size differences), but like the morphometric traits, the cell size differences between the sexes of this species were not significant. Intraspecific sex differences in cell size were not noted by Stevenson *et al.* (1995) in their survey of cell and organ sizes in Hawaiian *Drosophila* species, apparently because the sexes of individuals were not recorded.

Just why adult cell sizes should differ so markedly between the sexes and among *Drosophila* species is not immediately obvious. Larger body sizes could be achieved, if favored by selection, simply by more cell proliferation during an extended developmental period. Various rates of growth and development are found among the ecologically diverse Hawaiian *Drosophila* species (Kambysellis and Craddock, 1997), and these varied life histories undoubtedly play some role in the observed interspecific body size differences. Regulation of cell size and body size is complex and subject to both molecular and ecological constraints. For this reason, our lab is using the extraordinary array of Hawaiian *Drosophila* species to explore some of the genetic and developmental factors that have contributed to evolution of species differences in cell size and thus body size; further data from these investigations will be presented elsewhere.

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Immune response in the *tu-pb* melanotic tumor strain of *Drosophila melanogaster.*

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Introduction

Drosophila, like other insects, has developed an efficient host defense against microbial infection that induces both humoral and cellular reactions (Boman, 1995; Hoffman *et al.*, 1996; Hoffman and Reichart, 1997). The humoral reactions consist of proteolytic cascades, namely coagulation and phenoloxidase cascades, which lead to localized melanization and blood coagulation; they also consist of the rapid and transient synthesis of a battery of antimicrobial peptides. It has been reported that some genes controlling embryonic development [*dorsal (dl)*, *Toll* and *cactus*] are expressed in larval and adult fat bodies, where their RNA expression is enhanced upon injury (Lemaitre *et al.*, 1995). Upon bacterial challenge, the Dorsal protein (Dl), normally localized in the cytoplasm of the fat body, is rapidly imported into the nucleus; this nuclear uptake of Dl occurs spontaneously in mutants exhibiting melanotic tumors. The cellular immune system, hemocytes and

lymph glands are less characterized. The cellular immune reactions consist of phagocytosis and encapsulation of invading microorganisms by circulating blood cells (Nappi and Vass, 1993). In larvae, hemocytes originate from the hematopoietic organ, the lymph glands, and can initially be classified as crystal cells or plasmatocytes (Shresta and Gateff, 1982). The function of crystal cells appears to involve the release of the crystalline inclusions to provide substrates and enzymes to phenoloxidase for the melanization reaction. Plasmatocytes, instead, perform a phagocytic function like mammalian macrophages or neutrophils. When a larva undergoes an immune challenge, plasmatocytes are induced to engage in phagocytosis or differentiate into adhesive lamellocytes (Rizki and Rizki, 1992). They form multilayered capsules around foreign invaders and get melanized by activities of crystal cells. Mutations in *cactus* or *Toll*, or constitutive expression of Dorsal, can induce lamellocyte differentiation and cause the formation of melanotic capsules around self tissue which results in a melanotic tumor phenotype (Govind, 1996; Qiu *et al.*, 1998). Generally, melanotic tumors are thought to be a reaction to abnormal development and it has been proposed that all melanotic tumor mutants can be categorized in two classes: class 1, in which melanotic tumors, associated with an apparently normal immune system, respond to abnormal tissues; and class 2, in which melanotic tumors are associated with defects of the immune system, lymph glands and hemocytes (Watson, 1991).

In this study, we analyzed some aspects of the immune response in a melanotic tumor mutant, *tu-pb*, of *Drosophila melanogaster* (Di Pasquale Paladino and Cavolina, 1983).

tu-pb is an atypical melanotic tumor mutant in which the tumor manifestation is exclusively restricted to the head of the adult and melanotic masses, absent during the larval stadium, are evident just after esclosion. The *tu-pb* phenotype depends on at least two different loci, one on the 2nd chromosome, the other, recessive, on the 3rd chromosome located between *sr* (62) and e^s (70.7), presumably close to e^s: Only genotypes including both the two large *tu-pb* autosomes elicit tumor manifestation.

A hematopoietic defect could be involved in the melanotic tumor formation in the *tu-pb* mutant: results obtained from crystal cell counts indicate that *tu-pb* 3rd instar larvae have fewer free floating crystal cells in their hemolymph (O.9%) than Oregon-R wild-type larvae (5-10%). The scarcity of circulating crystal cells appears to be a consequence of a retention of fully differentiated crystal cells in the hematopoietic organ (Di Pasquale Paladino *et al.*, 1988). Here we have analyzed the main properties related to the immune response (induction of antimicrobial peptides, subcellular localization of Dl protein, and phenoloxidase activity) in the *tu-pb* mutant and in two lines, obtained by appropriate crosses, carrying separately the 2nd and the 3rd *tu-pb* chromosome. We have further examined survival rate and bacterial proliferation after infections.

