Leptopilina heterotoma and L. boulardi: Strategies to Avoid Cellular Defense Responses of Drosophila melanogaster

T. M. RIZKI,* R. M. RIZKI,* AND Y. CARTON†

*Department of Biology, University of Michigan, Ann Arbor, Michigan 48109, U.S.A., and †Laboratoire de Biologie et Genetique Evolutives, CNRS, 91190 Gif sur Yvette, France

RIZKI, T. M., RIZKI, R. M., AND CARTON, Y. 1990. Leptopilina heterotoma and L. boulardi: Strategies to avoid cellular defense responses of Drosophila melanogaster. Experimental Parasitology 70, 466–475. Eggs of three strains of the cynipid parasitoid Leptopilina heterotoma and a Tunisian strain (G317) of L. boulardi are not encapsulated by hemocytes of Drosophila melanogaster hosts, but the eggs of a Congolese strain (L104) of L. boulardi are encapsulated. To determine the reason for the difference in host response against the parasitoid eggs, lamellocytes (hemocytes that encapsulate foreign objects and form capsules around endogenous tissues in melanotic tumor mutants) were examined in host larvae parasitized by the five Leptopilina strains. Parasitization by the three L. heterotoma strains affected the morphology of host lamellocytes and suppressed endogenous melanotic capsule formation in melanotic tumor hosts. L104 did not alter the morphology of host lamellocytes nor block tumor formation in melanotic tumor mutant hosts. The morphology of some lamellocytes was affected by G317 parasitization but host lamellocytes were still capable of forming melanotic tumors and encapsulating dead supernumerary parasitoid larvae. Therefore, the eggs of strains affecting lamellocyte morphology are protected from encapsulation by the host's blood cells. L. heterotoma eggs float freely in the host hemocoel but L. boulardi eggs are attached to host tissue surfaces. Lamellocytes cannot infiltrate the attachment site so the capsule around the L104 egg remains incomplete. The wasp larva uses this gap in the capsule as an escape hatch for emergence. © 1990 Academic Press, Inc.

INDEX DESCRIPTORS AND ABBREVIATIONS: Leptopilina heterotoma; L. boulardi; Drosophila melanogaster; Parasitoid; Cellular defense; Hemocytes; Encapsulation; Melanotic tumor strain (tu).

INTRODUCTION

The lamellocytes of Drosophila melanogaster larvae parasitized by the endoparasitic wasp Leptopilina heterotoma are destroyed by a factor from an accessory gland which the parasitoid female injects along with its eggs into the host hemocoel (Rizki and Rizki 1984). Within a few hours after a Drosophila larva is parasitized by L. heterotoma, the host lamellocytes lose their typical discoidal shape and their ability to adhere to each other to form clumps. Since the lamellocytes are the hemocytes that form capsule walls around foreign bodies, the destruction of these cells assures a safe environment for the development of the parasitoid eggs. This mechanism of abrogating the host's cellular defense system may account for the fact that L. heterotoma

can successfully exploit a number of Drosophila species as its host. At least 10 different host species for this parasitoid in its holarctic distribution area have been examined (Carton et al. 1986). The destruction of capsule-forming hemocytes of the host can also explain the observations of Walker (1959) and Nappi (1975, 1977) that encapsulation of endogenous tissues by hemocytes to form melanotic masses in melanotic tumor (tu) mutant hosts is blocked by parasitization.

Unlike L. heterotoma, L. boulardi favors D. melanogaster as its host (Carton and Kitano 1981; Carton et al. 1987). This difference in host specificity raises the question whether L. boulardi uses the same mechanism as L. heterotoma to suppress host cellular defense responses. More intriguing is

the fact that the eggs of the L104 strain of *L. boulardi* collected in Brazzaville, Congo, are encapsulated in *D. melanogaster* hosts (Carton 1988) but the eggs of the *L. boulardi* G317 strain from Nasrallah, Tunisia, are not (Carton 1984). The difference in encapsulation frequencies of the eggs of the two strains might be due to differences between the eggs themselves or to factors external to the eggs that influence host responses to parasitization. One purpose of the present study was to compare the lamellocytes of *Drosophila* hosts parasitized by these two wasp strains. A second purpose was to determine whether *L. boulardi* and *L. heterotoma* utilize the same strategy to ward off host cellular defense responses. This question was examined by comparing *Drosophila* hosts parasitized by three strains of *L. heterotoma* with hosts infected by the two *L. boulardi* strains. Earlier work examined the Leiden strain of *L. heterotoma* (Rizki and Rizki 1984). The present investigation included this strain and the Mallorca and Storrs strains of *L. heterotoma.*

