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Genetic Analysis of Contributions of Dorsal Group and JAK-Stat92E Pathway Genes to Larval Hemocyte Concentration and the Egg Encapsulation Response in Drosophila

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ABSTRACT

Drosophila larvae defend themselves against parasitoid wasps by completely surrounding the egg with layers of specialized hemocytes called lamellocytes. Similar capsules of lamellocytes, called melanotic capsules, are also formed around "self" tissues in larvae carrying gain-of-function mutations in *Toll* and *hopscotch*. Constitutive differentiation of lamellocytes in larvae carrying these mutations is accompanied by high concentrations of plasmatocytes, the major hemocyte class in uninfected control larvae. The relative contributions of hemocyte concentration *vs.* lamellocyte differentiation to wasp egg encapsulation are not known. To address this question, we used *Leptopilina boulardi* to infect more than a dozen strains of host larvae harboring a wide range of hemocyte densities. We report a significant correlation between hemocyte concentration and encapsulation capacity among wild-type larvae and larvae heterozygous for mutations in the Hopscotch-Stat92E and Toll-Dorsal pathways. Larvae carrying loss-of-function mutations in Hopscotch, Stat92E, or dorsal group genes exhibit significant reduction in encapsulation capacity. Larvae carrying loss-of-function mutations in dorsal group genes (including *Toll* and *tube*) have reduced hemocyte concentrations, whereas larvae deficient in Hopscotch-Stat92E signaling do not. Surprisingly, unlike *hopscotch* mutants, *Toll* and *tube* mutants are not compromised in their ability to generate lamellocytes. Our results suggest that circulating hemocyte concentration and lamellocyte differentiation constitute two distinct physiological requirements of wasp egg encapsulation and Toll and Hopscotch proteins serve distinct roles in this process.

CELLULAR immune responses in higher eukaryotes entail specific acts of cell proliferation and differentiation. In humans, cellular immune responses include innate mechanisms such as the phagocytosis of microorganisms by macrophages, as well as mechanisms of adaptive immunity, exemplified by the clonal expansion of antigen-specific lymphocytes. While there is almost no evidence among insects of adaptive immunity, insects nonetheless possess powerful innate immune mechanisms that can serve as models for innate immunity in humans. In addition to humoral defense mechanisms involving secretion of antimicrobial peptides into the hemocoel (Tzou *et al.* 2002), insects also possess cellular defense mechanisms. These latter defense responses include phagocytosis of microbes and encapsulation of parasites (Hoffmann *et al.* 1999; Meister and Govind 2003). Encapsulation of parasites or of other foreign substances has been documented for the moth *Heliothis viriscens* (Vinson 1971), the cockroach *Periplaneta americana* (Ravindranath and Anantaraman 1977), the mosquito *Armigeres subalbatus* (Guo *et al.* 1995), and the fruit fly *Drosophila melanogaster* (Rizki and Rizki 1992). During encapsulation, specialized host blood cells (hemocytes) completely envelop invading macroscopic parasites such as parasitoid wasps and restrict the development of the parasite (reviewed in Lavine and Strand 2002). This complex response is composed of a series of discrete steps, in which molecular and/or cellular effectors must not only recognize a macroscopic parasite, but also trigger the proliferation, differentiation, and mobilization of specific hemocytes to effectively protect the host.

Drosophila is an excellent model for the genetic dissection of the encapsulation response. Different species of Drosophila are natural hosts for a number of parasitoid wasps, such as *Leptopilina boulardi* and *Asobara tabida*. Unparasitized third-instar *D. melanogaster* larvae have only two types of circulating hemocytes: plasmatocytes, which are phagocytic and comprise 90–95% of circulating hemocytes; and crystal cells, which are thought to carry the phenol oxidase proenzyme as well as substrate(s) necessary for melanin synthesis (Rizki 1957; Rizki and Rizki 1980, 1984; Rizki *et al.* 1980, 1985; Shrestha and Gateff 1982; Lanot *et al.* 2001). Hundreds of hemo-

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cytes are also present in the larval lymph gland, a hematopoietic organ that is subdivided into three or four paired lobes arranged symmetrically about the dorsal vessel. Lymph gland hemocytes are predominantly of the plasmatocyte and crystal cell lineages at various stages of maturation, with the most mature cells present in the anteriormost pair of lobes (Shrestha and Gateff 1982; Lanot *et al.* 2001).

Injection of an egg by an avirulent strain of *L. boulardi* (*G486*) into the hemocoel of a first- or second-instar *D. melanogaster* larva induces a striking series of changes in the subsequent third-instar host hematopoietic system. Soon after parasitization, circulating hemocyte concentration of the host increases (Russo *et al.* 2001). After a parasitized host molts into third instar, the lymph gland also exhibits a burst of hemocyte proliferation, an over threefold increase in the number of mature crystal cells, and the differentiation of large numbers of a third hemocyte class, the lamellocytes (Lanot *et al.* 2001; Sorrentino *et al.* 2002). The lymph gland then disperses, releasing hundreds of hemocytes into circulation. Ultimately, the wasp egg is encapsulated in one to many layers of hemocytes, predominantly lamellocytes, but also including plasmatocytes and crystal cells. The encapsulated wasp egg then melanizes, a process most likely mediated by phenol oxidase released by crystal cells (Rizki and Rizki 1984, 1990; Russo *et al.* 1996).

Even though clear changes in hemocyte behavior after wasp infection, both in the lymph gland and in circulation, have been clearly documented, many aspects of the egg encapsulation process are largely unknown. One of the more important and least understood aspects of wasp egg encapsulation is the mechanism that governs egg recognition after its entry into the larval hemocoel. The mechanism by which immune effector cells subsequently coordinate to construct a cellular capsule is also not understood. Bodian *et al.* (2003) have proposed the existence of signaling mechanisms that involve lymph gland precursors and that lead to the proliferation and differentiation of immune effectors. Some insights into the regulation of encapsulation have come from the analysis of Drosophila mutants that either are deficient in the encapsulation response or exhibit constitutive encapsulation of self tissue in the absence of parasitization. For instance, loss-of-function alleles of *Rst(2)Lb* and *Rst(2)At* are associated with suppression of encapsulation capacity in response to parasitization by the wasps *L. boulardi* and *A. tabida*, respectively. It has been proposed that the gene products of these loci are required for wasp egg recognition (Benassi *et al.* 1998; Hita *et al.* 1999).

Constitutive melanized encapsulation of self tissue (also referred to as "melanotic tumors") is observed in the absence of wasp parasitization in Drosophila larvae carrying mutations (*e.g.*, $Toll^{10b}$, $cactus^{E8}/cactus^{D13}$, and $hopscotch^{Tumorous\text{-}lethal}$) that cause the overproliferation of hematopoietic precursors and their differentiation into lamellocytes, suggesting that sufficient hemocyte concentration, lamellocyte differentiation, or both can trigger successful encapsulation (reviewed in Meister and Govind 2003). Toll, a transmembranal receptor and a member of the Toll-like receptor (TLR)/IL-1R family (Rock *et al.* 1998), was originally identified as one of the maternal-effect "dorsal group" genes required for the determination of dorsal-ventral (DV) polarity in the Drosophila embryo. Activation of embryonic Toll requires activation of its ligand, Spätzle. The activation of Spätzle is dependent on the functions of the extracellular serine proteases Snake and Easter. Activated Toll interacts with a cytoplasmic protein complex that includes Tube and Pelle, resulting in the degradation of Cactus. Cactus is an IκB-family protein and sequesters the Rel-family transcription Dorsal in the cytoplasm in the absence of the Toll signal. The Toll signal therefore promotes the nuclear localization of Dorsal. In the larva, Toll, Tube, Pelle, Cactus, and Dorsal, as well as Dif (another Rel-family transcription factor regulated by Cactus) are expressed in larval lymph gland hemocytes. Loss of function of Toll, Tube, or Pelle proteins results in significant reductions in circulating hemocyte concentration. Importantly, dominant, gain-of-function *Tl* alleles (*e.g.*, Tl^{10b}) and strong loss-of-function or null combinations of *cact*, both of which cause a constitutive upregulation of nuclear localization of maternally deposited Dorsal protein in the embryo, also cause an increase in hemocyte concentration in the larva. This overabundance phenotype is linked to increased mitotic activity of hemocytes (Qiu *et al.* 1998).

Like the Toll-Dorsal segment of the DV pathway, the Hopscotch-Stat92E (Hop-Stat) pathway controls many biological processes in Drosophila, including hematopoiesis (Hou *et al.* 2002). Hopscotch is a member of the JAK family of nonreceptor tyrosine kinases; some JAK proteins have roles in mammalian hematopoiesis (Ward *et al.* 2000). In Drosophila, activation of the receptor with which Hop is associated is believed to result in activation of apposed Hop molecules and subsequent phosphorylation and nuclear translocation of the transcription factor Stat92E. Dimerized Stat92E translocates to the nucleus, binds to target promoters, and regulates the activity of target genes (Hou *et al.* 2002). The Hop-Stat signal is constitutively upregulated by the product of the dominant gain-of-function allele $hop^{Tum\text{-}l}$. Consistently, loss of one functional copy of *stat92E* significantly reduces the penetrance of the $hop^{Tum\text{-}l}$-induced melanotic tumor phenotype in adult flies (Luo *et al.* 1997), while a loss of function of a Stat92E inhibitor protein, Dpias, increases the frequency of $hop^{Tum\text{-}l}$-induced tumors (Betz *et al.* 2001). These findings suggest that the Hop-Stat92E pathway is involved in lamellocyte differentiation. Not surprisingly, $hop^{Tum\text{-}l}$ hosts are highly immune competent and exhibit a higher likelihood of wasp egg encapsulation [against *Pseudeucoila bochei* (Nappi and Streams 1969) and against *L. boulardi G486* (Sorrentino *et al.* 2002)] as compared to their control siblings.

