

Evidence of a mutualistic relationship between an algal epibiont and its host, *Daphnia pulicaria*

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Abstract

We examined seasonality, intensity, and level of epibiont infection by the green alga *Korshikoviella gracilipes* on the zooplankton community over 2 yr in the high mountain Río Seco lake. *Daphnia pulicaria* was the preferred substrate for the epibiont, whose life cycle was exclusively completed on this taxon. The density of epibiont dispersal stage and the epibiont prevalence and burden on crustacean zooplankton were directly related to *D. pulicaria* density in both years. Laboratory experiments showed that attached epibionts had a negative effect on *Daphnia* by increasing the weight and sinking rates of infected animals and a positive effect by increasing its reproductive rates, with a neutral effect on survivorship. On the other hand, the epibiont dispersal stage was actively and intensively grazed by *Daphnia*, which implies a benefit for *Daphnia* and a cost for *K. gracilipes*. Thus, both species derived benefits and costs from this relationship. Our results indicate a positive cost-benefit balance for both epibiont and host in such a way that a mutualistic relationship can be suggested. This epibiont-host interaction may play an important role in population and community regulation in Río Seco lake.

The presence of epibionts on marine and freshwater zooplankton is a very common phenomenon. Numerous studies have shown that epibionts can influence zooplankton individual and population dynamics in many ways, although the available data show considerable variability. Negative effects have been described on host reproduction (Green 1974; Xu and Burns 1991; Weissman et al. 1993; Stirnadel and Ebert 1997), survival (Allen et al. 1993; Weissman et al. 1993), swimming abilities (Weissman et al. 1993), and feeding (Green 1974; Kankaala and Eloranta 1987), as well as on increased sinking rates (Herman and Mihursky 1964; Allen et al. 1993) and vulnerability to predation (Willey et al. 1990; Chiavelli et al. 1993; Threlkeld and Willey 1993; Willey and Threlkeld 1993). On the other hand, it has also been reported that there is little or no effect on host reproduction (Allen et al. 1993; Threlkeld and Willey 1993), respiration (Ikeda 1977; Allen et al. 1993), excretion (Ikeda 1977), or molting rate (Weissman et al. 1993). Finally, several studies suggested possible benefits for the hosts from the epibiont cleaning up the host surface (Holland and Hergenrader 1981), from epibiont excretion of specific nutrients (Holland and Hergenrader 1981), and from the host feeding on its epibiont (Green 1974; Van Dover et al. 1988; Threlkeld and Willey 1993; Al-Dhaheri and Willey 1996). Field and experimental evidence of this last possibility has only recently been obtained (Bartlett and Willey 1998; Poltz et al. 1998).

In addition to the advantage of substrate availability for growth and reproduction, epibionts are assumed to derive benefits from interaction with the host through access to light and nutrient availability zones (Green 1974; Kankaala and Eloranta 1987) and through nutrient supply from the host's excreta, feeding current, and exoskeleton (Ikeda 1977; Holland and Hergenrader 1981; Nagasawa 1987; Wahl 1989). On the other hand, the epibiont must deal with the ephemerality and dispersion of its habitat (Threlkeld and Willey 1993; Threlkeld et al. 1993) and with possible losses due to host predation (Bartlett and Willey 1998).

Epibionts may influence the zooplankton population dynamics and plankton community structure (Allen et al. 1993; Chiavelli et al. 1993; Weissman et al. 1993). However, recent papers have pointed out that the study of epibiosis in zooplankton is poorly developed and that further analyses on the functionality of these relationships are required (Wahl 1989; Threlkeld and Willey 1993; Threlkeld et al. 1993; Carman and Dobbs 1997). There is a spectrum of possible functional relationships, ranging from parasitic to mutualistic interactions. Because there are many kinds of epibionts (bacteria, fungi, algae, protozoans, and rotifers), the result of these interactions is greatly conditioned by the nature of the epibiont. On the other hand, each specific epibiont-host relationship is a dynamic interaction whose outcomes could vary between and within lake systems and would depend on many factors, such as epibiont and host densities, abiotic conditions, interactions with other epibionts and zooplanktonic species, or the presence and density of zooplankton predators (Xu and Burns 1990; Allen et al. 1993; Chiavelli et al. 1993; Threlkeld et al. 1993; Weissman et al. 1993; Willey and Threlkeld 1993).