Materials and Methods

Stocks: Fly cultures and crosses were grown on standard fly medium at 25°C. Oregon-R flies were used as a standard wild type strain. The melanotic tumor mutant, *tu-pb*, and two lines, L5 and L5a. These lines were obtained by substitution of the 3rd or 2nd *tu-pb* chromosome with a wild type one. The modified genotypes were obtained by the plan of crosses reported in Figure 1. Strain Tl^{9Q}/+ was used as positive control in immunofluorescence experiments and phenoloxidase activity assay.

Injury and survival experiments: Bacterial challenge was performed by pricking larvae at 3° instar, pupae, or adults with a thin needle previously dipped into a concentrated bacterial pellet of *Enterobacter cloacae b 12* acid nalidixic resistant strain, kindly supplied by Ylva Engstrom, Arrhenius Laboratories for Natural Sciences Department of Molecular Biology, Stockholm University. Groups of 25 adults, aged 2-4 days, were challenged and transferred to a fresh vial. Flies

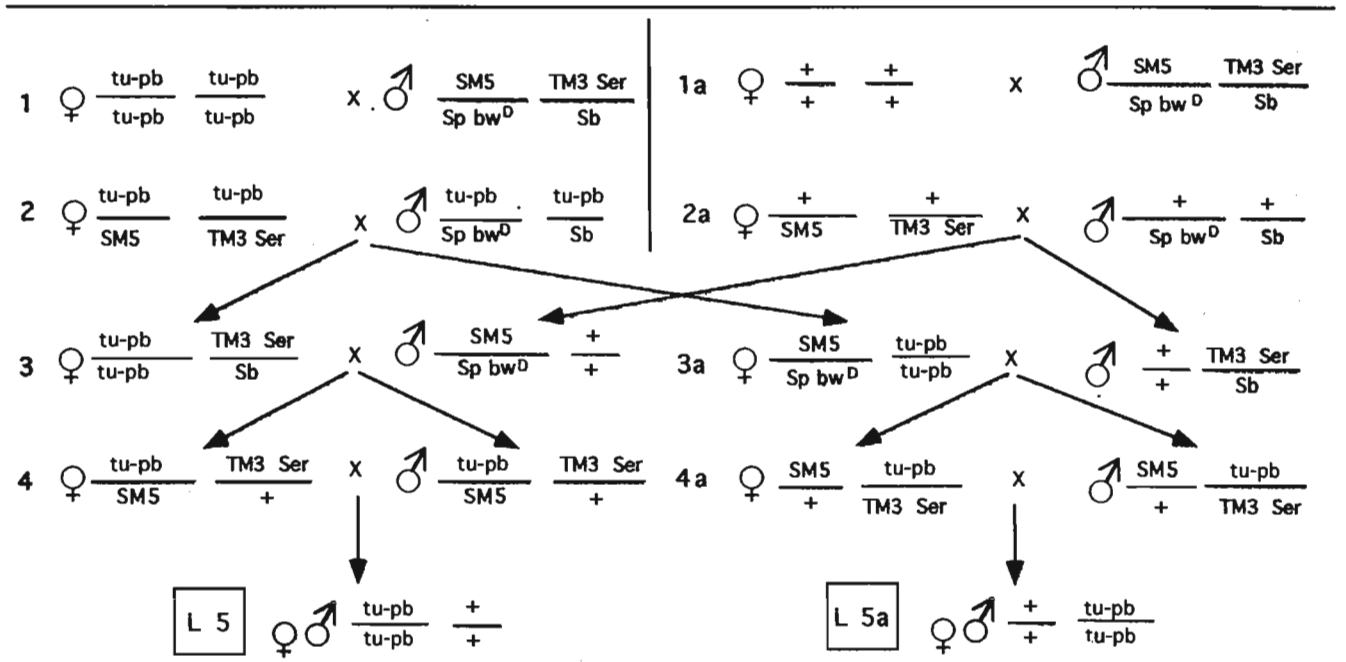

Figure 1. Plan of crosses to obtain line L5 and L5a, which respectively carry the 2nd or the 3rd chromosome separately. Symbols are described in Lindsley and Zimm (1992).

that died within 3 hr (about 3% of the total) after the challenge were not considered in the analysis. Survival rates were calculated during the 120 hr after challenge.