The availability of Toll-Dorsal and Hop-Stat pathway mutants provides an opportunity to test whether hemo-

TABLE 1

Loci and alleles tested

Locus	Allelic combination(s)	lof/ GOF?	Presumed effect on signal
	Hopscotch-Stat92E pathway		
oustretched (*os*)	os^{o}/Y	lof	↓
hopscotch (*hop*)	hop^{msv1}/Y	lof	↓
	hop^{M4}/Y	lof	↓
	$hop^{Tum\text{-}l}/Y$	GOF	↑
stat92E	$stat92E^{HJ}$	lof	↓
	DV pathway		
snake (*snk*)	snk^{233}/snk^{073}	lof	↓
easter (*ea*)	ea^{1}/ea^{3}	lof	↓
	ea^{1}/ea^{1}	lof	↓
spätzle (*spz*)	spz^{rm7}/spz^{rm7}	lof	↓
Toll (*Tl*)	Tl^{r632}/Df	lof	↓
	Tl^{r632}/Tl^{r444}	lof	↓
	Tl^{r444}/Df	lof	↓
	$Tl^{1\text{-}RXA}/Tl^{r444}$	lof	↓
	$Tl^{10b}/+$	GOF	↑
tube (*tub*)	tub^{238}/tub^{238}	lof	↓
pelle (*pll*)	pll^{385}/pll^{078}	lof	↓
cactus (*cact*)	$cact^{E8}/cact^{D13}$	lof	↑
dorsal (*dl*)	dl^{1}/Df	lof	↓

Loci at which specific loss-of-function (lof) and gain-of-function (GOF) allelic combinations were tested are shown. Presumed effect on signal is: ↓, downregulation; ↑, upregulation.

cyte concentration (largely plasmatocytes in circulation) affects encapsulation efficiency. An effect of hemocyte density on lamellocyte differentiation induced by wasp parasitization should be independent of the presence of preexisting circulating lamellocytes, as lamellocytes are absent in the hemocoel of uninfected wild-type larvae (Lanot *et al.* 2001). Previous experiments involving parasitization of six different wild-type species of Drosophila (including *D. melanogaster*) by *A. tabida* demonstrated a positive correlation across host species between mean hemocyte density and encapsulation capacity (Eslin and Prévost 1998). However, the heterogeneity of hemocyte types in these species is not characterized and it is not known if this correlation can be extended to other Drosophila parasites. Here, we subjected more than a dozen strains of *D. melanogaster* larvae (Table 1) spanning a 10-fold range of hemocyte concentrations to parasitization by *L. boulardi* to determine if hemocyte concentration correlates with the efficiency of encapsulation. Further, experiments on the differentiation of lymph gland lamellocytes in loss-of-function mutants allow us to dissect the relative roles of the Toll and Hop-Stat pathways in the egg encapsulation process.

MATERIALS AND METHODS

Insect stocks: Drosophila stocks were as follows: wild type, Canton-S; Hop-Stat pathway, f^{1} B^{1} os^{o} car^{1}/*Binsinscy* (Umeå Stock Center), *y* hop^{msv1}/*Basc*, *y* hop^{M4}/*Basc*, *ry* $stat92E^{HJ}$ *e*/*ry* $stat92E^{HJ}$ *e* (*hop* and *stat92E* stocks were provided by C. R. Dearolf and H. Luo); dorsal group, *ru st* snk^{233} *e ca*/*TM6C Sb Tb*, *st* snk^{073} *e*/*TM6C Sb Tb* (both *snk* stocks were from Nüsslein-Volhard lab, Tübingen), ru^{1} h^{1} th^{1} st^{1} cu^{1} ea^{1}/*TM6C Sb Tb st* ea^{3} *e*/*TM6C Sb Tb* (Bloomington Stock Center), *ru th st ri roe* p^{p} *e* spz^{rm7}/*TM6C Sb Tb* (D. Morisato), *ca* Tl^{r632}/*TM6C Sb Tb*, Tl^{r444} *st e*/*TM6B Tb*, *ru h st e* $Tl^{1\text{-}RXA}$/*TM6C Sb Tb*, tub^{238} *st*/*TM6B Tb*, pll^{385} *ca*/*TM6B Tb*, and ndl^{046} pip^{386} tub^{238} pll^{078} *ru th st ri e ca*/*TM6B Tb* (referred to as *nptp*). All *Tl*, *tub*, and *pll* stocks were provided by K. V. Anderson. *y w*; $cact^{E8}$/*CyO* y^{+}, *y w*; $cact^{D13}$/*CyO* y^{+}; *y w*; dl^{1} cn^{1} sca^{1}/*CyO* y^{+}, and *y w Df(2L)TW119 cn*/*CyO* y^{+} are as described in Qiu *et al.* (1998). When necessary, backgrounds of the above stocks were changed to facilitate genotyping of larvae (Sorrentino 2003). The recombinant msn^{03349} *ca* Tl^{r632} chromosome was generated from msn^{03349}/*TM6B Tb* and *ca* Tl^{r632}/*TM6C Sb Tb* stocks by standard crossing techniques. msn^{03349} contains a *PlacZ* insert. While β-galactosidase expression is not limited to hematopoietic tissue, among hemocytes, only lamellocytes express β-galactosidase. Flies carrying a recombinant chromosome were identified by *claret* eyes (*TM6C* carries *ca*) and the ability to produce larvae exhibiting β-galactosidase activity. Female sterility and production of dorsalized embryos were confirmed by examining eggs laid by msn^{03349} Tl^{r632}/*Df(3R)*ro^{80b} females generated in a cross. For simplicity's sake we refer to heterozygous and +/*Y* sibling larvae and $stat92E^{HJ}$/+ half-sibling larvae as "control" larvae. *L. boulardi* provided by P. Chabora (Queens College, City University of New York) were designated as strain "*Q*." Y. Carton provided a different, avirulent strain, *L. boulardi G486*.

Egglays and wasp parasitizations: Drosophila egglays took place at 25° in vials containing standard yeast/cornmeal/agar fly food that had been sprinkled with dry yeast. Egglay duration was as follows: stock egglays were allowed to take place for 2–8 hr, depending on the fecundity of females; egglays of crosses between two stocks, because they generally involved fewer females than stock egglays did, were allowed to take place for 24 hr. Larvae were exposed to females of *L. boulardi* beginning 48 hr after the initiation of the egglays. Exposure period was 24 hr.

Determination of mean circulating hemocyte concentration and lamellocyte percentage: Individual larvae were washed twice in phosphate-buffered saline (PBS) and once in 95% ethanol; larvae were then transferred to glass slides wiped with 95% ethanol. Larvae were opened using fine forceps (Style 5; T-4662; Sigma, St. Louis). Hemolymph that accumulated around the larval carcass was taken up using a 10.0-μl-capacity polypropylene micropipette tip attached to a 10.0-μl-capacity micropipettor. All hemocyte counts were performed as described in Qiu *et al.* (1998), with modifications: for each sample, a 2-μl drop of halocarbon oil (no. 27; Halocarbon Products) was placed onto a hemocytometer grid and the hemolymph sample was injected onto the hemocytometer under the oil. The number of hemocytes within a hemolymph volume of 2.4×10^{-2} mm^{3} was determined and multiplied by 41.67, yielding hemocytes per microliter. Lamellocyte percentage was assessed relative to the total number of hemocytes.

Lymph gland dispersal and lamellocyte differentiation: To assess lamellocyte differentiation, lymph glands from control and mutant larvae carrying the msn^{03349}/+ marker, after being fixed were then incubated for 18 hr at room temperature in a standard X-gal staining solution. Because β-galactosidase expression due to the msn^{03349} allele is also detectable in larval brain tissue, brains from third-instar msn^{03349}/+ and Canton-S larvae served as positive and negative controls, respectively, for the staining procedure. β-Galactosidase expression in the larval brain also allowed us to identify msn^{03349}-carrying recombinant chromosomes. Determination of dispersal was performed as described (Sorrentino *et al.* 2002).

Wasp egg encapsulation assay controls and protocol: Previous work by others has shown that the presence of the dominant *Rst(2)Lb*$^{+}$ allele (which endows larvae with resistance to *L. boulardi*; Carton and Nappi 1997) varies among tested stocks (Carton and Boulétreau 1985; Hita *et al.* 1999). While we did not assess the status of the *Rst(2)Lb* gene in the individual stocks used in our study, we did, as with comparisons of circulating hemocyte concentration (CHC), assay the effect of a mutation on wasp encapsulation capacity by making use of sibling control hosts. Additional controls included the use of multiple and/or heteroallelic combinations (as we did with *snk*, *ea*, *pll*, *Tl*, *cact*, and *dl* stocks) and testing for similar effects on encapsulation by multiple allelic backgrounds (as we did with *hop*, *ea*, and *Tl* stocks). The encapsulation assay was performed exactly as described (Sorrentino *et al.* 2002). In the case of the *cact* assay only, we used a virulent *L. boulardi* wasp strain (*Q*) obtained from Peter Chabora. Finally, as parasitization by *L. boulardi G486* did not evoke an encapsulation response in the balancer class of larval offspring of the cross *y w/y w; b Df(2L)TW119 cn/CyO y*$^{+}$ × *y w/Y; dl*^{1} *cn*^{1} *sca*^{1}*/CyO y*$^{+}$, we could not assess the effect of loss of function of *dl* on encapsulation capacity.