In Río Seco lake, a small, high mountain lake in Southern Spain, the green alga *Korshikoviella gracilipes* regularly attaches to crustacean zooplankton individuals and especially to *Daphnia pulicaria* (Sánchez-Castillo 1987; Cabrera-Fernández 1996). Río Seco lake provides an excellent system to study the epibiont-host relationship, because there is no

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Daphnia predator to complicate the relationship, the plankton community is extremely simple, and the level of epibiont infestation on *Daphnia* is regularly high.

The present article describes field and laboratory studies conducted to elucidate the nature of the *K. gracilipes*–*D. pulicaria* relationship and its effect on the life histories and population dynamics of both in the context of the lake plankton community. *D. pulicaria* and *K. gracilipes* are major constituents of the simple lake plankton community, so their relationship affects the dynamics of the entire plankton community (Cabrera-Fernández 1996).

We examined the seasonality, intensity, and level of epibiont infection among the zooplankton community over 2 yr in relation to changes in zooplankton and phytoplankton seasonality and physical and chemical conditions. We also performed laboratory experiments to study the effects of the epibiont on *D. pulicaria* survival and reproduction and its effects on *D. pulicaria* weight and sinking rates.

Methods

Study site—Río Seco is a small (1,920 m²), oligomesotrophic, and shallow ($z_{\max} = 2.90$ m) lake of glacial origin, located at 3,040 m above sea level in the Sierra Nevada mountains (southern Spain). The lake is ice covered from around October–November until June–July. The lake is without fish and presents total Secchi disk visibility during the ice-free period.

The plankton community of the lake is extremely simple. The phytoplankton community consists of ~10 nanoplanktonic species, and the dominant species are *Chromulina nevadensis*, *Ochromonas* sp., *Dictyosphaerium chlorelloides*, and the zoospores of the epizoan chlorophyte *K. gracilipes*. The dominant zooplanktonic species are the calanoid *Mixodiaptomus laciniatus* and the cladoceran *D. pulicaria*.

***K. gracilipes* life cycle**—*K. gracilipes* presents a complex life history (Sánchez-Castillo 1987). The asexual life cycle of *K. gracilipes* is composed of five different stages: zoospore, chlorangoid, ankyroid, adult, and cyst.

The zoospores are biflagellate cells (length 7.98 ± 1.30 μm [$\bar{x} \pm \text{SD}$], width 3.90 ± 0.75 μm ; biovolume 69.00 μm^3 , $n = 1,495$) living as free-swimming planktonic organisms. The zoospore stage is the dispersal stage of the epibiont life cycle. Once a zoospore encounters a substrate organism, it attaches by the apical region. This unicellular attached stage is the chlorangoid. Chlorangoids then develop into ankyroids by transverse division and cell elongation. Ankyroids are, therefore, bicellular organisms (length 26.06 ± 3.03 μm , width 3.98 ± 0.64 μm ; $n = 306$), with the appearance of individuals belonging to Genus *Ankyra*. Ankyroids reach the adult stage by elongation through transverse divisions. The adult stage (length 140.69 ± 64.65 μm , width 10.14 ± 2.85 μm ; biovolume $14,447.70$ μm^3 , $n = 543$) is the primary reproductive phase of the *K. gracilipes* life cycle. Each cell of the adult organism is able to produce zoospores by mitotic division. Under certain conditions, the adults produce cysts with resistant walls that probably overwinter on the lake bottom.

The chlorangoid stage was found attached to all the zoo-

plankton species in the lake, although mainly on *M. laciniatus* and *D. pulicaria*. However, the ankyroid and adult stages were found almost exclusively on the legs of *D. pulicaria*, so that *K. gracilipes* only completes its life cycle on *D. pulicaria*.

