INCORPORATION OF BODY COMPONENTS OF DIVERSE MICROORGANISMS BY LARVAL MOSQUITOES

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ABSTRACT. A pulse-purge schedule of exposure to labeled microorganisms was used to compare their digestibility by larval mosquitoes. Larvae were placed for an hour in suspensions of diverse axenically grown microorganisms that had been labeled with radioactive carbon (in the form of glucose or glycine). The guts of these mosquitoes were then purged with nonlabeled Sephadex® particles for 30 min, and retained radioactivity was measured. Larvae imbibed no dissolved material. Larval mosquitoes differ in their capacity to derive label from algae (*sensu lato*), and certain algae contribute more label to these mosquitoes than do others. The nature of any algal food, as well as the feeding habits and developmental stage of the larva, influence its capacity to derive label from algae. This pulse-purge method of analysis can assist in the selection of algal "vectors" suitable as vehicles for transgenic larvicide. Although larval mosquitoes fail to assimilate the contents of *Palmellacoccus* cells with which they are confined, as much as ⅓ of the body contents of a *Euglena gracilis* cells become incorporated into their bodies. Because larval mosquitoes internalize more material from *Euglena* than they do from various other algae, these microorganisms provide a promising candidate vehicle for transgenic *Bacillus thuringiensis israelensis*.

INTRODUCTION

A variety of approaches have been used to evaluate the digestibility of microorganisms by larval mosquitoes. Ingested material can be identified visually (Laird 1988, Walker et al. 1988) or by plating out samples of gut contents (Thiery et al. 1991). The digestive process itself, however, negates the usefulness of any technique that depends upon viability and subsequent growth. Digestibility can be compared indirectly by maintaining larvae monoxenically in pure algal cultures and observing rate of larval development. This laborious method is useful solely with microorganisms that can be grown in pure culture and that provide all nutrients required by the developing larvae. A method is needed for determining digestibility of algae and other microorganisms by mosquitoes that is applicable to any such food particle and that directly measures incorporation.

To compare the incorporation of various microorganisms by diverse mosquitoes, larvae were held in standardized suspensions of candidate foods in a pulse-purge schedule of exposure. Larval mosquitoes were exposed to ^{14}C-labeled microorganisms, then purged and their residual radioactivity measured. We compared the amount of label transferred from candidate food particles to the larval mosquitoes that ingested them.

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MATERIALS AND METHODS

The *Culex pipiens* Linn. mosquitoes used in this work were isolated in 1991 in Boston, MA, and maintained autogenously thereafter. The anautogenous "Rock" strain of *Aedes aegypti* (Linn.) also was used. Larvae were fed Purina guinea pig chow and maintained in covered pans.

The microorganisms used were chosen because their cell envelopes differed in a manner that might affect their digestibility by mosquitoes (Table 1). Each is ubiquitous and abundant in the freshwater bodies of water commonly infested by mosquitoes. Each multiplies rapidly in culture. The 7 selected species represent 5 of the dozen or so classes classically considered to include the algae. The blue-green "algae" (Cyanophyta), however, should more properly be considered as bacteria (Cyanobacteria), and *Euglena* is commonly considered to be a protozoan (Laird 1990).

Microorganisms were grown axenically in the media listed in Table 1 except that the *Ochromonas* medium was prepared without liver extract. Each culture was maintained on a rotary shaker at 25°C with illumination of 32 μ E m² sec supplied by equal numbers of cool-white and red fluorescent tubes. Cultures were harvested by centrifugation. Cultures were checked for bacterial contamination on the day before each experiment by plating on standard LB agar medium and were examined microscopically. No cultures were found to be contaminated.