Statistical analysis: The test for normal distribution of hemocyte counts is as follows. Individual CHC and ln CHC values (x_i) were standardized with respect to the observed mean value [(x_i − sample mean)/(sample standard deviation)]. We defined value intervals ("bins") for CHC as having a width of 2000 cells/μl and for ln CHC as having a width of 0.3000, with the mean value located in the center of its bin. Additional bins extended away from the bin containing the mean value in decrements/increments of 2000 cells/μl (CHC) or 0.3000 (ln CHC). A total of nine bins were defined. We compared the frequency distribution of observed values to the expected frequency of values assuming a normal distribution about the observed mean. Frequency distributions of values were considered consistent with normality if the χ^2 value for the comparison of observed and expected frequency distributions was less than the critical value at $9 - 1 = 8$ d.f. Means of ln CHC values were compared using Student's *t*-test: $t = (x_1 - x_2)/(s_1^2/n_1 + s_2^2/n_2)^{1/2}$. Degrees of freedom were $\nu = \{(s_1^2/n_1 + s_2^2/n_2)^2/[((s_1^2/n_1)^2/(n_1+1)) + ((s_2^2/n_2)^2/(n_2+1))]\} - 2$. n_i is the sample size, x_i is the sample mean, and s_i is the sample standard deviation. Wasp egg encapsulation capacities of control and mutant classes were compared by determining a binomial distribution function defined by the probability (p) of encapsulation of the control class (the decimal version of the encapsulation capacity). The area under the curve from 0 to $f(x; n, p) = \{(n!)/[(x!)(n-x)!]\}(p^x)(1-p)^{n-x}$, in which n is the mutant sample size and x is the number of mutant larvae that are positive for encapsulation, represents the cumulative probability of obtaining any mutant encapsulation capacity value from 0 to the observed value and was calculated by Microsoft Excel 98. If this cumulative value was ≤0.05, the mutant value was considered significantly less than the control value. Since this is a one-tailed test, it could test only for significant reductions. Thus, in the cases of *os* and *cact*, values for mutant encapsulation capacities were used to define p, and control values for x and n were used. Lymph gland lamellocyte differentiation and lymph gland dispersal frequencies were similarly compared, using binomial distributions defined by the class (either control or mutant) that had the higher value. Correlation analysis of ln CHC and encapsulation capacity was performed by determining the best-fit linear equation, $y = bx + a$, as described in Crow *et al.* (1960): $b = (n\Sigma xy - \Sigma x\Sigma y)/(n\Sigma x^2 - (\Sigma x)^2)$; $a = (\Sigma y - b\Sigma x)/n$, $r = [n\Sigma xy - (\Sigma x)(\Sigma y)]^2/\{[n\Sigma x^2 - (\Sigma x)^2][n\Sigma y^2 - (\Sigma y)^2]\}$, in which n is the number of data points and x and y are the values for mean ln CHC and encapsulation capacities, respectively. We did not assume that either parameter is necessarily dependent on the other. Correlations were considered significant if the correlation coefficient r was greater than or equal to the critical value for $P(|r|)$ with two variables and $n - 2$ d.f. (Crow *et al.* 1960, Appendix, Table 7).

RESULTS AND DISCUSSION

Distribution of CHC values is log-normal: CHC, like any quantitative trait, is highly variable (*e.g.*, control mean raw CHCs exhibit a nearly sevenfold range of values; see Table 2). When making comparisons among experimental classes, it is important that observed data satisfy the implicit assumptions of a given statistical model. Comparison of mean raw CHC values for controls and mutants (discussed in the next section) by Student's *t*-test rests upon the assumption that both control and mutant values are drawn from normally distributed populations of values.

To test the validity of this assumption of normality, we first plotted the frequency distribution of CHC values of wild-type Canton-S larvae ($n = 24$). We observed that the distribution of Canton-S CHC values (Figure 1, hatched bars) apparently does not conform to normality: The distribution is skewed to the right, the mean value (4379 cells/μl) does not fall into the modal class, and the distribution fails the χ^2 test for normality ($\chi^2 = 27.8283$; 8 d.f.; critical value at $P = 0.05$ is 15.51; see test for normality in materials and methods).

To reduce the likelihood that our observations of Canton-S CHC values were due to chance, we plotted the frequency distribution of CHC values of 110 control larvae from five genotypic classes that had been subjected to the same treatment with respect to egglay period and examination time (see Figure 1 legend). In this analysis, we found that the frequency distribution of these "pooled control" CHC values is also not consistent with a normal distribution (again, the distribution is skewed to the right; Figure 1). However, the frequency distribution of pooled control CHC values does conform to a log-normal distribution, in which it is the natural logarithms of raw CHC values (ln CHC) that are normally distributed (Figure 2; mean ± standard deviation, 8.5910 ± 0.5058; $\chi^2 = 1.3970$; 8 d.f.).

While biological parameters can conform to normal distributions, log-normal distributions are often better predictors of frequency distributions of some biological parameters than are normal distributions, particularly when the mean value of an index is low with respect to the high limit of the range of possible values, when variability is high, and when zero is the lowest possible index value (Limpert *et al.* 2001). Examples of this include the distribution of sizes of senile plaques in Alzheimer's disease patients (Hyman *et al.* 1995) and the definition of groups of women at low and high risks of breast cancer based on polygenic inheritance (Pharoah *et al.* 2002). Our observations, made on a large sample of control animals, suggest that raw CHC

TABLE 2

Raw CHC values (mean ± standard deviation)

Control genotypes	n	Raw CHC (cells/μl)	Mutant genotypes	n	Raw CHC (cells/μl)
Canton-S	24	4,379 ± 3,224			
+/*Y*	30	7,368 ± 6,212	os^{o}/Y	30	5,365 ± 4,224
+/*Y*	14	1,917 ± 1,531	hop^{msv1}/Y	16	4,532 ± 4,790
+/*Y*	12	3,118 ± 3,007	hop^{M4}/Y	11	12,769 ± 7,273
$stat92E^{HJ}/+$	20	12,193 ± 6,735	$stat92E^{HJ}/stat92E^{HJ}$	21	12,957 ± 8,447
$snk^{223}/+$, $snk^{073}/+$	30	4,302 ± 2,466	snk^{223}/snk^{073}	30	5,358 ± 2,844
$ea^{1}/+$, $ea^{3}/+$	28	6,267 ± 3,823	ea^{1}/ea^{3}	24	5,209 ± 4,456
$ea^{1}/+$	30	5,735 ± 2,856	ea^{1}/ea^{1}	30	2,995 ± 2,101
$spz^{rm7}/+$	12	9,414 ± 4,012	spz^{rm7}/spz^{rm7}	12	3,632 ± 2,125
$Tl^{5BRE}/+$, $Df/+$	12	3,771 ± 892	Tl^{5BRE}/Df	18	1,222 ± 683
$tub^{238}/+$, $nptp/+$	11	6,381 ± 3,182	$tub^{238}/nptp$	11	1,375 ± 970
$pll^{rm8}/+$, $nptp/+$	11	6,051 ± 3,011	$pll^{rm8}/nptp$	10	2,506 ± 1,137
$cact^{E8}/+$, $cact^{D13}/+$	11	3,898 ± 1,527	$cact^{E8}/cact^{D13}$	10	41,556 ± 8,412
$dl^{1}/+$, $Df/+$	30	4,827 ± 3,650	dl^{1}/Df	20	9,849 ± 6,855

Mean raw CHC values ± standard deviation (cells/μl) for control (left column) and mutant (right column) larvae are shown. n, sample size. Mean ln CHC values are presented in Figure 3.

values within the log-normal distribution are physiologically acceptable. Furthermore, to assess the effect of a recessive mutation on CHC values, comparison with external controls is of limited use and it is more appropriate to utilize values from control sibling larvae.