The attached stages of the epibiont are limited to the duration of the crustacean intermolt period. When molting occurs, the epibionts are discarded with the exoskeleton. Chlorangoids detach from the exoskeleton and become free-swimming, dispersing to colonize a substrate organism again. Most of the adults produce zoospores on a massive scale, whereas others produce cysts.

Field samples—Plankton was collected at five points in Río Seco lake from July through November in both 1996 and 1997. The lake area was divided into five zones of about same size, and one sample was collected at a random point within each one. Phytoplankton (100 ml) and zooplankton (10 liters) were collected from a homogenized volume of water pumped up (centrifuged electric pump) at each sampling point. Nutrient samples (1 liter) and water temperature measurements were only taken from the point at which the lake is deepest. Phytoplankton samples were immediately fixed in acetic Lugol's solution. Zooplankton were filtered through a 40- μm mesh and immediately fixed with 4% sucrose formalin for analyses. Zooplankton was also collected from July through November in 1998 (see below, experiments on *Daphnia* reproduction).

Laboratory analyses—Dissolved nutrient concentrations were analyzed after filtering water through Whatmann GF/C filter paper. The analytical methods used were: Murphy and Riley (1962) for soluble reactive phosphorus (SRP) and total phosphorus after persulfate digestion, the phenolphthorite method (Solórzano 1969) for ammonium, Strickland and Parsons (1972) for nitrite, and ion chromatography for nitrate. The dissolved inorganic N:total P molar ratio (DIN:TP) was calculated as an indicator of P or N limitation (Morris and Lewis 1988).

Phytoplankton samples were allowed to settle in counting chambers before examination with a Leitz inverted microscope, according to the technique of Utermöhl (1958). Algal samples were analyzed for species identification and cell density. At least 30 cells of each phytoplankton species were measured every sampling day. Phytoplankton biovolume was estimated by use of geometrical formulae and converted to biomass under the assumption of a density of 1 mg fresh weight mm^{-3} . Zooplankton individuals were identified according to species and development stage and counted in all samples of 1996, 1997, and 1998. Presence of ephippial or parthenogenetical eggs was recorded in the 1997 and 1998 samples (see below, experiment on *Daphnia* reproduction).

The zooplankton of 1996 and 1997 was examined for *K. gracilipes* epibionts at each sampling point on each sampling day, in a total of 59 samples in 1996 and 75 samples in 1997. The analyses focused on *D. pulicaria* and *M. laciniatus*. All the daphnids in samples with <100 individuals and all the *M. laciniatus* in samples with <50 individuals were examined for epibionts. In larger samples, we randomly selected and analyzed at least 100 daphnids and 50 *M. laci-*

niatus individuals. A total of 991 and 4,129 daphnids and of 2,652 and 4,336 *M. laciniatus* individuals were examined for epibionts in 1996 and 1997, respectively. A minimum of 35 individuals of each remaining taxon (if a taxon represented >1% of the zooplankton community biomass) were examined for epibionts.

Epibiont prevalence, defined as the percentage of zooplankton individuals with epibionts (Willey et al. 1990), was calculated separately for chlorangioids and for ankyroids/adults on each infected zooplankton individual. Epibiont burden, defined as the number of epibionts found on a substrate organism (Threlkeld et al. 1993), was assessed by use of distinct scales for *D. pulicaria* and *M. laciniatus* because of their very different epibiont densities. We differentiated between the chlorangioid burden and the ankyroid/adult burden. For *D. pulicaria*, the chlorangioid burden was assessed by ranking individuals on a scale of 0–5, with 0 = no epibionts present and 5 = extremely heavy infestation. The ankyroids and adults were ranked as follows: 0 = no cells present; 1 = 1–50 cells; 2 = 50–100 cells; 3 = 100–200 cells; 4 = 200–500 cells; 5 = >500 cells. For *M. laciniatus*, the chlorangioid burden was also assessed by ranking individuals on a scale of 0–5, with 0 = no epibionts present and 5 = maximum infestation. A score of 5 on the *M. laciniatus* scale is, at most, equivalent to a score of 2 on the *D. pulicaria* scale. Ankyroids and adults on *M. laciniatus* were so rare that we simply counted their number, which was always less than three.

