

## Epibiosis of *Colacium* on *Daphnia*

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Received June 8, 1997, Accepted March 12, 1998

### Abstract

The epibiotic, euglenoid flagellate, *Colacium* Ehrenberg, attaches to the cuticle of the cladoceran, *Daphnia* O.F. Muller, and other crustacean arthropods, in freshwater habitats. When *Daphnia* molts, the epibionts are shed with the castskin, becoming free-swimming, and must reattach to another substrate organism. In laboratory experiments, the reciprocal impacts of each of the members of this interaction were examined. Epibiont population density is depressed in the presence of *Daphnia* as a result of predation on the motile flagellates, in dispersal stage, and *Daphnia* exhibits enhanced fitness in the presence of the epibionts. But the attached epibionts reproduce during the intermolt period of *Daphnia* and losses of the motile stages of the epibiont, due to predation, are offset, when attached, by reproduction.

Keywords: *Colacium*, *Daphnia*, epibiosis, fitness, substrate organism

### 1. Introduction

Epibiosis is an association between two non-related organisms, one of which, the epibiont, spends a part of its life attached to the other, the substrate organism. We studied the reciprocal nature of the epibiotic interactions of the

Presented at the Second International Congress of Symbiosis, April 13-18, 1997, Woods Hole, MA

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cladoceran, *Daphnia* (host or substrate organism) and the euglenoid flagellate, *Colacium* (epibiont). The system is interesting and dynamic, as the cladoceran host molts at the end of each instar and discards the exoskeleton along with its resident epibiont community. This results in the presentation of newly exposed exoskeleton surfaces for colonization. Persistence of the epibiotic interaction requires that the epibionts regularly disperse, find, and recolonize another substrate (organism).

Adaptations specific to this epibiotic mode of life are expected to include the ability to detect and attach to a suitable living substrate, recognition of the imminent molting of the substrate organism, and the ability to respond to disruption of a substrate by assuming the free-swimming mode for dispersal (Willey and Giancarlo, 1986; Vacchiano et al., 1992; Al-Dhaheri and Willey, 1996). But persistence of the epibiotic population are also affected by interactions that occur during epibiont dispersal when the cells are motile and free in the water column. *Daphnia* are heterotrophic feeders, and have been reported to consume *Colacium* cells (Green, 1974; Naylor et al., 1992). This will result in a cost to the epibiotic cells, but should benefit the substrate organism (host) by enhancing its nutrient availability and increasing its fitness. Thus, the persistence of the epibiotic interaction involves a balance of reciprocal costs and benefits for both members of the association.

## 2. Materials and Methods

The substrate organism *Daphnia laevis* Birge, a cladoceran, was collected at the Chicago Botanical Garden in 1991. All individuals used in the experiments were selected from a clonal population derived from a single female with parthenogenetic eggs. Cultures were maintained in open air, glass containers with aged tap water augmented by weekly additions of double distilled, deionized water, and given twice weekly additions of dry yeast (Frank, 1957). Cultures were non-axenic and undetermined species of bacteria and yeast were present.

The epibiont used was *Colacium vesiculosum* Ehrenberg, an euglenoid flagellate, (Class Euglenophyceae, Order Euglenales) which was obtained from the University of Texas culture collection – first isolated by Pringsheim (Johnson, 1934). Culture medium was an equal mixture of Cerophyll (Sigma, St. Louis) at a density of 3 g per l and soil-water-pea-AlgaGro prepared according to Ward and Willey (1981). The blended solution was autoclaved, and 12 ml were placed each in an experimental, Pyrex, Petri dish (diameter of 5 cm and a depth of 1.5 cm).

Epibionts and *Daphnia* were maintained separately in culture. All culture growth and all experiments were conducted under fluorescent lighting with a light/dark regime of 14/10 hours. Temperature varied from 19°C to 25°C, with a mean temperature of 22°C. Experimental dish location was randomized (according to a random number table; Sokal and Rohlf, 1981) directly under the light source at a distance of 36.5 cm, and at an intensity of 30.1 lumens (during the light phase of the L/D cycle) measured by a Weston Illuminator Meter, Model 756.