Identification of mutations that alter mean CHC: To identify mutations that significantly (Student's t-test; see MATERIALS AND METHODS) alter mean CHC, we assayed larvae carrying mutations in the Hopscotch-Stat92E pathway (*i.e.*, *os*, *hop*, and *stat92E*; Os is a putative ligand for the Hop-associated receptor; HARRISON *et al.* 1998) or dorsal group genes (*i.e.*, *snk*, *ea*, *spz*, and *dl*). Because the dominant gain-of-function mutation $hop^{Tum\text{-}l}$ produces a constitutively active protein and causes a dramatic increase in mean ln CHC (SILVERS and HANRATTY 1984; LUO *et al.* 1995), we anticipated that loss-of-function mutations that downregulate the Hopscotch-Stat92E signal would have an opposite effect on ln CHC. However, this was not the case. While os^{o}/Y and $stat92E^{HJ}/stat92E^{HJ}$ mutants have mean ln CHC values statistically indistinguishable from those of their respective control siblings, the mean ln CHC values for two hypomorphic *hop* mutants, hop^{msv1}/Y and hop^{M4}/Y (7.9695 ± 0.9463, n = 16, and 9.2701 ± 0.6685, n = 11, respectively; Figure 3), are significantly higher than those for their control siblings (7.3301 ± 0.6687, n = 14, and 7.7654 ± 0.7365, n = 12,

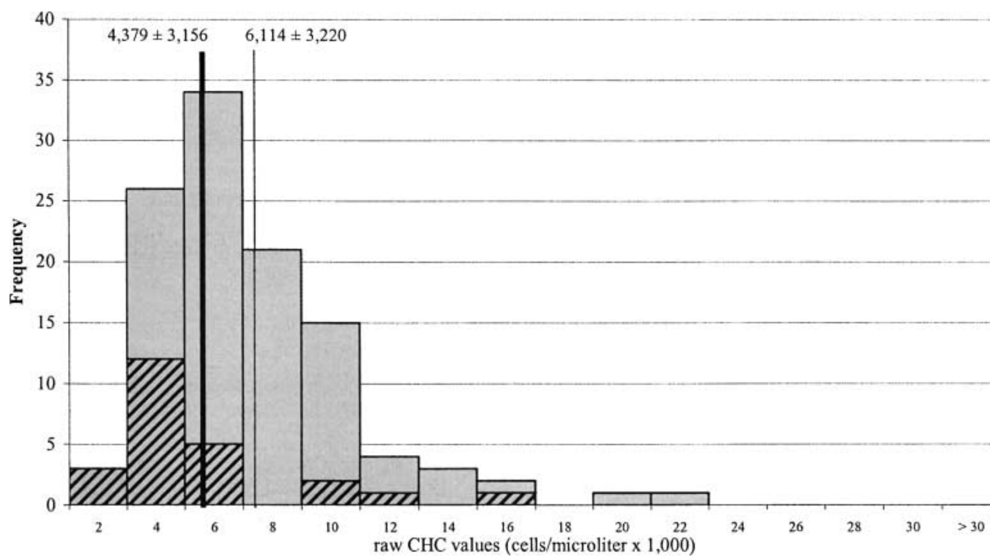

FIGURE 1.—Frequency distributions of raw CHC values for wild-type Canton-S larvae (n = 24) and pooled control larvae (n = 110). Canton-S larvae (hatched bars) were examined on day 5 after 2-hr egglays. For the pooled control larvae (shaded bars), we pooled raw CHC values from 110 control larvae that had been subjected to the same treatment (24-hr egglays; examination on day 6): $os^{o}/Binsinscy$ × Canton-S, $stat92E^{HJ}/stat92E^{HJ}$ × *TM3/TM6B*, $snk^{233}/TM6C\ Sb\ Tb$ × $snk^{073}/TM6C\ Sb\ Tb$, $ea^{1}/TM6B\ Tb$, and $ea^{1}/TM6B\ Tb$ × $ea^{3}/TM6B\ Tb$ crosses (see Table 2). The number of raw CHC values falling into the leftmost bin (*i.e.*, CHC ≤2000 cells/μl) for both Canton-S and pooled controls is the same, at n = 3. Intersection of vertical lines with the x-axis represents the mean CHC value for Canton-S (thick line; 4379 ± 3156 cells/μl) and pooled control (thin line; 6114 ± 3220 cells/μl) larvae, which, inconsistent with normal distributions, are both not in the modal value intervals (bins). x-axis, bins for CHC values; numbers represent the highest CHC value in the bin immediately above it.

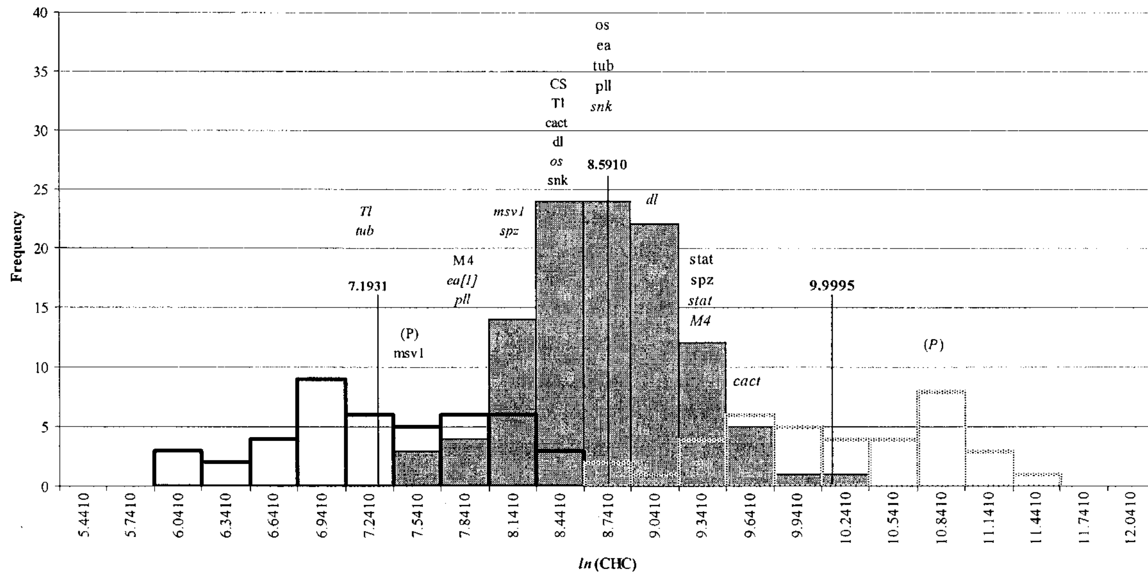

Figure 2.—Natural logarithms of CHC values among pooled control (n = 110), reduced-CHC (n = 44), and tumorous larvae (n = 38). Unless otherwise specified below, ln CHC values were generated in this report. x-axis, bins for ln CHC values; each bin traverses an interval of 0.3000. Each number on the x-axis represents the highest value in the bin immediately above it. Shaded bars, pooled control values; open bars with solid outlines, low-CHC mutants; CHC values were originally reported in Qiu *et al.* (1998) for Tl^{5BRE}/Df, tub^{238}/tub^{238}, pll^{rm8}/pll^{035}, and $tub^{238}\ pll^{rm8}/tub^{238}\ pll^{035}$ mutants. Open bars with lightly shaded outlines, tumorous mutants; ln CHC values for $hop^{Tum-l}/+$ (Sorrentino 2003), $Tl^{10b}/+$ (Sorrentino 2003), and $cact^{E8}/cact^{D13}$ (Qiu *et al.* 1998) larvae are shown. Intersections of vertical lines with the x-axis indicate the mean ln CHC values (actual values are above each line) for the pooled controls, low-CHC mutants, and tumorous mutants of the location. Gene symbols above specific bars indicate bins into which mean ln CHC values (calculated by taking the mean of the natural logarithm of individual CHC values) for control (roman typeface) and mutant (italic) larval classes would be placed. Raw CHC values are listed in Table 2. (P), control larvae for *daughterless-GAL4 UAS-Pvf2* and *e33C-GAL4 UAS-Pvf2* larvae, 1500 ± 500 cells/μl (Munier *et al.* 2002); (*P*), *daughterless-GAL4 UAS-Pvf2* and *e33C-GAL4 UAS-Pvf2* larvae, 40,000 ± 10,000 cells/μl (Munier *et al.* 2002). Because individual CHC values were not available to us, ln CHC values for (P) and (*P*) were calculated by the less precise method of calculating the natural logarithms of the mean CHC values reported in Munier *et al.* (2002).

respectively). Yet these increased values do not fall outside of the control distribution of ln CHC values (Figure 2).

Among tested dorsal group mutants, mean ln CHC is significantly reduced in *Tl*, *tub*, and *pll* mutant larvae, while it is significantly greater in *cact* larvae (Qiu *et al.* 1998; Figure 3). As for the remaining dorsal group backgrounds, we observed that there is no significant difference between the mean ln CHC of snk^{233}/snk^{073} mutant larvae and that of their heterozygous siblings, while there is a reduction of mean CHC in *ea* and *spz* mutants (Figure 3). While the weak heteroallelic ea^{1}/ea^{3} combination has no effect on mean ln CHC (8.1785 ± 0.9582; n = 24) when compared to the sibling control value (8.5876 ± 0.5642; n = 28), ln CHC of larvae carrying the null ea^{1}/ea^{1} genotype (7.7147 ± 0.8723; n = 30) is significantly less than that of heterozygous siblings (8.5463 ± 0.4778; n = 30). Furthermore, the mean ln CHC of spz^{rm7}/spz^{rm7} larvae (7.9535 ± 0.8409; n = 12) is also less than that of $spz^{rm7}/+$ siblings (9.0557 ± 0.4701; n = 12). Finally, larvae carrying a null allelic combination of *dl*, dl^{1}/Df, exhibited a significant increase in mean ln CHC (8.9364 ± 0.7723; n = 20; Figure 3) with respect to control sibling larvae (8.1770 ± 0.8553; n = 30). However, the ln CHC values for *ea*, *spz*, and *dl* mutants are well within the control range of values defined in Figure 2.