In the present text, prevalence and burden refer to chlorangioids. Prevalence and burden of ankyroids/adults is specified as adult prevalence or adult burden.

D. pulicaria survival and reproduction—Two laboratory experiments were performed in a 2 × 2 factorial design with epibionts (+E, present; –E, absent) and food (+F, present; –F, absent) as independent factors. The aim of these experiments was to examine the effect of epibionts on the survival and reproduction of *D. pulicaria*. Food was included as a treatment because the epibiont effect appears to interact with food availability (Xu and Burns 1991; Weissman et al. 1993; Bartlett and Willey 1998).

For the experiments, *D. pulicaria* individuals were collected with vertical plankton net tows from Río Seco lake on 18 October 1997 and 10 September 1998. Experimental animals were collected at the times when the intensity and prevalence of epibiont infection were high. After 24 h of acclimation in the laboratory, adult females without parthenogenetic or ephippial eggs were selected by size (see below) and sorted as infected and uninfected individuals under the microscope. Each animal was placed individually into a 100-ml glass tube containing 75 ml of filtered and autoclaved lake water. Thirty-six randomly chosen individuals were prepared for each treatment, yielding a total of 144 individuals per experiment. Experimental infected animals were those showing maximum infection level with >1,000 adult epibionts attached to their legs. Uninfected experimental animals were those that had molted during the previous 24 h of acclimation period in the laboratory and had been isolated from sources of epibiont infection (lake water or infected molts), whereas heavily infected animals were those

nearly ready to molt. To evaluate the effect of epibiont on reproduction, we started the experiment with newly molted animals, to equalize the energy reserves and potential reproductive rate. The sizes of experimental animal were $1,775 \pm 128 \mu\text{m}$ ($\bar{x} \pm \text{SD}$) in 1997 and $1,809 \pm 123 \mu\text{m}$ ($\bar{x} \pm \text{SD}$) in 1998. The experimental incubation temperature and the light:dark relationship were the same as the lake conditions at the time of collection: 8°C and a L:D cycle of 11:13 h in 1997 and 13°C and a L:D cycle of 12.30:11.30 h in 1998. Once a week, one third of the water in the cultures was replaced with fresh filtered and autoclaved lake water. Exoskeletons of *Daphnia* were left in the experimental tubes, to facilitate epibiont infection. Each experiment lasted 20 d, counted from the first animal molt in the laboratory.

The green alga *D. chlorelloides*, isolated from Sierra Nevada lakes and cultured in Z8 medium (Skulberg and Skulberg 1990) in continuous culture, was used as food for the animals. Food was added as needed to avoid depletion below the incipient limiting concentration (Geller 1975). The animals were examined daily with an inverted microscope at 40× magnification for survival, egg production, and molts. Infected and noninfected animals remained so throughout the experimental period.

D. pulicaria sinking rate and weight—The sinking velocity was measured in *Daphnia* obtained from the lake on six sampling days through August, September, and October 1998. Infected (chlorangioid burden 3.56 ± 0.09 , $\bar{x} \pm \text{SE}$; adult burden 4.41 ± 0.05 ; $n = 68$) and uninfected (chlorangioid burden 0.60 ± 0.06 ; adult burden 0.01 ± 0.01 ; $n = 119$) animals without eggs were selected and included in commercial carbonated water for 1 min to narcotize them. The individual sinking rate was immediately determined in a 1-liter graduated cylinder filled with commercial water. Each individual was gently placed on the top of the water column and allowed to sink. We recorded the time taken by each animal to sink through two 10-cm intervals and used the mean value to calculate the sinking rate. The length of the *Daphnia* was then measured from the base of the tail spine to the top of the head.

