate × mean peak amplitude) of their song (Greig & Greenfield, 2004), we also calculated this parameter from the values given by the initial analysis described above. Additional studies have shown that an index represented by a linear model of all three acoustic parameters can explain a substantial proportion of variation in male attractiveness to females (Jang & Greenfield, 1998; cf. Scheuber *et al.*, 2004). However, the model specifying this attractiveness index differs between populations in terms of the relative contributions of the individual acoustic parameters (Zhou *et al.*, 2008), and we did not conduct female choice trials in all of our populations to allow determination of the correct model for each one. Therefore, we do not analyse reaction norms for an overall attractiveness index in our study.

Quantitative and ecological genetic analyses

To estimate the potential influence of population on the four song and two developmental parameters, we implemented a 4-way mixed-model ANOVA in which the environmental rearing condition (temperature) was a fixed effect, whereas population, sire and dam were treated as random effects (see Fry, 1992). We implemented a general linear model (GLM) for ANOVA to account for the lack of a fully balanced design. Sires were nested within populations, dams were nested within sires, and the environmental rearing condition was crossed with population. This analysis also allowed us to estimate the influences of sire and of the interaction between sire and the environmental rearing condition on song and developmental parameters. The interaction values served as an approximate indication of GEI (cf. Zhou *et al.*, 2008). Similarly, we noted the interaction between population and environmental rearing condition as well as that between year and environmental rearing condition. We considered these interaction values as approximate indications of the potential for interpopulation and interyear differences to influence ecological crossover.

Because most of the aforementioned data were not distributed normally, we applied a Box–Cox transformation to all data, which removed approximately 50% of the departures from normality (departures defined by a Kolmogorov–Smirnov statistic with $P < 0.05$). We then checked for equality of variances among the four populations for each of the six parameters, and we proceeded with the 4-way ANOVA in those cases where variances were equivalent ($P > 0.05$, Bartlett's test). Variances were markedly unequal for two song parameters, pulse rate and mean asynchrony interval, and we therefore did not perform the ANOVA procedure on them. Rather, we analysed the differences in these two song parameters among populations nonparametrically by determining the median values for each sire within a given rearing condition and then applying the Kruskal–Wallis test for equality of medians.

We implemented the same 4-way, mixed-model ANOVA approach to estimate the potential influence of year on song and developmental parameters in the longitudinal part of our study. Year, sire and dam were treated as random effects. As mentioned above, sires were nested within years, dams were nested within sires, and the environmental rearing condition was crossed with year. All data were transformed (Box–Cox transformation) as before to improve normality, and equality of variances among years was checked. Variances for male and female body mass, male and female development and male pulse rate were not equal in the 2 years, and we therefore checked for the effect of year on these parameters by the Kruskal–Wallis procedure.

We recognized the possibility that the two rearing conditions may have inadvertently biased our sampling of adults – and ultimately our measures of reaction norms and crossover – via differential survival during immature development. To check this possibility, we performed the same 4-way mixed-model ANOVA procedure on survival, defined as the proportion of 2nd-instar larvae placed in cups that later eclosed to adults.

For each of our ANOVA models, we also calculated the variance components corresponding to all of the random factors as well as narrow-sense heritabilities (h^2) and standard errors of h^2 for these factors. Variance components and heritabilities were determined via the restricted maximum likelihood (REML) procedure in JMP version 9.0, and standard errors of h^2 were calculated by the method of Dickerson (1969).

By mating each sire with multiple dams, the potential maternal and genetic influences of a given dam on a sire's offspring under a particular rearing environment were greatly reduced. Thus, we could consider the average song or developmental parameter value of a

sire's offspring as an estimate of his genotypic value in a particular environment (see Zhou *et al.*, 2008). We relied heavily on this critical assumption in our analysis of ecological crossover.