Bacterial proliferation assays: Groups of 50 flies were challenged as described above and after 2 or 24 hr, they were pulverized with a Dounce homogenizer in 10 mM $MgSO_4$. The homogenates were diluted and plated on LB agar and nalidixic acid at a concentration of 25 mg/ml for *E. cloacae b12* selection. Plates were incubated at 37°C overnight and colonies were counted (Ausubel *et al.*, 1994).

RNA preparation and analysis: Wandering third instar larvae, pupae, or 48hr old adult flies, challenged or not, were collected and stored at -80°C until extraction. Total RNA extraction was performed with the RNA Trizol (GIBCO/BRL) method. Northern blotting experiments were performed as in Lemaitre *et al.* (1995).

The following probes were used: diptericin cDNA (Wicker *et al.*, 1990), cecropinC cDNA (Tryselius *et al.*, 1992), and rp49 cDNA (O'Connel and Rosbach, 1984) as control. The probes were constructed by PCR using primers based on the published sequences.

Immunolocalization of Dorsal in fat body: Fat bodies were dissected in PBS and fixed in 3.7% paraformaldheyde, 2 mM $MgSO_4$, 1mM EDTA, and 0.1M PIPES buffer for 15 min. They were washed three times for 5 min in PBS and permeabilized by a 2 hr incubation in PBT A (1% BSA, 0.1% Triton X-100 in PBS). An antidorsal rat polyclonal antibody (kindly supplied by C.A. Rushlow, Department of Biological Science, Columbia University, New York) was applied to the fat bodies at a 1:500 dilution in PBT A and incubated overnight at 4°C. The preparation was then washed three times for 30 min in PBT B (0.1% BSA, 0.1% Triton X-100 in PBS) with 2% sheep serum. The second antibody was a fluorescein isothiocynate-conjugated anti-rat IgG (Boehringer Mannheim, Germany). It was pre-absorbed on fixed fat bodies then diluted 1:128 and applied to fat bodies in PBT B for 1 h at room temperature. The preparation was washed three times in PNBT and