We also measured the individual dry weight of the animals used for the sinking rate experiments. Each experimental animal was placed in a previously weighed aluminum plate, allowed to dry at 60°C during 24 h and immediately weighed on a Mettler UMT2 microbalance ($d = 0.1 \mu\text{g}$).

Statistical analysis—Field variables were normalized by logarithmic transformation or by arcsine-square-root transformation, in the case of the epibiont prevalence percentages (Sokal and Rohlf 1995, pp. 413, 419). Pearson's correlations between daily values (mean of the five daily sampling points) of biotic and abiotic variables were used to examine possible relationships between them. Annual comparisons of variables were tested by one-way ANOVAs on the mean daily values of each variable. The pattern of spatial distribution of zooplanktonic species and zoospores of *K. gracilipes* was analyzed by Taylor's model (Elliot 1977): $\log s^2 = a + b \log x$, where s^2 is the variance, x is the mean daily number of organism per liter or per milliliter, and a and b are fitted constants. The spatial correlation between zoospore

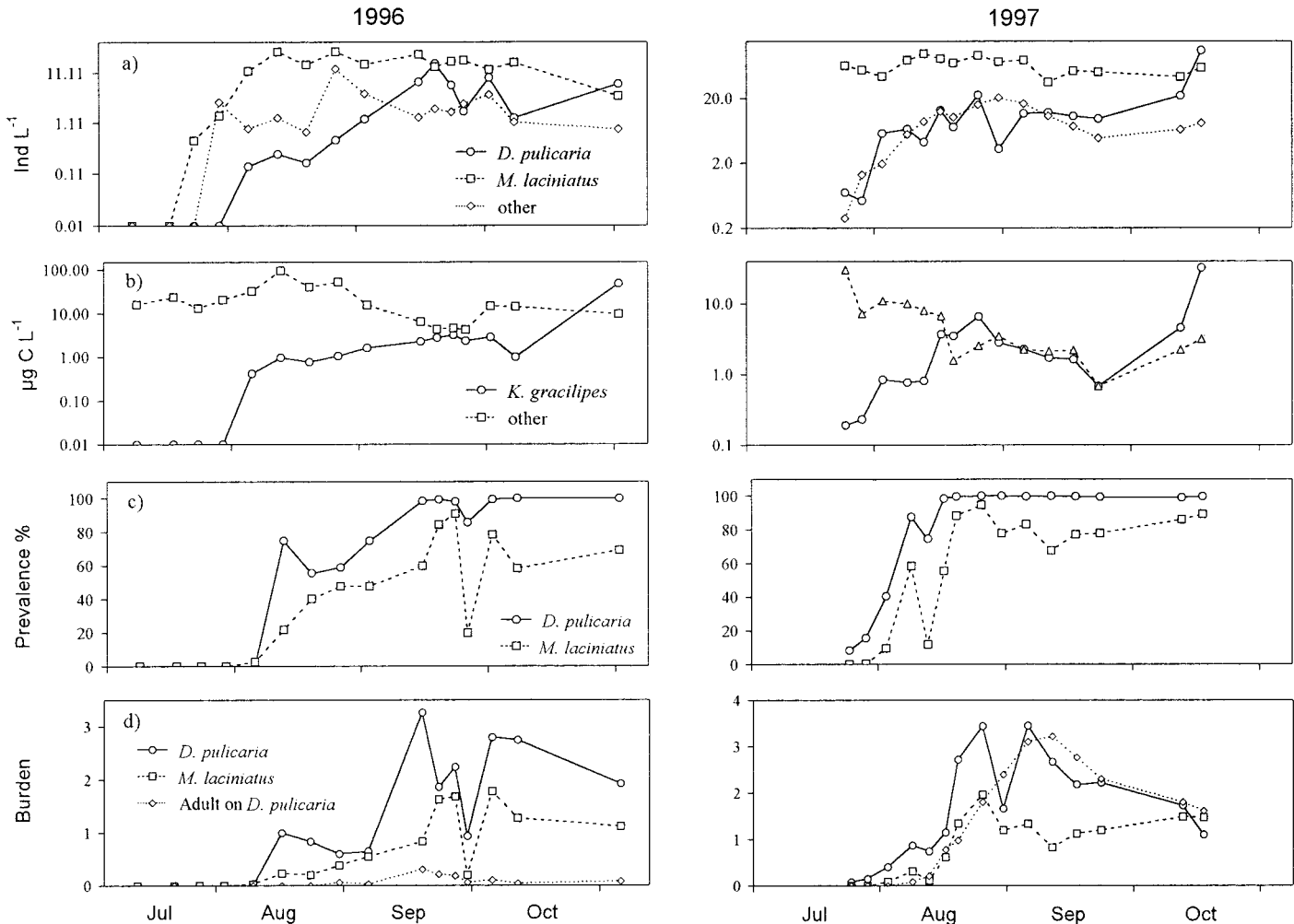


Fig. 1. (a) Zooplankton abundance and composition during 1996 and 1997 ice-free period. Values are daily average for five sampling points. Other consists mostly of *Diaptomus cyaneus*, *Chydorus sphaericus*, and rotifer species. Values <0.01 are presented as values $=0.01$. (b) Phytoplankton abundance and composition during 1996 and 1997 ice-free period. Values are daily average for five sampling points. Other consists mostly of *C. nevadensis* and *D. chlorelloides*. Values <0.01 are presented as values $=0.01$. (c) Daily prevalence of *K. gracilipes* on *D. pulicaria* and *M. laciniatus* during the ice-free period of 1996 and 1997. (d) Daily chlorangioid burden on *D. pulicaria* and *M. laciniatus* in 1996 and 1997 and daily adult burden on *D. pulicaria* in both years.

density and zooplanktonic species densities was examined by a Mantel test for matrix similarity (Sokal and Rohlf 1995, p. 813), by use of the Monte-Carlo procedure. Each specific matrix was made on the Euclidean distances between sampling point densities.

