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Genetic basis for reproductive diapause is correlated with life history traits within the *Culex pipiens* complex

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Abstract

The evolution of late season reproductive arrest (diapause) among female *Culex pipiens* mosquitoes allows them to overwinter in temperate climates, while females of the sibling species *Culex quinquefasciatus* do not exhibit the diapause phenotype. We present results for quantitative trait loci (QTL) analyses of two independent segregating populations derived from crosses between *C. pipiens* and *C. quinquefasciatus*. QTL for diapause and three life history traits were identified and compared for genome positions and gene effects. Using a combination of composite interval mapping, single-marker analysis and all possible subsets regression, we identified multiple QTL for each trait, totalling 14 and 17 QTL for each population, respectively. Individual QTL across traits often mapped to similar genome locations, suggesting these traits may be controlled in part by genes with pleiotropic effects or multiple tightly linked genes. The majority of QTL were intermediate in magnitude in that they explained 10–25% of the phenotypic variation. The majority of QTL showed overdominance effects. We suggest that this could impact natural populations, as increased heterosis across hybrid zones may allow populations to adapt to environmental conditions via stabilizing selection, and yet maintain species identity outside these regions because of strong morphological integration, wherein related traits evolve as an integrated unit.

Keywords: heterosis, overdominance, photoperiod, QTL, West Nile virus.

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Introduction

Diapause is a state of programmed developmental arrest that facilitates survival under adverse environmental conditions. Individuals in diapause exhibit characteristic changes in morphology, physiology and behaviour compared to nondiapausing individuals (Tauber *et al*., 1986; Danks, 1987). Adult diapause is a common adaptation among some insects for overwintering in temperate climates, and involves arrested reproductive development, typically evidenced in females as delayed ovary development following adult eclosion (Danks, 1987). Diapause status in receptive individuals is controlled by the interaction between environmental cues, namely photoperiod and temperature, and insect hormone activity (Beck, 1980). Short daylength coupled with low ambient temperatures result in reduced juvenile hormone secretion by the corpus allatum, which suppresses reproduction (Denlinger, 1985).

The *Culex pipiens* complex represents a group of closely related mosquito species that are distributed nearly worldwide (Vinogradova, 2000). The major species are delineated largely by their geographical distribution, with *C. pipiens* (identified as *C. pipiens pallens* in the Far East) largely associated with temperate zones and *C. quinquefasciatus* largely associated with tropical zones. Members of the *C. pipiens* complex have been implicated as primary vectors for the West Nile virus in North America (Fonseca *et al*., 2004), and diapausing females can serve as reservoirs for West Nile virus to re-initiate vertebrate infections in the spring (Nasci *et al*., 2001; Farajollahi *et al*., 2005). Interspecies hybrids are known to occur in areas where their distributions overlap (Barr, 1957; Jakob *et al*., 1979, 1980; Tabachnik & Powell, 1983; Pryor & Daly, 1991; Cornel *et al*., 2003; Fonseca *et al*., 2004). Investigations of hybrid zones have confirmed that, although significant introgression occurs, fitness clines largely related to temperature gradients are evident (Urbanelli *et al*., 1995, 1997; Humeres *et al*., 1998).

Culex pipiens females are induced into ovarian diapause, wherein ovarian follicles remain largely undeveloped, by decreasing photoperiod and low ambient temperatures in late summer or autumn (Eldridge, 1968; Sanburg & Larsen, 1973). Additionally, females in diapause are characterized

by reduced blood avidity, fat body hypertrophy and a general state of inactivity (Eldridge, 1966, 1987). Diapause can be reversed in these females by oral or topical treatment with juvenile hormone analogues, indicating that diapause induction and maintenance is determined by activity of the corpora allata (Spielman, 1974). Conversely, *C. quinquefasciatus* females are not subject to environmental influence and do not exhibit ovarian diapause under even very short photoperiod conditions (Eldridge, 1968; Wilton & Smith, 1985).

The molecular genetic basis for adult diapause induction in insects has not been elucidated. Recent studies have shown that some genes involved with bloodmeal processing are down-regulated, while some genes involved in lipid sequestration are up-regulated in female *C. pipiens* following exposure to short day length as fourth instar larvae or early pupae (Robich & Denlinger, 2005). Studies with both the *C. pipiens* complex (Spielman & Wong, 1973; Wilton & Smith, 1985) and *Drosophila melanogaster* (Williams & Sokolowski, 1993) have clearly demonstrated that adult reproductive diapause is under genetic control, wherein the nondiapause condition is inherited largely as a simple autosomal dominant trait. However, in *Drosophila* some studies suggest that while a single major effect gene with at least partial dominance determines diapause, several minor effect genes probably have a significant influence on the observed phenotype (Lakovaara *et al*., 1972; Lumme *et al*., 1975).

In this study, we investigated the genetic basis for adult diapause in *C. pipiens* as a quantitative trait and report the results from two independent mapping experiments designed to identify quantitative trait loci (QTL). In addition, for the same populations we identified QTL for several life history traits characteristic of the *C. pipiens* complex species. This allowed us to compare the genome positions of these QTL with those that determine diapause and therefore test the hypothesis that some of the major QTL-determining traits characteristic of the individual species should reside in the same genome locations as major QTL for diapause. Our expectation was based on a premise that these QTL contain either tightly linked independent genes that affect the various phenotypes or that single genes within these QTL exhibit a pleiotropic influence across phenotypes.

