The Genetic Basis of the Interspecific Differences in Wing Size in Nasonia (Hymenoptera; Pteromalidae): Major Quantitative Trait Loci and Epistasis

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Manuscript received June 5, 2001
Accepted for publication March 6, 2002

ABSTRACT

There is a 2.5-fold difference in male wing size between two haplodiploid insect species, *Nasonia vitripennis* and *N. giraulti*. The haploidy of males facilitated a full genomic screen for quantitative trait loci (QTL) affecting wing size and the detection of epistatic interactions. A QTL analysis of the interspecific wing-size difference revealed QTL with major effects and epistatic interactions among loci affecting the trait. We analyzed 178 hybrid males and initially found two major QTL for wing length, one for wing width, three for a normalized wing-size variable, and five for wing seta density. One QTL for wing width explains 38.1% of the phenotypic variance, and the same QTL explains 22% of the phenotypic variance in normalized wing size. This corresponds to a region previously introgressed from *N. giraulti* into *N. vitripennis* that accounts for 44% of the normalized wing-size difference between the species. Significant epistatic interactions were also found that affect wing size and density of setae on the wing. Screening for pairwise epistatic interactions between loci on different linkage groups revealed four additional loci for wing length and four loci for normalized wing size that were not detected in the original QTL analysis. We propose that the evolution of smaller wings in *N. vitripennis* males is primarily the result of major mutations at few genomic regions and involves epistatic interactions among some loci.

An important question in evolutionary biology is whether adaptation involves the accumulation of many genetic changes with small phenotypic effects or, at least initially, few genes with large phenotypic effects (macromutations *sensu* Orr and Coyne 1992). An obvious way to test which hypothesis is correct is to determine the genetic basis of adaptive traits that vary between closely related species or different populations of the same species.

Most interspecific studies of the genetic basis of quantitative traits have been performed on Drosophila hybrids and demonstrated a polygenic basis of these characters (reviewed in Coyne and Orr 1998). These results are not necessarily incompatible with the view that major genes with large phenotypic effects (*e.g.*, >10% explained phenotypic variance; Tanksley 1993, Figure 5, p. 219) play a significant role in the evolution of adaptive traits. One hypothesis for the evolution of adaptive traits is that major mutations generate major phenotypic changes in the trait but simultaneously generate negative pleiotropic effects. Subsequent selection on modifying genes ameliorates the negative effects of major mutations (Clarke 1997).

With the advent of complete genomic maps and new statistical methods for mapping quantitative trait loci (QTL), we can now estimate the minimum number of "genetic factors" (Via and Hawthorn 1998, p. 353) affecting specific traits and pinpoint their locations within the genome. Additionally, it is possible to directly estimate the effects of individual QTL on the phenotypic variance and the effects of different alleles at specific QTL. Several QTL studies of interspecific differences of adaptive quantitative traits have found QTL with large effects (Laurie *et al.* 1997; Macdonald and Goldstein 1999).

Epistasis is here defined as a nonadditive phenotypic effect of interacting genes. Although the role of epistasis in evolutionary and quantitative genetics has been of great theoretical interest, little is known about the relative importance of epistasis during speciation (*e.g.*, Coyne *et al.* 2000; Goodnight and Wade 2000) or the influence of epistasis on quantitative traits (Cheverud and Routman 1995). This is because of the inherent difficulties of measuring epistatic genetic variance using classical quantitative genetics (but see Cheverud and Routman 1995; Wolf *et al.* 2000). However, new analytical methods like QTL provide direct experimental access to estimates of epistatic interactions (*e.g.*, Lark *et al.* 1995; Yu *et al.* 1997). Detection of epistasis is easier in haploids because the number of possible genotypes is reduced (increasing statistical power). Therefore, whole genomes can be readily scanned for genetic interactions in haploids. For example, two-locus (pairwise) interactions can be detected by analyzing the phenotypic values of the four genotypes; epistasis is indicated by significant

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deviation from additive effects of individual loci (Lark *et al.* 1995; Chase *et al.* 1997). The comparable analysis in diploids would involve nine genotypes, and recessive interactions would occur at low frequencies in the mapping population, making detection more difficult. We have taken advantage of the haploidy of males in our system to investigate both additive and epistatic effects on wing size in the haplodiploid insect genus Nasonia.

The Nasonia species complex consists of three closely related species: *N. vitripennis*, *N. giraulti*, and *N. longicornis* (Darling and Werren 1990). *N. giraulti* occurs in eastern North America where it parasitizes protocalliphora fly pupae in bird nests, and *N. vitripennis* is cosmopolitan and occurs microsympatrically with *N. giraulti* (Darling and Werren 1990) in parts of its range. It is estimated that the species have been separated for ~0.8 millon years (Campbell *et al.* 1993; J. H. Werren, unpublished data), which is comparable to the youngest known Drosophila species pair, *Drosophila mauritiana* and *D. sechellia* (0.6–0.9 mya; Kliman and Hey 1993; Macdonald and Goldstein 1999). Nasonia species are reproductively isolated, in part by infections with different strains of the bacterium Wolbachia, which cause cytoplasmic incompatibilities (Breeuwer and Werren 1990; Bordenstein *et al.* 2001). However, Nasonia that have been cured with antibiotics of their Wolbachia endosymbionts (Breeuwer and Werren 1990, 1995) are capable of producing viable and fertile hybrid offspring. However, there is some mortality among the sons of F_1 females in crosses between *N. giraulti* and *N. vitripennis* due to recessive genetic incompatibilities (Breeuwer and Werren 1995; Gadau *et al.* 1999). *N. vitripennis* males have small vestigial wings that are ~40% the size of male wings in *N. giraulti*.

Weston *et al.* (1999) conducted hybrid crosses with five eye color mutant strains of *N. vitripennis* (corresponding to the five chromosomes of Nasonia) to assess the effects of each chromosome on a wing-size trait of an F_2 hybrid. They found an effect of three chromosomes on wing size and tight linkage of one or more wing-size loci with the eye color mutation "or123" on linkage group (LG) IV. They also introgressed this region from *N. giraulti* into the genetic background of *N. vitripennis*. The introgressed region, containing either one or a few tightly linked genes, accounted for 44% of the species difference in normalized wing size between *N. vitripennis* and *N. giraulti*.