MATERIALS AND METHODS

Aberrant tissues in larvae of *melanotic tumor (tu)* strains of *D. melanogaster* are encapsulated by lamellocytes to form cellular masses that later melanize and are referred to as melanotic tumors (Rizki and Rizki 1986). Unlike nontumorous larvae, *tu* larvae have many lamellocytes (Rizki 1957). The surfaces of lamellocytes in larvae in which encapsulation is in progress bind wheat germ agglutinin conjugated to fluorescein isothiocyanate and light up as speckled. Since this speckling is not found in lamellocytes of larvae where encapsulation is not occurring, the speckled surface property indicates that lamellocytes are competent for encapsulation (Rizki and Rizki 1983). Larvae of *tu* strains have many lamellocytes competent for encapsulation so the use of a *tu* strain excludes possible complications associated with induction of lamellocyte differentiation.

Larvae of two *tu* strains as well as the *Ore-R* wild type strain were used as hosts in this study. The *tu-W* strain was used to evaluate the effect of parasitization on melanotic tumor formation because the melanotic tumors in this strain are large and readily visible by external examination of the larvae and pupae (Rizki and Rizki 1979). Tumor penetrance in the *tu-W* line

(S85) used in the study was above 95% at 24°C. The *tu-Sz^{ts}* mutant strain (line m) was used for evaluating the effects of parasitization on lamellocytes since larvae of this strain have a high percentage of free-floating lamellocytes (Rizki and Rizki 1980). The *tu-Sz^{ts}* mutant is temperature sensitive and melanotic masses appear only when the larvae are kept above 26°C. Therefore, studies with *tu-Sz^{ts}* larvae were performed at 27°C. Host larvae were grown on cream of wheat/molasses medium seeded with live Fleischmann's yeast. Larval ages were recorded from time of hatching from the egg.

The L104 strain of *L. boulardi* was collected in the suburbs of Brazzaville, Congo, and the G317 strain originated in the oasis of Nasrallah, Tunisia. The Leiden (Holland) and Mallorca (Spain) strains of *L. heterotoma* were obtained from Dr. K. Bakker of the University of Leiden and the Storrs (United States) strain of this species was provided by Dr. A. J. Nappi, Loyola University. *L. heterotoma* strains were reared on the *tu-Sz^{ts}* strain of *D. melanogaster* at 24°C. Adult wasps were maintained in plastic vials containing two small feeder glass vials, one with water and the other with 50% honey solution. The strains of *L. boulardi* were reared on the Brazzaville strain of *D. melanogaster* (Carton and Claret 1982) at 24°C and the adults were maintained as described for the *L. heterotoma* strains.

Drosophila larvae on filter paper strips moistened with 0.2% glucose solution were exposed to parasitoid females in 3.5 × 10-cm vials for 2 hr (Rizki and Rizki 1984). Control groups of larvae were maintained on filter papers with glucose solution for 2 hr. After the wasps were removed from the vials, the *Drosophila* larvae were returned to regular food.

RESULTS

Melanotic tumor formation in parasitized hosts. Whether parasitization affects the formation of melanotic tumors was examined by exposing 46- to 48-hr-old (early third instar) *tu-W* larvae to *Leptopilina* females and subsequently examining the *tu* hosts for the presence of melanotic masses. Sib larvae not exposed to parasitoids were maintained as controls. Since encapsulated tissues in *tu-W* larvae generally blacken in the late third larval instar, larvae were examined for melanotic masses at this stage. Larvae with visible melanotic masses were dissected to determine whether they were parasitized. Individuals in which melanotic masses were not externally visible were dissected after pupariation to search for

parasitoids and small melanotic masses that had escaped detection by external examination.

The data summarized in Table I show that melanotic tumor formation was suppressed in *tu-W* larvae parasitized by the three *L. heterotoma* strains. Parasitization by *L. boulardi* L104 or G317 parasitoids did not interfere with the formation of melanotic tumors. The melanotic tumors in parasitized hosts were generally as large as those in uninfected larvae.

