Reproductive characteristics of the flower-breeding *Drosophila hibisci* Bock (Drosophilidae) in eastern Australia: within-population genetic determinants of ovariole number

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Genetic variation for ovariole number in the flower-breeding *Drosophila hibisci* was studied within populations obtained from three separate sites in the centre of the species distribution along the east coast of Australia. Heritability for ovariole number (adjusted for body size), derived from isofemale lines from each site, was estimated to be $h^2 \pm \text{SE} = 0.564 \pm 0.160$. The variance of ovariole number within sites ($\sigma_{\text{within}}^2 = 2.039$) was comparable to the variance between sites ($\sigma_{\text{between}}^2 = 2.048$) obtained from an earlier study of populations sampled over 14° of latitude. Two isofemale lines (from within one site) that differed by an average of 4.6 ovarioles were used to generate F₁, F₂ and backcross generations. Analysis of mean ovariole number for these generations showed that only additive gene effects were important and that dominance, digenic epistasis and maternal effects were not significant. This within-population result contrasted with earlier results between populations that revealed additive and digenic epistasis for the same trait. High heritability within populations and the relatively large within-population variation for ovariole number suggest that substantial microhabitat variation is influencing this fitness-related trait.

Keywords: *Drosophila hibisci*, epistasis, *Hibiscus*, isofemale lines, line-cross analysis, ovariole number.

Introduction

A comparison of genetic variation within and between populations of the same species provides insights into the amount of genetic diversification and the potential for salient evolutionary events such as speciation. What constitutes 'within' and 'between' can be as simple as separate groups of individuals occupying different spatial localities. Random interbreeding within the groups and sufficient noninterbreeding between groups provide the conditions necessary for genetic and biological diversification. To some degree the microhabitat used by organisms can influence the genetic structure of populations and determine the within vs. between distinction, although temporal isolation also can affect genetic groupings, especially when breeding cycles are

out of phase. Whether it is space, time or some combination of these, their actions on genetic exchange among groups are pervasive in ecological and evolutionary processes.

Traits closely coupled to fecundity and survival are expected to undergo natural selection and thus show little heritability within populations (Falconer, 1989). Much of the variation for fitness-related traits is thus expected to result from environmental causes, nonadditive genetic variation and/or genotype by environment interactions. Genetic variation between populations could be ascribed to these factors as well but may also include additive genetic components that would be exposed to natural selection only if the isolated populations were brought together in secondary contact.

We have been investigating the reproductive characteristics of the flower-breeding *Drosophila hibisci* in eastern Australia. The fly shows considerable clinal variation among populations, over 14° of latitude, in the

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number of ovarioles per female (Starmer *et al.*, 1997). Crosses between two divergent populations, approximately 1150 km apart, have shown that at least part of the among-population variation is attributable to different genetic determinants (additive and epistatic gene activity) as well as environmental influences such as temperature and genotype by environment interactions (Starmer *et al.*, 1998). In this paper, we examine the within-population quantitative genetics of ovariole number in *D. hibisci*. We used isofemale lines obtained from three separate populations of *D. hibisci* within the centre of its distribution in eastern Australia to estimate heritability of ovariole number. Line-cross analysis was also used to determine the importance of genetic effects, including additivity, dominance, epistasis and maternal effects, in influencing variation in ovariole number within a single population. The results are contrasted to the earlier estimates of genetic effects on variation in ovariole number between populations (Starmer *et al.*, 1998).

The study organism, *D. hibisci*, is restricted to the blossoms of native *Hibiscus* species and, as a consequence, its distribution is coincident with the plant distribution. Courtship and mating occur within open *Hibiscus* blossoms (Polak *et al.*, 1998), and females oviposit into the petal tissue at the base of the corolla. Larvae feed and develop in decaying blossoms under or near the parent tree, and pupation probably occurs in litter surrounding fallen blossoms. The plants grow at forest margins, in disturbed areas, along ravines, waterways and roads. The plant distribution is patchy and results in a substructured population for *D. hibisci*.