To label food particles, harvested microorganisms were washed twice with fresh medium, lacking sugar. The centrifuged pellet was weighed, and a 50-mg sample was resuspended in 1.0 ml sugarless medium. Cells were labeled with 5 μCi of labeled U-[14C] glucose (ICN, Irvine, CA), or

Table 1.   Microorganisms used in this study.

| Alga class | Species | Envelope | | Medium |
		Wall	Base	
Cyanobacteria	*Anabaena variabilis*	Rigid	Peptidoglycan	Rippka et al. (1979)
Rhodophyta	*Cyanidium caldarium*	Gelatinous	Pectin	Beale and Chen (1983)
Chlorophyta	*Chlorella vulgaris*	Rigid	Cellulose	Weinstein and Beale (1985)
	Scenedesmus bijugatus	Rigid	Cellulose	Weinstein and Beale (1985)
	Palmellacoccus protothecoides	Rigid	Sporapollenin	Weinstein and Beale (1985)
Euglenophyta	*Euglena gracilis*	Discontinuous	Protein	Beale et al. (1981)
Chrysophyta	*Ochromonas danica*	—	—	Starr and Zeikus (1987)

of 2-[^{14}C] glycine in the case of *Anabaena variabilis*. After 12 h of incubation at room temperature, the cells were washed twice with 10 ml of tap water and resuspended in 1 ml of tap water.

To pulse-label larval mosquitoes, 300 μl of washed cell suspension containing 16.7 mg of food particles was added to vessels containing 10 ml of tap water and 10 larval mosquitoes that had been held for 1 h in tap water. Each such assay was run 3 times. After 1 h, larvae were washed and food particles suspended in the medium were removed by straining. To purge larvae of labeled food particles, they were washed in 50 ml tap water and transferred to vials containing 10 ml tap water and 60 mg Sephadex® G-25 beads (mean diam 20 μm). After 30 min, larvae were separated from the beads by straining and washed once again in 50 ml tap water. To measure radioactivity retained by these larval mosquitoes, they were drained and placed in 10 ml Aquasol-2 scintillation liquid for at least 12 h. The decomposed larvae were counted by scintillation. The total amount of retained food-derived label was calculated by dividing the amount of label in 10 washed and purged larvae by the amount originally added to the medium. This parameter was expressed as a percent.

RESULTS

In a preliminary series of observations, we determined how rapidly larvae accumulate label from algae to which they were exposed. Thus, 4th-instar larval *Culex* were held for various periods of time in a suspension of labeled (95 kcpm per sample) alga (*Euglena*), then purged in Sephadex for an hour and the quantity of the retained label assayed. Label accumulated most rapidly in the bodies of these mosquitoes during the first 10 min after initial exposure and tended to plateau after a half hour or so (Fig. 1). An hour of exposure to labeled algae, thereafter, would permit estimates of the ability of a larva to accumulate label.

Another preliminary series of experiments was designed to determine how long larvae should be purged to insure that no labeled algal particles remain in their guts. In this experiment, 4th-instar larval *Cx. pipiens* were placed in a suspension of labeled (95 kcpm per sample) *Euglena* for an hour and subsequently held in Sephadex for various periods of time; the quantity of label retained in their bodies was then assayed. Label diminished most rapidly from the bodies of these mosquitoes during the first 10 min after purging began and remained constant thereafter (Fig. 2). A half-hour of exposure to inert Sephadex appears to be sufficient to flush labeled algae from the guts of larval mosquitoes.

We then determined whether mosquitoes differ in their capacity to derive label from an array of diverse microorganisms. Thus, starved 3rd-
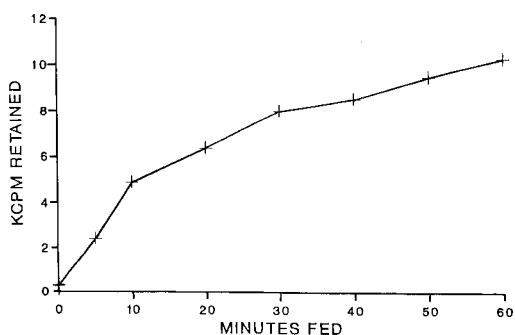

