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Epixenosomes: Peculiar Epibionts of the Hypotrich Ciliate *Euplotidium itoi* Defend Their Host against Predators

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ABSTRACT. *Euplotidium itoi* harbors on its dorsal surface peculiar episymbionts (referred to as epixenosomes) equipped with a complex extrusive apparatus. In the laboratory, *E. itoi* stocks without epixenosomes behave and reproduce like symbiotized stocks. The hypothesis that epixenosomes play a defensive role against predators was tested by comparing the behavior of *Litonotus lamella* when preying upon *Euplotes crassus, E. itoi* without epixenosomes, and *E. itoi* with epixenosomes. *Litonotus* discharges its toxicysts upon direct-cell-to cell contact, and paralyzes the three types of prey with the same efficiency. Nevertheless, *Litonotus* can ingest *Euplotes, Euplotidium* without epixenosomes, and to a certain extent, *Euplotidium* with epixenosomes whose ejecting capability has been inhibited, while it never eats *Euplotidium* with unaltered epixenosomes. In each prey-type, about 60% of the individuals attacked by *Litonotus* toxicyst discharge are able to recover their normal behavior once transferred into pure sea water. This percentage for *E. itoi* with epixenosomes that are never eaten by the predator corresponds to the probability of survival. This probability is lower for the other two prey-types in which the prey engulfed by the predator do not have the chance to recover. These data support the hypothesis and suggest the involvement of the epixenosome's ejecting apparatus in a defensive function.

Key Words. Alloxan, episymbiosis, extrusive apparatus, extrusome, predator-prey interaction, symbiosis.

BACTERIA epibionts of protists are widespread. The functional role of these relationships has been identified only in a few cases being found on intestinal flagellates, free-living flagellates, and sand-dwelling ciliates. In ciliates, the presence of bacteria on the external surfaces is considered a characteristic property of the sulfide fauna (Fenchel and Finlay 1991). The hypotrich *Euplotidium* harbors on its dorsal surface peculiar organisms referred to as "epixenosomes" (Verni and Rosati 1990) as their real nature (prokaryotic or eukaryotic) has not yet been established (Rosati 1999). During the reproductive stages, the epixenosomes have a bacterial-like morphology and divide like prokaryotes, but in the mature stage they have a greater structural complexity with which a functional compartmentalization is associated (Rosati, Giambelluca and Taiti 1996). The most prominent structure is a complex extrusive apparatus (Rosati, Lenzi and Verni 1993) whose ejection is triggered by external signals mediated by membrane receptors (Rosati et al. 1997). Ejection is easily recognized under the light microscope by the appearance of rigid filaments (~ 40 μm long) nearby the ciliate. For this reason, epixenosomes were previously described as exocytotic organelles (Ito 1958; Tuffrau 1985); once ejected, they look like discharged trichocysts, i.e. extrusomes whose defensive function against predators has been demonstrated recently in *Paramecium* (Harumoto and Miyake 1991; Miyake and Harumoto 1996).

Natural populations of *E. itoi* are always found in association with epixenosomes. Every *E. itoi* specimen analyzed soon after collection, in the course of several years, carried the typical band of epixenosomes (Rosati 1999). In the laboratory *E. itoi* stocks tend to lose epixenosomes when a moderate starvation slows their cell cycle, although they maintain their symbionts indefinitely when regularly fed. The lack of epixenosomes does not modify either the behavior or the fission rate of the ciliate (Giambelluca and Rosati 1996). The observation that, in a laboratory environment, *E. itoi* can live and reproduce even without epixenosomes, led us to hypothesize that, in the natural environment, the presence of the episymbionts could play a crucial role, such as defense against predators. The contemporaneous availability of *E. itoi* stocks with and without epixenosomes allowed us to experimentally verify this hypothesis. *Litonotus lamella,* a raptorial feeding ciliate that shares its habitat with *E. itoi,* was chosen as the predator in this study. The feeding behavior of *L. lamella* involves several basic steps (Ricci and Verni 1988; Ricci, Morelli, and Verni 1996): the detection of prey from a distance, the discharge of toxicysts upon direct cell-to-cell contact, the search for the stricken prey, and the ingestion of the prey. As the whole process has been widely described and analyzed from different perspectives mainly using *Euplotes crassus* as prey (Morelli and Verni 1996; Ricci and Verni 1994; Ricci, Morelli and Verni 1996; Verni 1985; Verni and Gualtieri 1997), the hypothesis that epixenosomes function as defensive devices was tested by comparing *E. crassus, E. itoi* without epixenosomes, and *E. itoi* with epixenosomes as prey for *L. lamella.* The results strongly supported the hypothesis.