Materials and methods

Adults and larvae of *D. hibisci* in flowers of *Hibiscus heterophyllus* were collected in early September 1997 at three field sites: (i) Bungwahl Creek (bck), N.S.W., 32.056°S, 152.442°E, 65 m elevation; (ii) Bellingen (bel), N.S.W., 30.419°S, 152.824°E, 40 m elevation; (iii) Nimbin (trd), N.S.W., 28.595°S, 153.241°E, 190 m elevation. Isofemale lines were started from these collections with a single pair (male and female) of either wild-caught adults or adults reared from the field flowers. Each pair was kept in a cage supplied every day with glasshouse-grown *H. heterophyllus* blossoms. Progeny from these pairs were given 1–2 blossoms each day in their cage of origin to maintain and increase the population size of the lines. Twenty-three lines were successfully established (9 bck, 9 bel and 5 trd). Females from the second generation of each isofemale line were allowed to mature in their cages before being characterized. Thorax length was measured from the

anterior end of the thorax to the posterior end of the scutellum. Reproductive tissues were dissected into a solution of physiological saline (PBS: 16 g NaCl, 0.4 g KCl, 0.4 g KH₂PO₄, 2.3 g Na₂HPO₄, 200 mL H₂O, brought to a pH of 6.5 with NaOH) and fixed for 1 min or less in FAA (6:16:1:30, formalin: absolute alcohol: glacial acetic acid: distilled water). Ovarioles were teased apart and counted for each ovary. Measurements were made on the width (*w*) and length (*l*) of the egg (if present) in the uterus. Egg volume was calculated as $(1/6) \pi w^2 l$ (Atkinson, 1979; Montague *et al.*, 1981). Up to 24 females from each line were dissected. In cases where lines were not productive, or were eventually lost, fewer females were dissected. Dissection of females of each line was interspersed over a one month period.

One site (bel) was sampled four times over a three-week period (on 22 and 28 October, 1 and 12 November 1997) for comparison to a similar sample over 4 weeks taken the year before (Starmer *et al.*, 1998). The ovariole numbers from these eight collections were used to estimate the year-to-year and collection-to-collection variation within the site. Variance component estimates were obtained using the SAS procedure VARCOMP (SAS, 1989).

Because ovariole number was a function of female thorax length ($r = 0.787$, d.f. = 437, $P < 0.001$), ovariole number was corrected for size using an analysis of covariance with thorax length as the covariate (continuous size factor). In general, the thorax size correction was applied to ovariole number in the context of the particular comparison and thus corrected means for ovariole number depend on the comparison. The heritability of individual differences (h^2) and its standard error for (i) corrected ovariole number, (ii) thorax length, (iii) egg width, (iv) egg length, and (v) egg volume, was estimated using the method described by Hoffman & Parsons (1988), but corrected by deleting the square of the denominator in their equation for the standard error of h^2 . ANOVA of between lines within sites, and estimation of variance components for between and within isofemale lines was carried out using LSMLMW (Harvey, 1990).

After initial characterization, two lines from the Nimbin (trd) population were chosen for line-cross analysis (Lynch & Walsh, 1997). These lines (trd48 and trd35) showed substantial divergence (size-adjusted mean ovariole number of 21.53 and 16.94, respectively; $t_{40} = 5.71$, $P < 0.00001$) and were crossed to form 10 lines: four nonsegregating lines made up of the two parental, F₁ and reciprocal F₁; six segregating lines made up of two F₂s and four backcross generations. The two F₂ lines were pooled because they did not differ significantly in corrected ovariole number [F₂ (1), 18.47

and F_2 (2), 18.02; $t_{39} = 0.65$, $P = 0.52$) and could not be used to estimate unique genetic effects. Because one of the backcrosses (BC48r) did not yield sufficient flies it was eliminated and only eight of the nine lines were used in the line-cross analysis. Two models based on generation means analysis (Mather & Jinks, 1982) were used to estimate progeny and maternal genetic contributions to the line means. The full progeny model included composite genetic effects of additive [d], dominance [h], and digenic epistatic effects of additive by additive [i], additive by dominance [j] and dominance by dominance [l]. The full maternal model included progeny composite effects of additive [d] and dominance [h] as well as maternal additive [$d.m$] and maternal dominance [$h.m$] effects.