Fig. 1.   Rate of accumulation of label in 10 larval *Culex pipiens* mosquitoes that were permitted to feed on ^{14}C-labeled *Euglena gracilis*. Each test contained 10 larvae and was run 3 times.
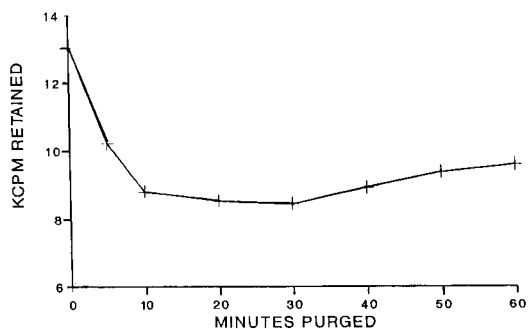
Fig. 2. Rate of loss of label from 10 larval *Culex pipiens* mosquitoes that were purged by exposure to inert Sephadex beads after they were permitted to feed on ^{14}C-labeled *Euglena gracilis*. Each test contained 10 larvae and was run 3 times.

Table 3. Proportion of total ^{14}C inoculum retained in the bodies of larval *Culex pipiens* exposed to radiolabeled microorganisms for 1 h and purged for 30 min with inert Sephadex beads. Each test includes 10 larvae and was run 3 times.

Kind of food	^{14}C (kcpm)/ 10 ml medi- um	Instar	% label retained	
			Mean	Range
Euglena	780	2nd	2.7	2.4–3.0
		3rd	18.3	17.9–18.7
		4th	35.9	32.8–39.0
Cyanidium	780	2nd	3.2	3.0–3.4
		3rd	6.3	6.0–6.6
		4th	7.6	7.2–8.0
Chlorella	780	2nd	1.3	1.1–1.5
		3rd	3.6	3.2–3.9
		4th	6.2	6.0–6.4

instar larvae of diverse genera were held for an hour in the presence of various labeled algae. Their gut contents were then purged, and the label that they retained was measured. Larval *Cx. pipiens* retained far more label from *Euglena gracilis* than from other algae that were tested, and far less from *Palmellacoccus protothecoides* (Table 2). Other algae contributed intermediate amounts of label. Larval *Ae. aegypti,* in contrast, retained far more label from *Anabaena* and from *Scenedesmus* than did *Cx. pipiens*. Replicates were similar. We concluded that larval mosquitoes differ in their capacity to derive label from algae and that certain microorganisms contribute more label to these mosquitoes than do others.

The capacity of larvae of different instars to derive label from an array of diverse food particles was compared. Thus, starved larval *Cx. pipiens* were held in the presence of various labeled particles. Their gut contents were then purged, and the label that they retained was measured. More label was retained by relatively mature larvae than by less mature larvae, regardless

of the food that was used (Table 3). The differential capacity of mature larvae to retain label was greater when the larvae were exposed to *Euglena* than to other microorganisms that were tested. Replicates were similar, varying by no more than 10% of the mean value. This observation indicates that the ability of a larva to digest and assimilate food particles varies with the nature of that food and with the species and stage of the mosquito.

We considered the possibility that larvae may acquire dissolved label directly from the medium. Toward this end, we dissolved 5μCi of ^{14}C-glucose in 10 ml of water containing 10 larval *Cx. pipiens* and washed the larvae after 1 h. They were exposed to Sephadex beads for 30 min and washed once again. The experiment was repli-

Table 2. Proportion of the total ^{14}C inoculum retained in the bodies of 3rd-instar larval mosquitoes that were exposed to diverse radiolabeled microorganisms for 1 h and subsequently purged for 30 min with inert Sephadex beads. Each test included 10 larvae and was run 3 times.

Kind of food	^{14}C (kcpm)/10 ml medium	% label retained by			
		Culex pipiens		*Aedes aegypti*	
		Mean	Range	Mean	Range
Euglena	780	17.7	16.9–18.5	—	—
Cyanidium	707	9.8	9.2–10.4	—	—
Ochromonas	129	8.8	8.3–9.2	7.1	6.9–7.2
Chlorella	623	6.2	6.0–6.4	—	—
Scenedesmus	369	5.9	5.4–6.4	10.8	10.5–11.1
Anabaena	155	5.1	4.8–5.4	18.9	18.2–19.6
Palmellacoccus	59	0.3	0.2–0.4	—	—

cated twice. Only background levels of radio-activity (<0.01% of applied label) were detected in these mosquitoes. We concluded, therefore, that these mosquitoes imbibed no dissolved material.