MATERIALS AND METHODS

All species used in this study were originally collected in tide pools along the sea shore near Leghorn (Italy) and then cultured in our laboratory. *Litonotus lamella* was fed on *Euplotes crassus* (Ricci and Verni 1988) and *E. crassus* was fed on the green flagellate *Dunaliella salina. Euplotidium itoi* was cultured with mixtures of the diatom *Pheodactylum tricornutum* and the flagellate *Chilomonas.* The food organisms were added in different amounts to different stocks. In this way more or less rapid fission rates were obtained. Before starting the experiments described below, specimens from each stock were checked for the presence of epixenosomes with the light microscope and with the scanning electron microscope. Stocks E2b and E13c were chosen from among those without and with epixenosomes, respectively. All experiments were carried out in glass depressions at 22 ± 1° C.

Experiment 1. In order to compare the feeding rates of *L. lamella* against *E. crassus, E. itoi* without epixenosomes (E2b), and *E. itoi* with epixenosomes (E13c), 30 starved *L. lamella* were placed in each of three glass depressions, each containing 0.2 ml sea water and 10 specimens of one different potential prey-type. After 10, 20, 40, 70, 80, 100, 120, 130, 140, 160, 180, 190, 200, 220, and 240 min, surviving individuals were counted in each depression. The procedure was repeated 15 times. The mean value ($\bar{x}$) and Standard Error (SE) were then calculated.

Experiment 2. The predatory behavior of *L. lamella* against *E. crassus, E. itoi* E2b, and *E. itoi* E13c was analyzed step-by-step by placing a single *L. lamella* in 0.25 ml of ~ 6,000 individuals/ml culture of each potential prey-type. In each case, the number of paralyzed individuals and the number of individuals engulfed by the predator within 20 min were recorded by direct observation. The observations were repeated 21 times for each prey-type. In parallel, specimens of each prey-type were taken away soon after they were paralyzed by the predator

