Feeding Behaviour of *Didinium nasutum* on *Paramecium bursaria* with Normal or Apochlorotic Zoochlorellae

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(Received 9 October 1979)

Clonal *Didinium nasutum* previously reared on either *Paramecium aurelia*, *P. bursaria* (normal and apochlorotic zoochlorellae-containing stocks), *P. caudatum*, or *P. multimicronucleatum* were presented with either 15 cells each of normal or apochlorotic *P. bursaria* or 30 cells of either stock in 1 ml depression slides. Prey-choice, search period and handling (= ingestion) times were recorded. Didinia chose apochlorotic prey twice as often as normal paramecia. They located and attacked apochlorotic cells more rapidly than normal prey, and they ingested 'bleached' paramecia more quickly than normal cells. There was a consistent pattern of relatively increased search and handling times for primary control didinia reared on paramecia other than *P. bursaria*, compared with the experimental predators. The frequency distributions of both search and handling times were normal. Predatory efficiency of didinia attacking apochlorotic paramecia was significantly greater (> 10 times) than that exhibited for normal cells as measured by the escape frequency of prey. Similarly, the greatest loss of zoochlorellae by regurgitation or premature defecation occurred after normal prey were ingested. These results support the hypothesis that the mutualistic zoochlorellae within *P. bursaria* tend to discourage predation by *D. nasutum* by releasing distasteful metabolites that repel them. The reciprocal nature of this ciliate-zoochlorella symbiosis includes a protective function for the latter partner.

INTRODUCTION

Considerable attention has been devoted to various aspects of the symbiosis between the ciliate protozoon *Paramecium bursaria* and its zoochlorellae (Karakashian, 1975; Trench, 1979). Historically, emphasis has been on evolutionary aspects of this association (Karakashian & Karakashian, 1965), the genetic basis for maintenance and transmission of zoochlorellae among host cells (Siegel, 1960), the metabolic exchange of micro- and macronutrients between algae and ciliates (Brown & Nielsen, 1974; Muscatine *et al.*, 1967), the ultrastructural basis of this relationship (Karakashian *et al.*, 1968; Vivier *et al.*, 1967), and the circadian rhythm of the host cell's behaviour imposed by its algal symbionts' photosynthetic metabolism (van Wagtenonk, 1974). Recently, Weis (1969, 1977, 1978) has investigated the regulation of algal densities, infectivity, and their synchronous development within host ciliates.

However, despite the long-held belief (Sandon, 1932) that *Didinium nasutum* would not eat *P. bursaria*, no report has yet speculated that its mutualistic zoochlorellae may serve a repellent function against their host's ciliate protozoon predators. This is especially surprising considering the well-known production of noxious and distasteful (to humans at least) metabolites by the closely related free-living *Chlorella* spp. (Taub, 1974; Hellebust, 1974). This genus has also been incriminated as a cause of invertebrate deaths (Prescott, 1968; Collins, 1978).

The possible antipredator function of zoochlorellae was suggested by observations of

Berger (1980) that *D. nasutum* was imprinted on *P. bursaria* only with great difficulty. Even after being trained to consume them, such didinia frequently regurgitate and/or defecate zoochlorellae and adhering prey cytoplasm. Also, the highest percentage of unsuccessful feeding attempts by didinia on paramecia occurred when *P. bursaria* were attacked. Berger (1980) reported a pronounced avoidance of *P. bursaria* by starved didinia when it was the 'unfamiliar' prey-species in prey-choice experiments.

The present study tests the hypothesis that didinia are specifically repelled by paramecia containing normal zoochlorellae, but are capable of ingesting bleached hosts. A preliminary report has been published elsewhere (Berger, 1979a).

METHODS

Stocks of *Didinium nasutum* and four species of *Paramecium* were obtained from Carolina Biological Supply Co., Burlington, N.C., U.S.A. Clonal paramecia were cultured at 22 to 24 °C in 20 × 150 mm screw-capped tubes containing 0.05 % (w/v) Cerophyll powder (Cerophyll Laboratories, Kansas City, Mo., U.S.A.) in Millipore-filtered spring water from Mississauga, Ontario, Canada. Didinia were cloned and first fed *P. multimicronucleatum* in culture tubes for six weeks, then subcloned and trained to feed on either *P. aurelia*, *P. bursaria* or *P. caudatum* cultured on a mixed microbiota.