We implemented an analysis of ecological crossover to provide a specific estimate of the potential contribution of GEI to preserving V_A for male song. For each of the four song parameters and both thermal rearing conditions, we calculated the mean value for the offspring of a full-sib family and then averaged these mean values for all full-sib families produced by a given sire. Considering these mean sire values as genotypic values, we then noted whether crossover occurred between cool and warm rearing conditions for all possible genotypic pairs within a given population; the four sire values analysed in a genotypic pair are termed a 'quadruple'. We used these 'presence vs. absence' data to estimate the proportion of crossover expected for a song parameter in a particular population. Similarly, we noted whether crossover occurred between cool and warm rearing conditions for all possible genotypic pairs within a given year for the population (ON-N) that was studied longitudinally, that is, in 2007 and 2008. Here, these data allowed us to estimate the proportion of crossover expected for a song parameter in a particular year. In all cases, we also determined the statistical significance of crossover by applying a modification (Baker, 1988; Cornelius *et al.*, 1992) of the Azzalini & Cox (1984) for assessing changes in rank order in each quadruple.

To assess the potential contribution that spatial variation in reaction norms could make to ecological crossover in a given population should some level of interpopulation migration occur, quadruples that comprised pairs of populations and environments (rearing temperatures) were also examined. We considered all possible genotypic pairs between that focal population and one of the three others in the study and scored crossover by the presence vs. absence criterion and the modified Azzalini–Cox rank-order test. These values were determined for all six pairs of populations and for all four song parameters and two developmental parameters. Thus, we estimated the proportion of crossover expected for a parameter should a migrant arrive from another population in the area. We then compared these estimates with the within-population estimates described above to evaluate the relative importance of spatial heterogeneity. In a similar manner, we made analogous estimates of quadruples for our longitudinal study by considering all possible genotypic pairs between the ON-N population in 2007 and in 2008 and scoring crossover for all four song parameters and two developmental parameters at the two temperatures. Here, we estimated the proportion of crossover expected for a parameter should these generations encounter one another. As described above, we compared these estimates with the within-year estimates for the ON-N population and thus evaluated the relative importance of temporal heterogeneity.

Results

Population, year, genotype effects, and interactions

We found significant differences between the four populations studied in 2007 for several of the song parameters measured. Pulse rate ($P < 0.01$; Kruskal–Wallis test), mean peak amplitude ($P = 0.04$; 4-way ANOVA; Table 1) and acoustic power ($P = 0.007$; ANOVA; Table 1) differed between populations. Mean asynchrony interval differed under one rearing condition (warm; $P = 0.04$; K–W test) but not under another (cool; $P = 0.28$; K–W test). Neither body mass nor developmental rate (=reciprocal of the developmental period) in males or in females differed significantly between the four populations.

We found fewer significant differences between the 2 years studied at the ON-N site for song parameters, but on the other hand, we observed differences in developmental parameters. Pulse rate differed between years under one rearing condition (warm; $P < 0.01$; K–W test) but not under the other (cool; $P = 0.41$; K–W test). Neither mean peak amplitude, mean asynchrony interval, nor acoustic power differed significantly between years (Table 2). Both body mass and developmental rate, in males and in females, differed significantly between the 2 years studied ($P < 0.01$; K–W tests).

Our ANOVA revealed significant differences between genotypes (sire effects) for mean peak amplitude and acoustic power (Tables 1 and 2). Significant interaction values were observed for acoustic power in the four populations studied in 2007 (rearing condition × sire (population); $P = 0.038$; Table 1) and for mean asynchrony interval in the populations studied at the ON-N site in 2007 and 2008 (rearing condition × sire (year); $P = 0.047$; Table 2). We also observed significant interaction values (rearing × sire (population) for all four developmental parameters in the 2007 study (Table 1) as well as for the interaction between rearing condition and population (male developmental rate, $P = 0.001$; female developmental rate, $P = 0.002$; female body mass, $P = 0.019$; mean peak amplitude, $P = 0.019$; Table 1) and between rearing condition and year (mean peak amplitude, $P < 0.001$; acoustic power, $P = 0.001$; Table 2).