In experiment 1, where the effect of *Daphnia* on the mobile forms of its epibiont was studied, individual *Daphnia* were used without regard to sex, or whether they carried eggs. Densities of the free-swimming forms of the epibiont and of the substrate organism were crossed. The initial population densities (IPD) of the epibionts were low (1600 cells/dish), and high (4800 cells/dish). They were placed in the Petri dishes, on the 14/10 L/D cycle, for 7 days (2 molt periods). The *Daphnia* IPD's were none (0), low (1), and high (2) (see Table 1). Cells were counted by filtering the medium through 1.2  $\mu\text{m}$  pore-size, gridded Millipore filters (total 246 grid squares per filter). Algal cells were then counted in 10 of these squares (selected randomly), and the results were averaged to obtain a mean number of epibionts per dish (see Table 1).

In experiment 2, where the effect of the epibiont on its substrate organism was examined, individual female *Daphnia* (with no eggs), were selected from the largest size class in the culture vessel, ranging from 1.88 mm to 2.15 mm length (crown of head to base of spine), with a mean length of 2.00 mm. Prior to being placed in the experimental dishes, individual *Daphnia* were sequentially washed in four bowls of double distilled, deionized water to remove excess bacteria and yeast cells. However, some bacteria and yeast cells were assumed to have entered the dishes attached to the carapaces of the individual *Daphnia*. Data were collected twice daily, for 33 days: number of neonates per female *Daphnia*, length of each neonate, clutch size, frequency of molt, as well as survivorship and net growth of adult daphnids (see Table 2).

In experiment 3, substrate preference of the epibiont for cuticle, on living *Daphnia*, over the shed castskin was determined by placing a single *Daphnia* each in a Petri dish with an IPD of 1,000 motile epibiotic cells on the 14/10 L/D cycle at 30 lumens (during the light period). Daily records, over 7 days, were made of epibiont burden per *Daphnia*, and per castskin, as well as dates when each *Daphnia* molted. Attached epibiotic cells were counted with a Zeiss microscope at a magnification of 200 $\times$ , and recorded categorically (see Threlkeld and Willey, 1993).

Statistical analysis used one-way ANOVA's from SYSTAT (Wilkinson, 1992).

### 3. Results

In experiment 1, under low and high IPD's, *Colacium* populations were significantly reduced by the presence of the substrate organism (Table 1,  $P < 0.01$ ) over the 7 days of the experiment. The significant reduction in the epibiont population appeared to be caused by predation, with the result that the epibiotic cells were serving as a nutrient source for *Daphnia* (see Green, 1974). Direct observation of *Daphnia* and *Colacium* interactions revealed that individual streams of motile epibiotic cells were drawn inside the carapace of *Daphnia*, during the course of its feeding, and the gut of *Daphnia* became green indicating ingestion of *Colacium*.

Experiment 2 focussed on the possible impact of this predation with the prediction that the increased food source would enhance various measures of *Daphnia* fitness, including reproduction, adult growth and survivorship (Table 2). Over the length of experiment 2 (33 days), there was no significant increase in number of neonates per *Daphnia* ( $P = 0.075$ ), clutch size (number of neonates per clutch;  $P = 0.16$ ), number of clutches per *Daphnia* ( $P = 0.26$ ), nor in molt frequency ( $P = 0.107$ ). However, there was a significant increase ( $P < 0.01$ ) in the size of the neonates in the treatments containing the epibionts. Adult *Daphnia* lived an average of 6.4 days longer in treatments containing epibiotic cells than in treatments without the cells. Despite a small sample size, this was a statistically significant difference ( $P < 0.05$ ; Table 2). Only a small number of adult *Daphnia* ( $N = 3$  per treatment) could be measured for growth during the experiment. No differences ( $P = 0.267$ ) in adult size were observed.

Experiment 3 demonstrated that colonization of the substrate organism's living cuticular surface was rapid with a burden of 4.5 (35 attached cells) developing within the first 24 hours after the *Daphnia* molt. The subsequent burden on the living cuticle increased modestly, but showed a precipitous drop of 2.6 (17 cells) just after the exoskeleton was shed (see Day 0-1, Table 3) and became a castskin. This very significant difference in burden between substrates suggests a preference, by the colonizing epibionts, for attachment to the living, cuticular substrate rather than the castskin.