Two subclones derived from the original *P. bursaria* 'Carolina' stock were maintained separately for nine weeks; one had normal zoochlorellae (exposed to sunlight and fluorescent room illumination at 22 to 24 °C); the second was placed in a dark incubator at 20 °C. Both cultures were fed mixed microflora and *Chilomonas paramecium*. The 'dark' cultures were exposed to light for less than 5 min per week when being subcultured. After 60 d no chlorophyll was visible in their zoochlorellae, whose abundance was reduced to about 25 % of their normal intracellular population density. When removed from their host paramecia, these algae exhibited no visible chlorophyll under 1000× magnification. The *P. bursaria* grown in the dark had the cytoplasmic coloration of comparably-sized paramecid species. This stock is designated as 'apochlorotic'; that grown in light as 'normal'.

A fifth clone of *D. nasutum* was trained on apochlorotic paramecia two weeks prior to the first preference experiments. This clone had been fed normal *P. bursaria* previously. The successful training period was shorter than that required for didinia fed normal *P. bursaria* after a diet of *P. multimicronucleatum* (Berger, 1980). This fifth clone and the four described previously were used in the following trials.

Experiments were conducted in 1.0 ml Millipore-filtered spring water in the central hemispherical cavity of a glass three-spot depression slide. All didinia were deprived of prey for approximately 24 h before use to ensure that they would be receptive to prey. No didinia in a precystic state or displaying any predivision morphogenetic reorganization were used. Fifteen normal and apochlorotic paramecia were placed separately into the two outer wells of the depression slide at 22 to 24 °C. Excess culture medium was pipetted off and the cells were rinsed with 0.75 ml filtered spring water at least three times to remove extraneous microbial prey. The 30 paramecia were transferred to the centre well containing spring water and left for 5 min, or until normal locomotory behaviour resumed. A single *D. nasutum* (previously imprinted on either *P. aurelia*, normal or apochlorotic *P. bursaria*, *P. caudatum* or *P. multimicronucleatum*) was introduced at the righthand margin of the centre well along with a minimal volume of its culture fluid, and a stopwatch was started concurrently. Paramecia could not be added to the *D. nasutum* because this procedure resulted in non-random forced mechanical contact with the predator (due to the stream of culture fluid causing prey to hit the proboscis of didinia). The time required by didinia to contact (= attack, as defined by extrusion of pexicysts and/or toxicysts from their proboscis) and secure either a normal or apochlorotic prey cell was recorded as their 'search period'. 'Handling time', defined as the total ingestatory period from initial proboscis contact to complete disappearance of a prey cell within a *D. nasutum* and the subsequent reclosure of its cytosome, was recorded using a second stopwatch. The *D. nasutum*'s choice of prey-species was also recorded. The mean frequency of unsuccessful attacks was so low (3 %) that they were included in the results for search period, but not for handling time or prey preference. These experiments were controlled in two ways: the primary control didinia were reared on *P. aurelia*, *P. caudatum* or *P. multimicronucleatum* and, thus, had no prior dietary experience with the experimental prey; the secondary controls were didinia from the five imprinted stocks presented either 30 normal or 30 apochlorotic *P. bursaria* separately in a well-slide. These experiments were replicated in 1974 and 1975 using fresh stocks of paramecia and didinia each time. As no significant differences were observed between yearly replicates, the results thus obtained were pooled to form the sample set of 500 successful trials. Statistical procedures are described in Sokal & Rohlf (1969).

Table 1. *Prey preference of Didinium nasutum when presented with normal and apochlorotic Paramecium bursaria simultaneously*

Prior diet of <i>D. nasutum</i>	Percentage eaten	
	Normal <i>P. bursaria</i>	Apochlorotic <i>P. bursaria</i>
<i>P. bursaria</i>		
normal	41	59
apochlorotic	21	79
<i>P. aurelia</i> *	33	67
<i>P. caudatum</i> *	37	63
<i>P. multimicronucleatum</i> *	35	65
Totals	33.4	66.6

* Primary control didinia, see Methods.