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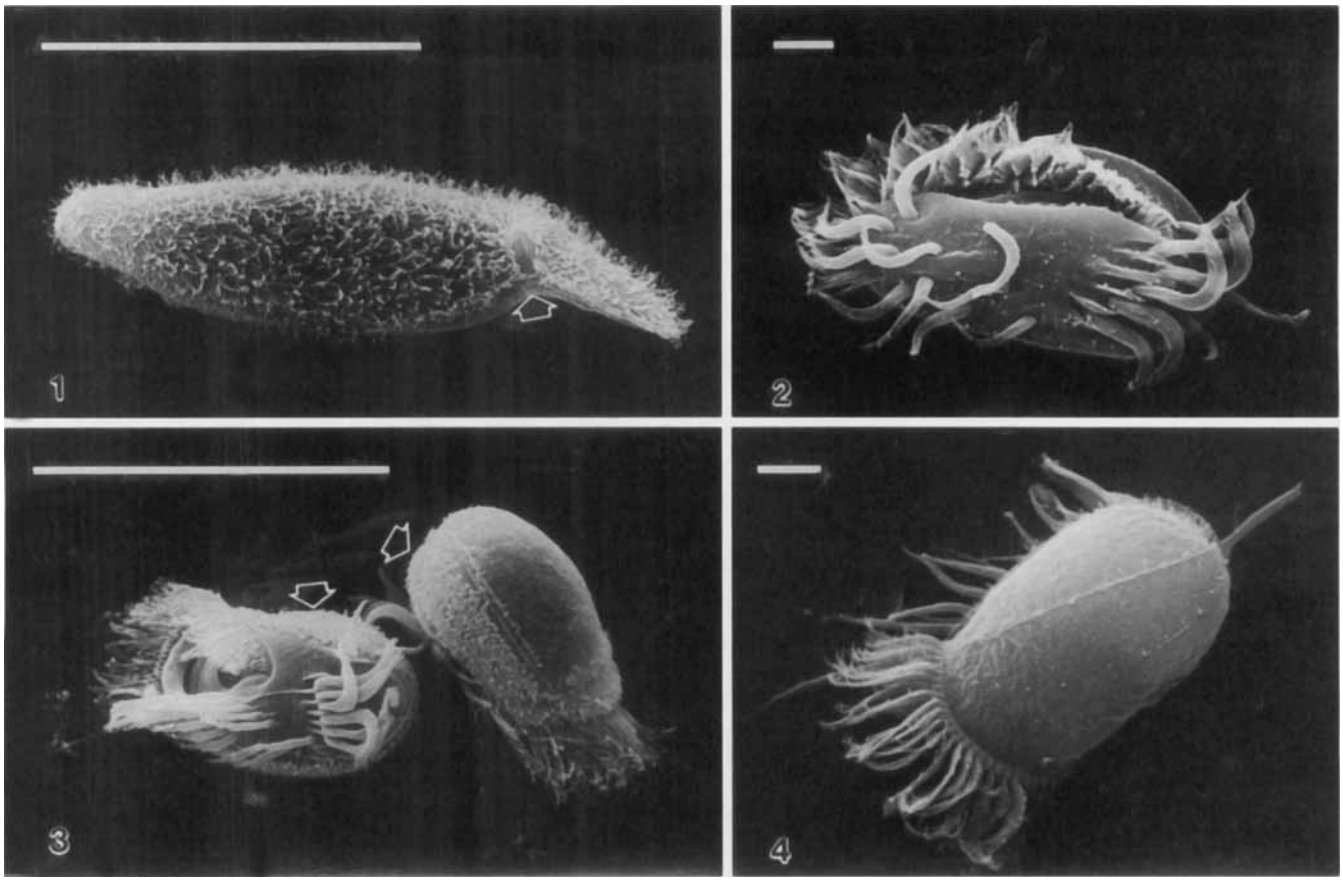

Fig. 1–4. Scanning electron micrographs of *Litonotus, Euplotes* and *Euplotidium*. **1**. *Litonotus lamella*. Ventral view. Arrow points to the cytostome. Bar = 100 μm. **2**. *Euplotes crassus*. Ventral view. Bar = 10 μm. **3**. Two *Euplotidium itoi* with epixenosomes (stock E13c) showing the ventral surface (the one at the left) and the dorsal surface, respectively. Arrows indicate epixensomes inserted along a lateral band. Bar = 100 μm. **4**. *E. itoi* without epixenosomes (stock E2b). The cortical region corresponding to the epixenosomal band is smooth and empty. Bar = 10 μm.

and transferred to pure sea water. The number of individuals that recovered their normal behavior was counted after 60 min.

Experiment 3. The possibility that the ejection of epixenosomes is involved in defending *E. itoi* against *L. lamella* was investigated by using alloxan treatment (Rosati et al. 1997). Samples of both *E.itoi* E2b and *E. itoi* E13c were concentrated by mild centrifugation and treated by the addition of alloxan (Sigma A-8128, St. Louis, MO USA) at 6.67 mM final concentration. After 15 min, the samples were resuspended in pure sea water, concentrated, and resuspended again to eliminate any residual alloxan. Then 10 untreated *E. itoi* E2b, 10 alloxan-treated *E. itoi* E2b, 10 untreated *E. itoi* E13c, and 10 alloxan-treated *E. itoi* E13c were placed in different slide depressions each containing 0.2 ml sea water with 30 *L. lamella*. Every 10 min over 2 h the number of surviving prey was counted. The procedure was repeated 15 times for each potential prey-type.