Lamellocytes of Drosophila hosts. To examine the effect of parasitization on host lamellocytes, parasitoid females were allowed to lay eggs in early third instar *tu-Sz^{ts}* larvae. For each experiment, one batch of larvae was not exposed to wasps and this group was the source for control hemocytes. Hemocyte samples were taken from host larvae 4–6 hr after they had been exposed to parasitoid females, and hemocytes from control groups were examined at this same time.

Lamellocytes from larvae with L104 eggs were discoidal and indistinguishable from the lamellocytes of uninfected larvae (Figs. 1A and 1B). These cells also resembled normal lamellocytes in their tendency to adhere to each other. Clumps of lamellocytes were routinely found in hemolymph samples taken from larvae with L104 eggs and uninfected *tu* larvae. Lamellocyte clumps were also present in larvae parasitized by

G317 wasps. However, not all of the lamellocytes in hemolymph samples from larvae with G317 eggs had the normal discoidal shape. Some of the lamellocytes were distorted. Others were elongated and had blunt or flared ends (Fig. 1C). The affected lamellocytes in larvae with G317 eggs did not generally develop the fine, pointed tips that were common in lamellocytes from larvae parasitized by *L. heterotoma* strains (Figs. 1D–1F). There were no apparent differences among affected lamellocytes from larvae with Mallorca, Storrs, or Leiden eggs. The present study also confirmed that lamellocyte clumps are rare in hemolymph samples from larvae parasitized by *L. heterotoma* (Rizki and Rizki 1984).

Lamellocytes of hosts with L104 eggs were plentiful and normal the day following infection. There was no evidence of lamellocyte destruction in hemolymph samples from larvae infected by G317 or L104 wasps. However, small blebs of cytoplasmic contents and masses of cellular debris were present in the hemolymph samples from larvae containing *L. heterotoma* eggs and larvae. These observations confirm the earlier report of lamellocyte destruction in hosts parasitized by the Leiden strain of *L. heterotoma* (Rizki and Rizki 1984).

An additional experiment was undertaken to determine the frequency of normal and affected lamellocytes in larvae parasitized by G317. For this study, G317 females

TABLE I
Effect of Parasitization on Melanotic Tumor Formation in *tu-W* Larvae

	No. of larvae	% Tumorous
Parasitized by *L. heterotoma*		
Leiden	116	2%[a]
Mallorca	148	6%[a]
Storrs	113	3%[a]
Parasitized by *L. boulardi*		
G317.1	106	100%
L104.1	79	100%
Unparasitized control	223	98%