The objective of this study was to conduct a QTL analysis of wing-size differences in *N. vitripennis* × *N. giraulti* hybrid males, using a linkage map based on 91 randomly amplified polymorphic DNA (RAPD) markers (Gadau *et al.* 1999). This analysis permits us to more precisely map wing-size loci, to measure the magnitude of individual QTL effects, and to investigate epistatic interactions among loci affecting wing size. The results are compared to the previous work using five visible markers and an introgression strain (Weston *et al.* 1999). The QTL analysis of the likely adaptive wing-size difference between two Nasonia species reveals the genetic architecture that underlies this difference.

MATERIALS AND METHODS

Nasonia stocks and mapping population: Two inbred and endosymbiont (Wolbachia) free strains of *N. vitripennis* (ASYMC) and *N. giraulti* (R16A) were used to generate 15 genetically identical hybrid F_1 females. These females produced 178 males that were used for constructing a genomic map and for QTL analysis (see Gadau *et al.* 1999 for the construction of a linkage map of Nasonia). The *N. giraulti* strain R16A is an introgression strain with a *N. giraulti* nuclear genome in a *N. vitripennis* cytoplasm (see Breeuwer and Werren 1995 for details). R16A was used to avoid the known nuclear-cytoplasmic incompatibility between *N. vitripennis* and *N. giraulti* (Breeuwer and Werren 1995).

Measurements and transformations: Measurements were done on the forewings as described in Weston *et al.* (1999). Wing length is the distance from the distal end of the wing to the mass of dark connective tissue at the proximal end. Wing width is measured as the widest part, perpendicular to the length measurements. Interocular distance (Weston *et al.* 1999) is the distance between the eyes at the first ocelli. It is correlated with relative body size in Nasonia (Skinner 1983) and was used to normalize the wing-size measurements. All measurements are given in ocular units [1 unit = 0.02 mm; note the conversion given in Weston *et al.* (1999) was reported incorrectly by a factor of 10].

Wing setae in Nasonia are small, slender hairs evenly distributed over the whole area of the forewing and are probably homologous to the setae of *D. melanogaster* described as bristles by Dobzhansky (1929). Seta density in a region just below the end of the stigmal vein of the forewing was measured in the F_2 males by counting the number of setae in a 0.0156-mm^2 grid. Every seta with its base within the grid was counted, including those on the underlying side of the wing. Besides the two basic wing measurements, wing length and wing width, we also used a derived composite measurement—normalized wing multiple (wing length × wing width/interocular distance)—which was also used by Weston *et al.* (1999). Normalized wing multiple was introduced since this measurement eliminated the correlation between wing size and body size in both species (Weston *et al.* 1999 and Table 2).

Linkage analysis: The mapping population and linkage map used for the QTL analysis were the same as in Gadau *et al.* (1999). This linkage map was based on the segregation of 91 RAPD markers in 178 males derived from 15 F_1 females. Linkage group designations were the same as in previously published maps (Saul 1993; Gadau *et al.* 1999). The average distance between two markers in the linkage map was 8.4 cM. The relationship between physical distance and map unit is 0.41 Mb/cM (Gadau *et al.* 1999). Having haploids as a mapping population for QTL analysis has multiple advantages: (1) The effect of an allele is directly measurable because there are no dominance interactions among alleles of the same locus; (2) epistatic interactions between nuclear loci are easier to analyze because in two-way interactions only four genotypes are possible; and (3) linkage phase can be determined in each individual even if dominant markers like RAPDs are used.

QTL analysis: MapQTL 4.0 (Van Ooijen *et al.* 1999) was used to identify QTL for all traits. First a standard interval mapping was done to identify the major QTL. Then multiple-QTL-model (MQM) mapping, implemented in MapQTL, was used to fit more than one QTL at a time. The MQM-mapping procedure uses markers closest to the QTL as cofactors to

take over the role of the QTL. Thus, the cofactors will reduce the residual variance, increase the power in the search for other segregating QTL, and enhance the accuracy of QTL mapping (Jansen 1993, 1994; Zeng 1993, 1994; Jansen and Stam 1994). Van Ooijen (1999) obtained statistical thresholds for QTL analysis by large-scale simulations. He distinguished between chromosome-wide (suggestive linkage) and genome-wide thresholds (significant linkage) that control the type I error. The statistical threshold for a suggestive QTL (controls for a chromosome-wide type I error at $\alpha = 0.05$) for our map was LOD = 1.9 (Van Ooijen 1999). Markers with LOD scores >1.9 in an interval mapping procedure were used as cofactors during the consecutive MQM mapping. If the LOD value for a QTL linked with the cofactor dropped below 1.9 during the MQM mapping it was removed from the cofactor list and MQM was run again. This procedure was repeated until the cofactor list remained stable. The genome-wide LOD thresholds for a significant QTL at the 5 and 1% false-positive rates in Nasonia according to Van Ooijen (1999) were 2.9 and 3.7, respectively. Additionally, suggestive and statistically significant QTL were statistically confirmed using the standard permutation test for interval mapping (Churchill and Doerge 1994) incorporated in MapQTL 4.0 (Van Ooijen *et al.* 1999).

Epistat (Chase *et al.* 1997) was used to search for epistatic interactions of QTL. This program searches the whole genome for significant interactions between QTL and uses log-likelihood ratios to compare the likelihood of explaining the effects by null, additive, or epistatic models (for a detailed description of the underlying algorithms see Chase *et al.* 1997). The program organizes genetic mapping data and quantitative trait values into graphic displays that illustrate the individual effects of single loci as well as the interactions between any two loci. The program is available for download at the following site: http://64.226.94.9/epistat.htm.

Mapping procedure of conditional QTL: First an automated search option was used to find all conditional QTL for all traits exceeding a predetermined threshold [the default settings of the program were used: *i.e.*, 5.0 log-likelihood ratio (LLR) of an additive model *vs.* a nonadditive model for the null threshold and 6.0 LLR for the additive threshold; minimal group size was 10 (Chase *et al.* 1997)]. Then, we discarded all interactions between linked markers because linkage confounds detection of epistatic interactions in this program. The remaining interactions were analyzed with a Monte Carlo program implemented in Epistat (Chase *et al.* 1997) to test for the statistical significance. Each Monte Carlo simulation was specific to a given trait and pair of loci. Subpopulations were chosen randomly without replacement from the total population. Log-likelihood ratios for the additive model were calculated on the basis of these distributions. A total of 1,000,000 trials were done for each interaction. We transformed the *P* value found in the Monte Carlo simulation (the probability that a single trial exceeded the observed value) into $1 - (1 - P)^5$ to account for searching through five linkage groups (Lark *et al.* 1995). We did not correct for the number of markers ($n = 91$) because we considered only epistatic interactions between markers of different linkage groups to avoid problems with confounding effects of linkage between markers of the same linkage group.