Distribution of CHC values of mutants: Next, we analyzed the distributions of ln CHC values of mutants with reduced or elevated hemocyte concentrations (relative to their sibling controls; Student's t-test). The goal of this analysis was to examine if there is any overlap between control and "mutant" distributions and to determine if any mutant values fall outside of the control lognormal distribution depicted in Figure 2. The frequency distribution of ln CHC values obtained from 44 larvae of loss-of-function *Tl*, *tub*, *pll*, and *tub pll* backgrounds (Qiu *et al.* 1998) shows that this distribution is consistent with a normal distribution (χ^2 = 8.0937; 8 d.f.). Furthermore, not only is the mean value of 7.1931 ± 0.6674 significantly lower than the mean value for pooled controls (Student's t-test; $P < 0.001$), but also it lies completely outside of the control distribution (Figure 2). Finally, while there is considerable overlap between this distribution and the control distribution, 24/44 of the reported (Qiu *et al.* 1998) values lie outside of the control range.

CHC values of previously reported tumorous mutants ($cact^{E8}/cact^{D13}$, Qiu *et al.* 1998; $Toll^{10b}/+$ and $hop^{Tum-l}/+$,

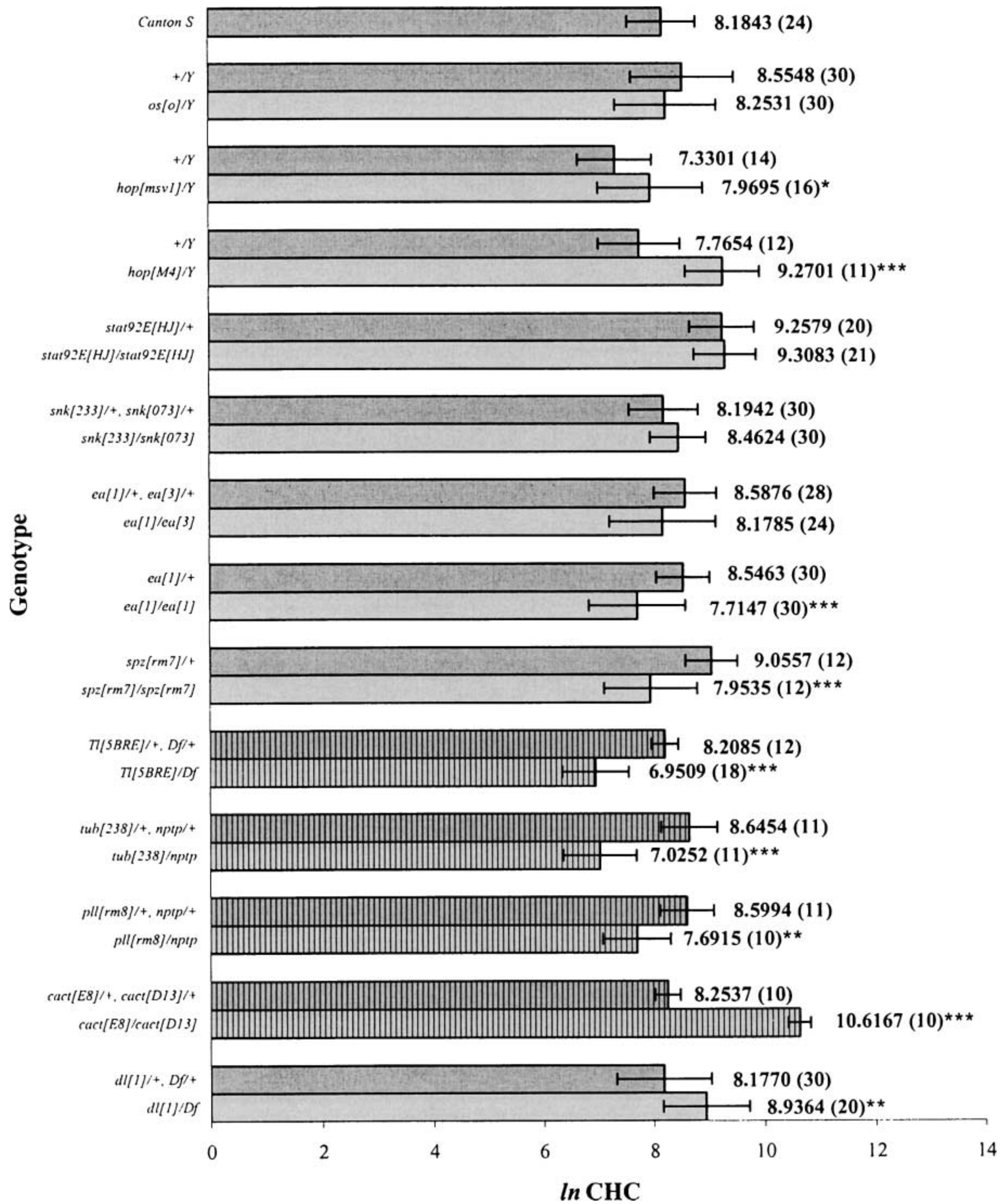

Figure 3.—Means of natural logarithms of CHC values of Hopscotch-Stat92E pathway and dorsal group mutants. In each pair of bars, the darker top bar represents the mean ln CHC value for control larvae, and the lighter bottom bar, that for mutant larvae. Numerical values of mean ln CHC are presented to the left of each bar. Numbers in parentheses indicate the number of larvae examined. Values obtained in this study are represented by shaded bars; values for *Tl*, *tub*, *pll*, and *cact* stocks were originally reported by Qiu *et al.* (1998) and are represented by striped bars. Error bars indicate one standard deviation. Significant differences between control and mutant means of values of ln CHC are indicated: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Sorrentino 2003; $n = 38$) are significantly higher (Student's *t*-test) than those of their respective controls, but display a tremendous spread of CHC values among a relatively small sample size. Nonetheless, the frequency distribution of ln CHC values for this group is consistent with normality ($\chi^2 = 6.6501$; 8 d.f.). The mean value (9.9995 ± 0.7011) for this distribution is significantly greater (Student's *t*-test; $P < 0.001$) than that for the pooled controls (Figure 2) and falls into the rightmost bin of the control distribution. As a result, a consider-

able number (16/38) of the tumorous values lie outside of the control distribution. Thus, assuming a log-normal distribution of CHC values is valid even if CHC values have been significantly altered by genetic mutation. However, we cannot rule out the possibility that the two mutant distributions could be explained in terms of other distribution patterns, were additional CHC values to be obtained in the future.

Together, these results suggest that it is possible to define distinct ranges of CHC values that are "low," control, and "high." We can define low ln CHC values as those that exist outside and to the left of the control distribution (CHC $\leq$ 1395; ln CHC $\leq$ 7.2410). Similarly, high CHC values are those that are outside and to the right of the control distribution (CHC $>$ 28,029; ln CHC $>$ 10.2410). Thus, the mean ln CHC values for *Tl* (6.9509) and *tub* (7.0252; Qiu *et al.* 1998) satisfy our definition for low. However, the mean ln CHC values for *pll* (7.6915) and *tub pll* (7.4360; Qiu *et al.* 1998) mutants fall within the lower end of the control distribution. As for tumorous larvae, the ln CHC value for $cact^{E8}/cact^{D13}$ mutants (10.6167; Qiu *et al.* 1998) can be considered high. ln CHC values for nontumorous larvae that still exhibit elevated hemocyte densities, such as transgenic *daughterless-GAL4 UAS-Pvf2* and *e33C-GAL4 UAS-Pvf2* larvae (mean ln CHC = 10.5966; Figure 2), can also be classified as high. However, the ln CHC value for $hop^{Tum\text{-}l}/+$ larvae (9.7928; Sorrentino 2003) cannot. The remainder of the mean ln CHC values listed in Figure 3 (which includes those for all control sibling larvae) lies within the control range of ln CHC values.

The control distribution suggests that wild-type/control larvae can tolerate a fairly wide range of CHC values. Whether statistically significant differences in ln CHC induced by mutations that still fall within this control distribution have a bearing on the wasp egg encapsulation response is considered next.

Wasp encapsulation capacity: To test the effects of loss-of-function mutations on encapsulation capacity, we performed wasp encapsulation assays using avirulent *G486* wasps on mutant larvae of the same genetic backgrounds tested in the mean CHC assay.

First, loss of function of *hop* or *stat92E* results in significant reduction in encapsulation capacity. hop^{msv1}/Y larvae exhibit an encapsulation rate of 15.60% (n = 141), a significant ($P < 0.05$; one-tailed comparison of binomial distribution; see materials and methods) reduction in likelihood by a factor of one-third, as compared with the sibling control value of 23.70% (n = 907). hop^{M4} has a stronger effect than hop^{msv1}: hop^{M4}/Y mutants exhibit an encapsulation response of 7.11% (n = 479), less than one-fourth ($P < 0.001$) the control value of 29.26% (n = 1480; Figure 4).

Homozygous loss-of-function mutation in *stat92E* also suppresses encapsulation: The encapsulation rate of $stat92E^{HJ}/stat92E^{HJ}$ larvae is 26.55% (n = 437), slightly over half the heterozygote control value of 48.99% (n = 149). Thus, whereas only Hop is implicated in regulating mean CHC, both Hop and Stat92E are implicated in the encapsulation response. Importantly, suppression of encapsulation occurred in backgrounds in which CHC was either unaltered ($stat92E^{HJ}/stat92E^{HJ}$) or significantly greater (hop^{-}). Finally, we observed that os^{o}/Y mutants exhibit no suppression of encapsulation capacity when compared to sibling controls (Figure 4). Such observations suggest that the Os protein is likely not involved in the encapsulation response. Other Outstretched/Unpaired-like cytokines have been identified in the genome (Agaisse *et al.* 2003; Bodian *et al.* 2003), and it is possible that one or more of these proteins have a role in activation of the JAK-STAT pathway in hematopoiesis.