DISCUSSION

Our evidence that different microorganisms contribute more of their substance to larval mosquitoes than do others is based on a novel assay system. Previous studies on larval nutrition have approached the problem in different ways: 1) microscopic analysis of material contained in larval guts (Merritt et al. 1992), 2) experiments in which organisms removed from the guts of mosquito larvae were cultured in selected media (Thiery et al. 1991), and 3) observations on the growth of larvae exposed to selected nutrients provided in isolation (Marten 1986). The usefulness of microscopic analysis for this purpose is limited because material that is readily digested would more rapidly disappear than would poorly digested material. This method, therefore, biases the results away from highly nutritious material and toward the indigestible (Laird 1988). The culture method of analysis responds to this problem, but unjustifiably assumes that anything that is killed in the gut is digested. The method based on larval growth responses is limited by its indirect nature and by the necessity of providing only a single nutrient source. Harvestable, digestible, and absorbable material, for example, would appear not to be utilized if it failed to provide a complete source of nutrition or if it were toxic. Our more direct and quantitative pulse-purge method of comparison rapidly provides information that is not confounded by any failure of larvae to thrive.

The structure of the envelope of a microorganism would profoundly affect its digestibility by mosquitoes (Ragan and Chapman 1978). Those microorganisms that have rigid walls might be most resistant. Cellulose components characterize the walls of the various *Chlorella* and *Scenedesmus* species, peptidoglycans that of *Anabaena*, and sporopollenin (Marten 1986), a highly stable carotenoid polymer, that of *Palmellacoccus*. Peptidoglycans also characterize the cell walls of bacteria. Other materials, such as silicon, are prominent in the walls of algae that were not used in these experiments. The gelatinous pectin-based envelope of the *Cyanidium* species also contains cellulose. The euglenoids, which are covered by a proteinaceous envelope, would be more digestible, and the naked algae, such as *Ochromonas*, would be most vulnerable to digestion. Our ranking of digestibility is consistent with the structure of the envelope of the food particle. Indeed, we find that larval mos-

quitoes are unable to digest *Palmellacoccus* cells (Marten 1986). Marten considered this "inert" property of these algae to be so dominant that he advocated their use in source reduction, an application that is discounted by Laird (1988). Marten considered *Scenedesmus* to be similarly indigestible, but our experiments and Laird's field observations suggest otherwise. *Euglena* is exquisitely digestible.

In addition to assessing the amount of alga-derived material internalized by larval mosquitoes, our pulse-purge technique permits us to estimate transit time of the gut contents. Direct observation suggests that living *Chlorella* or yeast pass the guts of larval *Cx. pipiens* in less than an hour (Dadd 1971). Some 90% of radiolabeled *Bacillus thuringiensis israelensis* spores pass the guts of larval *Ae. aegypti* within 20 min when purged with nonlabeled algae (Khawaled et al. 1992). We find that transit time may be even more rapid; label appears to stabilize within 10 min in the guts of *Cx. pipiens*. Sephadex purging eliminates virtually all label associated with non-digested algae within this span of time, demonstrating that any retained label must be associated with the tissue of the larva.

Different microorganisms may become distributed in a column of water in a manner that influences the ability of larval mosquitoes to harvest them. Immotile particles tend to adhere to solid surfaces or to the air–water interface, whereas the vagility of other kinds of microorganisms permits them to remain suspended. The feeding strategies of different kinds of larval mosquitoes vary correspondingly: *Culex* filter feed and *Aedes* browse (Clements 1992). This may explain our observation that larval *Aedes* more effectively harvest *Scenedesmus* and *Anabaena* than do *Culex*; neither of these microorganisms are motile. Interestingly, larval *Culex* more effectively incorporate the body components of *Euglena* than those of *Ochromonas*, even though both are highly motile. Motility, therefore, cannot be the sole factor affecting the ability of larval mosquitoes to incorporate the body components of their food particles.