RESULTS

Morphometrics of wing- and head-size measurements: A comparison between the wing-size measurements of males from both parental strains (ASYMC and R16A; Figure 1) and F_2 hybrid males showed that the average values of the F_2 males were intermediate between the two parental phenotypes (Table 1). The phenotypic correlations between the different traits in the 178 F_2 males are given in Table 2. Surprisingly the phenotypic correlations between the different wing traits were rather low (Table 2), which could indicate that these traits are determined by different sets of genes. The nonsignificant correlation coefficient (0.03) of head size and normalized wing multiple indicates that we effectively removed the influence of body size on wing size by using normalized wing multiple.

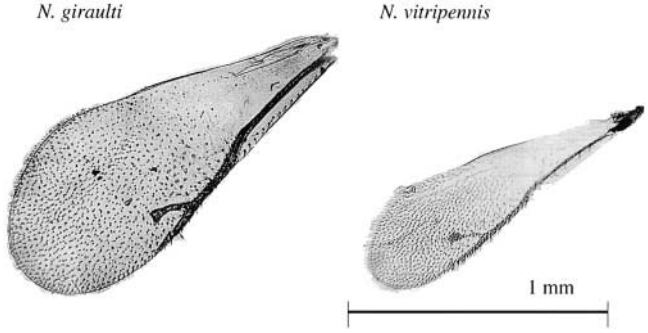

Figure 1.—Comparison of forewing size of the two parental species *Nasonia vitripennis* (line ASYMC) and *N. girault* (line R16A). Note the reduced wing venation typical for species of the hymenopteran family Pteromalidae and the differences in seta density.

QTL mapping: Significant QTL (genome-wide type I error $P < 0.01$; Table 3) were detected for each of the four wing trait measurements: wing length (two), wing width (one), normalized wing multiple (three), and seta density (five). Additionally, we detected epistatic interactions for all traits except wing width (Table 4). All epistatic interactions were conditional. Conditional QTL have no significant effect individually but show a significant phenotypic effect if a particular allele is present at a second unlinked locus. Hence, the effect of a "conditional QTL" is conditional on the genetic background of an individual.

Contrary to expectations based on the phenotype of the parental species, we found some QTL in which the vitripennis allele was associated with the larger wing phenotype (Table 3, footnote *a*). Similar results (the "high" allele comes from the "low" line) have been found repeatedly in QTL mapping studies (Hunt *et al.* 1995; Lark *et al.* 1995; Page *et al.* 2001) and are normally thought to result from fixation of low alleles in the high line during selection or speciation. They are referred to as "transgressive alleles" (Tanksley 1993).

Wing-size QTL: Overall, the initial QTL search for the three wing traits (wing width, wing length, and normalized wing multiple) revealed a few genomic regions (QTL) of large effect. The LOD scores for these QTL are typically well above the 0.01 genome-wide acceptance threshold. Furthermore, permutation tests indi-

TABLE 1

Basic measurements of the 178 F_2 males used for the QTL analysis and the two parental strains

Trait	F_2 (mean ± SD)	ASYMC[c]	R16A[c]
Wing width[a]	10.3 ± 1.9	7.3 ± 0.7	14.0 ± 0.9
Wing length[a]	27.4 ± 3.4	25.3 ± 2.0	34.5 ± 2.9
Interocular distance[a]	9.1 ± 0.9	9.2 ± 0.8	9.9 ± 0.7
Normalized wing multiple[a]	31.3 ± 8.1	20.1 ± 2.3	49.1 ± 4.5
Seta density[b]	103 ± 30	133 ± 22	46 ± 7

[a] All measurements are in ocular units (1 unit = 0.02 mm).
[b] Seta density is given in number of bristles in a square of 0.0156 mm^2.
[c] Values for the two parental strains R16A and ASYMC were taken from Table 1 and for seta density from the text (p. 589) in Weston *et al.* (1999).

cate that we could detect QTL with effects of phenotypic variance of 4–6%. For example, our threshold value for detection of wing width QTL is LOD 2.3 (Table 3) and allows detection of a QTL explaining as little as 5.5% of the phenotypic variance. However, only one QTL was detected, which had a LOD score of 17.63 (38% of phenotypic variance explained). Therefore, we can confidently say that there is a region of very large effect on wing width and no evidence of intermediate magnitude QTL for this trait.

A QTL search for wing length revealed two QTL (LOD scores 3.98 and 5.56, respectively) whereas QTL with LOD scores as low as 3.4 could be detected with $P < 0.01$ probability. One of these two QTL, occurring on LG II, apparently involves transgressive alleles; the *vitripennis* allele has a wing length significantly greater than that of the *giraulti* allele. Two QTL on LG III and IV explained 13.5 and 11.8% of the phenotypic variance of wing length, respectively, in our mapping population. The QTL for wing length on LG IV explained 38.1% of the phenotypic variance for wing width and 22% of the phenotypic variance of normalized wing multiple (Table 3). As seen in Figure 2 and Table 3, there also appears to be a three-way epistatic interaction affecting wing length among regions on LG II (marker 407-1.01), LG IV (tightly linked markers 323-0.98 and A20-1.5), and LG V (marker P4-1.46). The two markers on LG IV map very closely to the major wing length QTL (all markers map within a 2.9-cM region; 1 cM = 0.41 Mb) and therefore possibly represent the same locus or tightly linked loci.

The normalized wing multiple was used to measure overall wing size normalized for body size. For this trait, one QTL of large effect was found on LG IV (LOD 11.39, 22.0% phenotypic variance explained, see also Figure 3) and two QTL of smaller effect (LOD 4.00 and 3.32) were found on LG I and LG III, closer to the detection threshold ($P < 0.01$, LOD 3.2). The QTL for normalized wing multiple on LG IV corresponds to the region of large effect linked to the visible marker *or123*, described by Weston *et al.* (1999); *or123* was previously mapped to the same region (Figure 1, marker 320-2.1f, LG IV; Gadau *et al.* 1999). This region was introgressed from *N. giraulti* into *N. vitripennis* and found to account for 44% of the wing-size difference between the species.