ANOVA showed that the two rearing conditions did not bias our results via differential survival (Table 3). Moreover, we found only one factor that had a significant influence on survival in the two analyses: The dam factor was marginally significant ($P = 0.05$) in the analysis of the four populations sampled in 2007. Variance components and narrow-sense heritabilities for the random factors in the models that analysed the four populations sampled in 2007 and the one population sampled in both 2007 and 2008 are given in supplementary Tables S1-S8. Heritability values are comparable to those reported in previous studies of other North American *A. grisella* populations (Supporting information Tables S7, S8; cf. Collins *et al.*, 1999; Brandt & Greenfield, 2004).

Table 1 Results of mixed-model 4-way ANOVA (general linear model; MINITAB version 13) on developmental parameters (DP^{-1}, developmental rate; BM, body mass at eclosion) and song parameters (PA, mean peak amplitude; AP, acoustic power of male song) in four Louisiana populations of *Achroia grisella*. The two different environmental rearing conditions (29 °C and 22 °C) represent the fixed factor, and the population, sire and dam represent random factors. All data were treated by the Box–Cox transformation prior to analysis. Pulse rate (PR) and mean synchrony interval (AI) of male song are not included owing to nonhomogeneous variance and are analysed nonparametrically (Kruskal–Wallis test; see text).

		DP^{-1} (females)		BM (females)		DP^{-1} (males)		BM (males)		PA (male song)		AP (male song)	
Source	d.f.	MS	F	MS	F	MS	F	MS	F	MS	F	MS ($\times 10^6$)	F
Population	3	8388.0	3.71	680.4	1.85	6836.7	2.79	36.64	1.07	6042.4	4.69*	30.620	**6.30****
Sire (population)	74†	647.4	1.25	159.1	1.17	807.2	1.35	33.0	**1.87****	694.6	**1.82****	4.687	**1.63***
Environmental rearing condition	1	400.442.8	**203.34****	71.255.7	**242.88****	406.680.3	**206.57****	14.195.65	**998.70*****	36.866.2	**43.80****	4.727	2.29
Population × environmental rearing condition	3	1982.3	**5.57****	295.0	3.52*	1970.9	**6.15****	14.22	1.13	842.6	**3.52***	2.064	1.14
Environmental rearing condition × sire (population)	74†	363.8	**2.72*****	84.8	**1.63****	323.1	**1.79*****	12.65	**1.40***	240.1	1.14	1.830	**1.31***
Dam (population sire)	154‡	291.5	**2.18*****	194.1	**2.00*****	459.3	**2.54*****	14.17	**1.57*****	354.2	**1.69*****	2.467	**1.77*****
Error	3627§	133.8		52.0		180.7		9.05		209.8		1.397	

*$P < 0.05$; **$P < 0.01$ and ***$P < 0.001$.
†d.f. for male traits = 76.
‡d.f. for male traits = 158.
§d.f. for BM (females) = 3607; d.f. for male traits = 3836.
Boldface F statistics are significant ($P < 0.05$) following the Holm (1979) correction (sequential Bonferroni correction) for multiple tests.

Table 2 Results of mixed-model 4-way ANOVA (general linear model) on male song parameters (PA, mean peak amplitude; AI, mean asynchrony interval; AP, acoustic power of male song) during two consecutive years (2007 and 2008) at the ON-N *Achroia grisella* population. The two environmental rearing conditions (29 °C and 22 °C) represent the fixed factor, and the year, sire and dam represent random factors. All data were treated by the Box–Cox transformation prior to analysis. Developmental rate (DP^{-1}), body mass at eclosion (BM) and pulse rate (PR) of male song are not included owing to nonhomogeneous variance and are analysed nonparametrically (Kruskal–Wallis test; see text).