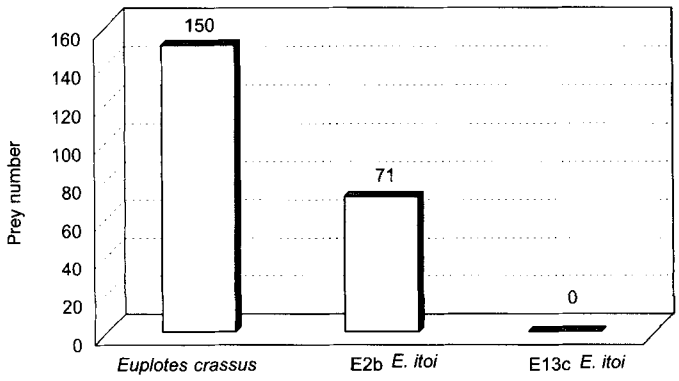

Fig. 5. Total numbers of each prey type, *Euplotes* and *Euplotidium* without (E2b) and with (E13c) epixenosomes, eaten by *Litonotus lamella* during 15 replicates in which 10 prey individuals were in the presence of 30 predators for 4 h (Experiment 1).

Scanning electron microscopy. Specimens of all species were fixed with either 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 and postfixed in 2% OsO_4 in artificial sea water or in 2% OsO_4 in sea water alone. They were then placed on poly-L-Lysine hydrobromide-coated coverslips, dehydrated in ethanol, and critical point dried, coated with gold, and examined with a JEOL/JSM-5410.

RESULTS

The predator *L. lamella* and the potential prey-types *E. crassus, E. itoi* without epixenosomes (stock E2b), and *E. itoi* with epixenosomes (stock E13c) are shown in Fig. 1–4.

Experiment 1. Feeding rate of the predator against *E. crassus, E. itoi* without epixenosomes, and *E. itoi* with epixenosomes. The observations, in which the prey (paralyzed or not) still present were counted, were repeated at intervals so that the toxicyst discharge and its paralyzing effect could be followed and correctly interpreted. The rates of feeding on the

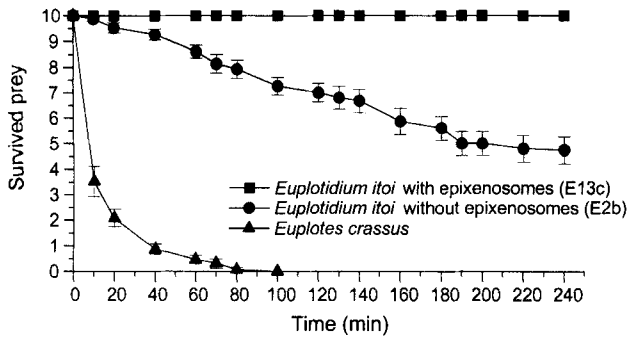

Fig. 6. Comparison of the feeding rates of *Litonotus lamella* against the three different prey-types. Each symbol represents the mean ± SE of 15 replicates. Note that *Euplotes crassus* was eaten earlier and more efficiently than *Euplotidium itoi* E2b, while all *E. itoi* E13c were still surviving at the end of the experiment.

three potential prey-types by *L. lamella* clearly differed. Significant differences appeared when the total numbers of individuals, belonging to each type of prey, eaten by predators during 15 observations were compared: 150 *Euplotes* (i.e. all those available), 71 *E. itoi* E2b, and 0 *E. itoi* E13c (Fig. 5). *Euplotes crassus* was eaten more quickly than *E. itoi* E2b: most *Euplotes* were eaten within 10 min and none escaping predation within 100 min; under the same conditions, *E. itoi* E2b was eaten more slowly and at a lower rate with slightly less than 50% ($\bar{x}$ = 4.73; SE = 0.52) escaped predation after 4 h; and *E. itoi* E13c were never eaten—all of them could be found in the container, still alive although almost all paralyzed, at the end of the experiments (Fig. 6).