Results

Linkage analysis

RFLP marker linkage maps covering all three linkage groups were constructed for both the *C. quinquefasciatus* Vero Beach × *C. pipiens* South Bend (Vero/SB) and *C. pipiens* Gose × *C. quinquefasciatus* Vero Beach (Gose/Vero) F_1 intercross populations. With the Vero/SB population, the map constructed with nine markers spanned 88.8 cM based on 96 female progeny. With the Gose/Vero population, the map constructed with 13 markers spanned 161.2 cM based on 192 female progeny. The two maps contained seven common markers. Both maps were in good agreement with that of Mori *et al*. (1999). As sex determination in the *C. pipiens* complex is determined by a single autosomal locus on chromosome 1 (Gilchrist & Haldane, 1947), few if any of the paternal genotype were obtained for any marker on this linkage group (see Severson *et al*., 1993). Further, for unknown reasons, recombination frequencies in the *C. pipiens* complex are very low across chromosome 1 when compared, for example, to *Aedes aegypti* (Mori *et al*., 1999).

Phenotype analysis

Ratios of the dorsal and ventral arms of the phallosome of the male genitalia (DV/D ratios) for the three strains were 1.15 ± 0.33 (Vero), 0.15 ± 0.06 (SB) and 0.32 ± 0.09 (Gose). These ratios are consistent with expectations for *C. quinquefasciatus* and *C. pipiens*, respectively. Individual strain characteristics for females under diapausing and nondiapausing conditions are shown in Fig. 1. As expected, the two *C. pipiens* strains (SB and Gose) showed similar phenotypes across each trait and rearing conditions. Also as expected, the *C. quinquefasciatus* strain (Vero) did not respond to diapause inducing conditions, as evidenced by the general consistency in follicle size (Fig. 1A). In contrast, both SB and Gose exhibited highly reduced follicle sizes (e.g. undeveloped) under diapausing conditions. Differences in wing vein cell-to-stem (CS) ratios were completely discriminatory for *C. quinquefasciatus* versus *C. pipiens* (Fig. 1C). Differences in wing length and development time were less evident, with Vero being significantly smaller ($P < 0.01$, Fig. 1B) and slightly but not significantly quicker to develop ($P > 0.05$, Fig. 1D) than either SB or Gose.

QTL analysis

With an experimentwise threshold likelihood ratio (LR) value of $P = 0.05$, composite interval mapping (CIM) analysis identified one QTL on chromosome (chr.) 1 influencing follicle size in Vero/SB and three QTL in Gose/Vero (one on chr. 1, two on chr. 2) (Fig. 2A–B and Table 1). However, the stringency of the experimentwise threshold, while reducing the likelihood of declaring false positive QTL (type I error), also increases the potential for rejecting true positive QTL (type II error) (Churchill & Doerge, 1994). In addition, the power of our QTL analyses in Vero/SB was negatively impacted by the sample size ($n = 96$) as well as the uneven distribution of markers ($n = 9$), resulting in some large interval sizes. In contrast, for Gose/Vero the sample size was ~twofold larger ($n = 192$) and marker ($n = 13$) distribution was more even. Therefore, we also examined results for comparisonwise analysis of individual markers and all possible subsets (APS) regression analysis. We considered

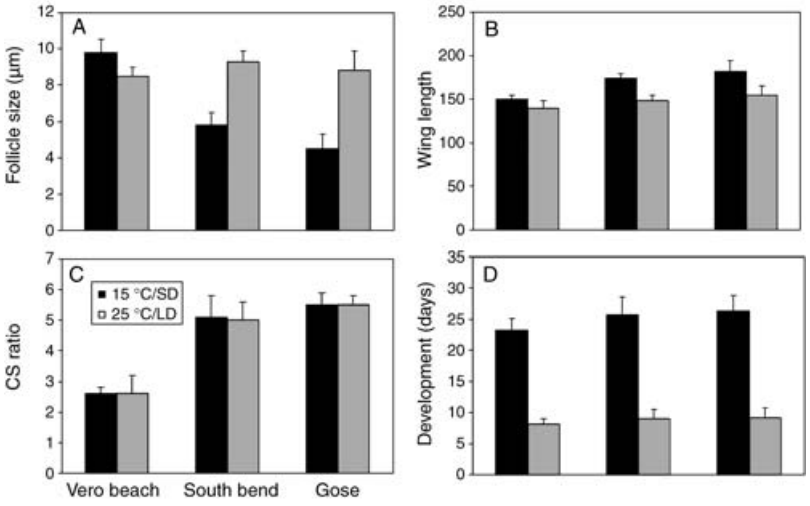

Figure 1. Means + SD for traits by mosquito strains reared at high temperature and long photoperiod versus low temperature and short photoperiod conditions: (A) follicle size (μm), (B) wing length, (C) CS ratio (wing cell-to-stem vein ratio), (D) developmental time (days).