		PA (male song)		AI (male song)		AP (male song)	
Source	d.f.	MS	F	MS ($\times 10^3$)	F	MS ($\times 10^6$)	F
Year	1	4.4	0.00	51.7	1.25	1.049	0.04
Sire (year)	35	813.0	**1.94***	163.6	1.02	4.579	**1.94***
Environmental rearing condition	1	50.342.6	6.54	1.221.0	44.42	35.593	1.65
Year × environmental rearing condition	1	7702.6	**31.10*****	27.5	0.19	21.518	**14.20****
Environmental rearing condition × sire (year)	35	249.0	1.36	149.4	1.44*	1.519	1.19
Dam (year sire)	74	357.3	**1.95*****	114.5	1.10	2.139	**1.67*****
Error	1748†	183.5		103.8		1.279	

*$P < 0.05$; **$P < 0.01$ and ***$P < 0.001$.
†d.f. for AI = 1818.
Boldface F statistics are significant ($P < 0.05$) following the Holm (1979) correction (sequential Bonferroni correction) for multiple tests.

Ecological crossover

By analysing all possible 'quadruples' (binary combinations of genotypes reared under warm and cool conditions) for a given developmental or song parameter in a population, we estimated the expected incidence of crossover. This method showed that approximately one-third of the quadruples experienced crossover, defined by an intersection of reaction norms. Values ranged from a low of 22% to a high of 62% among the four populations studied in 2007 and the six parameters measured in male moths (Table 4). When we restricted

Table 3 Results of mixed-model 4-way ANOVA (general linear model) on survival from the 2nd-instar larva to the adult in *Achroia grisella*. Fixed and random factors are as specified in Tables 1 and 2. All data were treated by the Box–Cox transformation prior to analysis.

		Survival	
Source	d.f.	MS	F
a. Data taken from 4 populations sampled in Louisiana during 2007			
Population	3	264 173	1.97
Sire (population)	74	95 963	1.18
Environmental rearing condition	1	16 470	0.17
Population × environmental rearing condition	3	97 071	1.66
Environmental rearing condition × sire (population)	74	58 554	0.81
Dam (population sire)	154	94 859	1.31*
Error	154	72 394	
b. Data taken from the ON-N population sampled during both 2007 and 2008			
Year	1	3.806	0.29
Sire (year)	35	6.977	1.34
Environmental rearing condition	1	2.359	0.22
Year × environmental rearing condition	1	10.684	2.29
Environmental rearing condition × sire (year)	35	4.663	0.86
Dam (year sire)	74	5.982	1.10
Error	74	5.432	

*$P < 0.05$

crossover to those quadruples for which the intersection of reaction norms was significant by the modified Azzalini–Cox rank-order test, the values dropped considerably, ranging from 0 to 13% among the populations and parameters (Table 4). These percentages are comparable to findings on crossover in previous studies of *A. grisella* (Jia *et al.*, 2000; Danielson-François *et al.*, 2006; Zhou *et al.*, 2008).

We did not observe higher percentages of crossover among quadruples in which the two genotypes represented two different populations (Table 4, lower six rows) than among within-population quadruples (Table 4, upper four rows). This result held for crossover scored either as the simple intersection of reaction norms ($P = 0.32$, $t = 1.01$, d.f. = 51) or as a significant change in rank order according to the modified Azzalini–Cox test ($P = 0.83$, $t = 0.21$, d.f. = 40). Similarity of percentages also held when we made these comparisons for a given developmental or song parameter ($P > 0.15$, t-tests). We did not observe markedly lower percentages of crossover in between-population quadruples including genotypes from the ON-N and ON-S populations, which were separated by only 2 km, than in quadruples including genotypes from two populations separated by greater distances (see Fig. 1).

On the other hand, we did observe a generally higher incidence of crossover for song parameters among quadruples in which the two genotypes represented two different years (2007 and 2008; Table 5, bottom row) than among within-year quadruples at the ON-N population (Table 5, upper two rows). In particular, we found that for three of the four song parameters measured,

Table 4 Incidence of ecological crossover for two developmental (DP^{-1}: developmental rate; BM: body mass at eclosion) and four song parameters (PR, pulse rate; PA, mean peak amplitude; AI, mean asynchrony interval; AP, acoustic power) in males sampled from four *Achroia grisella* populations (CR, HR, ON-N and ON-S) during 2007. The number of quadruples analysed represents all binary combinations of genotypes (sire families); each genotype reared under two different environmental conditions, 22 °C and 29 °C. Quadruples were composed in two ways: (i) within population, in which the two genotypes in a binary combination were taken from the same population (upper four rows); (ii) between population, in which the two genotypes were taken from the two different populations specified in the left column (data listed in lower six rows).