RESULTS

The prey preferences of five imprinted clones of *D. nasutum* when offered two stocks of *P. bursaria* are presented in Table 1. They overwhelmingly (2:1) preferred apochlorotic paramecia. Even those didinia whose prior diet was normal *P. bursaria* chose 'bleached' cells significantly more than 'green' prey (Student's *t*-test: $P < 0.001$). As expected, didinia trained on apochlorotic paramecia displayed the most pronounced preference for such prey.

The time required for the five stocks of *D. nasutum* to locate and attack normal or apochlorotic prey is presented in Table 2. The pooled frequency distribution of these search periods was normally distributed. Several significant patterns emerge from inspection of these results: (1) the shortest search times were exhibited by the experimental and secondary control didinia previously reared on both stocks of *P. bursaria*; (2) the primary control didinia required about twice as long to attack these two stocks whether presented as prey-pairs or as uniform secondary controls; (3) when prey-pairs and secondary controls were compared, the experimental and primary control didinia differed between the normal and apochlorotic stocks; (4) didinia attacked apochlorotic prey in the shortest time.

A detailed examination by a single classification analysis of variance (1-way ANOVA) of the statistically significant relationships existing among these subtreatments revealed that: (1) column 1 (normal prey-pairs) differs from column 3 (apochlorotic prey-pairs), $P < 0.001$; (2) the search periods for the two secondary control groups (columns 2 and 4) differ, $P < 0.02$; (3) within columns 1 to 4, all primary controls differ in search period from their corresponding experimental didinia (all four *P* values < 0.001) as prey-pairs or secondary controls; (4) the experimental didinia that chose apochlorotic prey (column 3) differ in search period from those that attacked normal cells (column 1), $P < 0.001$; (5) the experimental and primary control didinia fed only normal cells (column 2) were significantly slower in attacking them than those fed only apochlorotic paramecia (column 4, all four *P* values < 0.001).

Comparisons of the results (by a 1-way ANOVA) presented in column 1 versus column 4 and column 2 versus column 3, or proportions thereof, although statistically significant (all at the $P < 0.001$ level) are not included as they are not biologically meaningful in the present experimental context.

Two statistically non-significant relationships were apparent from this analysis: (1) in neither case were the search periods for didinia fed prey-pairs or appropriate secondary control prey (columns 1 versus 2 and 3 versus 4) different; (2) when considered separately, the values for experimental and primary control didinia are not different between prey-pairs and appropriate secondary controls (portions of columns 1 versus 2 and 3 versus 4).

The handling or ingestion times of the five didinial clones are presented in italics in Table 2. The general behavioural pattern of *D. nasutum* for this variable is the same as for search

Table 2. Search period and handling (=ingestion) times of *Didinium nasutum* when fed normal and apochlorotic *Paramecium bursaria*

Results for search periods are shown in roman type and those for handling times are in italics. Prey pairings refer to mixed normal and apochlorotic prey (see text). In secondary controls, *D. nasutum* was presented with 30 normal cells or 30 apochlorotic cells.

Search period and handling time (s)												
Prior diet of <i>D. nasutum</i>	Normal <i>P. bursaria</i>						Apochlorotic <i>P. bursaria</i>					
	Prey pairings			Secondary controls			Prey pairings			Secondary controls		
	$\bar{X}$	S.E. $\bar{X}$	N	$\bar{X}$	S.E. $\bar{X}$	N	$\bar{X}$	S.E. $\bar{X}$	N	$\bar{X}$	S.E. $\bar{X}$	N
<i>P. bursaria</i>												
normal	49.7	5.4	42	47.0	6.6	26	40.1	2.2	60	40.7	2.9	25
	26.6	1.8	41	22.7	2.0	25	20.1	2.1	59	19.5	1.8	25
apochlorotic	41.3	1.9	21	40.0	2.1	25	30.7	1.3	80	29.9	2.3	25
	20.1	2.7	21	21.7	3.4	25	16.7	1.9	79	17.0	1.8	25
Primary controls												
<i>P. aurelia</i>	91.1	13.4	35	87.8	11.4	29	79.7	8.3	67	77.7	10.1	25
	35.9	3.0	33	38.1	3.1	25	28.8	1.4	67	27.6	1.6	25
<i>P. caudatum</i>	81.0	9.6	40	77.6	10.3	31	75.4	6.1	63	74.8	12.0	25
	38.1	3.8	37	39.9	4.1	25	32.1	2.8	63	33.0	4.6	25
<i>P. multimicronucleatum</i>	74.7	10.7	37	75.1	12.3	27	69.8	4.9	65	75.0	10.6	25
	36.6	4.4	35	35.6	5.0	25	30.7	3.0	65	34.1	3.9	25
Totals	69.4	4.3	175	66.7	4.2	138	58.2	2.3	335	59.6	3.8	125
	32.3	1.5	167	31.6	1.6	125	25.4	1.4	333	26.2	1.4	125