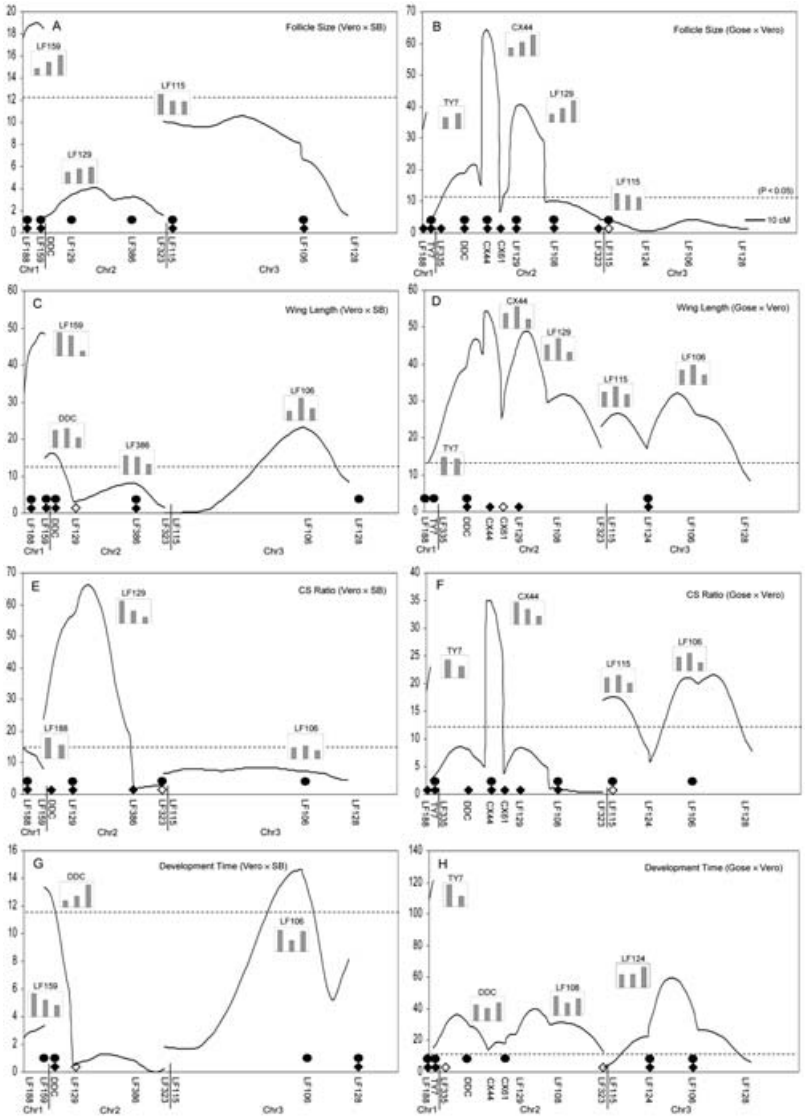

Figure 2. Likelihood ratio profiles identifying quantitative trait loci (QTL) for the four traits. The horizontal line represents the experimentwise threshold value ($P = 0.05$) for identifying a QTL. Diamonds indicate single markers exceeding the comparisonwise threshold value (for solid $P = 0.01$, for open $P = 0.05$). Circles indicate best set of markers identified by all possible subsets regression ($P = 0.05$). Histogram inserts show the mean phenotype for the *Culex pipiens* homozygous genotype, heterozygote and *C. quinquefasciatus* homozygous genotype, respectively, for both the Vero Beach (Vero) × South Bend (SB) and Gose × Vero populations. For some chromosome 1 markers, means are presented only for the heterozygote and *C. quinquefasicatus* genotypes, respectively, with Vero/SB, and for the *C. quinquefasciatus* homozygous genotypes and heterozygote genotypes, respectively, with Gose/Vero. (A, B) follicle size; (C, D) wing length; (E, F) CS ratio (wing cell-to-stem vein ratio); (G, H) development time for the Vero/SB and Gose/Vero populations.

Table 1. Quantitative trait loci (QTL) identified for four traits by composite interval mapping in two F_2 populations from crosses among the *Culex pipiens* complex