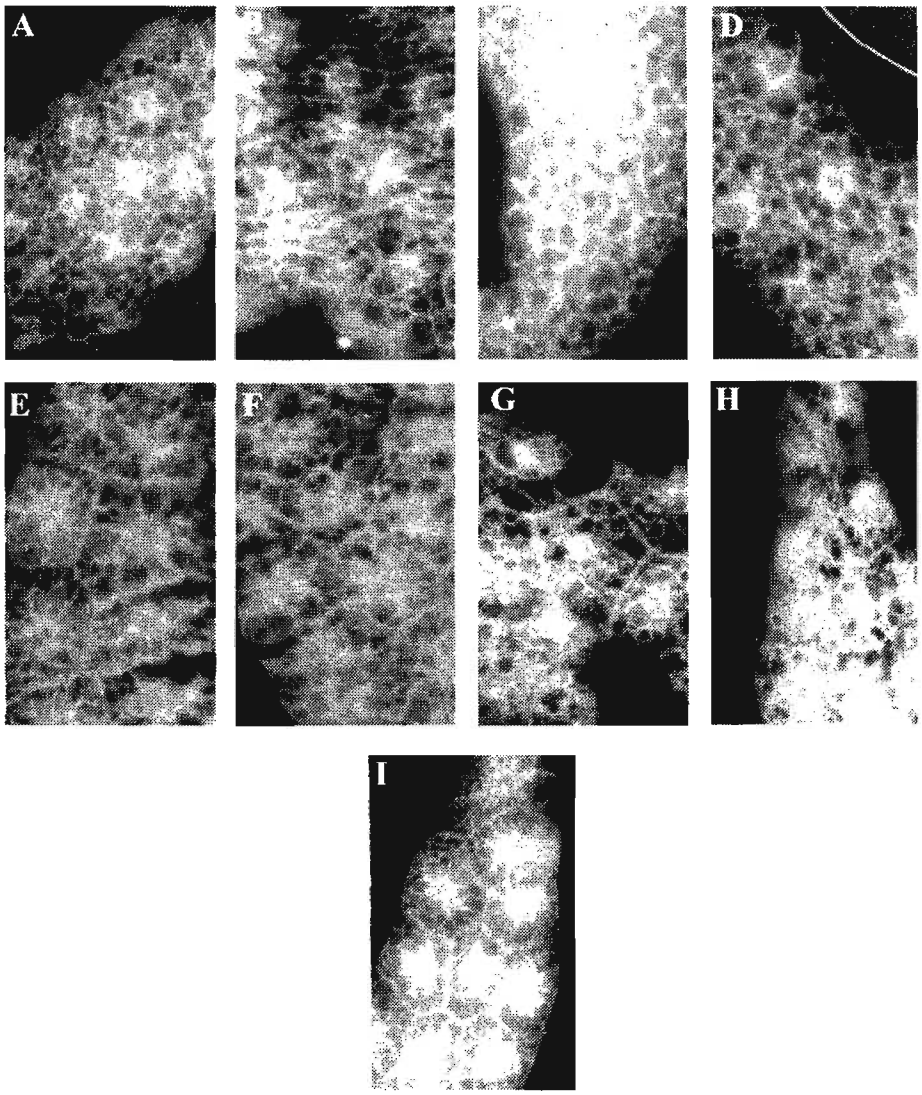

Figure 2. Immunolocalization of Dorsal in *Drosophila* fat body. Fat bodies dissected from unchallenged larvae of *tu-pb* (A), Oregon-R (C), line L5 (E), line L5a (G). Fat body dissected from challenged larvae of *tu-pb* (B), Oregon-R (D), line L5(F), line L5a (H). Fat body from unchallenged Tl^{9Q}/+ larva used as positive control (I). Staining is nuclear in unchallenged and challenged *tu-pb* and derivatives (L5 and L5a lines). Staining is nuclear only after injury in Oregon-R larvae. Fat body was dissected and treated as reported in Materials and Methods. Photographs were taken with a Nikon (100× magnification).

twice in PBS. It was mounted in 9:1 mixture of glycerol plus PBS and examined with a Nikon fluorescence microscope.

Quantification of phenoloxidase activity: The assay was performed as in Braun *et al.*, (1998): 3 ml of hemolymph samples were added to 50 ml of 10mM phosphate buffer (pH 5.9) containing 0.01M L-3,4-dihydroxyphenylalanine (Sigma), and the OD at 470 nm was recorded every minute for 30 minutes.

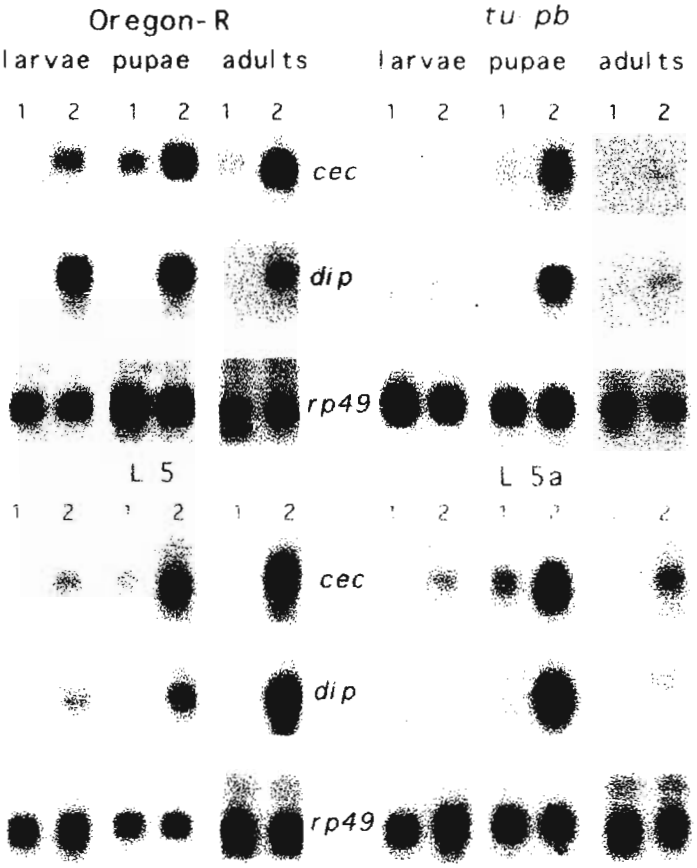

Figure 3. Induction of immunity genes *dip* and *cec C*. Induction of genes encoding antimicrobial peptides in third instar larvae, pupae, and adults of *tu-pb* and its derivatives (L5 and L5a lines). Oregon-R was used as control. 20 mg of total RNA were analyzed by Northern blotting. Blots were hybridized successively with the following probes: *dip* (diptericin), *Cec* (Cecropin C) and rp49 as control. (Line 1, unchallenged animals, line 2, challenged animals).