We then examined the wasp encapsulation capacities of dorsal group mutant larvae (Figure 4). Just as it has no effect on mean CHC, the snk^{233}/snk^{073} combination has no significant effect on encapsulation capacity, when compared to the value for heterozygous siblings. Strikingly, encapsulation capacity is significantly reduced in larvae mutant for the subsequent contiguous series of genes in the dorsal group. Encapsulation capacity is significantly reduced by ea^{1}/ea^{3} (0.76 $\times$ control values), ea^{1}/ea^{1} (0.44 $\times$ control value), and spz^{rm7}/spz^{rm7} (0.60 $\times$ control value). We had previously observed that larvae carrying a *trans*-heterozygous null allelic combination of *Tl* (Tl^{9QRE}/Tl^{5BREQ}) exhibited successful encapsulation of *G486* (49.44%, n = 267), but reciprocal lethal markers on the balancer chromosomes of parental stocks eliminated the control siblings as embryos. However, larvae carrying any of four loss-of-function combinations of *Tl* (*e.g.*, Tl^{r632}/Df, 0.31 $\times$ control) exhibited significant reductions of encapsulation capacity with respect to their sibling controls. Consistently, encapsulation capacity is also strongly reduced by tub^{238}/tub^{238} (0.21 $\times$ control value) and pll^{385}/pll^{078} (0.06 $\times$ control value). We were unable to assess the effect of dl^{1}/Df on encapsulation capacity, as we observed a 0% encapsulation response for both the control and mutant classes. It is not clear why animals of the control class [dl^{1}/CyO or *Df(2L)TW119/CyO*] did not exhibit any encapsulation. The effect of the *dl* mutation on CHC is also puzzling and it is possible that these anomalous effects are due to other, unknown genetic factors in this background.

As expected, loss-of-function *cact* has an opposite effect on encapsulation; the $cact^{E8}/cact^{D13}$ combination significantly increases the likelihood of encapsulation almost ninefold (8.84 $\times$ control value). Thus, among tested stocks, wherever loss-of-function mutations produce a significant decrease in mean CHC (*ea*, *spz*, *Tl*, *tub*, *pll*) they also produce a significant reduction in encapsulation, and vice versa (*cact*).

Examination of encapsulation capacity values reveals two important trends. First, we were somewhat surprised to observe such considerable variability in encapsulation capacity among wild-type and control larvae: Among

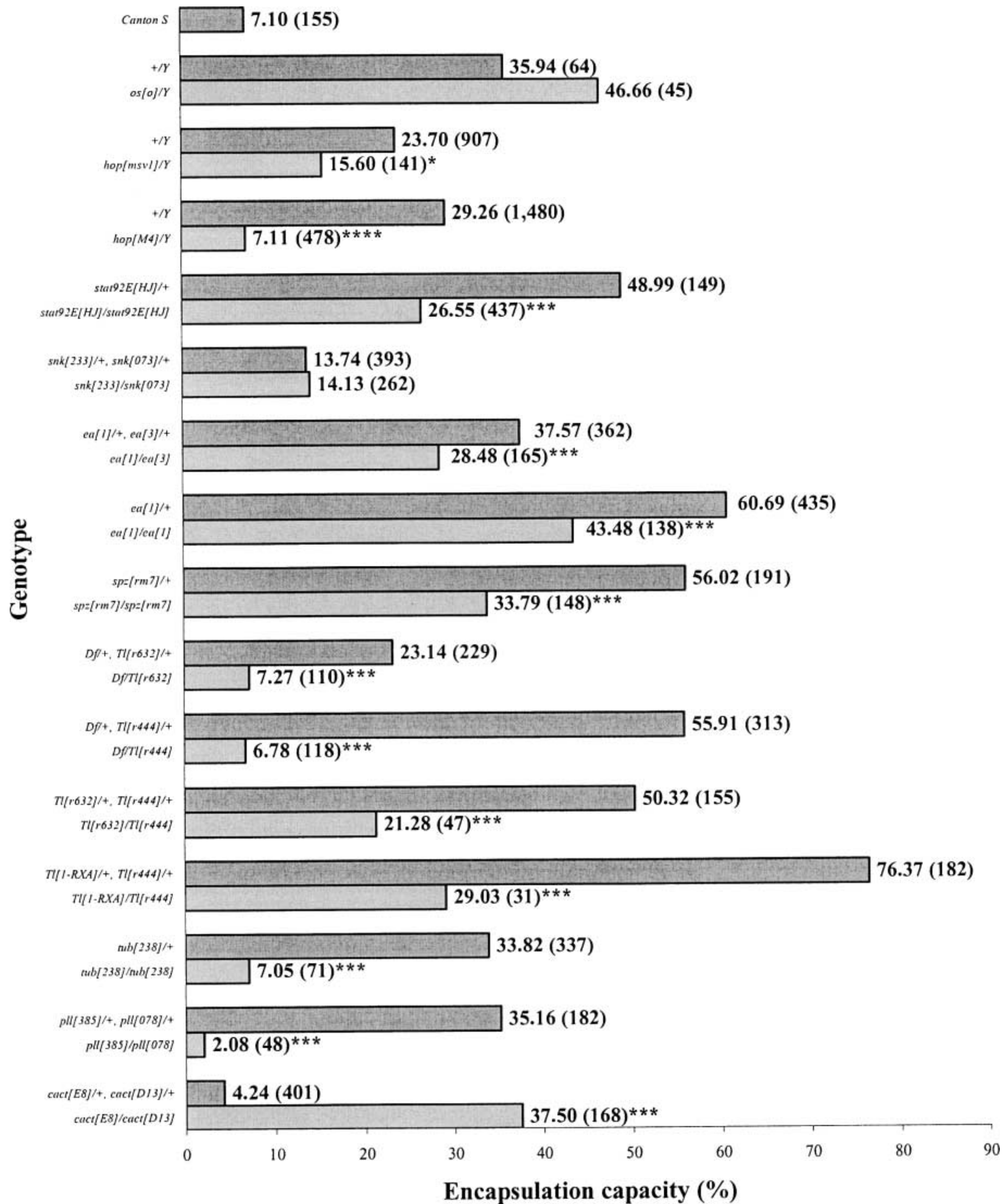

FIGURE 4.—Encapsulation capacities of Hopscotch-Stat92E pathway and dorsal group mutants, in response to parasitization by *L. boulardi G486*. In each pair of bars, the darker top bar represents the control value, and the bottom lighter bar indicates the mutant value. Numerical values for encapsulation capacities are presented to the left of each bar. Numbers in parentheses indicate the number of wasp-parasitized larvae examined. For the *cact* backgrounds only, a different wasp strain, *L. boulardi Q*, was used. Significant differences between control and mutant values are indicated: *, $P < 0.05$; ***, $P < 0.01$; ****, $P < 0.001$.

16 control strains, encapsulation capacities varied from 7.10% for Canton-S to 76.37% for $Tl^{l\text{-}RXA}$/+ and Tl^{r444}/+. Even though encapsulation capacity among mutants was just as variable (ranging from 2.08% for *pll/nptp* to 46.66% for os^{o}/Y) as among controls, a statistically significant reduction in encapsulation capacity among mutants was still observable. Second, encapsulation capacity in almost all of the stocks tested in this study is rather low compared to the nearly 100% encapsulation capacities of Rlb^{+}/Rlb^{+} larvae reported by HITA *et al.* (1999). For

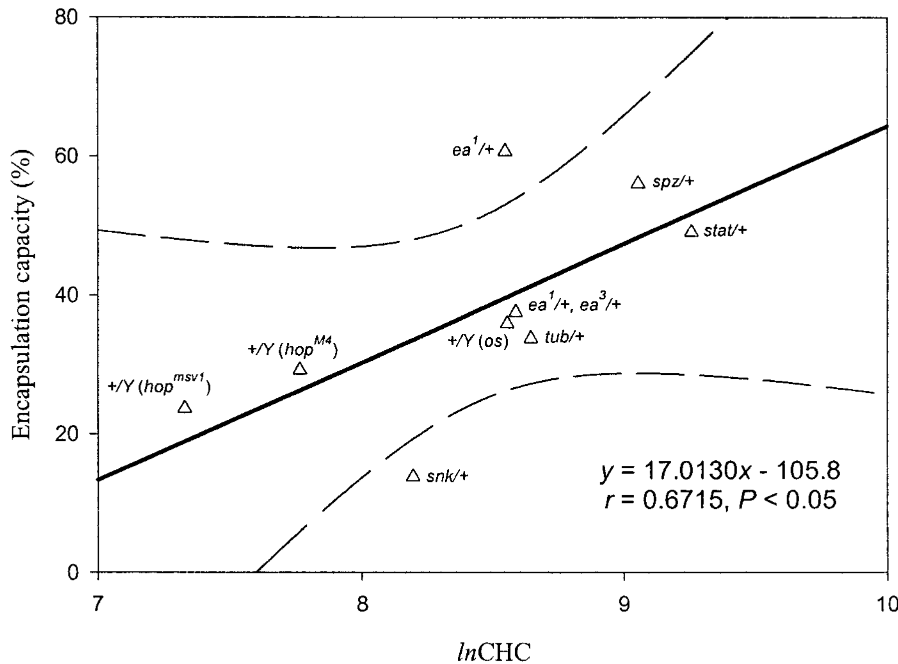

FIGURE 5.—Correlation analysis of pooled control values for mean CHC and encapsulation capacity. Control data points exhibit a significant ($P < 0.05$) correlation ($r = 0.6715$; two variables; 7 d.f.) only between mean ln CHC and encapsulation capacity. Dashed lines represent 99% confidence limits of the correlation function.

example, 11 of the 16 control backgrounds that we tested exhibited encapsulation capacities of <50% (Figure 4).