Trait	Chromosome	Position*	Flanking markers	Maximum LR	1-LOD interval†	R^2	Gene effect		Gene action‡	Degree of dominance§
							Additive	Dominant		
Vero Beach (Vero) × South Bend (SB) population										
Follicle size	1	4.01	LF188–LF159	18.95	0.0–8.0	16.75	1.63	NA	NA	NA
Wing length	1	6.01	LF188–LF159	48.59	0.0–8.0	37.23	–7.46	NA	NA	NA
	2	4.01	*DDC*–LF129	16.17	0.0–8.9	12.6	–3.89	4.25	D	1.09
	3	66.75	LF106–LF128	23.27	54.5–76.6	12.01	–2.11	4.35	OD	2.06
CS ratio	1	0	LF188–LF159	14.79	0.0–8.0	8.57	–0.26	NA	NA	NA
	2	21.68	LF129–LF386	66.27	17.6–26.2	67.24	–0.37	0.12	PD	0.32
Development	2	0	*DDC*–LF129	13.33	0.0–8.5	11.27	0.43	–0.36	D	0.84
	3	64.01	LF106–LF128	14.59	45.6–73.7	14.1	0.08	–0.50	OD	6.25
Gose × Vero population										
Follicle size	1	2.01	LF188–TY7	38.31	0.0–2.0	11.57	–1.28	NA	NA	NA
	2	28.84	CX44–CX61	64.49	26.8–31.3	22.91	–1.25	–0.51	PD	0.41
	2	44.59	LF129–LF108	40.47	41.6–50.8	14.97	–1.08	–0.34	PD	0.31
Wing length	2	28.84	CX44–CX61	53.87	26.6–31.4	25.87	–1.12	6.76	OD	6
	2	46.59	LF129–LF108	48.92	42.4–51.7	27.67	–12.11	7.6	PD	0.62
	3	8.01	LF115–LF124	26.76	0.0–16.6	17.15	–1.65	5.78	OD	3.5
	3	36.33	LF124–LF106	31.99	29.2–44.6	21.11	–0.97	6.11	OD	6.3
CS ratio	1	2.01	LF188–TY7	23.09	0.0–2.0	10.84	0.23	NA	NA	NA
	2	28.84	CX44–CX61	34.91	26.8–32.2	16.28	0.25	0.07	PD	0.28
	3	6.01	LF115–LF124	17.63	0.0–15.7	9.1	–0.01	0.21	OD	21.1
	3	53.77	LF106–LF128	21.59	33.2–61.8	14.75	–0.04	0.3	OD	7.5
Development	1	2.01	LF188–TY7	120.92	0.0–2.0	42.18	3.46	NA	NA	NA
	2	12.01	LF335–*DDC*	36.35	7.0–16.4	14.07	0.43	–1.98	OD	4.6
	2	48.59	LF129–LF108	40.06	44.5–54.1	13.54	0.99	–1.82	OD	1.83
	3	32.33	LF124–LF106	59.67	28.6–38.2	23.23	–0.23	–1.76	OD	7.65

*Position across chromosome in centiMorgans.

†Region flanking individual QTL peaks in which LOD scores decline by one LOD.

‡Mode of gene action determined as the absolute value of d/a where additive = 0.0–0.20, partial dominance = 0.21–0.80, dominance = 0.81–1.20 and overdominance > 1.20 (after Stuber *et al.*, 1987; Babu *et al.*, 2006). For Vero × SB, mode of gene action was determined relative to the *C. quinquefasciatus* allele, while for Gose × Vero, mode of gene action was determined relative to the *C. pipiens* allele.

§Absolute value of dominance divided by additive (d/a) gene effect.

NA, not applicable. As sex determination in culicine mosquitoes is autosomal and located on chromosome 1, paternal homozygotes for markers on this chromosome are infrequently observed among females, thus preventing analysis of dominance effects (Severson *et al.*, 1993).

CS, wing vein cell-to-stem ratio; LR, likelihood ratio.

markers with significant associations with individual traits by either analysis as identifying tentative QTL. Comparisonwise analysis identified an association with the LF115 and LF106 loci for Vero/SB and the LF115 locus for Gose/Vero on chr. 3. APS results were consistent with QTL regions identified by experimentwise and comparisonwise analyses, but included chr. 2 marker loci (LF129 and LF386) for Vero/SB (Table 2). The combined analyses support the presence of four QTL (one on chr. 1, two on chr. 2 and one on chr. 3) in both populations, despite the different genetic backgrounds.

CIM analysis of wing length identified three QTL in Vero/SB (one each on chr. 1, 2 and 3) and four QTL in Gose/Vero (two each on chr. 2 and 3) determining wing size (Fig. 2C–D, Table 1). Comparisonwise analysis with Vero/SB identified an association with the LF386 locus on chr. 2. APS analysis included the TY7 and LF188 loci on chr. 1 with Gose/SB and LF386 on chr. 2 with Vero/SB (Table 2). The combined analyses support the presence of four QTL (one on chr. 1, two on chr. 2, one on chr. 3) in Vero/SB, and five QTL (one on chr. 1, two on chr. 2, two on chr. 3) in Gose/Vero.

CIM analysis identified two QTL (one each on chr. 1 and 2) in Vero/SB and four QTL (one on chr. 1, one on chr. 2, two on chr. 3) in Gose/Vero determining CS ratio (Fig. 2E–F, Table 1). APS analysis included LF106 on chr. 3 with Vero/SB (Table 2). The combined analyses support the presence of three QTL in Vero/SB (one on chr. 1, one on chr. 2, one on chr. 3), and four QTL in Gose/Vero (one on chr. 1, one on chr. 2, two on 3).

CIM analysis of development time identified two QTL (one on chr. 2, one on chr. 3) in Vero/SB and four QTL (one on chr. 1, two on chr. 2, one on chr. 3) in Gose/Vero (Fig. 2G–H, Table 1). Comparisonwise analyses were consistent with experimentwise analyses for both populations. APS analysis included the LF159 locus on chr. 1 in Vero/SB (Table 2). The combined analyses support the presence of three QTL in Vero/SB (one on chr. 1, one on chr. 2, one on chr. 3) and four QTL in Gose/Vero (one on chr. 1, two on chr. 2, one on chr. 3).