In both models, least-squares procedures were used to estimate model parameters contained in vector $\mathbf{y}$ and their variances from the diagonal of their variance covariance matrix $\mathbf{S}$ (Mather & Jinks, 1982; Lynch & Walsh, 1997). The estimates of $\mathbf{y}$ and $\mathbf{S}$ are obtained as $\hat{\mathbf{y}} = (\mathbf{C}^T \mathbf{V}^{-1} \mathbf{C})^{-1} \mathbf{C}^T \mathbf{V}^{-1} \mathbf{x}$ and $\hat{\mathbf{S}} = (\mathbf{C}^T \mathbf{V}^{-1} \mathbf{C})^{-1}$ where $\mathbf{C}$ is the coefficient matrix (Table 1) for the contribution of effects to each line mean, $\mathbf{V}$ is the diagonal matrix of the error variances of each line mean, and $\mathbf{x}$ is the vector of observed line means. All parameters of each model were estimated in a full model to provide a guide to which parameters might be important in reduced models. Estimates of parameters are used to predict the line means as the algebraic sum of the contribution of each parameter associated with the expected genotype of that line (Mather & Jinks, 1982). Goodness of fit of each model was tested by a χ^2 , calculated as $\chi^2 = \mathbf{x}^T \mathbf{V}^{-1} \mathbf{x} - \mathbf{x}^T \mathbf{V}^{-1} \mathbf{C} \hat{\mathbf{y}}$ (Hayman, 1958). The degrees of freedom for this χ^2 are the number of line means minus the number of parameters estimated in the model.

F -statistics were used to evaluate the improvement in the goodness of fit for other parameters (Graybill, 1961). This was accomplished by using:

$$F_{p_2 - p_1, n - p_2} = \frac{(\chi^2_{(n - p_1)} - \chi^2_{(n - p_2)}) / (p_2 - p_1)}{\chi^2_{(n - p_2)} / (n - p_2)},$$

where n is the number of line means, p_1 is the number of parameters in the basic model and p_2 is the number of parameters of the expanded model.

In the analysis of generation means (or means of line crosses) the loci involved are assumed to be in Hardy–Weinberg and linkage equilibrium. In addition, loci that differ between the two parental lines are assumed to be unlinked. The parental lines can carry the same alleles but because the analysis estimates the net difference between additive effects, average dominance and the net directional epistasis, the alleles that are the same only need to differ in frequency (Lynch & Walsh, 1997). However, because we crossed progeny of isofemale lines we assume most of the genetic differences are homozygotes for different alleles affecting the trait of interest. The composite effects of the model summarize the total effects over all loci such that some alleles will contribute positive and others negative increments to the phenotype. In essence, the models are arbitrary for the number and distribution of loci involved.

Results

The least-squares means and standard errors for each line were calculated for ovariole number (corrected for thorax length), thorax length, egg width, length and volume. The site means for these variables are listed in Table 2. A nested analysis of variance showed the line within site effect to be significantly different for all variables and the site effect to be nonsignificant (Table 3).

Given that dissections were interspersed over 1 month and that two people were involved in the dissections, we analysed the effects of date of dissection and person (within isofemale lines/site) for corrected ovariole number and thorax length. The person effect was nonsignificant for both variables (ovarioles: $F_{12,274} = 1.64$, $P = 0.08$; thorax length: $F_{12,276} = 0.91$, $P = 0.53$), whereas date of dissection was significant for both variables (ovarioles: $F_{123,274} = 1.36$, $P = 0.02$; thorax length: $F_{123,276} = 2.21$, $P = 0.0001$).

Heritability estimates for the five variables are given in Table 4 along with a test of significance ($H_0: h^2 = 0$). Ovariole number, thorax length and egg width had significant heritabilities ($P < 0.05$) but egg length and volume did not ($P > 0.05$).

Line means for the line-cross analysis of corrected ovariole numbers are listed in Table 5. Insufficient eggs were obtained in the line crosses to conduct the analysis

Table 1 Coefficient matrix $\mathbf{C}$ for line-cross analysis

	m	[d]	[h]	[i]	[j]	[l]	[$d.m$]	[$h.m$]
trd35	1	−1.0	0.0	1.00	0.00	0.00	−1	0
BC35r	1	−0.5	0.5	0.25	−0.25	0.25	−1	0
BC35	1	−0.5	0.5	0.25	−0.25	0.25	0	1
F_1	1	0.0	1.0	0.00	0.00	1.00	1	0
F_2	1	0.0	0.5	0.00	0.00	0.25	0	1
F_{1r}	1	0.0	1.0	0.00	0.00	1.00	−1	0
BC48	1	0.5	0.5	0.25	0.25	0.25	0	1
trd48	1	1.0	0.0	1.00	0.00	0.00	1	0