Table 1. Effect of *Daphnia* on *Colacium* population size (mean number/dish  $\pm$  S.D.  $\times 10^3$ )

IPD motile <i>Colacium</i>	0 <i>Daphnia</i>	1 <i>Daphnia</i>	2 <i>Daphnia</i>	P
Low (1600 cells)	228.6 $\pm$ 105.5 N = 5	74.5 $\pm$ 54.1 N = 6	31.6 $\pm$ 29.9 N = 5	$P < 0.001$
High (4800 cells)	437.9 $\pm$ 146.1 N = 5	142.4 $\pm$ 138.6 N = 6	113.4 $\pm$ 131.4 N = 5	$P < 0.01$

N = Number of *Daphnia*

Table 2. Effect of *Colacium* on *Daphnia* (mean  $\pm$  S.D.  $\times 10^3$ )

	<i>Colacium</i> present	<i>Colacium</i> absent	P
Number of neonates/ <i>Daphnia</i> <sup>1</sup>	19.2 $\pm$ 11.7 N = 6	13.0 $\pm$ 6.7 N = 7	0.075
Length of neonates (mm)	0.807 $\pm$ 0.11 N = 102	0.773 $\pm$ 0.08 N = 79	<0.01
Clutches/ <i>Daphnia</i> <sup>1</sup>	3.3 $\pm$ 2.1 N = 6	2.7 $\pm$ 1.3 N = 7	0.26
Neonates/clutch <sup>1</sup>	5.7 $\pm$ 3.0 N = 6	4.8 $\pm$ 2.7 N = 6	0.16
Molt frequency (days)	2.6 $\pm$ 0.3 N = 7	2.9 $\pm$ 0.5 N = 7	0.107
Adult <i>Daphnia</i> survivorship (days from start of experiment)	21.0 $\pm$ 8.5 N = 7	14.6 $\pm$ 3.5 N = 7	<0.05
Net adult <i>Daphnia</i> growth (mm)	0.33 $\pm$ 0.35 N = 3	0.43 $\pm$ 0.19 N = 3	0.82

<sup>1</sup>Difference in sample size (N) is explained by exclusion of one *Daphnia*, in treatments containing epibionts, which failed to produce eggs. N = Number of *Daphnia*.

Table 3. *Colacium* burden<sup>1</sup> by substrate

Days after molt <sup>2</sup>	Castskin	Living cuticle	P
0	4.6 $\pm$ 1.1 N = 26	0.0 $\pm$ 0.0 N = 20	<0.001
1	2.0 $\pm$ 1.4 N = 26	4.5 $\pm$ 1.3 N = 20	<0.001
2	2.3 $\pm$ 1.4 N = 23	4.4 $\pm$ 1.3 N = 19	<0.001
3	3.2 $\pm$ 1.7 N = 23	5.3 $\pm$ 0.5 N = 7	<0.01

<sup>1</sup>Burden is categorical: 0 = no cells, 1 = 1-10 cells, 2 = 11-20 cells, 3 = 21-30 cells, 4 = 31-40 cells, 5 = 41-50 cells, and 6 = 51-60 cells attached as epibionts. <sup>2</sup>Days after molt: time since *Daphnia* shed its castskin and how much time was available for colonization of either the castskin or the living cuticle. Day zero reflects burden at the time of molt. N = Number of *Daphnia*.

#### 4. Discussion

As Table 1 demonstrates, the presence of *Daphnia* reduces the population

density of *Colacium* by as much as 80%. While in the motile (dispersal) stage, *Colacium* is thus vulnerable to predation by *Daphnia*. The attached stage appears to be relatively protected (see Janzen, 1985), and *Colacium* derives a benefit from its ability to reproduce during the intermolt period of *Daphnia*. It also is possible that attachment may provide a nutritional benefit for *Colacium* because it should be able to use the daphnid waste product, ammonia, as a source of nitrogen (Cook, 1968; Rahat, 1992). Such a possibility needs further investigation.

*Daphnia*, through its predation on *Colacium*, receives nutritional benefits which translate into increases in individual fitness for the adult daphnids and their brood, specifically increase in size of the neonates (Table 2). Thus, the association is one of exploitation with a benefit for the substrate organism. However, attachment of the green epibionts has been shown possibly to increase the substrate organism's (plus the attached epibiont's) cost due to susceptibility of the associated pair to predation by visually orienting fish, as well as to increase in possible expenditure of energy for swimming due to increased drag caused by the surface cells (Green, 1974; Willey and Threlkeld, 1993). The role of molting is important to the substrate organism to prevent a potentially dangerous buildup of attached cells to its surface.