Table 2. Best set of markers identified by all possible subsets (APS) regression for four traits in two F_2 populations from crosses among the *Culex pipiens* complex

Marker	Linkage group	Partial regression coefficient	SE	*t*-value	*P**
Vero Beach (Vero) × South Bend (SB) population					
Follicle size (multiple R^2 = 0.335)					
LF188	1	1.527	1.037	1.47	0.144
LF159	1	1.425	0.910	1.58	0.119
LF129	2	0.878	0.545	1.61	0.111
LF386	2	0.776	0.510	1.52	0.132
LF115	3	–1.125	0.492	–2.28	0.025
LF106	3	–0.940	0.451	–2.08	0.040
Wing length (multiple R^2 = 0.495)					
LF159	1	–8.943	2.443	–3.66	0.0004
LF188	1	–4.703	2.794	–1.68	0.096
DDC	2	–4.408	1.634	–2.70	0.008
LF386	2	–5.733	2.012	–2.85	0.006
LF128	3	–3.728	1.447	–2.58	0.012
CS ratio (multiple R^2 = 0.497)					
LF188	1	–0.498	0.135	–3.69	0.0004
LF129	2	–0.740	0.097	–7.65	0.0001
LF323	2	–0.153	0.099	–1.54	0.126
LF106	3	–0.219	0.086	–2.54	0.013
Development (multiple R^2 = 0.228)					
LF159	1	–0.379	0.239	–1.59	0.117
DDC	2	0.763	0.219	3.49	0.0008
LF106	3	–0.346	0.214	–1.62	0.109
LF128	3	0.828	0.249	3.33	0.001
Gose × Vero population					
Follicle size (multiple R^2 = 0.517)					
TY7	1	2.534	0.387	6.55	0.0001
DDC	2	0.951	0.494	1.92	0.056
CX44	2	1.281	0.649	1.97	0.050
LF129	2	1.065	0.628	1.70	0.092
LF108	2	1.110	0.378	2.93	0.004
LF115	3	–0.579	0.029	–2.00	0.047
Wing length (multiple R^2 = 0.141)					
LF188	1	9.606	3.935	2.44	0.016
TY7	1	–11.830	3.972	–2.98	0.003
DDC	2	–6.006	1.575	–3.81	0.0002
LF124	3	–5.027	1.573	–3.20	0.002
CS ratio (multiple R^2 = 0.274)					
TY7	1	–0.468	0.102	–4.57	0.0001
CX44	2	–0.465	0.090	–5.16	0.0001
LF108	2	–0.125	0.088	–1.42	0.157
LF115	3	–0.220	0.077	–2.86	0.005
LF106	3	–0.212	0.082	–2.57	0.011
Development (multiple R^2 = 0.537)					
LF188	1	–1.980	1.046	–1.89	0.060
TY7	1	–4.940	1.056	–4.68	0.0001
DDC	2	1.667	0.549	3.03	0.003
CX61	2	–1.342	0.563	–2.39	0.018
LF124	3	1.011	0.474	2.13	0.340
LF106	3	1.223	0.479	2.56	0.011

*In APS, individual markers may not have significant *P*-values, but are selected in the best model for explaining the greatest amount of phenotypic variance for each trait (see Statistical methods).
CS, wing vein cell-to-stem ratio.

QTL magnitude and mode of action

The phenotypic variation explained by individual QTL using CIM ranged from 8.6 to 67.2% across all four traits (Table 1). Following Burke *et al.* (2002), we used arbitrary thresholds of <10, 10–25 and >25% to delineate QTL magnitude as minor, intermediate and major, respectively. The majority of CIM-identified QTL (16 out of 23; 70%) showed intermediate magnitude, while only two minor and five major QTL were identified. Major QTL were identified for wing length in both populations, and for CS ratio in Vero/SB and development time in Gose/Vero.

As Zmapqtl provides estimates of the additive (a) and dominance (d) effects, we were able to estimate degree of dominance (d/a) with five QTL for the four traits in Vero/SB and 12 QTL in Gose/SB (Table 1). Note that Zmapqtl

Table 3. Correlations among four traits in two F_2 populations from crosses among the *Culex pipiens* complex

	Wing length	CS ratio	Development
Vero Beach (Vero) × South Bend (SB) population			
Follicle size	−0.318*	−0.063	−0.118
Wing length		0.388***	−0.147
CS ratio			−0.135
Gose × Vero population			
Follicle size	−0.199*	−0.308***	−0.226*
Wing length		0.396***	−0.236**
CS ratio			−0.024

CS, wing vein cell-to-stem ratio.
*$P < 0.01$, **$P < 0.001$, ***$P < 0.0001$.

calculates a and d effects relative to alleles from the 'A' parent in the cross; therefore, gene effects were calculated for the effects of the *C. quinquefasciatus* allele with Vero/SB and for the effects of the *C. pipiens* allele with Gose/Vero. QTL identified on chr. 1 by CIM were not included because, as indicated elsewhere (see Experimental procedures), for most marker loci on this linkage group no paternal homozygotes were present in our segregating populations because sex determination is autosomal. Ten out of 17 (59%) CIM-identified QTL exhibited an overdominance mode of gene action. For six out of seven QTL with partial dominance or dominance gene action, the effect was in the 'expected' direction (see 'best' marker histogram insets, Fig. 2). That is, the relationships between the observed individual genotypes and phenotypes were consistent with the mean phenotypes of the parental strains (Fig. 1) with *C. pipiens* > or < *C. quinquefasciatus* (depending on the trait) with heterozygotes being intermediate. Note that the histograms in Fig. 2 were organized such that for both populations, genotype data were ordered *C. pipiens* homozygotes, heterozygotes, *C. quinquefasciatus* homozygotes, respectively. Of interest, the tentative QTL for follicle size, the LF115 locus on chr. 3, exhibited an effect opposite to the 'expected' direction (*C. pipiens* > *C. quinquefasciatus*) in both populations (Fig. 2A–B). In addition, the *DDC* locus on chr. 2 exhibited an opposite effect to the 'expected' for developmental time in Vero/SB, although some caution in interpretation is warranted as the parental strains showed slight but not significant differences in the trait (Fig. 1). Gene action and direction of effect were largely consistent across populations when we compared QTL for individual traits across the same genome regions (Fig. 2). This included all comparisons of CIM-identified QTL in one population to comparisonwise or APS-identified tentative QTL regions in the other population.