Experiment 2. Toxicyst discharge, predation index, and predation efficiency. The predatory behavior of single *L. lamella* against an excess of the three different prey-types (~ 6,000 specimens/ml in each case) was followed step-by-step over 20 min. Thus, the entire predatory process was reconstructed. The majority of *E. crassus* and *E. itoi* E2b were either paralyzed or eaten while *E. itoi* E13c were only paralyzed (Fig. 7). This translated into increasing survival probabilities for these different prey-types (Table 1). In a parallel series, about

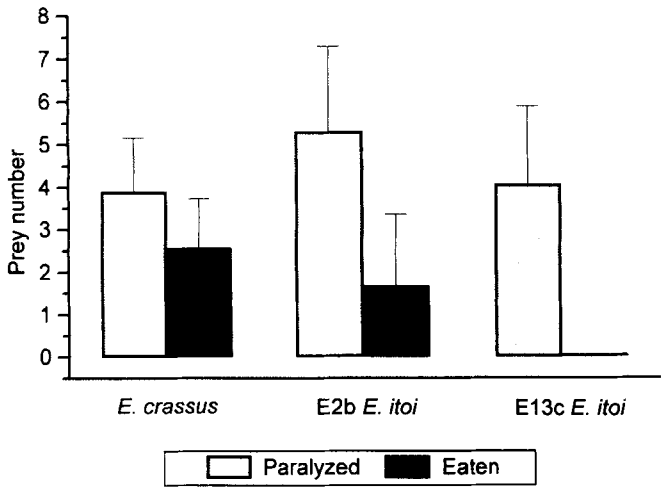

Fig. 7. Mean and Standard Deviation (SD) of paralyzed and eaten individuals of each prey-type, *Euplotes crassus* and *Euplotidium itoi* without (E2b) and with (E13c) epixenosomes, during 21 replicates in which a single predator was exposed to an excess of prey (Experiment 2). Note that *E. itoi* E13c were paralyzed but not eaten.

Table 1. Presumptive survival probability of paralyzed *Euplotes crassus* and *Euplotidium itoi* without (E2b) and with (E13c) epixenosomes against *Litonotus*.

Prey-types	Paralyzed total number	Eaten total number	Not eaten irreversibly damaged[a]	Not eaten able to recover[b]	Survival probability
E. crassus	82	54	11	17	0.21
E2b *E. itoi*	111	35	30	46	0.41
E13c *E. itoi*	85	0	34	51	0.60

[a] 40% of paralyzed cells not eaten.
[b] 60% of paralyzed cells not eaten.

60% of each prey-type, paralyzed upon discharge of *L. lamella* toxicysts, recovered normal behavior in a short time, once transferred to pure sea water. The remaining 40% appeared to be irreversibly damaged (Table 1).

The following indices were calculated to compare the predation behavior of *Litonotus* on the potential prey-types: 1) toxicyst discharge index (DI) as the number of discharging *L. lamella*/total number of *L. lamella;* 2) discharge efficiency (DE) as the number of paralyzed prey-individuals/number of discharging *L. lamella;* 3) Predation index (PI) as the number of *L. lamella* that engulfed prey/total number of *L. lamella*; 4) predation efficiency (PE) as the number of prey-individuals eaten/number of *L. lamella* that ingested them. There were no significant differences in DI and DE with respect to the three different prey-types (Table 2); thus, all *L. lamella* discharged their toxicysts upon direct contact against *E. crassus, E. itoi* E2b, and *E. itoi* E13c and paralyzed them with a comparable efficiency. On the other hand, obvious differences emerged when PI was considered: while *Litonotus* tested against *E. crassus* engulfed all the prey it paralyzed (PI = 1) not all *L. lamella* that discharged their toxicysts against *E. itoi* E2b were able to ingest the paralyzed prey (PI = 0.67) (Table 2). However, those that ingested this kind of prey did so with the same efficiency observed against *Euplotes*: their PEs were not significantly different (t = 2.02; p = 0.05) (Table 2). Finally, in agreement with the results of Experiment 1, no *Litonotus* succeeded in ingesting *E. itoi* with E13c (PI = 0) (Table 2).