Correlation analysis of CHC and encapsulation capacity: To determine whether CHC could have a bearing on encapsulation capacity, we performed correlation analysis on control and mutant larvae. For each of nine control classes (we used mean ln CHC and encapsulation capacity values only if *both* values were obtained from larvae carrying the identical genotype), we plotted mean ln CHC (*x*-axis) against encapsulation capacity (*y*-axis). Because $cact^{E8}/cact^{D13}$ mutants already carry a large number of preexisting lamellocytes (Qiu *et al.* 1998), it is not possible to test such mutants for any correlation between hemocyte density and the likelihood of lamellocyte differentiation in response to wasp parasitization; thus *cact* data were not included in this analysis. The results, including best-fit functions, are presented in Figures 5 and 6.

Analyses of control and mutant data points reveal that in the control group, encapsulation capacity increases as mean ln CHC increases (Figure 5). In fact, the best-fit function that we obtained ($y = 17.0130x - 105.8$) is linear and is significant ($r = 0.6715$; $P < 0.05$; the critical value for 95% significance at 7 d.f. is 0.666; Crow *et al.* 1960). Thus, regardless of whether larvae are controls for Hop-Stat or dorsal group mutations, they behave as members of a single group.

Importantly, this correlation is not true when all nine mutant data points are analyzed together ($r = 0.0819$; $P > 0.05$; Figure 6). A closer inspection of individual mutant data points reveals an interesting pattern: If we consider dorsal group mutants alone, we find a qualitative correlation between CHC and encapsulation (the number of dorsal group data points considered here is insufficient to detect significance). The lack of correlation between ln CHC and encapsulation capacity among tested mutants as a whole (Figure 6) is therefore likely due to the effects of the *hop* and *stat92E* data points. These results suggest that CHC alone is not a sufficient determinant of encapsulation capacity and that components of the Toll and Hopscotch pathways have differential effects on the encapsulation response.

Lymph gland lamellocyte differentiation in wasp-induced cellular encapsulation: Loss-of-function mutations in *hop*, *Tl*, or *tub* suppress encapsulation capacity. To determine whether these mutations have different effects on parasite-induced lamellocyte differentiation, we compared the immune reactivity of lymph glands of *G486*-parasitized hop^{M4}/Y, Tl^{r632}/Df, and tub^{238}/tub^{238} larvae. In general, parasitization results in a characteristic lymph gland response, in which 3 days after parasitization, lymph glands of infected larvae exhibit lamellocyte differentiation accompanied by dispersal (Lanot *et al.* 2001; Sorrentino *et al.* 2002). We assayed parasitized *hop* and *Tl* larvae for parasite-induced lymph gland lamellocyte differentiation by crossing in a copy of msn^{03349} (msn^{03349} is a marker for lamellocytes; see materials and methods). Lymph glands dissected from parasitized mutant and control animals were subjected to staining for β-galactosidase activity; residual lamellocytes that were not released from the lymph gland stained positive. Furthermore, lymph glands from parasitized *hop*, *Tl*, and *tub* larvae were examined for evidence of dispersal (*e.g.*, the basement membrane surrounding lymph gland hemocytes is no longer contiguous and shows clear signs of disruption). We considered a lymph gland to be immune reactive if we observed partial or complete dispersal of

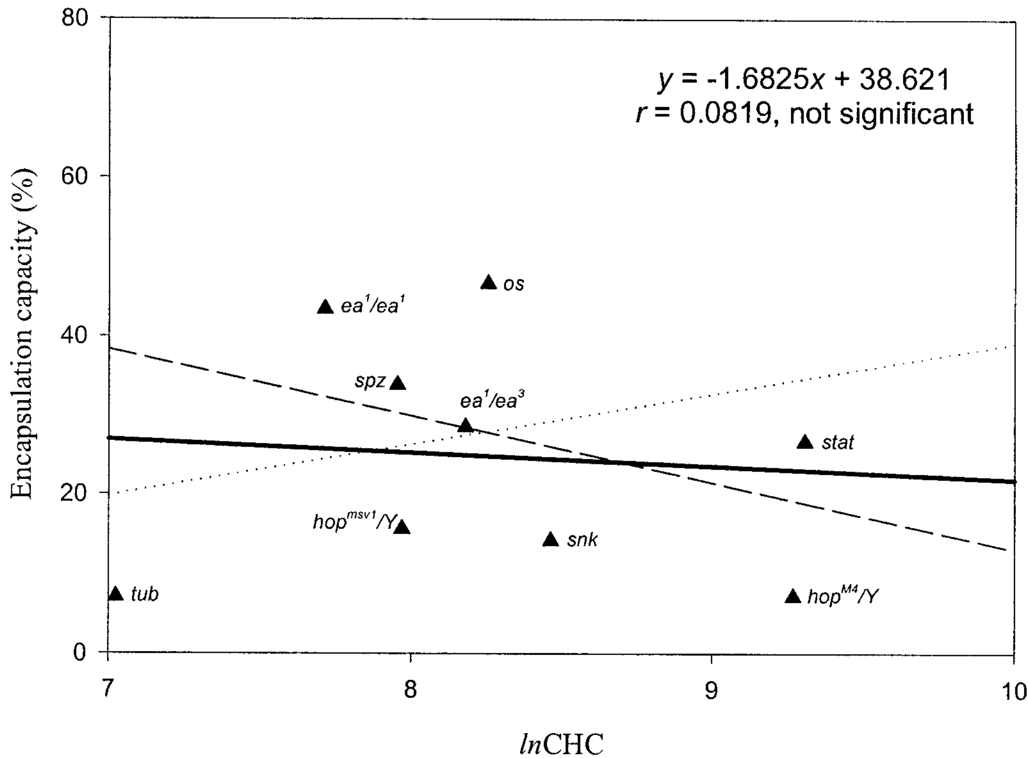

FIGURE 6.—Correlation analysis of pooled mutant values for mean CHC and encapsulation capacity. Pooled mutant data points do not exhibit a significant ($P > 0.05$) correlation between the two parameters (solid line; $r = 0.0819$; two variables; 7 d.f.). Correlation analyses based on Hopscotch-Stat92E pathway mutants and dorsal group mutants considered separately, both of which are not significant (in part because of insufficient sample size), are represented by dashed and dotted lines, respectively.

the lymph gland, paying close attention to the integrity of the basement membrane and the presence of lamellocytes in the vicinity of the dispersed lymph gland. In addition, in the case of *hop* and *Tl* larvae, we examined dissected lymph glands for presence of positive β-galactosidase staining. Lymph glands that were negative for dispersal (and β-galactosidase expression) were considered unreactive.

In *G486*-parasitized hop^{M4}/Y; $msn^{03349}/+$ mutants, we observed a significant reduction in lymph gland immune reactivity, as compared to the same in control siblings (Table 3; Figure 7, A and B). Among control siblings, 10 of 30 lymph glands exhibited dispersal, and 10 of the remaining 20 intact lymph glands stained positively for β-galactosidase activity; thus 66.67% (20/30) control sibling lymph glands were immune reactive. Strikingly, among lymph glands from hop^{M4}/Y; $msn^{03349}/+$ larvae, minimal evidence of lymph gland dispersal was observed in 1 of 30 lymph glands from *G486*-parasitized hop^{M4}/Y; $msn^{03349}/+$ larvae, and only 1 of the remaining 29 intact lymph glands was positive for lamellocyte-specific β-galactosidase staining. Thus only 3.45% (2/30) mutant lymph glands were immune reactive, a highly significant ($P \ll 0.001$) reduction in lymph gland immune reactivity.

After confirming that the recombinant msn^{03349} Tl^{r632} chromosome that we generated could still significantly suppress encapsulation capacity *in trans* to $Df(3R)ro^{80b}$ (controls, 22.07%, $n = 281$; mutants, 10.76%, $n = 158$; $P < 0.01$), we examined the lymph glands of *G486*-parasitized msn^{03349} $Tl^{r632}/+$ *Df* larvae. In contrast to our observations of hop^{M4}/Y; $msn^{03349}/+$ larvae, we found no significant effect of Tl^{r632}/Df or tub^{238}/tub^{238} on lymph gland immune reactivity (Table 3; Figure 7, C and D). Sibling control larvae (msn^{03349} $Tl^{r632}/+$ + or $tub^{238}/+$) exhibited expectedly high immune reactivity: 85.19% (23/27) of msn^{03349} $Tl^{r632}/+$ + lymph glands examined were immune reactive (15 of 27 lymph glands exhibited signs of dispersal, while 8 of the remaining 12 intact lymph glands were positive for β-galactosidase activity). However, lymph gland immune reactivity among msn^{03349} $Tl^{r632}/+$ *Df* mutants, (78.57%; 22/28), was not significantly different from that of the controls (20 of 28 lymph glands exhibited signs of dispersal, and 2 of the remaining 8 intact lymph glands were positive for β-galactosidase activity). Like the *Tl* mutants, we found no significant difference in lymph gland dispersal between $tub^{238}/+$ (73.91%; $n = 23$) and tub^{238}/tub^{238} (82.14%; $n = 28$) animals (Table 3).