A QTL for normalized wing multiple on LG III appears to be transgressive. That is, males with the *giraulti* allele actually have wings significantly smaller than those of males with the *vitripennis* allele. This involves the same marker showing a transgressive effect for wing length, which may explain the effect. The QTL for normalized wing multiple on LG I and III explain 7 and 5.4%, respectively, of the observed phenotypic variance. Interestingly, the region on linkage group I that explained 7% of the observed variance of normalized wing multiple had no effect on wing length or wing width. Furthermore, the region on LG III that influenced wing length had no effect on wing width or normalized wing multiple.

Using the program Epistat (Chase *et al.* 1997) we screened for pairwise epistatic (nonadditive) interactions occurring between markers on different linkage groups. As previously explained, haploid males facilitate detection of epistatic interactions between loci. The analysis for wing length revealed a region on LG II that interacts with the primary QTL on LG IV (Table 4). The *vitripennis* allele at this locus significantly increases wing size relative to the *giraulti* allele, but only when combined with the *giraulti* allele at the primary QTL on LG IV. The locus on LG II has no effect on wing

TABLE 2

Phenotypic correlation of the traits used in the QTL analyses

	Wing length	Head size	Wing multiple	Seta density
Wing width	0.57***	0.27**	0.86***	0.31***
Wing length		0.34***	0.77***	0.11
Head size			0.03	0.16
Wing multiple				0.33***

Given are the correlation coefficients and the significance level. **$P < 0.01$; ***$P < 0.001$.

TABLE 3

Significant QTL using MapQTL (Van Ooijen *et al.* 1999)

Trait genome-wide LOD threshold 0.05/0.01	MQM mapping (linkage group, *marker*, LOD score)	% explained phenotypic variance	Mean *vitripennis* allele	Mean *girault* allele
Wing length 2.5/3.1	III, *323-0.99*, 3.98[a]	13.5	27.57	24.90
	IV, *306-0.75f*, 5.56	11.8	26.18	28.98
Wing width 2.3/3.0	IV, *306-0.75f*, 17.63	38.1	9.36	11.26
Normalized wing multiple 2.6/3.2	I, *316-0.87*, 4.00	7.0	28.92	33.26
	III, *323-0.99*, 3.32[a]	5.4	31.82	25.92
	IV, *76-1.03f*, 11.39	22.0	27.88	35.64
Seta density 2.6/3.5	I, *209-0.85*, 5.97	7.3	93.45	79.31
	II, *317-0.62*, 7.03	12.1	94.75	77.97
	III, *P1-1.48f*, 17.61	22.2	101.35	71.41
	IV, *76-1.03f*, 11.38	14.8	96.53	76.23
	V, *34-1.03*, 5.00	5.1	91.29	79.24
Head width 2.6/3.1	I, *D07-0.85*, 5.90	9.4	18.71	17.44
	II, *30-1.11f*, 5.01	9.6	18.54	17.30
	IV, *213-0.47*, 6.40	10.6	17.70	18.89
	V, *356-0.5*, 4.38	6.2	18.72	17.44

LOD thresholds for 0.05 and 0.01% genome-wide error rates were determined by a permutation test and are given for each trait separately.

[a] QTL that show an effect opposite to the expected phenotype.

length when combined with the *vitripennis* allele at the primary QTL. Thus, it can be described as a "conditional" epistatic interaction or a conditional QTL.

Two significant epistatic interactions were detected for normalized wing multiple. One involves regions on LG V and LG IV (Table 4). The LG V region shows a much larger wing size for its *girault* allele in combination with the *girault* allele on LG IV, but a weak effect on wing size when the LG IV region has the *vitripennis* allele. The conditional nature of these QTL may explain the failure to detect their effect in the original primary QTL analysis (see DISCUSSION for details). The LG IV marker (A20-1.5) maps 24.1 cM from the major QTL on LG IV, and it is possible that it is the same locus given the uncertainties involved in map locations of QTL. To investigate this possibility, we determined the nonadditive interaction between the major QTL on LG IV (76-1.03f) and the LG V marker; no significant epistatic interactions were found between these two loci (LLR = 0.58, $P \gg 0.05$). Therefore, we conclude that these represent two different loci on LG IV: one that interacts epistatically with a locus on LG V and one that has an effect on its own.

The epistatic interactions between regions on LG I (76-0.42) and LG IV (76-1.29) also probably represent two new wing-size loci. The marker on LG IV is >50 cM from the major QTL on this linkage group, and the major QTL on LG I does not show a significant epistatic interaction with the major QTL on LG IV (LLR 4.26, LLR > 6.0 statistical threshold). This epistatic interaction appears to be synergistic: The *girault* allele at each locus increases wing size, but *girault* alleles at both loci have a markedly larger effect on wing size. It is possible that the locus on LG IV (A20-1.5) involved in this interaction is the same as that involved in the interaction between LG IV and LG V: a three-way epistasis. However, these two regions are >50 cM apart, arguing against this interpretation. Taken together, the data suggest six to seven loci affecting wing size, with one region of large effect (22% of explained phenotypic variance) and two additional sets of epistatically interacting loci.

We also conducted QTL analyses for the wing width/wing length (ww/wl) ratio and normalized wing width and wing length (each divided by interocular distance). These analyses gave fundamentally the same results. QTL for ww/wl ratio were found on LG IV and LG III, mapping near the QTL for normalized wing size. Normalized wing width and normalized wing length yielded the same QTL as for the unnormalized trait. Therefore, the location of wing-size QTL is insensitive to various normalization procedures.

Seta density: The density of setae on the wing is strongly correlated with the wing cell size in Drosophila and presumably also for Nasonia. Seta density also increases with the measurements of body size in Nasonia, indicating that larger males have larger wing cells. We, therefore, investigated QTL for seta density in hybrid males. We found significant QTL for seta density on all five LGs (Table 3) and detected three epistatic QTL (Table 4; Figure 2). In each case, the *girault* allele significantly decreases seta density (*i.e.*, increases wing cell size) relative to the *vitripennis* allele. Highly significant QTL were found on LG IV (LOD 11.38, 14.8% explained phenotypic variance) and LG III (LOD 17.61,