Results

Immunolocalization of dorsal in fat body of tu-pb mutant and derivatives: Since Dl localization is constitutively nuclear in melanotic tumor mutants, we were curious to examine the subcellular localization of Dl in fat bodies of unchallenged and challenged *tu-pb* larvae and its derivatives L5 and L5a. We have observed that either larvae *tu-pb* (Figure 2, A-B) or larvae L5 (Figure 2, E-F) and L5a (Figure 2, G-H), carrying respectively the 2nd or 3rd chromosome *tu-pb*, show marked nuclear staining of Dl also in absence of challenge. In the wild-type Oregon-R strain, Dl is predominantly cytoplasmic in fat bodies derived from unchallenged wild type larvae (Figure 2, C) and nuclear in challenged ones (Figure 2, D) (Reichart *et al.*, 1993). As positive control we used the Tl^{9Q}/+ mutant, which exhibits melanotic tumor phenotype and shows Dl constitutively nuclear (Figure 2, I).

Expression of antibacterial genes in larvae, pupae, and adults: To understand whether the costitutively nuclear localization of Dorsal has some effect on the inducible antimicrobial response, such as reported for *dl* or *tl* mutants (Lemaitre *et al.,* 1995), total RNAs from larvae, pupae, and adults of control and challenged wild type, *tu-pb*, L5 and L5a mutants, were probed with cDNAs corresponding to diptericin and cecropin C peptides, rp49 serving as a control. Upon challenge, in wild type, the two genes were strongly induced at all stages (Figure 3, A). In contrast, the induction of two genes was impaired in *tu-pb* adults and larvae. Only in *tu-pb* pupae both genes were strongly induced after challenge. However, the inducibility was not totally abolished in challenged *tu-pb* adults where a low hybridization signal remained detectable (Figure 3, B). In L5 line (carrying the 2nd chromosome *tu-pb*) *cec C* and *dip* showed a pattern of expression similar to that of wild type, although lower in larvae and in pupae (Figure 3, C). However L5a line (carrying 3rd chromosome *tu-pb)* retained the inducibility for *cec* C while *dip,* as in *tu-pb*, was strongly inducted only in pupae (Figure 3, D).

Survival rates and bacterial proliferation assay after microbial infection: Owing to evidenced absence or reduced induction of *cec C* and *dip* in *tu-pb* flies, we performed survival assays to determine if *tu-pb* mutant and derivative might be less resistant to experimental infection by

microorganisms. For this purpose we monitored the survival rates of groups of adult flies (50 flies/group) after injury or bacterial challenge every day for 5 days. Our results (Figure 4) showed that all mutant strains (*tu-pb*, L5 and L5a) exhibited good resistance either to injury or to infection during the 120hr of observation and the survival rates (ranging from 65 to 85%) were not significantly different from wild type. Our results suggest that the mutations conferring *tu-pb* phenotype do not exert a dominant effect on the immune response after injury or bacterial infection and that a strong immune defense is elicited also in absence of *cec C* and *dip* induction.

To confirm a correlation between survival and growth inhibition of the pathogen in challenged flies, bacterial proliferation essays were also performed as previously described. Results, reported in Figure 5, show that the *tu-pb* mutant and its derivatives are able to inhibit bacterial growth as, or more, efficiently than wild type.