[a] Ten of the 14 tumors in hosts parasitized by *L. heterotoma* strains were small and not visible by external examination.
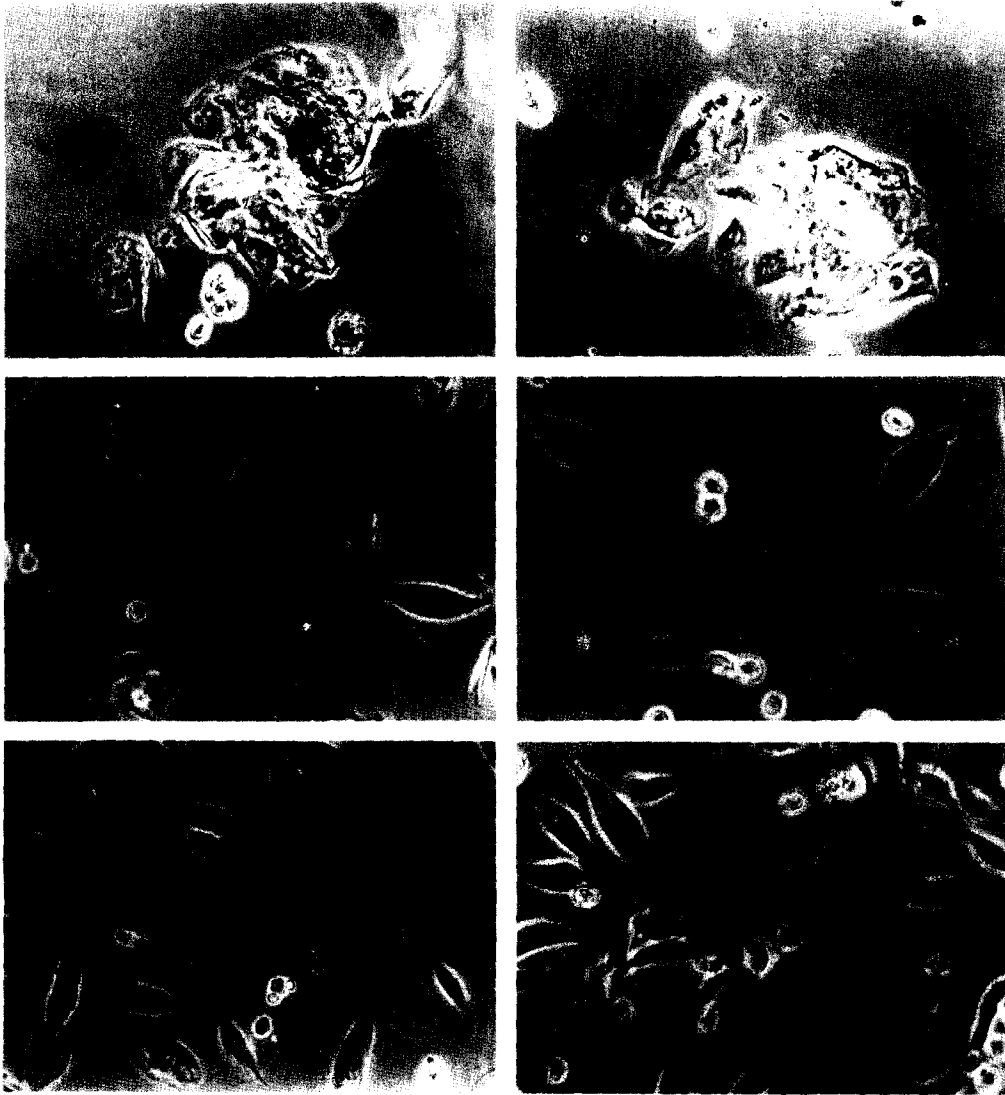
FIG. 1. Hemocytes from *tu-Sz^{ts}* larvae. (A) Cells from unparasitized larvae illustrating the adhesion of lamellocytes in a clump and the discoidal shape of a single lamellocyte (arrow). (B) Lamellocytes from larvae parasitized by L104 are indistinguishable from control cells in A. (C) Affected lamellocytes from G317 infected larvae. (D, E, and F) Lamellocytes from larvae parasitized by *L. heterotoma* Storrs, Mallorca, and Leiden strains, respectively. In all cases the lamellocytes are elongated. Bar = 100 μm.

were allowed to oviposit in 46-hr-old *tu-Sz^{ts}* larvae for 2 hr. The lamellocytes in hemolymph samples taken from these larvae at 4, 20, and 44 hr postinfection were classified as normal or affected, and the data are given in Table II. The fraction of af- fected lamellocytes remained high for the first day following infection and decreased to 6% by 44 hr postinfection. Lamellocyte frequency doubled between 4 and 20 hr, but by 44 hr it had returned to the lower frequency. Since lamellocytes are used in tu-

Hours after infection	No. hemocytes	% Lamellocytes	% Affected lamellocytes
4	3986	22	35
20	5030	40	39
44	2857	19	6

mor formation, a decrease in the circulating lamellocytes in the hemolymph of late third instar *tu* larvae is expected.

Encapsulation of parasitoid eggs. Within several hours after oviposition, hemocytes were apparent on the surfaces of L104 eggs in *tu-Sz^{ts}* larvae. The eggs were eventually encapsulated by layers of lamellocytes. The surfaces of G317, Mallorca, Storrs, and Leiden eggs remained free of hemocytes. *L. heterotoma* eggs were found floating in the hemocoel whereas most *L. boulardi* eggs were attached to host tissue surfaces (Fig. 2). Many of the latter were found adhering to the surfaces of the gut where it loops in the posterior part of the hemocoel. The adherence of G317 and L104 eggs to gut surfaces appeared to be similar, but the G317 eggs did not have hemocytes on their surfaces whereas the surfaces of L104 eggs were covered by lamellocytes.

Although the surfaces of the L104 eggs exposed to the host hemocoel were completely covered by host lamellocytes, hemocytes did not infiltrate between the egg surface and the host tissue surface. Thus, the capsules around L104 eggs remained incomplete, and parasitoid larvae were observed emerging from these ineffective capsules. The emerging wasp larva uses a springtail motion to free itself from the partially encapsulated egg membranes, and the unencapsulated portion of the egg surface is available to serve as an escape hatch. The capsules around free-floating L104 parasitoids were fully formed so it is clear that the absence of hemocytes from the egg surface apposed to host tissues re-

sults from mechanical exclusion of hemocytes rather than differences in the surface of the egg attached to the host tissues and the egg surfaces exposed to the host hemocoel.