	Number of quadruples analysed	DP^{-1}	BM	PR	PA	AI	AP
Within-population crossover							
CR	190	30.53 (0.53)	41.05 (5.79)	38.95 (3.68)	42.63 (3.16)	62.63 (13.16)	46.32 (1.58)
HR	190	27.37 (0.00)	35.79 (2.63)	38.96 (3.68)	28.95 (1.05)	54.74 (10.00)	22.63 (1.05)
ON-N	190	28.95 (2.11)	24.74 (0.53)	42.11 (5.79)	28.42 (4.74)	45.26 (3.68)	36.84 (4.74)
ON-S	190	38.95 (0.53)	46.32 (6.32)	35.79 (4.74)	34.74 (4.21)	47.37 (8.95)	44.74 (6.84)
Between-population crossover							
CR/HR	400	28.50 (3.00)	38.50 (2.00)	33.00 (8.25)	38.75 (5.50)	62.25 (9.50)	31.50 (1.75)
CR/ON-N	400	22.25 (2.00)	31.75 (1.75)	16.50 (1.50)	30.25 (2.50)	42.00 (3.50)	42.50 (4.75)
CR/ON-S	400	38.25 (1.50)	43.00 (3.75)	28.75 (4.00)	41.25 (4.75)	52.00 (9.25)	46.50 (5.75)
HR/ON-N	400	30.25 (1.00)	31.75 (2.25)	46.50 (9.00)	21.75 (1.75)	44.75 (4.75)	24.50 (1.25)
HR/ON-S	400	24.75 (1.00)	37.75 (5.75)	46.25 (6.75)	31.50 (2.50)	55.25 (8.00)	30.75 (4.00)
ON-N/ON-S	400	21.25 (1.00)	31.75 (4.75)	33.50 (3.25)	32.00 (2.00)	40.50 (6.25)	41.75 (3.00)

The values given under each of the six parameters are the percentages of all quadruples in which reaction norms crossed; parenthetic values are percentages of all quadruples in which ecological crossover was statistically significant ($P < 0.05$) by the modified Azzalini–Cox rank-order test (Baker, 1988).

Table 5 Incidence of ecological crossover for two developmental (DP^{-1}: developmental rate; BM, body mass at eclosion) and four song parameters (PR, pulse rate; PA, mean peak amplitude; AI, mean asynchrony interval; AP, acoustic power) in males sampled from the ON-N *Achroia grisella* population during both 2007 and 2008. The number of quadruples analysed represents all binary combinations of genotypes (sire families); each genotype reared under two different environmental conditions, 22 °C and 29 °C. Quadruples were composed in two ways: (i) within year, in which the two genotypes in a binary combination were taken from the same year (upper two rows); (ii) between year, in which the two genotypes were taken from the two different years (lowest row). The values given under each of the six parameters are the percentages of all quadruples in which reaction norms crossed; parenthetic values are percentages of all quadruples in which ecological crossover was statistically significant ($P < 0.05$) by the modified Azzalini–Cox rank-order test (Baker, 1988).

	Number of quadruples analysed	DP^{-1}	BM	PR	PA	AI	AP
Within-year crossover							
2007	190	28.95 (2.11)	24.74 (0.53)	42.11 (5.79)	28.42 (4.74)	45.26 (3.68)	36.84 (4.74)
2008	136	46.32 (9.56)	27.94 (3.68)	38.95 (0.07)	27.21 (0.07)	56.62 (11.03)	27.21 (1.47)
Between-year crossover							
2007/2008	340	0.00** (0.00)**	10.59** (0.09)**	42.65 (7.65)*	47.35** (12.35)**	48.82 (7.06)	44.12** (7.35)*

*$P < 0.05$ and **$P < 0.01$; test comparing two binomial proportions: the incidence of crossover in between-year quadruples and in within-year quadruples.