period, i.e. (1) didinia previously reared on both stocks of *P. bursaria* ingested prey most quickly; (2) the primary control didinia required about 1.7 times longer to swallow the two stocks, when compared to the experimental didinia, as prey-pairs or secondary controls; (3) the shortest handling times for all didinia involved swallowing apochlorotic prey; (4) when prey-pairs and secondary controls were compared, the experimental and primary control didinia differed in handling times between normal and apochlorotic stocks.

The statistically significant relationships among handling time sub-treatments in Table 2 are precisely comparable to those presented for the search period. The identical comparisons were made by 1-way ANOVA and each was found to have *P* values < 0.001. By substituting length of handling time for search periods, the interrelationships are exactly the same for this parameter. Furthermore, the identical statistically non-significant comparisons as determined for search periods apply equally to handling times. Lastly, the same significant, but biologically inapplicable relationships, exist for handling time as were listed above for search periods.

The predatory efficiency of *D. nasutum* when fed the two stocks of *P. bursaria* is given in Table 3. The highest escape frequencies involved normal paramecia. There were no significant differences between experimental and primary control didinia in this regard.

The paramecia's zoochlorellae and attached cytoplasm consumed were often partially regurgitated or defecated by didinia prior to their being even partially digested (Table 4). The majority of cytoplasmic prey loss occurred when normal prey were ingested. Among the 14% of didinia displaying such behaviour, nine times as many predators lost normal cells as lost bleached prey.

When didinia were placed in a depression slide with host-free zoochlorellae from normal or apochlorotic prey they did not attack them. They cruised near groups of algal cells and frequently swam rapidly away from them towards the surface film, as if they were avoiding a noxious stimulus. Control didinia exposed to macerated *P. aurelia* congregated near accumulations of cellular debris. There was no indication of avoidance of these algae-free prey by didinia.

Table 3. *Efficiency of prey capture by Didinium nasutum when fed normal and apochlorotic Paramecium bursaria*

<i>P. bursaria</i> stock	Frequency of escape by paramecia					
	Prey pairings		Secondary controls		Totals	
	N/n*	%	N/n*	%	N/n*	%
Normal	8/175	4.6	13/138	9.4	21/313	6.7
Apochlorotic	2/335	0.6	0/125	0	2/460	0.4
Totals†	10/510	2.0	13/263	4.9	23/773	3.0

* N, Number of total escapes; n, number of trials.

† Includes experimental and primary control didinia.

Table 4. *Loss of zoochlorellae in normal and apochlorotic Paramecium bursaria when consumed by Didinium nasutum*

<i>P. bursaria</i> stock	Fate of zoochlorellae*			
	Regurgitated		Defecated	
	N/n†	%	N/n†	%
Normal	68/292	23.3	29/292	9.9
Apochlorotic	7/458	1.5	4/458	0.9
Totals‡	75/750	10.0	33/750	4.4

* No *D. nasutum* regurgitated and defecated zoochlorellae from the same prey cell.

† N, Number of didinia losing zoochlorellae; n, number ingesting prey.

‡ Percentage of prey entirely consumed by didinia: normal, 66.8; apochlorotic, 97.6.

DISCUSSION

The prior dietary experience of *Didinium nasutum* determined its prey-choice under experimental conditions (Berger, 1979*b*, 1980) and demonstrated unequivocally that didinia preferred to consume paramecid species on which they had been fed previously. Furthermore, they located and swallowed such prey more rapidly than 'unfamiliar' congeneric prey. Both parameters were independent of the prey's size. Such observations conform to what Immelmann (1975) characterized as individuals with 'strong food preferences or . . . imprinting-like fixations'. The persistence of dietary imprinting through successive asexual generations has been discussed (Berger, 1980).