TABLE 4

Epistatic interactions

Trait/EQTL 1	EQTL 2		LLR nonadditive[a]	Additive *P* value/ transformed *P* value $(1 - (1 - P)^5)$[b]
Wing length				
	323-0.98 (IV)			
407-1.01 (II)	gir	vit		
gir	27.36	26.50		
vit	29.90	25.51	8.44	>0.0001/>0.0001
	A20-1.5 (IV)			
P4-1.46 (V)	gir	vit		
gir	30.92	25.97		
vit	28.17	26.54	6.25	0.0048/0.025
Normalized wing multiple				
	76-0.42 (I)			
76-1.29 (IV)	gir	vit		
gir	39.04	30.02		
vit	31.78	26.86	8.30	0.0091/0.045
	A20-1.5 (IV)			
P4-1.46 (V)	gir	vit		
gir	40.08	28.46		
vit	33.32	27.54	6.28	0.0019/0.009
Seta density				
	209-1.05 (IV)			
307-0.77 (V)	gir	vit		
gir	88.53	86.18		
vit	98.28	123.31	7.15	0.007/0.027
	209-1.05 (IV)			
315-2.46 (I)	gir	vit		
gir	90.18	86.11		
vit	97.55	117.83	9.34	0.0046/0.023
	315-0.53 (V)			
N16-0.8 (I)	gir	vit		
gir	92.58	96.63		
vit	95.39	140.15	12.74	0.0003/0.002

Epistatic interactions were detected for all traits except wing width. The mean phenotypic values for all four genotypes of the *e*pistatic *q*uantitative *t*rait *l*oci (EQTL 1 and EQTL 2) are listed. Underlined for each interacting pair are the genotypes with highest and lowest phenotypic values. gir, *N. giraulti*; vit, *N. vitripennis*.

[a] The LLR if an epistatic model is compared with an additive model (Chase *et al.* 1997).

[b] The *P* value associated with the LLR in column four derived from a Monte Carlo simulation (see materials and methods for details).

explaining 22.2% of the phenotypic variance). The major QTL on LG IV is the same marker identified for the major QTL of normalized wing size and is tightly linked to the QTL marker for wing width (Table 3; Figure 3). The LG III major QTL (22.2% of phenotypic variance) maps 19.2 cM from the transgressive QTL for wing length and normalized wing multiple. However, the seta density QTL is not transgressive but rather the *giraulti* allele is associated with a large reduction in seta density (presumed increase in cell size). It is perplexing why a QTL that has such a large effect on seta density (and therefore presumably on wing cell size) would not have any effect on wing size. Possible explanations for this observation are discussed later.

Three significant epistatic interactions were detected (Table 4; Figure 2). In each case, a double dose of *vitripennis* alleles greatly increases seta density. Two interactions involved a marker on LG IV (209-1.05) that is 21.0 cM from the major primary QTL for seta density on the same linkage group. The region on LG IV (209-

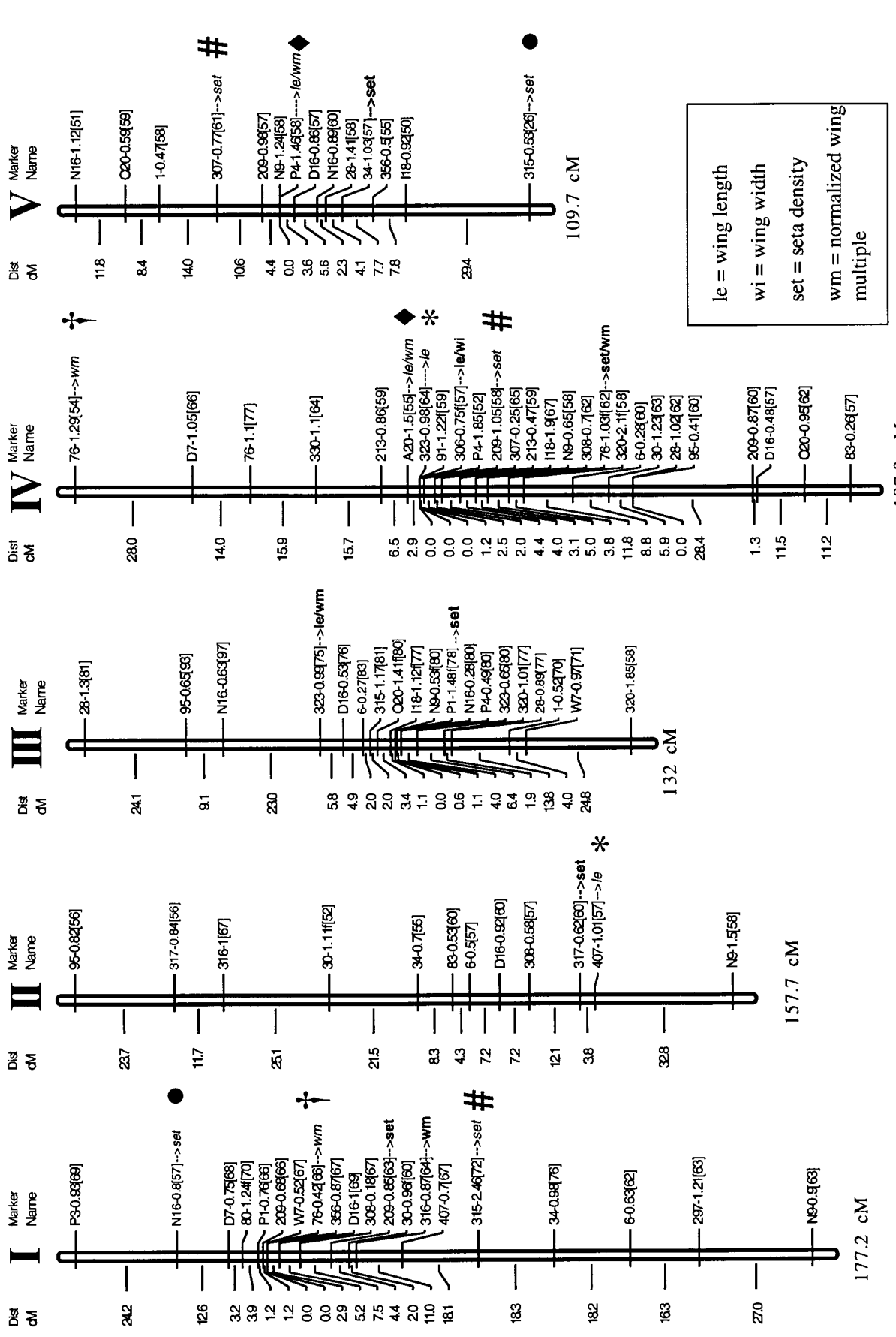

FIGURE 2.—Epistatic interactions between QTL for wing size superimposed on a linkage map based on the 178 hybrid males used for the QTL analysis (for details on the linkage map see GADAU *et al.* 1999). Symbols indicate interacting loci; for details on the effect see Table 4. All but one interaction (#) are two-way interactions. QTL detected by interval mapping are shown in boldface type and interacting loci in italics.