Although *Leptopilina* females may lay more than one egg in a host larva, only one wasp completes development in a host (Carton *et al.* 1986). Supernumerary larvae are found in the hemocoel of *Drosophila* hosts, but the mechanism for the death of the supernumerary parasitoids remains unknown. Dead supernumerary *L. boulardi* larvae are individually encapsulated, and individual capsules often aggregate to form a compound capsule in the caudal hemocoel of the host (Fig. 3).

Kopelman and Chabora (1984) suggested that the attachment of *L. boulardi* eggs to host tissue surfaces occurs subsequent to oviposition in the hemocoel. Our observations on the positions of the eggs in the host suggest that the female ovipositor is instrumental in depositing eggs on the host tissue surfaces. Parasitoid eggs were found entrapped beneath bundles of tracheae suggesting that they were originally placed in these positions (Fig. 4A). More convincing were cases in which the egg filaments (pedicels) were situated between the host tissue surface and overlying tracheoles. We also found an egg on one side of the gut with its filament protruding from the opposite side of the gut (Fig. 4B). In another case, the egg filament pierced the fat body, and another egg was found in the lumen of the gut. It is likely that the ovipositor pierced these host tissues during egg deposition, suggesting
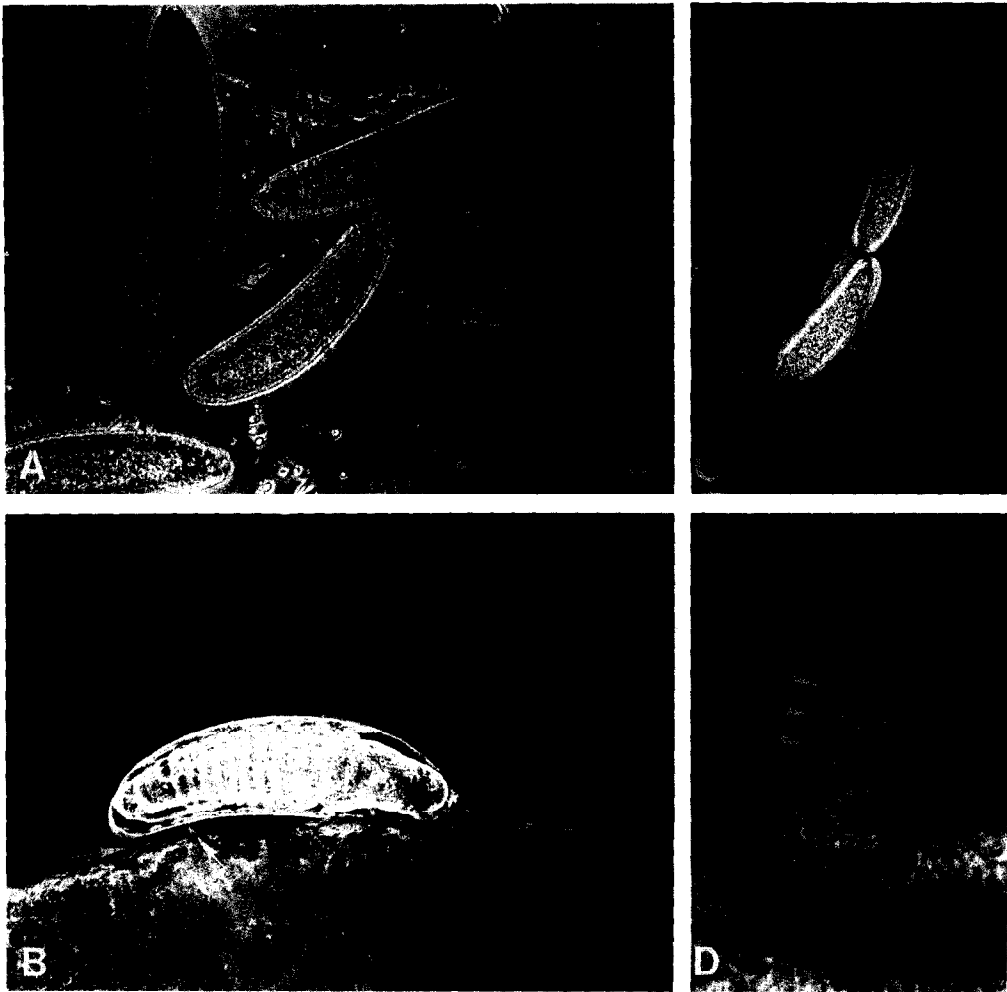
FIG. 2. (A) *L. heterotoma* (Storrs) eggs float freely in the host hemolymph. (B) *L. boulardi* (G317) egg attached to the surface of the host gut. The adhesion of the egg outer membrane to the gut surface can be seen by the contrasting stress lines indicated by the arrow. No host hemocytes adhere to the surfaces of the egg. (C) Two *L. boulardi* (L104) eggs are attached to the surface of the gut. Encapsulating hemocytes can be seen in the optical plane at the periphery of the egg. (D) After mechanical stretching of the looped region of the gut in C, one egg has been released from the surface and floated away. The other egg has been lifted from the gut surface showing that no hemocytes are attached to the surface of the egg that was adhering to the gut (arrows). Bar = 100 μm.