Trait analysis

With Vero/SB, phenotypic correlations of follicle size to wing length and wing length to CS ratio were significant (Table 3). With Gose/SB, all traits showed significant phenotypic correlations, with the exception of CS ratio to development time.

Using one-LOD support limits to define confidence intervals, we observed extensive overlap among QTL determining the individual traits (Table 1). Further, the predicted QTL positions (based on maximum LR) were very similar or even identical. This phenomenon is particularly evident within Gose/Vero, probably as a result of the more extensive marker distribution and large population size. For example, QTL at 28.84 cM on chr. 2 were predicted for follicle size, wing length and CS ratio.

Discussion

The evolution of the *C. pipiens* complex has allowed for their successful colonization of a broad environmental range from the extreme tropical to the extreme temperate zones. The primary environmental driver determining diapause status in adult female *C. pipiens* is decreasing daylength, with decreasing ambient temperatures probably enhancing the effect (Eldridge, 1968; Sanburg & Larsen, 1973). Adult females of the sister taxon, *C. quinquefasciatus*, do not enter diapause under any condition (Eldridge, 1968; Wilton & Smith, 1985). Here we identified QTL determining the diapause phenotype as well as three life history traits that reflect the biological diversity of the species complex, including wing length (as a proxy for body size), CS ratio and developmental time.

We employed a combination of statistical methods to identify QTL associated with each trait in two independent segregating populations (Vero/SB and Gose/Vero) including: CIM, single-marker analysis and APS. Based on all analyses, we identified a total of 14 QTL in Vero/SB and 17 QTL in Gose/Vero for the four traits as follows: diapause 4/4, wing length 4/5, CS ratio 3/4 and development time 3/4. Experimentwise thresholds in CIM are conservative (Churchill & Doerge, 1994); i.e. markers with real effects may be excluded. We have included QTL identified only by single-marker or APS but mark these as tentative to indicate that CIM did not detect them. It is noteworthy that only APS is expected to identify QTL alleles at different loci that significantly influence the phenotype only if they occur together. The power of our analyses, particularly within the Vero/SB population, was influenced by the sample size and marker distribution. However, we note that the predicted QTL were remarkably consistent between the populations for each trait, with CIM validation of each QTL in at least one of the populations, with the sole exception of follicle size on chr. 3, where both populations exhibited tentative QTL.

Although we observed multiple QTL for each trait throughout the genome, the individual QTL across traits often mapped to the same genome locations. This phenomenon is evident within both populations. Although different marker combinations were used between them

thus limiting direct comparisons, QTL positions clearly fall within the same genome regions even across populations. This nonrandom clustering of QTL across traits suggests evidence for pleiotropic effects of single genes or effects of tightly linked multiple genes. However, as our QTL interval sizes ranged from ~6–30 cM, additional fine-scale studies are needed to differentiate among these possibilities. Of note, we previously observed clustering of QTL for body size with those determining mosquito competence to transmit pathogens, including *Plasmodium gallinaceum* susceptibility in *Aedes aegypti* (Meece, 2002) and La Crosse virus transmission in *Ochlerotatus* interspecific hybrids (Anderson *et al*., 2005). These results suggest that some genes that are active during mosquito development may also significantly influence a variety of traits in adults. Still however, as not all QTL reflect overlap across traits, some uncoupling of traits would probably be expected across the species range, as has been reported with other traits in the *C. pipiens* complex, for example, in California (Cornel *et al*., 2003).

Our results demonstrate strong phenotypic and genetic correlations among traits, thus supporting our hypothesis that these traits are controlled at least in part by the same genes with pleiotropic effects or multiple tightly linked genes. Therefore, the traits probably reflect morphological integration, wherein functionally and developmentally related traits are expected to evolve as an integrated unit (Olson & Miller, 1958; Cheverud, 1982). While a diapause phenotype may outwardly seem unrelated to traits such as body size and development, it is reasonable to assume that genes critical to the individual traits may be associated with multiple biochemical pathways. Insect hormones undoubtedly provide the master coregulatory signals that coordinate gene expression affecting these apparently diverse phenotypes or in short, hormonal pleiotropy (Denlinger, 2002; Flatt *et al*., 2005; Koštál, 2006).