It is not surprising, therefore, that mean search and handling times (Table 2) of experimental didinia fed normal 'green' paramecia were less than those observed for primary controls. What dietary imprinting cannot explain (save in the case of didinia reared exclusively on bleached paramecia) is the significantly shorter times required for these two phases of the feeding process when didinia attacked apochlorotic prey. Similarly, prior dietary experience cannot, in itself, explain the overwhelming preference of these didinia for apochlorotic prey regardless of their previous diet. Such observations strongly suggest that either normal *P. bursaria* are distasteful or that apochlorotic paramecia are somehow attractive to didinia. As no evidence exists to support the latter hypothesis, and many previously published results (Prescott, 1968; Hellebust, 1974; Taub, 1974; Collins, 1978) point towards the former, it will be adopted here. What additional results support it? The significantly different escape frequencies between stocks of normal and bleached paramecia (favouring the latter theory) indicate the selective advantage normal paramecia possess over apochlorotic prey. Further evidence exists in the differential escape frequency relative to the prior feeding regime of the didinia: 7 out of 8 escapes by normal paramecia occurred when 'naive' primary control didinia were tested. Similarly, 12 out of 13 successful escapes by

secondary control normal paramecia also involved primary control predators. It can be inferred that most ciliate predators in Nature would not have encountered *P. bursaria* since their last asexual fission and, thus, would behave as did the primary controls in this study. Once imprinted, a ciliate predator would become adapted to the zoochlorellar repellent. Clearly, a distinct advantage accrued to *P. bursaria* with normal zoochlorellae. These escape frequencies (Table 3) are significantly higher than those previously observed (Berger, 1980).

In absolute terms, the mean search periods of primary control and experimental didinia were not significantly different from those observed when normal *P. bursaria* were attacked in mixed or single prey-species encounters (Berger, 1980). However, the mean search period for didinia that attacked normal paramecia was much longer and significantly different ($P < 0.001$) from that required by a stock previously fed on colpidia (Berger, 1979*b*). As its prey, colpidia, are about one-half the length of *P. bursaria*, a mean augmentation of 11 s in search period is surprising and suggests that prey repellency may be operative in this situation. This possibility is further strengthened by noting that the mean search period for apochlorotic prey (Table 2) is not significantly different from that noted in the colpidivorous stock (Berger, 1979*b*). The increased prey density of colpidia versus *P. bursaria* is not the decisive factor in this comparison. Clearly, didinia located bleached prey more quickly than green paramecia.

The handling time of didinia attacking normal *P. bursaria* was not significantly different from that recorded by Berger (1980) in single or mixed prey encounters. As would be predicted by their small size, colpidia are ingested significantly faster than paramecia (Berger, 1979*b*). However, a significantly shorter difference in handling time was exhibited by didinia swallowing apochlorotic paramecia in prey-paired or secondary control situations. As the cellular volume of bleached cells was essentially identical to that of normal prey, this decreased efficiency in ingesting the latter may be due to distasteful metabolites.

Chemotactic detection of prey by didinia has been confirmed independently by Seravin & Orlovskaya (1977) and Berger (1980). These quantitative analyses contradict the widely accepted anecdotal observations that didinia are random contact hunters (see, for example, Dragesco, 1962; Wessenberg & Antipa, 1970). The avoidance of exposed zoochlorellae by didinia indicated their ability to detect noxious soluble metabolites. The attractiveness of the cytoplasm of macerated *P. aurelia* to control didinia suggests that soluble prey factors are detectable by didinia. This chemotactic sensitivity has been further confirmed by the observation (G. W. Salt, personal communication) that didinia will attack and congregate about the excretory pore of asplanchnid rotifers fed paramecia. Presumably they are attracted by rotiferan excretory products derived from their paramecid prey.

Additional evidence as to the presumably distasteful nature of normal zoochlorellae may be noted in the significantly different regurgitation and premature (almost immediately after ingestion) defecation rates of zoochlorellae from ingested normal versus apochlorotic paramecia by didinia. Each time this loss of potential nutrients by didinia occurred almost 10 times more frequently with green paramecia than with bleached prey. Although absolute protection was not afforded the prey in these situations (obviously their cell membranes were dissolved), a presumably distasteful repellent factor resulted in significant dietary cytoplasmic loss (up to half the prey's volume) in some cases. Even bleached zoochlorellae elicited this response in some didinia suggesting that the repellent may not be a direct by-product of photosynthesis. As with escape frequency, most (about 67%) prey regurgitation and defecation was exhibited by naive primary control didinia. Apparently, successful imprinting on *P. bursaria* required adaptation to its repellent by didinia.