Size of the food particle may also affect incorporation of its components by mosquitoes. The diameters of all food particles used in these experiments fall within the limits that are readily ingested by last instar larvae (0.7–50 μm) (Dadd 1971, Merritt et al. 1992). All but *Anabaena*, however, are more or less spherical in form. Interestingly, larval *Aedes*, but not *Culex*, abundantly incorporate these *Anabaena* filaments. Threadlike helminths also serve as a ready food source for these mosquitoes (Dadd 1971). Perhaps larvae that feed by browsing more effec-

tively harvest spaghetti-like filamentous algae than do other mosquitoes.

In contrast to their more mature siblings, 1st-instar larvae fail to ingest particles larger than 2 μm. This is consistent with our finding that the body contents of *Euglena* are not readily incorporated by young larval *Culex*. In this manner, food selectivity applies to instar as well as kind of mosquito at issue.

The physical state of a larval mosquito may affect the quantity of material that it ingests. When starved, larvae ingest nearly twice as many particles as would otherwise be taken in (Rashed and Mulla 1989). To optimize in terms of quantity of ingested label, our mosquitoes were always deprived of food before each experiment. Addition of a phagostimulant, such as yeast extract, for example, also increases ingestion (Dadd et al. 1982). Thus, the quantity and nature of the particles present in the larval medium provide another particularly complex set of variables affecting the quantity of material ingested.

Although our method of analysis cannot differentiate "harvestability," "digestibility," and "absorbability," any microorganism that is poorly digested would provide a poor source of nutrients and a flawed vehicle for transgenic larvicidal toxin. Indeed, the results of our *Culex* experiments are consistent with these interpretations. Label was most abundantly harvested from the *Euglena, Ochromonas,* and *Cyanidium* species that were used. Surprisingly, our *Aedes* experiments indicate that the rigid-walled *Anabaena* cells are readily digested. Because bacteria comprise as much as 97% of the gut contents of certain species (Walker and Merritt 1991), larval mosquitoes would, presumably, possess a mechanism that specifically attacks peptidoglycans. A "vector" of a transgenic toxin should readily be digested by larval mosquitoes, and these include microorganisms that are naked, covered by a nonrigid envelope, or by a peptidoglycan-based wall.

The apparent pesticidal utility of transgenic plants that express *B. thuringiensis* toxins (McGaughey and Whalon 1992) has focused research attention on the possibility that particular algae might be used against larval mosquitoes as vehicles for transgenic *B. t. israelensis* (*B.t.i.*) toxin. Toxins of *B.t.i.* have been cloned into *Escherichia coli* or other bacteria preparatory to commercial production of larvicide by fermentation (Ward et al. 1984, Donovan et al. 1988). A similarly transgenic unicellular cyanobacterium (*Agmenellum quadruplicatum*) has been considered for direct larvicidal use because it propagates in nature (Angsuthanasombat and Panyim 1989, Murphy and Stevens 1992). Particular algae might be even more effective as vehicles for these toxins because they are self-propagating and abundantly harvested by mosquitoes. Such a larvicidal algal vehicle must readily be ingested by the target mosquito and effectively digested.

Larval mosquitoes appear to drink very little (Aly and Dadd 1989), and we confirm these observations. Indeed, larvae do not incorporate dissolved and labeled glucose. No lower limit on the size of the particles that a mosquito can ingest, however, has been established because convention dictates that any particles smaller than 0.5 μm are defined as "solutes" (Wotton 1990). Colloids and certain bacteria, in this usage, would be considered as "dissolved." Because larger particles are ingested in much larger quantities, the use of dissolved toxin seems counterindicated. It may be that the activity of certain commercial flowable formulations is limited because so much of their content is in small crystals.

Because filter-feeding larval *Culex* mosquitoes internalize more material from *E. gracilis* than they do from various other algae, this alga provides a candidate vehicle for transgenic *B.t.i.* to be directed against the full-grown larvae of these mosquitoes. Browsing larval *Aedes* mosquitoes that infest sunlit sites, on the other hand, could more readily be attacked using an *Anabaena* vector. Our pulse-purge method of analysis constitutes a practical technique for selecting such an algal "vector."

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