Evaluation of individual QTL effects is also quite intriguing. First, the majority of QTL showed intermediate effect magnitudes, indicating that the individual traits reflect complex interactions among several genes, and are not, as is often observed for QTL studies, determined largely by a single major effect QTL accompanied by effects of several minor effect QTL. Second, the finding that the majority of QTL in each population showed overdominance effects was unexpected. That is, for these QTL the heterozygote phenotype was outside the range for either parent. Although it is difficult to distinguish true overdominance, wherein the effect is the result of a single gene, from pseudo-overdominance, wherein the effect is the result of tightly linked genes with advantageous alleles in repulsion, the consistency of our results across two independent segregating populations with diverse genetic backgrounds provides firm support for true overdominance. However, as either situation results in hybrid heterosis, our findings raise some interesting possibilities for hybrid zones in natural populations that merits further research. Because our results are based on crosses involving laboratory strains with reduced genetic variability as a result of inbreeding, it is unclear whether the observed heterosis is a consequence of simple 'hybrid vigour' or would be directly applicable to expectations among field populations. Additional studies across hybrid zones are needed to determine if the heterosis observed with our QTL populations is actually evident and adaptive. We note that interspecific hybrids among overlapping *C. pipiens* and *C. quinquefasciatus* are well-documented (Barr, 1957; Jakob *et al*., 1979, 1980; Tabachnik & Powell, 1983; Pryor & Daly, 1991; Cornel *et al*., 2003; Fonseca *et al*., 2004), and a latitudinal shift in hybrid zone has been reported in California, apparently in response to changing environmental conditions (Urbanelli *et al*., 1997). Increased heterosis across these hybrid zones could allow populations to adapt to the prevailing environmental conditions via stabilizing selection, and yet maintain species identity outside these regions because of strong morphological integration as our lab results also suggest.

Advances in genomics and fine-scale genetic mapping have facilitated the nearly routine positional cloning of genes for individual QTL, even among organisms with very large complicated genomes (Flaherty *et al*., 2005; Keller *et al*., 2005; Bortiri *et al*., 2006). Given that a *C. quinquefasciatus* genome sequencing project is ongoing (http://msc.tigr.org/c_pipiens/index.shtml) and that the complete annotated genome sequences are already available for the yellow fever mosquito, *Aedes aegypti* (AAGE00000000), and the malaria vector mosquito, *Anopheles gambiae* (Holt *et al*., 2002), prospects for identifying genes within QTL for diapause and other important phenotypes in *C. pipiens* seem excellent.

Experimental procedures

Mosquito strains and rearing conditions

Three *C. pipiens* complex laboratory strains were used for these studies. *C. pipiens* South Bend strain was established from a single female collected in South Bend, Indiana, USA (41°42′N, 86°14′W), in 1998. The third laboratory generation was used in these studies. *C. pipiens* Gose strain (regionally identified as *C. pipiens pallens*) was initiated from mosquitoes collected in Gose, Nara, Japan (34°28′N, 135°42′E) and has been maintained in the laboratory for an unknown number of generations. *C. pipiens* Gose and South Bend are both diapausing strains. Our nondiapausing strain, *C. quinquefasciatus* Vero Beach, was established from the mosquitoes collected at Vero Beach, Florida, USA (27°35′N, 80°22′W). It also has been maintained in the laboratory for an unknown number of generations.

Genetic and phenotypic data were obtained for two F_1 intercross mapping populations. One population was prepared using *C. quinquefasciatus* Vero Beach strain females and *C. pipiens* South Bend strain males (Vero/SB). The second population was prepared using *C. pipiens* Gose strain females and *C. quinquefasciatus* Vero Beach strain males (Gose/Vero).

Each mapping population was generated by pair-wise matings, wherein a single male and five females from the appropriate strains were placed in 20 × 20 × 30 cm mesh cages within several hours of eclosion. Individual egg rafts were reared in separate rearing pans and P and F_1 generation pupae were separated by sex before adult eclosion. Mosquito larvae were reared on a suspension of dried beef liver powder. Adults were provided cotton soaked with 2% sugar solution *ad libitum*. Adult females were blood-fed on anaesthetized rats one week after eclosion. Our protocol for maintenance and care of experimental animals was reviewed and approved by the Institutional Animal Care and Use Committee at the University of Notre Dame. Animals are maintained and cared for in the Freimann Life Science Center, an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited facility. F_1 progeny were subsequently reared and allowed to pair-wise mate as described above. The P and F_1 generations were reared and maintained in an environmental chamber at 26 °C, 80% relative humidity and 16 h light: 8 h dark cycle with a 30 min crepuscular period at the beginning and end of each light cycle. Newly hatched larvae for the F_2 generation for each population were maintained in an environmental chamber at 80% relative humidity and 8 h light: 16 h dark cycle with a 30 min crepuscular period at the beginning and end of each light cycle. However, different temperature regimes were used, wherein the Vero/SB population was maintained at 21 °C and the Gose/Vero population was maintained at 15 °C. Differences in temperature regimes between the populations reflect insectary availability and were not intended to add temperature effects to our study. F_2 adult females were maintained for 14 days following eclosion in small mesh-covered containers (9 cm diameter × 9 cm height) and provided cotton soaked with 2% sugar solution *ad libitum*. After 14 days, F_2 females were frozen and stored at –80 °C.