pulse rate, mean peak amplitude and acoustic power, percentages of crossover, scored either as a simple intersection of reaction norms or as a significant change in rank order determined by the Azzalini–Cox test, were higher in between-year quadruples. For crossover scored by the Azzalini–Cox criterion, percentages found in between-year quadruples were significantly higher than those found in within-year quadruples for pulse rate ($P = 0.026$; comparison test of two binomial proportions), mean peak amplitude ($P < 0.001$) and acoustic power ($P = 0.022$). Importantly, this elevated incidence of crossover in between-year quadruples occurred despite the absence of a 'year effect' on the expression of peak amplitude or acoustic power (Table 2). A significant year effect was observed, however, on developmental parameters, and consistent differences between 2007 and 2008 moths are most likely responsible for the low incidence of crossover found in between-year quadruples for developmental rate and body mass (Table 5).

Discussion

Effects, interactions and crossover

Our breeding design and acoustic measurements revealed significant differences in several key male song parameters among the four populations sampled during 2007 in the vicinity of Baton Rouge, Louisiana (Table 1). In comparison with interpopulation variation, fewer differences in male song were found between years in the one population sampled in both 2007 and 2008 (Table 2). As in previous quantitative genetic studies of *A. grisella* populations from other regions in North America, ANOVA indicated significant sire effects on two of the key male song parameters, mean peak amplitude and acoustic power, suggesting that substantial V_A exists for these traits. Our analyses also revealed an interaction effect (rearing condition × sire (population)) on acoustic power, suggesting the existence of GEI for this trait and the potential for crossover. Other interaction effects on male song parameters were detected for rearing condition × population and for rearing condition × year. These two latter interactions suggest the potential for elevated levels of crossover between genotypes representing different populations or different years. But the actual measurement of crossover demanded specific analyses of the reaction norms.

As implied by the interaction values from our ANOVA, we measured a considerable incidence of crossover for the various male song parameters in all four populations (Table 3). That is, the rank order between mean sire (genotype) values for a particular song parameter often changed between rearing conditions such that the genotype exhibiting the 'superior' song – superiority being judged by female responses in independent tests – under one condition displayed the inferior song under the other. However, when we only considered changes in rank order that were significant, the amount of crossover was much lower for all parameters in each population. Nonetheless, these reduced levels of crossover might still be sufficient to help maintain the V_A observed in the various populations, variation that remains in spite of the directional selection that female preference apparently imposes on the male song parameters, because a high proportion of genotypes do experience some crossover interaction under this restrictive criterion. That is, the reaction norm of a given genotype generally intersects the reaction norm of one or more other genotypes sampled from the population.

Spatial and temporal heterogeneity

Given that a certain amount of crossover occurs for all of the key male song parameters and in all of the popu lations sampled, we are then faced with identifying the

factor(s) that could maintain the underlying diversity of reaction norm variants. We considered both spatial and temporal heterogeneity operating in the following contexts: (i) Owing to environmental differences as well as chance due to genetic drift, neighbouring populations may include different sets of reaction norm variants. When migration occurs, some of these variants may arrive at the focal population and add to its diversity, particularly as migration occurs subsequent to the development of the signal traits (Kokko & Heubel, 2008). (ii) Analogously, successive generations may be exposed to different environmental regimes, which select for different reaction norm variants. If generations occasionally overlap, the focal population would then include an elevated diversity of reaction norm variants. In both cases, this higher diversity may persist, albeit at a diminishing level, following the migration or generational overlap event. We tested the potential importance of both types of heterogeneity by evaluating the proportions of crossover that occurred in interpopulation and interyear quadruples. Whereas we did not find that crossover was any more likely to occur in between-population quadruples than in quadruples wherein both genotypes were taken from the same population; crossover was more frequent in between-year quadruples than in quadruples with genotypes from the same year. This latter observation implies that temporal hetero geneity represents a potential source of reaction norm diversity and crossover in *A. grisella* populations.