Table 2 Least-squares means (by site) of isofemales lines, range within site, and their standard errors (SE) for ovariole number, thorax length and egg characteristics of *Drosophila hibisci*

		BCK 9	BEL 9	TRD 5
Number of lines				
Ovarioles	Mean	19.59	20.21	19.82
	Range	17.42–22.65	17.90–21.41	17.05–21.76
	SE	0.204	0.183	0.305
Thorax length (mm)	Mean	0.942	0.908	0.922
	Range	0.862–1.007	0.884–0.941	0.855–0.966
	SE	0.007	0.007	0.011
Egg width (mm)	Mean	0.200	0.205	0.204
	Range	0.194–0.208	0.196–0.215	0.197–0.211
	SE	0.001	0.001	0.002
Egg length (mm)	Mean	0.618	0.611	0.621
	Range	0.611–0.627	0.584–0.636	0.594–0.640
	SE	0.003	0.003	0.006
Egg volume (mm ³)	Mean	0.0130	0.0135	0.0136
	Range	0.0123–0.0139	0.0123–0.0148	0.0123–0.0149
	SE	0.0002	0.0002	0.0003

BCK = Bungwahl Creek, NSW (32.056°S, 152.442°E, 65 m); BEL = Bellingen, NSW (30.419°S, 152.824°E, 40 m); TRD = Nimbin, NSW (28.595°S, 153.241°E, 190 m).

Table 3 Nested analysis of variance for ovariole number (corrected), thorax length and egg characteristics of *Drosophila hibisci*

Source	Ovariole		Thorax		Egg			
	d.f.	MS	d.f.	MS†	d.f.	Width MS‡	Length MS†	Volume MS§
Site	2	15.67	2	46.32	2	7.24	2.23	10.06
Line/Site	20	43.50***	20	25.48***	20	4.07***	2.03*	8.26*
Error	415	6.11	417	8.23	280	1.71	1.20	4.23

† $\times 10^{-3}$; ‡ $\times 10^{-4}$; § $\times 10^{-6}$.
* $P < 0.05$; *** $P < 0.001$.

on the egg characters, thus only the analysis for mean corrected ovariole numbers was possible. The estimates of parameters of the complete digenic epistatic progeny model (full digenic, Table 6) indicated that only additive effects are likely to be important in explaining the differences between line means. In fact, the goodness of fit for the additive model ($ova = m + [d]$) is not improved by incorporating dominance ($F_{1,5} = 1.57$, $P = 0.27$) or dominance and the three epistatic parameters into the model ($F_{4,2} = 0.76$, $P = 0.64$). Using both progeny and maternal effects showed additive effects of the progeny and mother as possibly important in explaining the line means. But the comparison of the

additive ($ova = m + [d]$) and mixed additive ($ova = m + [d] + [d.m]$) models indicated that including the maternal additive effect was not significant ($F_{1,5} = 4.48$, $P = 0.09$). It is thus apparent that the additive model is adequate to describe the difference between the two isofemale lines within the Nimbin population. The fit of this model to the line-cross means is illustrated in Fig. 1. These results support the assumption of no dominance used in the estimates of heritabilities from the isofemale line data.

The four collections made during October and November 1997 at the Bellingen site were compared to a similar set of collections made in the previous year

Table 4 Heritability (h^2) and standard error (SE) estimates for ovariole number (residual after regressing on thorax length), thorax length, egg width, egg length and egg volume for isolines of *Drosophila hibisci* from three different sites

Trait	h^2	SE	$P(H_0: h^2 = 0)$
Ovarioles (residual)	0.564	0.160	0.0011
Thorax length	0.211	0.091	0.0156
Egg			
Width	0.209	0.105	0.0343
Length	0.086	0.075	0.1348
Volume	0.134	0.088	0.0752

Table 5 Line means (least-square means after accounting for thorax length) of ovariole number from crosses involving *Drosophila hibisci* isolines 35 and 48 from within the Nimbin (trd) site