These results suggest that the suppression of encapsulation capacity by loss of function of *hop*, *Tl*, or *tub* is likely to be due to distinct requirements of these genes. The suppression of lymph gland response to parasitization in the hop^{M4} background is consistent with the observed reduction in hop^{M4}/Y encapsulation capacity and suggests that the Hopscotch protein is necessary for a parasite-induced signal for lamellocyte differentiation. As suggested by LUO *et al.* (1997), this signal for lamellocyte differentiation is most likely mediated by the transcription factor Stat92E: Loss of function of one copy of Stat92E suppresses the penetrance of the $hop^{Tum\text{-}l}$-induced melanotic tumor phenotype and Stat92E is constitutively activated in Drosophila cell cultures that overexpress

TABLE 3

Immune reactivity of lymph glands from hop^{M4}/Y, Tl^{r632}/Df, and tub^{238}/tub^{238} larvae parasitized by *L. boulardi G486*

Larval genotype	No. of lymph glands: Total	Unreactive	Immune reactive: Dispersed	Intact and LacZ$^+$	Immune reactive (%)	Mutant/control ratio
$+/Y; msn^{03349}/+$	30	10	10	10	66.66	
$hop^{M4}/Y; msn^{03349}/+$	30	28	1	1	3.45	0.05*
$msn^{03349}\ Tl^{r632}/+\ +$	27	4	15	8	85.19	
$msn^{03349}\ Tl^{r632}/+\ Df$	28	6	20	2	78.57	NSD
$tub^{238}/+$	23	6	17	Not done	73.9	
tub^{238}/tub^{238}	28	7	21	Not done	82.1	NSD

Immune reactivity of lymph glands from hop^{M4}/Y, Tl^{r632}/Df, and tub^{238}/tub^{238} larvae parasitized by *L. boulardi G486* is shown. Lymph glands from control and mutant larvae carrying one copy of msn^{03349} (see materials and methods) that were parasitized by *G486* were dissected and examined for minimal signs of dispersal and lamellocyte-specific LacZ staining. Larvae in the *tub* experiment were not assayed for β-galactosidase activity. See materials and methods for generation and selection of genotypes. Unreactive, no β-galactosidase staining visible (*hop* and *Tl* backgrounds only) and no evidence of dispersal; dispersed, at least minimal signs of dispersal (see materials and methods and results); intact, no signs of dispersal; LacZ$^+$, positive for β-galactosidase activity. Comparison of the percentage of control and mutant lymph glands that were immune reactive was made by determining cumulative binomial probability (see materials and methods); significant differences between proportions are indicated. NSD, no significant difference; $*P < 0.001$.

Hop$^{Tum\text{-}l}$. Our results are consistent with the proposed Stat92E-dependent lamellocyte signal: *stat92E* larvae are immune compromised and are unable to mount an efficient egg encapsulation response despite exhibiting control CHC levels. Additionally, mean circulating lamellocyte percentage in $hop^{Tum\text{-}l}/Y; stat92E^{HJ}/stat92E^{HJ}$ larvae that are tumor-free is ~1%, which is indistinguishable from the control value (Sorrentino 2003).

In contrast to Hop and Stat92E, Toll and Tube appear not to play a role in lamellocyte differentiation; rather,

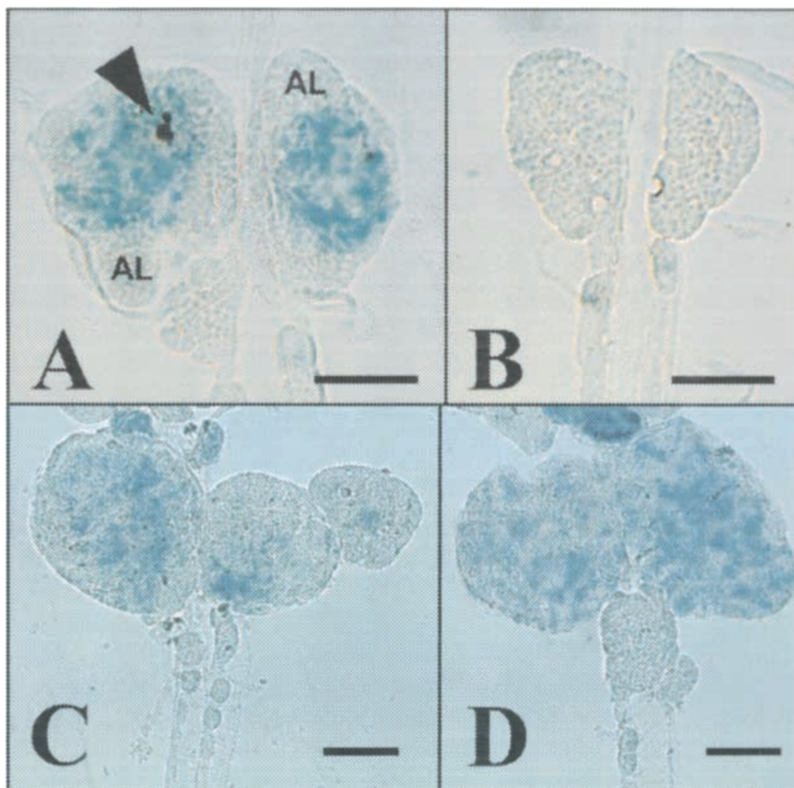

Figure 7.—Lymph glands from *G486*-parasitized larvae that exhibited no signs of any encapsulation response. (A) $+/Y; +/msn^{03349}$. Lymph gland is intact, but lamellocyte-specific β-galactosidase staining is abundant in both anterior lobes (ALs). Note the melanized encapsulations within the ALs (arrowhead). (B) $hop^{M4}/Y; +/msn^{03349}$. Lymph gland is intact, but is negative for any lamellocyte-specific LacZ staining. (C) $msn^{03349}\ Tl^{r632}/+\ +$. (D) $msn^{03349}\ Tl^{r632}/+\ Df(3R)ro^{80b}$. Control (C) and mutant (D) lymph glands exhibit similar extensive lamellocyte-specific β-galactosidase staining. Bars, 100 μm. See Table 3 for statistical analysis. Samples obtained from larvae are of the same *hop* and *Tl* backgrounds that were tested for ln CHC (Figure 3) and encapsulation capacity (Figure 4).

loss-of-function mutations in *Toll* or *tube* probably suppress encapsulation via other mechanisms. As *Toll* and *tube* larvae have very few circulating hemocytes, reduction in encapsulation in *Tl* and *tub* mutants might be due to defects in wasp egg recognition or a reduction in hemocyte proliferation that normally follows parasitization (Sorrentino *et al.* 2002). The effect of these mutations on crystal cells is unclear. While we cannot rule out the possibility that these mutations reduce encapsulation capacity by reducing the crystal cell population, this is an unlikely possibility, as *Black cells* mutant larvae without functional crystal cells are immune competent and can still successfully encapsulate wasp eggs (Rizki and Rizki 1990). The fact that lymph glands of loss-of-function *Tl* and *tub* mutant larvae can support lamellocyte differentiation suggests that the low CHC in *Tl* and *tub* larvae in itself does not hinder lamellocyte differentiation or the ability of the lymph gland to disperse after the wasp egg is introduced into the hemocoel. Given that gain-of-function *Tl* alleles induce lamellocyte differentiation, the lack of effect of Tl^{-} on lamellocyte differentiation is somewhat unexpected, and it is possible that lamellocyte differentiation is in some way secondarily activated in the Tl^{10b} background. Thus, the wasp egg encapsulation assay is a useful tool for evaluating the genetic requirements for lamellocyte differentiation.

In conclusion, our study shows that while there is substantial variation in hemocyte concentration in control larvae, this variation is consistent with a log-normal distribution. Such a distribution could be a result of the inherently logarithmic process of cell division. Using this quantitative method of CHC data analysis, we found that previously reported CHC values for mutant larvae that exhibit reduced or increased hemocyte densities are also log-normally distributed and that approximately half of each of these mutant distributions lie beyond the limits of the control distribution, allowing us to define ranges of CHC values as being low, control, and high. Second, encapsulation capacity in control and DV mutant larvae correlates with ln CHC. Evidence for biological significance of this correlation also comes from observations that *D. melanogaster* larvae selected for higher resistance against *A. tabida* have twice as many circulating hemocytes as compared to control larvae (Kraaijeveld and Godfray 1997; Fellowes *et al.* 1998). These observations support the notion that circulating hemocytes, possibly plasmatocytes, contribute to the efficiency of the egg encapsulation response. However, high CHC alone is insufficient to trigger encapsulation; lamellocytes must be present. For example, massive 20- to 300-fold increases in CHC involving plasmatocytes and crystal cells (Munier *et al.* 2002; Asha *et al.* 2003; H. Chiu and S. Govind, unpublished results), but not lamellocytes, are insufficient to trigger constitutive encapsulation of self tissue in the larva. The combined use of genetic and immune approaches used in this study demonstrates that different developmental signals independently contribute to the maintenance of the steady-state hemocyte concentration in circulation and the ability to differentiate lamellocytes. Together, these physiological parameters enable larval hosts to efficiently defend themselves against wasp infections.

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