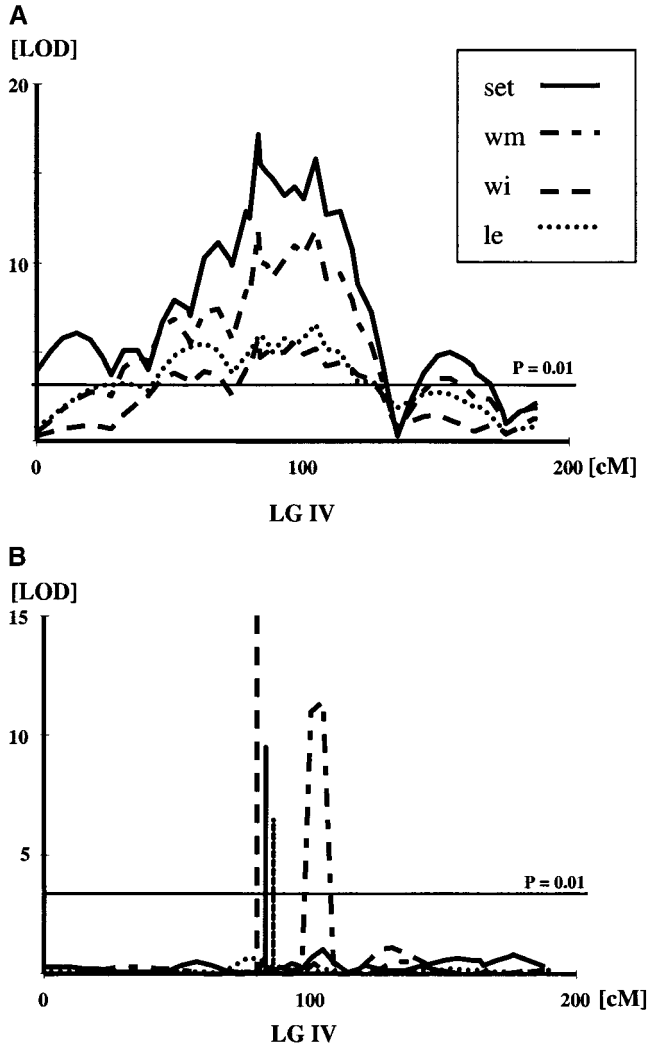

FIGURE 3.—Distribution of the LOD scores on LG IV for four wing traits (wm, normalized wing multiple; wi, wing width; le, wing length; set, seta density). (A) Results of a QTL analysis using the interval mapping algorithm of MapQTL (VAN OOIJEN *et al.* 1999). (B) Same data as in A analyzed with the multiple-QTL-model (MQM) mapping algorithm of MapQTL. Cofactors for the calculation were the markers listed in Table 3. The peaks for wing length, wing width, and seta density in B are at the same position (marker 306-0.75f) but were slightly shifted for better visibility. MQM significantly increased the possibility to localize a QTL and the result indicated a single significant QTL on LG IV for each trait and two different QTL for wing size, one influencing wing length, wing width, and seta density and a second affecting the normalized wing multiple only. The horizontal line indicates the threshold for a 1% genome-wide error rate. Note the *y*-axis in A ends at LOD 20 whereas in B it ends at LOD 15.

1.05) interacts with the regions on LG V and LG I. The marker (209-1.05) is only 4.1 cM from the epistatic QTL for wing multiple on LG IV (A20-1.05) that interacts epistatically with a region on LG V. Furthermore, the respective markers on LG V (307-0.77 and P4-1.46) are only 15.0 cM apart, and the epistatic interactions for seta density and wing multiple are both in the same directions. Therefore, the most likely explanation is that these conditional QTL for seta density and wing multiple represent the same QTL and these QTL interact epistatically to affect wing size through their effects on wing cell size. The second seta density epistatic interaction also involves the same marker on LG IV and a region on LG I. Thus, there is likely a three-way interaction affecting seta density (Figure 2). Although wing multiple also shows a LG IV-LG I epistatic interaction, the respective markers on LG IV are unlinked, as are those on LG I, arguing against involvement of the same loci. Finally, there is a third epistatic interaction for seta density, between a LG V region (315-0.53) and a LG I region (N16-0.8). The two LG V regions are unlinked as are the LG I regions, indicating different loci. In summary, the analysis of seta number reveals a region of very large effect plus a complex web of epistatic interactions; some loci also affect wing size.

Interocular distance: Head width was measured as the distance between the eyes at the ocellar region of the head, which was used as an index of body size for normalization of the wing multiple. We therefore investigated QTL involved in interocular distance. Four LGs contained significant QTL, each of which explained between 6.2 and 10.6% of the phenotypic variance among the hybrid males (Table 2). Three of these four QTL showed the effect where the allele of *N. vitripennis* was associated with the larger head size. We could also detect two epistatic interactions for interocular distance (results not shown).

These results may reveal alleles assorting for head shape or possible alleles affecting overall body size among the hybrid males. A subset of hybrid males was smaller than typical for the rearing conditions, indicating that negative epistatic interactions in some hybrid males could contribute to reduced body size. Consistent with this interpretation, one epistatic interaction showed significantly smaller head size for the two recombinant genotypes (*vitripennis-girault*i and *girault*i-*vitripennis*) and the other showed a significantly smaller head size for one recombinant genotype (*vitripennis-girault*i). However, because there is no correlation between head width and wing multiple (Table 1), such effects are unlikely to confound the identification of the wing-size QTL.

DISCUSSION

All QTL reported here were highly significant and exceeded the 0.01% genome-wide statistical threshold determined with two different methods: (1) a standard permutation test for interval mapping (CHURCHILL and DOERGE 1994) and (2) an alternative method described by VAN OOIJEN (1999). Both methods gave approximately the same statistical results. The method of Van Ooijen was slightly more conservative in that the thresh-

olds for controlling for a genome-wide type I error were more stringent (~0.1–0.2 LOD units).

The analyses of the genetic basis of seta density, wing shape, and wing size in *D. melanogaster* have a long history (Dobzhansky 1929; Milkman 1970) and have recently been revived by the use of new quantitative genetic techniques (Weber *et al.* 1999; Zimmerman *et al.* 2000). The results of these investigations indicated that wing size and shape in Drosophila are determined by multiple loci. Weber *et al.* (1999) estimated a minimum of 11 QTL on LG III in *D. melanogaster* while Zimmerman *et al.* (2000) estimated a minimum of 23 QTL for two intervein regions of the anterior compartment of the wing. Few of the QTL for wing size or wing shape had large phenotypic effects >1 SD or explained >10% of the phenotypic variance (Weber *et al.* 1999; Zimmerman *et al.* 2000).