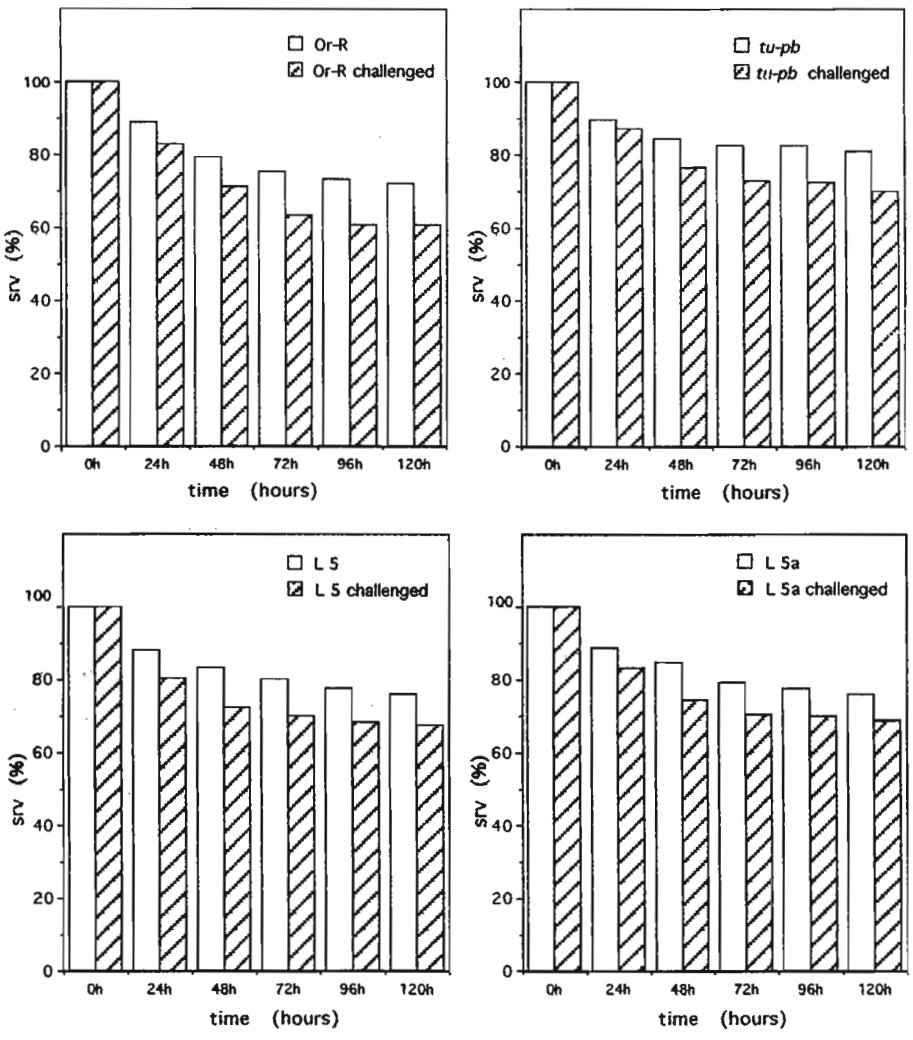

Figure 4. Survival of wild-type and mutant adults to bacterial infection. Bacterial challenge was obtained by pricking groups of 25 adults, aged 2-4 days, with a thin needle previously dipped into a concentrated bacterial pellet of *Enterobacter cloacae b 12*. As control flies were pricked with a sterile needle. Survival rates were calculated during the 120 hr after challenge. Panel A, Oregon-R wild type; panel B, *tu-pb* mutant; panel C, L 5 line; and panel D, L 5a line. Survival experiments were carried out in the same conditions for each genotype tested. At least four replicates were used for the determination of the survival rates.

Melanotic tumor formation and quantification of phenoloxidase activity: It is well known that in challenged wild type *Drosophila* larvae, melanization occurs within few minutes at the site of injury. We noted that melanization reaction also occurs in *tu-pb* larvae, moreover, in a previous investigation (Di Pasquale *et al.*, 1997) it had been evidenced that the rare *tu-pb* flies, derived from challenged larvae, exhibited a significantly higher level of this melanization reaction. In order to investigate a possible correlation between this melanization reaction and the activity of the phenoloxidase system, we

performed a quantitative essay of phenoloxidase activity in larvae and adults of *tu-pb* and derivative lines. Results (Figure 6) showed that phenoloxidase activity in hemolymph extracted from *tu-pb* larvae is lower than wild-type or Tl^{9Q}/+ mutant which normally exhibits melanotic tumor phenotype. On the contrary the phenoloxidase activity is much more efficient in *tu-pb* adult flies than in the wild-type adult ones. L5 and L5a lines show a melanization reaction and a phenoloxidase activity similar to the wild type both in larvae and adults.