Predation upon *Colacium*, by its substrate organism, must have placed selective pressure on the epibiont prey to recognize and anticipate molt so that they may detach from the shed castskin, and very quickly recolonize the freshly exposed cuticle of the same (or another nearby) substrate organism before they become prey. Dispersal is a dangerous period in the *Colacium* life cycle, and the shorter the distance from castskin to new attachment surface, the more successful the dispersal and colonization. Recognition of molt by the epibiont, perhaps cued by chemicals released during ecdysis, followed by extrusion of new flagella (see Killen et al., 1984), and perhaps some detachment from the cuticle just before, as well as after, it is shed would enable the newly motile, algal cells to achieve rapid dispersal and reattachment to the relative safety of a fresh substrate (Al-Dhaheri and Willey, 1996).

But why attach to an arthropod substrate organism? A benefit of epibiotic attachment to a living substrate may be its renewable nature. Competition for attachment to the periphyton and perilimnon is keen, as both are extensively colonized by other organisms which are either opportunistic colonizers or superior competitors for attachment space (Hughes, 1980). Thus, *Colacium* cells (freshly shed every 2.5–3 days; Table 2) may be excluded from non-arthropod attachment niche by other currently resident species. The only safe surface for immediate reattachment may be the substrate organism that has just molted (see Table 3).

*Colacium* is an euglenoid flagellate which requires attachment for reproduction. Cell division requires 2–4 hours, and occurs every 24 hours

(Leedale, 1967, 1968). As long as the benefits of rapid colonization and undisturbed reproduction, while attached, outweigh the costs of predation, *Colacium* can maintain a cyclic relationship of colonization, reproduction, dispersal, followed by recolonization. In turn, attachment of the epibionts to *Daphnia* may increase the substrate organism's cost, due to susceptibility to fish predation, but at each molt event, the epibionts are shed, become motile and available for food to a *Daphnia*, thus increasing its fitness and reducing its visibility to potential predators. There probably is a maximum burden before the number of attached epibionts becomes too much for the substrate organism. In a well balanced epibiosis, a general positive interaction for both organisms requires that average burden stay below the maximum level. This might be brought about by effects of epibiont proliferation being balanced by the pair becoming more visible to visually-orienting predators, or by increasing drag, sinking, and impeding of food collecting ability by *Daphnia*. It all works in the same selective direction - epibionts that are successful are those that are able to grow and reproduce, within each molt cycle, enough to offset losses during the dispersal stage. Epibionts that are too productive would tend to be eliminated by subsequent loss of their substrate organism; epibionts that are not sufficiently productive may not reproduce enough, during the intermolt, to offset losses due to grazing by their substrate organism.

The *Daphnia-Colacium* epibiosis is a dynamic, complex symbiotic interaction. The nature of cost and benefit to the associated organisms depends greatly on the stage in life history (motile or attached epibiont, freshly molted substrate organism), and on the external environment (presence of visually-orienting fish). The association, thus, is flexible and dependent on its reciprocal nature. Seasonal extinction of some epibiont populations may be linked to the population crash of their particular hosts. The specificity of these epibionts for particular substrate organisms suggest an evolved relationship, although epibionts of other species may be only opportunistic in nature. The *Daphnia-Colacium* relationship cannot be linked directly with parasitism or mutualism, but rather is a form of conditional symbiosis determined both by direct and indirect interactions at different periods throughout the association (see Bronstein, 1994).

## 5. Conclusion

The substrate organism, *Daphnia*, acts as a predator upon the motile stage of its epibiont, *Colacium*. This provides a nutritional supplement for *Daphnia* that can be measured as an increase in fitness in the size of neonates and adult survivorship. *Colacium* cells, which exhibit rapid colonization of the fresh cuticle and a preference for living cuticle over molted castskin, show the

ability of the epibionts to discriminate among substrates. The rate of population growth of *Colacium*, is reduced by the periodic predation, in its motile phase, by the substrate organism. The tradeoff between costs of predation and the benefits of survivorship and reproduction appears to be a positive one for both members of the epibiosis, permitting a stable, interspecific relationship which involves balanced beneficial and antagonistic interactions.

### Acknowledgements

We would like to thank our respective spouses, Norine Gutekanst and Robert Willey, for the support necessary to complete the work. The Department of Biosciences of the University of Illinois at Chicago provided resources. Thanks to Dr. Howard Buhse, who helped with the media; Dr. Joel Brown, who provided statistical advice; and Dr. Roy Taylor, at the Chicago Botanical Gardens, who kindly allowed us to collect the *Daphnia* with which we established the clonal population. Special thanks to an unidentified reviewer, and to Dr. S.T. Threlkeld, University of Mississippi, for critical review and valuable suggestions.

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