In contrast to that finding, Weston *et al.* (1999) showed that one or a few genes in a single region of LG IV explained 44% of the differences in wing size between the two hymenopteran sibling species, when introgressed from *N. giraulti* into *N. vitripennis*. Our QTL analysis of wing size and other wing characteristics provides a more detailed analysis of the genetic architecture of wing-size differences between the two Nasonia species. We corroborate the Weston *et al.* (1999) finding of a region of large effect on LG IV near the visible marker or123. Additionally, we were able to dissect the genetic basis of these traits in more detail, determine the magnitude of the effect of single QTL, and detect epistatic interactions affecting wing size and seta density on wings (Tables 3 and 4). In addition, both the coarse scale analysis using one visible marker per linkage group (Weston *et al.* 1999) and our finer scale QTL analysis revealed a major QTL on LG I.

The epistatic analysis also helped us to explain a conflicting result with the Weston *et al.* (1999) study. This study found a significant phenotypic effect of LG V in their initial cross between *N. vitripennis* and *N. giraulti* (Table 2 in Weston *et al.* 1999), whereas we could not detect a QTL for the normalized wing multiple on that LG using normal interval mapping or MQM mapping procedures. There was a significant phenotypic effect of LG V on normalized wing multiple in our mapping population [*t*-test on phenotypic distribution at marker 315-0.53 (LG V), $t = 2.959$, $P = 0.004$]. This failure to map a QTL on LG V was probably due to the increased statistical threshold of interval mapping compared to the simple *t*-test performed by Weston *et al.* (1999). Additionally, the additive phenotypic effect of the QTL on LG V was "masked" by an epistatic interaction between LG IV and LG V (Table 4). In this interaction the effect of the conditional QTL for normalized wing multiple on LG V was conditional on the presence of the *giraulti* allele on LG IV (Table 4, normalized wing multiple). Therefore, about one-half of the individuals in our mapping population were not informative for the interval QTL mapping (54% of the individuals at the primary locus at LG IV had the *vitripennis* allele; Figure 2). However, by controlling for two-way epistatic interactions, we were able to reveal a conditional QTL on LG V. Although Weston *et al.* (1999) were able to detect that LG V has a phenotypic effect on normalized wing multiple, only the QTL mapping and subsequent search for epistatic interactions revealed the mechanism of the genetic effect of LG V on normalized wing multiple.

Studies from a variety of plants and animals show that experimental manipulations of cell size result in a compensatory change in cell number (and vice versa), maintaining overall size of the tissue (Day and Lawrence 2000). This suggests a regulatory mechanism that adjusts cell size and cell number to keep tissue (*e.g.*, wing) size relatively constant. Wing setae in Nasonia are small, slender hairs evenly distributed over the whole area of the forewing. Therefore, it appears that the Nasonia seta densities are comparable to those of Drosophila, where seta densities (*sensu* Dobzhansky 1929; see also Fristrom and Fristrom 1993; Zwaan *et al.* 2000) are correlated with cell size.

One mechanism of wing-size reduction in *N. vitripennis* seems to be a reduction in wing cell size because seta density in *N. vitripennis* is ~2.9 times higher than that in *N. giraulti* and wing area of *N. giraulti* is ~2.5 times larger than that of *N. vitripennis*. Therefore, our data suggest that the major QTL affecting male wing size act primarily by regulating wing cell size. Furthermore, several of our QTL appear to affect both wing size and seta density. However, we did map a QTL on LG III with large effect (LOD 17.61, 22% explained phenotypic variance) on seta density (the *vitripennis* allele has much higher seta densities than the *giraulti* allele) that did not show a corresponding QTL for wing width. Furthermore, a relatively weaker QTL on LG III for wing length and normalized wing multiple (Table 3) actually has the opposite effect expected on the basis of seta density—the *vitripennis* alleles increase wing size. Given the large effect of the seta QTL on LG III, an explanation is needed. One explanation, that seta density is simply a correlate of body size, is not supported by the correlation analysis (Table 2). A second possibility is pseudolinkage. LG III shows a general bias toward recovery of *vitripennis* alleles, and two sets of recessive hybrid lethal interactions occur on this linkage group (Gadau *et al.* 1999). Therefore, it is possible that the occurrence of an apparent seta density effect on this linkage group is actually due to accumulated effects of QTL on other linkage groups linked to interacting recessive hybrid lethal loci. This warrants further investigation and emphasizes the need for follow-up genetic analysis to confirm results of QTL studies, particularly when performed in interspecies crosses.

A third possibility is that this is a locus affecting seta density independent of wing size. Most of the QTL for

seta density are probably best interpreted as QTL influencing cell growth in wings. Therefore, the genes underlying these QTL may be of interest as regulators of cell size. Likely candidate genes are those involved in the insulin-dependent pathway (Weinkove *et al.* 1999; Coelho and Leevers 2000). Some mutants affecting cell size in this pathway have been shown to also affect wing size in Drosophila (*e.g.*, *chico* and *S6K*). However, other mutations in Drosophila alter cell size or number without changing the ultimate size of the wing (FlyBase 1999). This and other evidence (*e.g.*, surgical addition or removal of cells in imaginal disks) suggest a regulatory mechanism that adjusts cell size and cell number to keep wing size relatively constant (Coelho and Leevers 2000). The major seta density QTL on LG III could be in this cell size-cell number regulation pathway (hence explaining lack of an effect on wing size) whereas the normalized wing multiple QTL are not.

Our results demonstrate that epistasis occurs between QTL for nearly all analyzed traits (Table 4). One would expect epistasis to be quite common due to the ubiquity of gene interactions at the molecular level, *e.g.*, pleiotropic gene action, gene regulatory pathways, and signal transduction pathways. However, surprisingly few significant epistatic interactions were found in other insect QTL studies (*e.g.*, Weber *et al.* 2001; but see Long *et al.* 1995, Lark *et al.* 1995, and Yu *et al.* 1997 for a different situation in plant breeding). This lack of epistatic interactions may have two explanations: (1) If looked for at all, many QTL studies just looked for epistatic interactions between significant QTL (*e.g.*, Fry *et al.* 1995; for a review of the Drosophila literature see Mackay 1996); and (2) using diploid organisms like Drosophila, one needs greater power for detecting pairwise epistatic interactions, both genetically and statistically (Mackay 1996), than for using a haploid mapping population like hymenopteran males. Therefore, haploid and haplodiploid organisms seem to be good model systems to study epistasis. Our study shows clearly that epistatic interactions can be readily detected in haplodiploids.