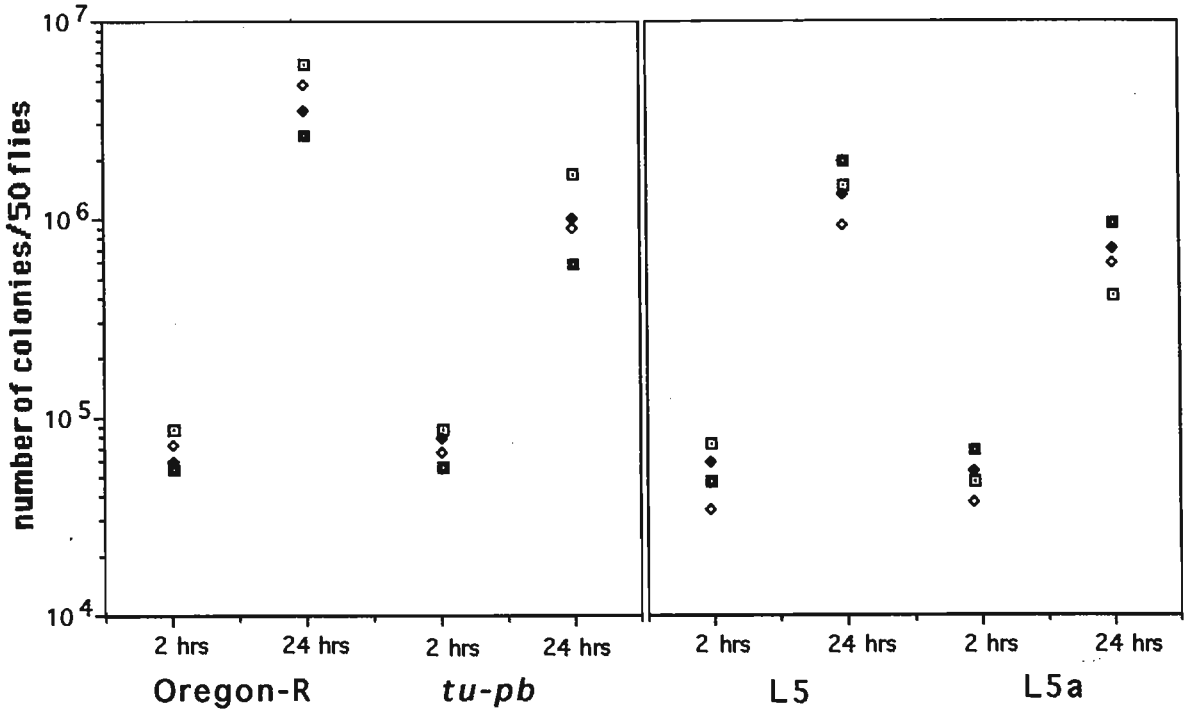

Figure 5. Bacterial proliferation assays. Groups of 50 mutants and wild-type adult flies were infected with a needle dipped into a concentred solution of nalidixic-resistant strain *E. cloacae b 12*. Flies were homogenized either at 2 or 24 hr after infection; bacterial counts were obtained by plating lysates on plates containing nalidixic acid (25 mg/ml). Each column displays the results of four independent experiments.

Discussion

Our data show also in *tu-pb* the correlation between the presence of the melanotic tumor phenotype and the occurrence of nuclear uptake of Dl, even in absence of challenge. In regard to the induction of *cec* and *dip* genes upon immune challenge during the different stages of life cycle in *tu-pb* mutants, the situation appears certainly more complex. In our experiments, we observed a severe loss of the induction of these genes in larvae and adults but not in pupae of *tu-pb* mutants. This condition is partially copied in L5a line, while line L5 exhibits a response more similar to the wild type. These results suggest that only the mutation localized on the third chromosome, close to e^s, is responsible for the absence of *dip* gene induction during the larval and adult stage, while *cec C* induction is controlled by both mutations. In fact, only genotype carrying both *tu-pb* chromosomes was impaired in *dip* and *cec C* induction. At the same time, the full capacity to induce expression of *cec* C and *dip* genes in response to challenge, that we have evidenced only in challenged *tu-pb* pupae but not in larvae nor adults, seems to indicate the existence of distinct regulatory pathways controlling the expression of these genes in various stages of development.