Line	Female	Male	n	Line-cross analysis	
				Mean	SE
trd35			24	16.489	0.386
BC35r	trd35	F_1	29	17.488	0.398
BC35	F_1	trd35	23	17.660	0.441
F_1	trd35	trd48	22	18.726	0.447
F_{1r}	trd48	trd35	32	17.768	0.375
F_2 (pooled)	$F_1 + F_{1r}$	$F_1 + F_{1r}$	41	18.114	0.325
BC48	F_{1r}	trd48	14	18.918	0.555
trd48			18	21.048	0.491

(Starmer *et al.*, 1998). The variance component estimate for between years was $\sigma^2_{\text{years}} = 0.345$ and for collections within year was $\sigma^2_{\text{collections/year}} = 0.472$.

Discussion

The variation in mean corrected ovariole number was much larger for isofemale lines within sites than for between sites (Table 3). The three sites were spatially separated by at least 182–397 km in the central part of the *D. hibisci* species distribution along the east coast of Australia. Earlier studies on population mean ovariole number indicate that the variability among sites over 14° of latitude ($\sigma^2_{\text{between}} = 2.048$) is the same as the variability, measured here, within sites ($\sigma^2_{\text{within}} = 2.039$) in the centre of the species distribution. One site near Bellingen, N.S.W. (bel) was sampled at weekly intervals during November 1996 (Starmer *et al.*, 1998) and at similar times in 1997 (this study). The variance estimates for corrected ovariole number of wild-caught females at Bellingen between years ($\sigma^2_{\text{years}} = 0.345$) and for collections within

Table 6 Estimates of genetic effects and standard errors (SE) from line-cross means for corrected ovariole number in *Drosophila hibisci*. Population mean: m . Genetic effects of progeny: additive [a], dominance [d], dominance by additive epistasis [i], additive by dominance epistasis [j], dominance by dominance epistasis [l]. Genetic effects of mother: maternal additive [h], maternal dominance [m]. Bold values indicate significant ($\alpha = 0.05$) parameter estimates

	Full model	Progeny	Additive and dominance	Additive	Mixed progeny and maternal	Mixed additive	Maternal
m	18.067 (1.947)	18.256 (1.834)	18.648 (0.269)	18.359 (0.151)	18.807 (0.301)	18.407 (0.153)	18.517 (0.199)
$[a]$	1.801 (0.427)	2.279 (0.312)	2.062 (0.274)	2.007 (0.271)	1.639 (0.365)	1.580 (0.358)	—
$[h]$	1.235 (4.832)	−0.478 (4.684)	−0.547 (0.423)	—	−0.492 (0.425)	—	—
$[i]$	0.702 (1.922)	0.513 (1.807)	—	—	—	—	—
$[j]$	−1.084 (1.655)	−1.853 (1.404)	—	—	—	—	—
$[l]$	−1.055 (3.317)	0.385 (2.955)	—	—	—	—	—
$[d,m]$	0.479 (0.292)	—	—	—	0.498 (0.262)	0.466 (0.256)	1.263 (0.199)
$[d,h]$	−0.307 (0.662)	—	—	—	−0.344 (0.310)	—	−0.388 (0.309)
χ^2 (d.f.)	—	2.779 (2)	5.344 (5)	7.019 (6)	1.198 (3)	3.701 (5)	21.555 (5)

Fig. 1 Line means of corrected ovariole number in *Drosophila hibisci* plotted as a function of the completely additive gene model (no dominance and no epistasis). Open circles are the expected means. Closed circles are the observed means.

year ($\sigma^2_{\text{collections/year}} = 0.472$) show that the within-site variation for the three sites studied here is about 4–5 times the short-term temporal variation (≈ 1 month) at one of them. This suggests that the variation at any one time within a population is greater than that over generations and that either substantial microhabitat variation is influencing ovariole number and/or significant genetic variation is maintained within populations.

The heritability estimate for female ovariole number (Table 4) indicates a significant potential for evolution of this trait within populations. This conclusion was supported by the line-cross analysis for Nimbin that showed neither dominance nor epistatic effects to be significant. The ovariole number analysis was carried out on corrected ovariole number after the covariation with body size (thorax length) was removed. It is worth noting that thorax length itself showed significant line-within-site effects (Table 3) and significant heritability ($h^2 = 0.211 \pm 0.091$, Table 4).