Experiment 3. Feeding rate of the predator against *E. itoi* E2b and *E. itoi* E13c treated with alloxan. *Euplotidium itoi* E13c were treated with alloxan so that their symbionts were deprived of their ejecting capacity. Alloxan-treated *E. itoi* E2b were used as controls to verify whether the treatment itself influenced the predator-prey interaction. No significant differences (Fig. 8) were observed in the predator feeding rate against treated and untreated *E. itoi* E2b (t = 0.77 at 120 min: P =

Table 2. Discharge and predation indices of *Litonotus* feeding on *Euplotes crassus* and *Euplotidium itoi* without (E2b) and with epixenosomes (E13c).[a]

	E. crassus	E2b *E. itoi*	E13c *E. itoi*
DI	1.0	1.0	1.0
DE	3.9 ± 1.2	5.3 ± 2.0	4.0 ± 1.8
PI	1.0	0.67	0
PE	2.6 ± 1.2	2.5 ± 1.5	0

[a] DI = number of discharging *L. lamella*/total number of *L. lamella*; DE = number of paralyzed prey-individuals/number of discharging *L. lamella*; PI = number of *L. lamella* that engulfed prey/total number of *L. lamella*; PE = number of prey-individuals eaten/number of *L. lamella* that ingested them.

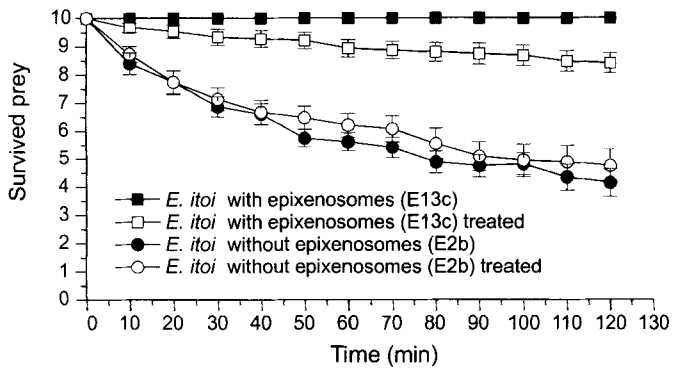

Fig. 8. The feeding behavior of *Litonotus lamella* against *Euplotidium itoi* E2b and E13c untreated and treated with alloxan. Each symbol represents the mean ± SE of 15 replicates. No significant differences exist between treated and untreated *E. itoi* E2b ($t = 0.77$, $p = 0.45$). While all untreated *E. itoi* E13c survived, some of the treated ones were eaten.

0.45); on the other hand, while all untreated *E. itoi* E13c were still surviving after 120 min (as expected on the basis of the experiments described above), some alloxan-treated-euplotidia of the same stock were eaten by *L. lamella* in almost all the experiments (Fig. 9).