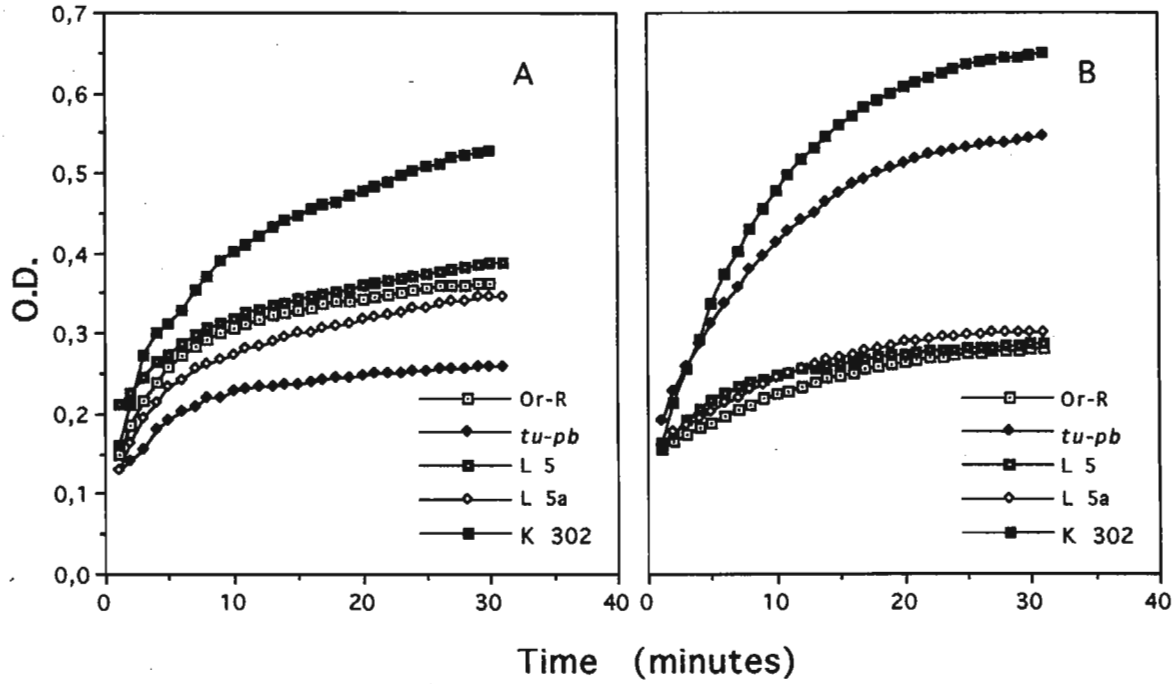

Figure 6. Assay of phenoloxidase activity. Quantification of phenoloxidase activity in *tu-pb* mutant and derivative L5 and L5a larvae (panel A) and adults (panel B). Oregon-R and Tl^{9Q}/+ (K 302) were used as control. The assay was performed as reported in Materials and Methods.

Nevertheless adults of all these genotypes exhibit a good survival rate after septic injury as well as a good capacity to block bacterial proliferation, thus behaving differently from *imd* mutants, which, showing a dramatically lowered challenge-induced synthesis of antibacterial peptides, exhibit a severely reduced survival rate when injected with bacteria (Corbo and Levine, 1996). Our results suggest the possible involvement of other defense processes which can be alternative to the antimicrobial peptide synthesis and may account for the survival of a high proportion of the challenged *tu-pb* individuals. This is not in disagreement with the observation that the *tu-pb* mutant is able to react efficaciously against bacterial infection; a similar resistance was observed in the *Black cell* mutants (lacking the humoral melanization reaction) and in the *domino* mutants (lacking hemocytes) (Braun *et al.*, 1998). The defense capacity of *tu-pb* might reside mostly in the activation of the prophenoloxidase cascade as well as in the cellular defense mechanism.

Results of our study on quantification of phenoloxidase activity are indicative of a lower activity of this enzyme in *tu-pb* larvae that instead strongly increases in adults. This may be related to crystal cell pattern modification evidenced in *tu-pb* which consists of a lower number of free-circulating crystal cells in the larval hemolymph, probably due to an abnormal retention of mature crystal cell in the hematopoietic organ (Di Pasquale Paladino *et al.*, 1988). This might explain the late formation of tumoral masses which characterizes *tu-pb* mutants. Therefore, we think that at the basis of the manifestation of the tumoral phenotype of *tu-pb* there may be an alteration of the crystal cells or of the lymphoglands, suggesting that this mutant enters into class 2, in which melanotic tumors are associated with immunity system faults, lymphoglands or hemocytes (Watson, 1991).

As to the two mutations which together confer *tu-pb* phenotype, the analysis of immune response in the two lines carrying separately the 2nd or 3rd chromosome *tu-pb,* indicate that the mutation localized on the 3rd chromosome seems to play a major role in conferring to the *tu-pb* mutant only some of the characteristics we have so far identified. In any case we think that the *tu-pb*

has revealed properties that make it a useful mutant for further investigations in the field of *Drosophila* immunity.

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New data on distribution of *Drosophila mercatorum* in inner regions of Eurasia.

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In Russia *Drosophila* (*Drosophila*) *mercatorum* Patterson et Wheeler, 1942 was first found in Novosibirsk city (Western Siberia) in 1990 (Ivannikov and Zakharov, 1994). Over the recent decade it has been found everywhere in the outskirts and the city of Novosibirsk. Since mid-1990s and until now, *Drosophila mercatorum* has been the most widely represented among synantropic *Drosophila* in Novosibirsk, which considerably outnumbered the other attendant species (*D. busckii, D. funebris, D. immigrans,* and *D. melanogaster*).

Novosibirsk city is situated in the geographical center of Russia - a country with vast territory covering most of the northern regions of the Eurasian continent. An assumption seems quite logical that occurrence of *D. mercatorum* in that area is not restricted to a single locality and the following facts testify to that. First, predominance of *D. mercatorum* over the other *Drosophila* species is evidence that, being a synantropic species, it can perfectly adapt to the conditions of continental Asia. Second, over the period from 1990 until now we have found the single individuals of *D. mercatorum* in inner areas of the continent - in Europe (Uman City, Ukraine, 1990) and Central Asia (Dushanbe, Tajikistan, 1994) (Ivannikov and Zakharov, 1995). However logical and valid the assumption about a wider occurrence of *D. mercatorum* in inner regions of Eurasia could be, until recently there has