The frequencies of the pooled didinial search periods were normally distributed with leptokurtotic tendencies as found previously (Berger, 1980), but differ from those observed for *D. nasutum* fed colpidia (Berger, 1979*b*) whose search periods were lognormally distributed. In neither the present nor the previous two didinial studies did the frequency

distributions of search time conform with theoretical distributions (negative exponential) postulated by Paloheimo (1971*a, b*) for a randomly hunting predator. Departures from this hypothetical distribution in this case are probably due to the brief acclimatization period exhibited by didinia following introduction into the depression well (Berger, 1980). A thorough discussion of predator search pertaining to carnivorous ciliates is given by Salt (1967). As found in previous studies, (Berger, 1979*b*, 1980), the frequencies of pooled prey-handling times are normally distributed.

It has been generally assumed that various species of paramecia (other than *P. bursaria*) avoid excessive predation by didinia in Nature by frequenting benthic refuges within detritus covering various freshwater substrates. Normal *P. bursaria* must inhabit the photic zones of their freshwater habitats to ensure that their zoochlorellae photosynthesize. They have been thought to escape predation by remaining relatively motionless on illuminated substrates thus avoiding random contact in the water column with actively swimming didinia (D. Spoon, personal communication). However, recent studies (Seravin & Orlovskaja, 1977; Karpenko *et al.*, 1977; Berger, 1979*b*) have demonstrated that didinia are more adaptive in their feeding behaviour than was previously suspected (Dragesco, 1962; Wessenberg & Antipa, 1970). Specifically, didinia are now known to be capable of attacking motionless prey (colpidia, *P. bursaria*, etc.) lying on various substrates. Thus, wild *P. bursaria* may be assumed to be in jeopardy when microsympatric with *D. nasutum*.

A considerable body of experimental and descriptive results exists (Prescott, 1968; Hellebust, 1974; Taub, 1974; Collins, 1978) that is consistent with the hypothesis that *Chlorella* spp. secrete distasteful and even toxic metabolites. As the symbiotic zoochlorellae within *P. bursaria* are congeneric with free-living species (Karakashian, 1975), it would not be surprising if they also secreted similar substances. Presumably their host cells would be adapted to withstand these substances. Inability of certain aposymbiotic strains of *P. bursaria* (Siegel, 1960) to maintain zoochlorellae could be due to their susceptibility as mutant stocks to these noxious substances. The results in this paper indicate that such zoochlorellae are capable of altering the feeding behaviour of *D. nasutum*. The net effect of such behavioural modifications is to lessen the predation pressure on *P. bursaria* by didinia. In evolutionary terms, a reduction of negative selection pressure, no matter how slight, will favour preservation of a species (Mayr, 1963; Dobzhansky *et al.*, 1977; Roughgarden, 1979). Therefore, it is apparent that by reducing predation pressure on their hosts, zoochlorellae ensure their continued survival as symbionts. The role of symbionts serving to protect their hosts has been frequently documented in metazoa (Caullery, 1952; Trager, 1970) but has not been considered previously in this ciliate-zoochlorellae association. Predator repulsion should be added to the repertoire of mechanisms evolved to avoid destruction of host cells (Trench, 1979).

Thus, the extent to which biological reciprocity exists between these symbiotic partners must be enlarged to include a protective antipredator role by zoochlorellae. Testing this protective hypothesis could involve field surveys of aposymbiotic hosts (presumably more vulnerable) to determine their abundance when wild didinia are present. It seems clear, at least under the experimental conditions employed in this study, that normal zoochlorellae significantly deterred predation by *D. nasutum* and by implication would reduce its effectiveness as a consumer of wild *P. bursaria*.

This research was supported by National Sciences & Engineering Research Council of Canada Operating Grant A-3473. I thank Mr James Elman for his technical assistance and Ms P. E. Bregman for typing the manuscript.

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