How might temporal heterogeneity function in natural populations of *A. grisella*? Our longitudinal study examined *A. grisella* sampled on two occasions separated by 1 year. The between-sample interval was likely several generations, and thus, the quadruples we analysed would not accurately represent the sort of intergeneration overlap that has been proposed for maintaining diverse reaction norms within a population. However, we note that if augmented crossover can occur between generations sampled from the same population and during the same time of year, albeit 12 months apart, equally augmented levels might be expected between moths of consecutive generations that had developed during different months – and conditions – of the same year. In the latter case, overlap of generations may occur when egg or larval development of the earlier generation is delayed due to weather, scarcity or low quality of food resources, or the specific location in or near a honeybee colony where eggs were laid or young larvae were developing. Such delays may lead to a coincidence of adults from both generations. Owing to the different selection regimes to which these insects had been exposed during early development, the surviving adults may represent somewhat different sets of reaction norm variants. Intergeneration overlap occurring under these circumstances would then yield increased crossover similar to that we observed under our partly artificial test.

Based on our findings, we have emphasized the potential importance of temporal heterogeneity in resolving the lek paradox by generating ecological crossover and ultimately maintaining V_A in mating signals, traits that are typically subject to directional selection. At the same time, we do not wish to dismiss the possibility that spatial heterogeneity functions in a similar way in other *A. grisella* populations, at other times, or in other species. Obviously, our results pertain to a single set of four *A. grisella* populations sampled during a single season. Whereas we selected these four populations based on their mutual isolation as well as their recent apicultural management, we do not know the full extent to which *A. grisella* adults migrate between honeybee colonies. Adult moths are capable of arriving at honeycomb baits placed in the field (M. D. Greenfield, unpublished data), and their propensity for migration may be greater than we had assumed. Consequently, our four populations, while exhibiting some differences (Table 1), had sufficiently similar sets of reaction norm variants that the level of interpopulation crossover did not exceed the within-population level. From a statistical perspective, we note that none of the tests indicating equivalence of crossover in between-population and within-population quadruples had power values > 0.80, suggesting that actual differences might yet occur. Moreover, the higher incidence of crossover in between-year than in within-year quadruples was based on a single comparison (2007 and 2008 at 1 population), and two of the song parameters (mean peak amplitude and acoustic power) for which differences were observed are not independent of each other. Thus, our conclusion on temporal heterogeneity must remain tentative at this point.

We also do not wish to overlook the other mechanisms that are likely to generate crossover and maintain V_A in sexually selected traits. In particular, our study has measured only male signals while ignoring the potential variation, phenotypic plasticity, GEI and crossover that may exist in female responses and preference functions. Some data show that these several features do exist in some *A. grisella* populations (Jang & Greenfield, 2000; Rodriguez & Greenfield, 2003), implying that 'superior' male signalling variants may not be superior from the perspective of all females. If reaction norm variants for female preference exist and respond to environmental conditions in ways that do not mirror the responses of male signalling variants, one can readily imagine the many situations in which variation in sexually selected traits would be maintained. Eventually, such plasticity in female response and preference traits will have to be both assayed and incorporated in theoretical simulations. While these additions are not expected to be easy, a full understanding of the evolution of sexually selected traits and the lek paradox cannot be accomplished without the joint consideration of signalling and preference traits.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1 REML estimates of variance components for male developmental and song traits using JMP version 9.0.

Table S2 REML estimates of variance components for female developmental traits using JMP version 9.0.

Table S3 REML estimates of variance components for male developmental and song traits using JMP version 9.0.

Table S4 REML estimates of variance components for female developmental traits using JMP version 9.0.

Table S5 REML estimates of variance components for survival (from 2nd instar to adult; males and females combined) using JMP version 9.0.

Table S6 REML estimates of variance components for survival (from 2nd instar to adult; males and females combined) using JMP version 9.0.

Table S7 Narrow sense heritability and its standard error (parenthetic value) for six male developmental and song traits as measured in the four Louisiana populations sampled in 2007 and the ON-N population sampled in 2008.

Table S8 Narrow sense heritability and its standard error (parenthetic value) for two female developmental traits as measured in the four Louisiana populations sampled in 2007 and the ON-N population sampled in 2008.

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