In most *Drosophila* species thorax length is strongly influenced by larval density or nutritional conditions that lead to varying amounts of nutrients available to larvae after they reach their critical size and commit to pupation (Robertson, 1963). In our experiments, each cage housing the lines was treated in the same manner

(i.e. started with approximately the same number of adults from the initial isofemale pair, provided with the same number and types of flowers each day, maintained at the same temperature, humidity and light conditions, and sampled in a similar manner over a 1-month period), but we did not know the egg density per flower each day or the exact population sizes of each isofemale cage. As a consequence, the observations for each line could be confounded by unknown cage effects. However, there were no significant effects for person dissecting or date-by-person interaction for either variable. A significant date effect for both thorax length ($P = 0.0001$) and size-adjusted ovariole number ($P = 0.02$) may reflect the quality of flowers, such as the amount of nectar or moisture content, that ultimately translated into larger or smaller females. However, the significance of the line-within-site effect is not changed by removing sums of squares due to days. Two additional observations argue against cage effects as the cause of differences in corrected ovariole numbers among the isolines. In the line-cross analysis, the two Nimbin isofemale lines differed by 4.6 ovarioles but had almost the same average thorax length (0.911 mm vs. 0.929 mm; $t_{41} = 0.54$, $P = 0.59$). Furthermore, additional females from line trd35 (used in the line-cross analysis) were dissected 3.5 months after the initial observations and the two trd35 samples showed no significant difference in corrected ovariole number (16.74 vs. 16.65; $t_{27} = 0.07$, $P = 0.94$), indicating little change after several generations in laboratory cages.

Earlier work (Starmer *et al.*, 1998) demonstrated that both genetic and environmental influences affected mean ovariole number in crosses between populations approximately 1150 km apart, with significant nonlinear interactions between larval growth temperature and composite dominance effects. These observations, coupled with the significant correlation of prior weather (rainfall and temperature) with mean ovariole number in the geographical study (Starmer *et al.*, 1997), suggest that both micro- and macro-climatic variation have strong influences on the trait. Other factors that might affect ovariole number in natural populations are: (i) characteristics of the flower used by the larvae, which include flowers of different native *Hibiscus* species, flower size, and microbial components of the decaying blossom; (ii) density of trees, i.e. number of available blossoms at a locality; (iii) density of adult flies; (iv) density of beetles that also use the blossoms for breeding; and (v) nematode parasite load in the population. All of these factors were studied in our geographical survey (Starmer *et al.*, 1997) and were shown not to be correlated with mean ovariole number across sites.

A comparison of the significant genetic effects within the Nimbin site with genetic effects estimated using two

widely separated populations (Starmmer *et al.*, 1998) indicated that both genetic models had significant additive effects, but only the between-site cross showed significant epistatic effects. The generality of this difference when comparing within- vs. between-population genetic activity is limited because of the singularity of the comparison, but the difference in genetic components does serve to show that, at least in this case, different gene combinations appear to be responsible for differences in the same trait in the two widely separated populations. Furthermore, the difference in mode of genetic activity reveals an epistatic system that should foster genetic divergence. This is consistent with the findings of Armbruster *et al.* (1997) for the pitcher-plant mosquito *Wyeomyia smithii* and supports the Wrightian theory that epistasis can lead to the formation of different adaptive gene combinations in spatially separated populations.

Other systems have demonstrated the same pattern, where crosses among isolated or divergent populations show significant gene interaction effects on the phenotype, whereas crosses of comparable local or non-divergent populations show that nonallelic interactions were not important (Simchen, 1967; Caten, 1979; Doebley *et al.*, 1995; Whitlock *et al.*, 1995). Two major hypotheses could explain this pattern. One is that an ancestral population with epistatic potential has become divided into subpopulations, some of which attain different adaptive peaks and thus different adaptive gene combinations via drift, selection and genotype-by-environment interactions. The other hypothesis is that the ancestral population has no potential epistasis and epistasis revealed in crosses between populations is a by-product of independent evolution via mutation and selection after subdivision. Our results and those cited above are unable to distinguish between these alternatives without more detailed information on the genetic architecture within and between populations.

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