Measurement of phenotypes

Individual F_2 progeny were evaluated for their diapause status and three traits associated with species discrimination. As a reference, we also evaluated these traits among females from each of our laboratory strains under both diapausing and nondiapausing conditions. Ratios of the two arms of the phallosome of the adult male genitalia (DV/D ratios) for each strain were calculated as this index represents a standard for species identification (Cornel *et al*., 2003). To determine diapause status, adult female ovaries were dissected in distilled water and the three most developed ovarian follicles were measured along their longitudinal axis using a dissecting microscope with an optical micrometer. We also removed one wing and measured wing length as the distance from the apical notch to the tip of the wing, but excluding the fringe scales, because wing length has been shown to be a reliable proxy for body size in mosquitoes (Van Handel & Day, 1989). In addition, we measured the length of wing veins r_2 and r_{2+3} to calculate the CS ratio as it has been shown to be greater in *C. pipiens* than in *C. quinquefasciatus* (Bekku, 1956). We also monitored the developmental time from egg hatch to adult eclosion as *C. pipiens* has been shown to exhibit longer developmental times than *C. quinquefasciatus* under both diapausing and nondiapausing conditions (Mori *et al*., 1988).

DNA extraction and genotyping

DNA extractions from individual females, digestion with *Eco*RI, Southern blotting and hybridizations were performed as described elsewhere (Severson, 1997). High stringency hybridizations were performed at 65 °C and membranes were washed for 15 min each at room temperature and at 65 °C in 2 × SSC/0.1% SDS followed by 15 min at 65 °C in 0. 2 × SSC/0.1% SDS. Most of the markers used were *Aedes aegypti* cDNA clones mapped as RFLP loci (Severson *et al*., 2002) that were previously used to construct a comparative linkage map for *C. pipiens* (Mori *et al*., 1999). Markers identified with the 'CX' prefix were random clones from a *C. quinquefasciatus* (Kuala Lumpur strain) cDNA library.

Statistical methods

Multipoint linkage analysis was performed using the MAPMAKER computer program (Lander *et al*., 1987) with a LOD of 3.0 as the threshold for significance. QTL controlling diapause induction and diapause-associated life history traits were identified using the QTL Cartographer computer package (Basten *et al*., 2001). Markers with significant partial regression coefficients were identified with the SRmapqtl function by forward–backward stepwise regression. QTL affecting each trait were identified by CIM (Zeng, 1993, 1994) using the Zmapqtl function with model 6. The comparisonwise and experimentwise LR thresholds for identifying a QTL were determined by permutation test (Churchill & Doerge, 1994). The data were permuted 1000 times, and critical LR values were determined for the $\alpha = 0.05$ significance levels. A likelihood ratio decline of ≥ 9.21 (equals a LOD decline of ≥ 2.0) between adjacent peaks on a linkage group was used to define linked QTL. The best estimate for QTL location was assumed to be the position having the largest LR value and one-LOD support limits were calculated around this value.

Zmapqtl also estimates additive (a) and dominance (d) effects of the detected QTL. Additive and dominance effects are calculated relative to alleles at the marker locus nearest to the predicted QTL from the designated 'A' parent in the cross. For additive effects, the BB homozygote effect is set to zero and AA effect = 2 × AB effect. For dominance effects, the BB homozygote effect is set to zero and AA effect = AB effect. Mode of gene action was determined as the absolute value of d/a where additive = 0–0.20, partial dominance = 0.21–0.80, dominance = 0.81–1.20 and overdominance > 1.20 (Stuber *et al*., 1987; Babu *et al*., 2006).

In addition to interval mapping methods for QTL analysis, we also used APS regression as implemented in SAS/STAT software, version 9.1.3 of the SAS System for Windows XP (SAS Institute Inc., 2000–2004). APS evaluates all possible combinations of markers. This method is computationally tractable only if the number of markers is small. At $n = 13$, the number of possible unique subsets is 8191, which requires only a minute or less for analysis. For comparison, 20 markers have more than a million unique subsets, 30 more than a billion and 40 more than a trillion. As regression statistics are calculated for every possible unique subset, there is no inherent bias in subset choice and no unexamined combination. The criterion for choosing the best set cannot be the coefficient of determination R^2 because R^2 is at a maximum when all the elements (all the markers in this case) are in the model. We identified the best subset using Mallow's C_p, a method that weighs the value of the variance explained against the number of variables 'in the model (Mallows, 1973; Freund & Littell, 1991). The C_p statistic is defined as follows:

$$C_p = \frac{RSS_p}{\hat{\sigma}^2} + 2p - n$$

where n = the number of observations, p = the number of variables in the regression, RSS_p = the residual sum of squares using p

variables and $\hat{\sigma}^2$ = an independent estimate of the error. The residual variance from the full model is used as the estimate of $\hat{\sigma}^2$. The best model is that which has the minimum value of C_p. This method differs significantly from subset selection methods that require each element in the model to have a significant effect when evaluated alone. None of the elements need be individually significant. The sole criterion is the performance of the entire subset when evaluated against the number of elements within it. The best model in this context will explain (1) the greatest amount of phenotypic variance with the least number of markers provided that C_p has a value approaching $(p + 1)$, at which point the value of C_p will be at or very close to a minimum. Marker genotypes were scored as 1 (nondiapausing parent type), 1.5 (heterozygote) or 2 (diapausing parent type). We included these analyses as interval mapping has low power across large intervals, and the experimentwise thresholds for interval mapping are conservative and likely to exclude valid QTL (Churchill & Doerge, 1994). Pearson correlation coefficients among phenotypes were determined using PROC CORR in SAS vs. 9.1.3.

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