that the *L. boulardi* female anchors its eggs to tissue surfaces. Adhesive material must secure the egg to the solid substratum.

The numbers of free and attached eggs in the *tu-Sz^{ts}* larvae parasitized by L104, G317, and Leiden wasps were counted. For this study, 46- to 48-hr larvae were exposed to female wasps for 2 hr and 10 larvae in each group were dissected beginning about 1.5 hr later. All (23) of the *L. heterotoma* eggs were free. A single G317 egg that had no chorion and no filament was free and 75 were attached to tissues. Among the hosts with L104 eggs, 6 eggs without the chorion were free (the collapsed chorions with encapsulating blood cells of 2 of these eggs
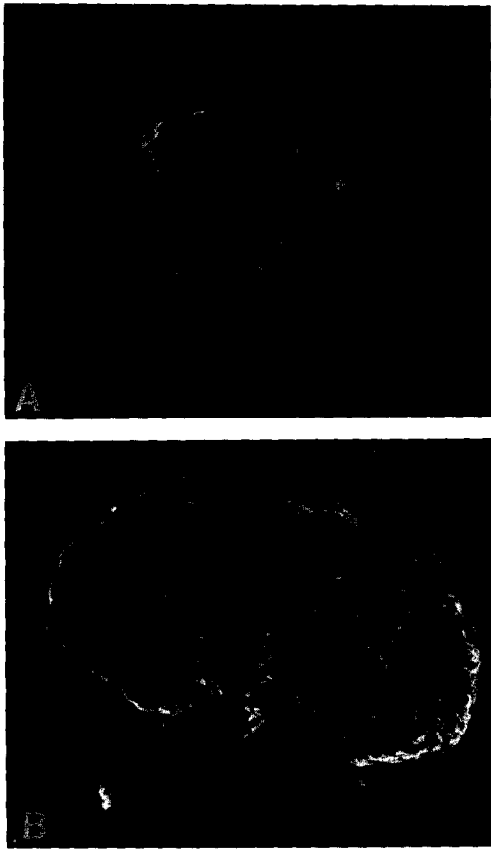
FIG. 3. Encapsulation of dead supernumerary G317 larvae. (A) A single larva surrounded by lamellocytes. (B) Aggregation of three encapsulated larvae in a single mass removed from the caudal hemocoel of the host. The capsules are not yet melanized. Bar = 100 μm.

were located attached to the gut), 1 free L104 egg with intact chorion had fat body cells attached to its side, indicating that it had broken free from its attachment to the fat body during dissection, and 58 L104 eggs were attached to host tissues. All of the attached L104 eggs had blood cells on their surfaces, but no hemocytes were found on the G317 or Leiden eggs. The observations of *L. boulardi* ruptured egg membranes and free eggs that were damaged agree with the suggestion that *L. boulardi* eggs are routinely anchored to host tissues and most free eggs of this species result during dissection of the hosts.