What is the adaptive significance of the male wing-size differences between the Nasonia species? One (*N. vitripennis*) of the three species in the genus Nasonia has lost its ability to fly due to a significant reduction of wing size (J. Gadau, unpublished data). We assume that the evolution of smaller wings in *N. vitripennis* was an active selection process rather than a process of accumulating loss-of-function mutations in an unused structure for several reasons. First, reduction in wing size in Nasonia is male specific. Therefore, one would have to argue that mutational degeneration in wing size in *N. vitripennis* involved only wing-size genes that were expressed in a sex-specific fashion, which seems unlikely. Second, wing size in Nasonia males can affect several aspects of fitness. Wing-size-dependent vibrations and movements play a role in the male courtship behavior (van den Assem and Werren 1994; Weston *et al.* 1999). These courtship differences could act as a prezygotic isolating mechanism under field conditions in sympatric populations. Additionally, wings in *N. vitripennis* are involved in male-male aggressive displays. Furthermore, the reduction in wing size in male *N. vitripennis* is associated with a reduction in flight muscle volume [*t*-test, $P = 0.001$; mean $\pm$ SD of normalized (by interocular distance) volume of the longitudinal flight muscle of males: *N. vitripennis*, 0.011 ± 0.003, $n = 5$; *N. giraulti*, 0.025 ± 0.003, $n = 5$]. Therefore it is likely that reduced wing size in *N. vitripennis* has resulted in increased resources for other functions during pupal development. However, the fitness consequences of smaller wing sizes have not yet been determined.

We believe that smaller wings and the associated loss of flight in *N. vitripennis* have evolved as a reaction to an increase in local male-male competition in this species. Evidence suggests that *N. vitripennis* experiences more male competition than does *N. giraulti* (Drapeau and Werren 1999). There are several examples in which males of species with wing-size polymorphism that retain their flight capabilities suffer from a decrease in their success in obtaining mates if they compete with males that have lost or reduced their flight ability (Crnokrak and Roff 1995; Fairbairn and Preziosi 1996). One model of the genetic basis of adaptive traits assumes that mutations with major effects are selected early in the evolution of adaptive traits and that subsequently modifying genes are selected that ameliorate the negative side effects of these major mutations (Clarke 1997). Our epistatic QTL could be such modifying genes. For example, epistatic QTL for normalized wing multiple depend on the presence of a *giraulti* allele at both loci to show a significant phenotypic effect (Table 4). Our results are not compatible with the infinitesimal model for the genetic basis of quantitative traits (Roff 1997, p. 7) because we found QTL with large phenotypic effects for nearly all of our traits. Since we have now independently confirmed major phenotypic effects of a single region on chromosome IV with three different methods [hybrid crosses with mutant markers and introgression of major wing region (Weston *et al.* 1999) and QTL analysis (this study)], it is very unlikely that this result is an artifact of QTL analysis or small sample size. Additionally, we found multiple loci that interacted nonadditively. This also violates one of the basic assumptions in classical quantitative genetics for the estimation of the number of genes underlying a quantitative trait (Wright 1968; Roff 1997).

As mentioned, a complication of this QTL analysis is the cosegregation of recessive hybrid lethal loci in our mapping population. Approximately 50% of F_2 haploid males in our cross die before adulthood (Breeuwer and Werren 1995). Four pairwise lethal interactions have been identified and mapped that contribute to the majority of this mortality (Gadau *et al.* 1999). The majority of these are asymmetric: Only one allele combination is lethal. Presence of these recessive interactions has not prevented detection of major QTL (and con-

firmation of one by introgression) for wing size. However, the F_2 QTL analysis may (1) not detect wing QTL linked to hybrid lethal alleles (although it did detect the major QTL for wing size on LG IV, which is linked to a hybrid lethal) or (2) be subject to spurious appearance of QTL that are linked to the viable allele at a hybrid lethal locus that interacts with a lethal locus at another LG. This latter effect is referred to as pseudolinkage (Weston *et al.* 1999). As mentioned, pseudolinkage could explain the apparent strong QTL for seta density on LG III, since this region also contains two sets of pairwise hybrid lethals that interact with the regions containing other setae QTL.

We view this QTL study as a starting point for a more detailed genetic dissection of wing size in Nasonia. Taking advantage of the haploid genetics and short generation time of this organism, we are now introgressing the different wing-size loci from *N. giraulti* into *N. vitripennis*, for fine-scale mapping and a more complete genetic analysis of wing-size evolution. Our long-term goal is positional cloning of the major wing-size genes in this system to investigate how divergent selection for a naturally evolving morphological trait acts at the molecular genetic level.

The genetic basis of the difference in wing size between the two Nasonia species, *N. vitripennis* and *N. giraulti*, seems to be few genes each with large effect. The demonstration of epistatically interacting QTL for all but one of our traits demonstrated that epistasis is a significant factor in the determination of wing size in *N. vitripennis*. However, the difference in wing size between *N. vitripennis* and *N. giraulti* is not determined simply by a few genes with large effects. Instead, a more realistic model might be that the evolution of smaller wings in *N. vitripennis* involved multiple genes with large phenotypic effects (defined as explaining >10% of the phenotypic variance), some with minor effects, so that we could not even detect them, and a significant proportion of nonadditive interactions within the genome.

We acknowledge Nida Meednu for useful comments during the development of this article. Assistance was provided by Helene Chan, Celina Kennedy, Seth Bordenstein, Berend-Jan Velthuis, Patrick O'Hara, and Jon Chen. J.H.W. and R.E.P. thank the Alexander von Humboldt Foundation Senior Scientist Awards, which led to the collaborations resulting in this work. The National Science Foundation is thanked for research support to J.H.W. (DEB-9981634) and R.E.P. J.G. thanks the Humboldt Foundation for a Feodor-Lynen Stipendium. Additional financial support for this project was provided for J.G. by the Deutschen Forschungsgemeinschaft SFB 554 (TP B-1 and GA-661). R.E.P. and J.G. acknowledge the Santa Fe Institute (SFI) for providing the opportunity to discuss these issues in a working group of the SFI.

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Communicating editor: R. Harrison