DISCUSSION

The results reported here strongly support the hypothesis that epixenosomes provide *E. itoi* with an efficient defensive tool against predation. Indeed, in our experiments, *Litonotus lamella* discharged its toxicysts upon direct cell-to-cell contact, with the same efficiency against *E. crassus, E. itoi* without epixenosomes, and *E. itoi* with epixenosomes. Nevertheless, while it was able to ingest the former two prey-types, it never ingested the latter. This means that something prevents the predator from finding and eating paralyzed *E. itoi* bearing epixenosomes. From our results, it appeared that the predatory efficiency of *L. lamella* towards *E. itoi* without epixenosomes is quite a bit lower than towards *E. crassus*. We used only *L. lamella* from populations grown in our laboratory and fed with *E. crassus*. So it is possible that, as demonstrated for *Didinium nasutum* (Berger 1979), *Litonotus* prefers the prey species upon which it has been reared. On the other hand *E. crassus* has been reported, together with *Euplotes vannus,* as the most suitable prey even for ex novo-collected *L. lamella* (Ricci, Morelli and Verni 1996). More surprising is the finding that the predatory behavior of *L. lamella* may also be different towards organisms of the same species, namely *E. itoi* E2b and *E. itoi* E13c. *Euplotidium itoi* with epixenosomes, although paralyzed upon toxicyst discharge, enjoyed a higher probability of escaping death, certainly higher than that of *E. itoi* without epixenosomes (Fig. 9, Table 2). Since the main difference between *E. itoi* E2b and *E. itoi* E13c stocks was the lack of episymbionts in the former, it may be concluded that epixenosomes themselves defend paralyzed *E. itoi* against engulfment. It may be assumed that, in the natural environment, where the toxic substance of the toxicysts can be rapidly dispersed, the percentage of paralyzed individuals able to recover corresponds to the one observed in the laboratory. Thus, the survival probability, following the attack of the predator, should be high for symbiotized *E. itoi,* even in their natural habitat.

How can these peculiar episymbionts play this defensive role? Predator-prey interactions are necessarily mediated by the potential prey's body surface. Harumoto and Miyake (1991) hypothesized that different surface antigens in *Paramecium* may have different protective values against a predator by averting its recognition to different extents. Epibiosis too should have an impact on predator-prey interaction by creating a new interface between the epibiotized organism and its environment. Investigations on marine invertebrates showed that some epibionts strongly affect predator's behavior (Whal, Hay and Enderlein 1997). The interface between *Euplotidium* and its environment varies depending on the presence or absence of epixenosomes; this might account for the different interactions with the predator. However, based on the results of Experiment 3 in which some alloxan-treated *E. itoi* E13c were ingested by *L. lamella* it can be inferred that the ejection itself plays a defensive function. Thus, it is possible that the toxicyst discharge by the predator functions as the stimulus triggering the ejecting process; the ejected tubes (Fig. 10) might perturb the "toxic area" in which *L. lamella* is supposed to release a substance capable of guiding it towards the stricken prey (Ricci and Verni 1994).

As previously demonstrated (Rosati et al. 1997) a mild treatment with alloxan, an inhibitor of the enzyme adenylate cyclase, prevents the ejection of the epixenosomal extrusive apparatus. The fact that the feeding behavior of *Litonotus* against