DISCUSSION

Leptopilina eggs are not encapsulated in *Drosophila* larvae whose lamellocytes show morphological alteration following infection. This is true for three strains of *L. heterotoma* and the G317 strain of *L. boulardi*. However, the mechanism for blocking encapsulation of *L. heterotoma* eggs and *L. boulardi* G317 eggs must differ since melanotic tumor formation is suppressed in *tu-W* hosts parasitized by *L. heterotoma* wasps but not in *tu-W* hosts infected by G317. In the case of *L. heterotoma* parasitization, the functional and structural integrity of the lamellocytes is lost (Rizki and Rizki 1984). Hence, there are no capsule-forming cells to participate in the formation of tumorous masses or in capsule formation around foreign objects, such as parasitoid eggs. Cytomorphic changes are apparent in some lamellocytes of hosts with G317 eggs. Presumably, the lamellocytes with no apparent morphological modifications form the endogenous melanotic capsules in parasitized hosts. On the other hand, participation of the affected lamellocytes in melanotic tumor formation has not been ruled out.

Several explanations may be proposed for the absence of encapsulation of the G317 egg and the concomitant encapsulation of host aberrant tissue in the same larva. The parasitoid egg surface may be coated with material that repels host hemocytes as reported for other parasitoid eggs (Salt 1965, 1980; Rotheram 1973; Davies and Vinson 1986). If this is the case, then the cytomorphic effects on some of the lamellocytes remain unexplained. An explanation that is consistent with the observed lamellocyte changes is that parasitization by G317 affects lamellocytes such that they are incapable of encapsulating parasitoid eggs but not other foreign objects. Since subpopulations of lamellocytes can be distinguished (Rizki and Rizki 1983; Nappi and Silvers 1984), perhaps only the
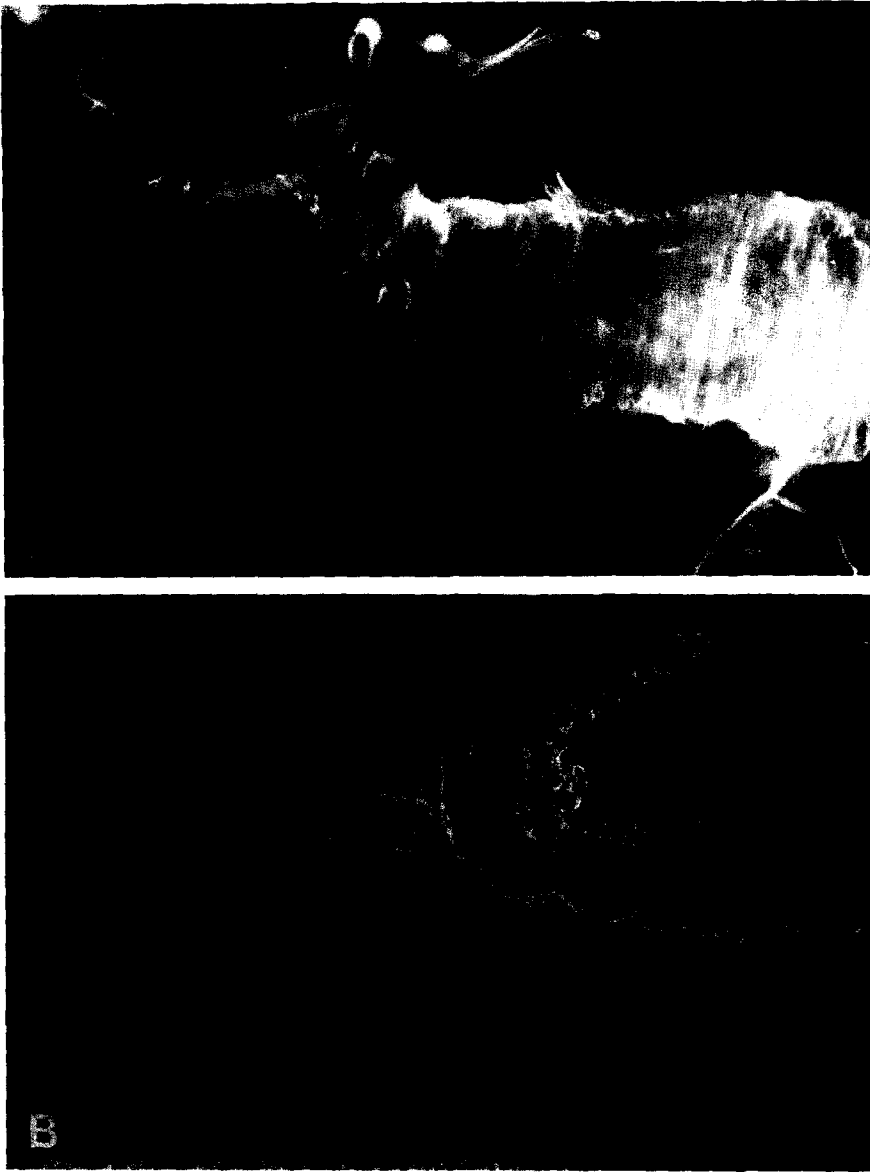
FIG. 4. (A) Two G317 eggs (e) embedded in the tracheal network of the host gut (g). The egg in the upper right is held by tracheae on both sides. The trachea on the far side of the egg (arrowhead) is not in the same focal plane as the egg. The filament of the second egg is anchored under a small trachea (arrow). (B) The filament (f) of a G317 egg piercing the gut (g) of the host larva. This positioning of the egg suggests that the female ovipositor pierced the host gut when the egg was extruded and the egg filament was positioned on the opposite side of the gut during retraction of the ovipositor. Displacement of the host gut cells can be seen at the proximal end of the filament. Bar = 100 μm.

lamellocyte subset capable of recognizing the parasitoid egg as a foreign body is inactivated by parasitization. Alternatively, parasitization by the G317 female may re-sult in differential inactivation of lamellocyte surface recognition sites such that the lamellocytes fail to recognize the wasp egg surface as nonself but distinguish dead su-

pernumerary larvae and the host aberrant tissues as nonself.

Lamellocytes in *Drosophila* larvae infected by L104 parasitoids retain a normal appearance and the ability of these cells to adhere to each other to form capsules is not affected. These hemocytes encapsulate both parasitoid eggs and prospective melanotic tumor sites in *tu* hosts. It appears that the survival of developing L104 parasitoids does not depend on interference with the encapsulation process itself but rather on the proper placement of the parasitoid egg within the host hemocoel so that lamellocytes are prevented from completely enclosing the egg. This mechanism of protecting parasitoid eggs from the harmful effects of the host's cellular defense system is mechanical rather than chemical. Presumably, it is a less effective means to combat destruction by the host's cellular defense system since the percentage of L104 wasps that complete development is lower than that of the G317 strain. It was earlier determined that, for the same level of infestation (100%), the rate of successful parasitism (percentage of adult wasps emerging) of the G317 strain is 89.8% (Carton *et al.* 1987) and that of the L104 strain is only 32% (Carton 1988), so one might conclude that the latter strain is poorly adapted to this host. Since G317 eggs are not encapsulated and L104 eggs are, it is reasonable to consider that encapsulation may limit the availability of nutrients to the L104 eggs.

The mechanism utilized by a parasitoid to avoid destruction by its host's cellular defense system must be genetically determined and have a wide norm of reaction with regard to the genetic variability of its favored host(s). The genetic variability of traits involved in the successful parasitization of *Drosophila* larvae by *L. boulardi* has been evaluated in a series of studies (Carton and Bouletreau 1984; Carton and Claret 1982; Carton and Kitano 1981; Carton *et al.* 1989). This parasitoid utilizes only *D. melanogaster* as its host, and in a few cases its

sibling species, *D. simulans,* for all of the populations studied (France, Tunisia, Italy, Brazil, California, Lesser Antilles) except the Brazzaville population which was found to infest seven different *Drosophila* species (Carton 1988). Eggs of both *L. boulardi* strains are attached to host tissue surfaces. If, as we have suggested, this placement of the egg is an adaptation for protection of the egg, then the destruction of the host's cellular defense system is not required for the survival of the parasitoid egg in this species. The survival of the parasitoid depends on its host's survival to the pupal stage, and an intact host cellular defense system confers a selective advantage for a parasitoid in the event of a secondary infection by another foreign body. It is interesting that the G317 parasitoid appears to have a dual indemnity that its egg will avoid destructive effects of host cellular defense responses. G317 eggs are attached to host tissue surfaces to guard against complete encapsulation by lamellocytes and, at the same time, host lamellocytes are affected to interfere with their encapsulating ability. Natural population studies with analyses of the responses of the *D. melanogaster* cellular defense system to *L. boulardi* strains will be useful to determine whether *L. boulardi* is evolving in the direction of the L104 or G317 survival strategy.

ACKNOWLEDGMENTS

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