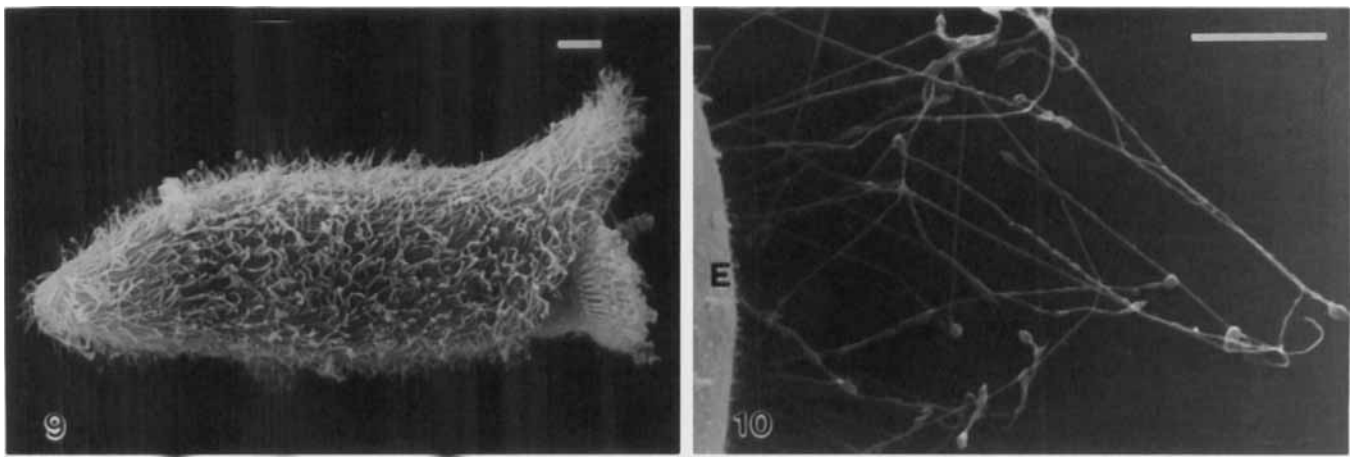

Fig. 9. Scanning electron micrograph of *Litonotus lamella* that has engulfed *Euplotidium itoi* without epixenosomes (E2b). Bar = 10 μm.

Fig. 10. Scanning electron migrograph of a portion of *Euplotidium itoi* E13c (E) with ejected epixenosomal tubes nearby. Bar= 10 μm.

untreated and alloxan-treated *E. itoi* E2b did not show significant differences indicates that the predator-prey interaction is not influenced by the treatment itself. Several types of antipredator defenses have been reported in ciliates. Predator-released signals are known to induce defensive phenotypic modifications in a variety of species (for review see Wicklow 1997). In a recent study, the benefits and the costs of predator-induced morphological changes in *Colpidium kleini* (Fyda and Wiackoski 1998) have been considered; the predator needs significantly more time and repeated attacks to catch and engulf the transformed prey but the prey pays a demographic price: the growth rate of induced phenotypes is reduced by about 25%. A defensive function of extrusomes, particularly trichocysts in *Paramecium,* has also been demonstrated against ciliates that feed in quite different ways. Trichocyst discharge from an attacking predator quickly dislocates the *Paramecium* out of range of the offensive organelles (Harumoto and Miyake 1991; Miyake and Harumoto 1996). An "economic" defensive system to facilitate the survival of the population has been reported in *E. crassus* (Morelli and Verni 1996). A small number of individuals of this species seems to produce and to emit into the medium a substance repulsive to the predator *Litonotus.* This system is active independently of the presence of the predator so there is no additional cost (Morelli and Verni 1996). As far as we know, the only case in ciliates in which protection against predators is apparently supplied by symbionts is that of *Paramecium bursaria* and its endosymbiotic zoochlorellae. The latter mutualistic organisms tend to discourage predation by *Didinium nasutum* by releasing distasteful metabolites that repel them (Berger 1980). In the present study, the defensive role of episymbiotic organisms has been demonstrated for the first time in ciliates. This defensive function could account for the apparent absence of *E. itoi* without epixenosomes in the tide pools from which all our experimental organisms were collected. Presumably, *E. itoi* may lose their symbionts in their natural environment as well (for example following a food supply reduction) and thus become suitable prey for *Litonotus* and, very likely, for other predators living in the same habitat.

The association between *Euplotidium* and epixenosomes is probably ancient and definitely well-established. At least in the case of *E. itoi* multiplication, redistribution between the two offspring cells, and the transformation of epixenosomes from the reproductive to the mature form are well-coordinated with the reproductive cycle of the ciliate (Giambelluca and Rosati 1996). Moreover, there is evidence that not only the ciliate cells influence epixenosomes, but also epixenosomes themselves influence the ciliate (Giambelluca and Rosati 1996; Rosati 1999). From the present study it appears that the reciprocal nature of this *Euplotidium*-epixenosomes symbiosis includes a protective function for the epixenosomes. The undoubted advantage conferred to *Euplotidium* by this function is very likely an important factor in stabilizing and maintaining such a specialized symbiotic relationship.

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