Survival was analyzed, by use of a survival test for censored data and multiple samples (Kirk 1997), with the STATISTICA program (Statsoft). Differences in survival functions between pairs of treatments were tested by use of nonparametric log-rank tests for homogeneity of survival functions.

Nonparametric tests were used to analyze differences in fecundity and intermolt time between treatments because of the nonnormal distribution of variables and the heterogeneity of variances. Differences in the number of ephippia produced per animal and, in the intermolt time between treatments, were compared with use of the Kruskal-Wallis ANOVA, Mann-Whitney *U* tests, corrected for multiple testing

with the sequential Bonferroni test (Rice 1989), were used for examining differences in fecundity between pairs of treatments.

Results

Zooplankton community seasonality—In 1996 and 1997, the most abundant zooplankton species were the cladoceran *D. pulicaria* and the copepod *M. laciniatus* (Fig. 1a). These species made up $>90\%$ of the total zooplankton biomass in both years. At the beginning of the ice-free period, the zooplankton community was dominated by nauplii of *M. laciniatus*. Later on, copepodites of *M. laciniatus* and small *D. pulicaria* individuals became more abundant, and, at the end of the growing season, the community was dominated by the last copepodite stages and adults of *M. laciniatus* and *D. pulicaria*. The densities of *M. laciniatus* and *D. pulicaria* were significantly higher in 1997 than 1996 (Table 1). *D.*

Table 1. Range and mean value of the abiotic conditions (during the period that *K. gracilipes* was observed in the lake) and biotic parameters in 1996 and 1997. The table contains the F ratio and significance level (*P*) of the ANOVA tests done to examine interannual differences of the variables (analyses done on the daily mean values and, for biotic parameters, over the period each variable was observed). All variables were normalized by log transformation except prevalence percentages, which were transformed to arcsine square roots.

Parameter	1996		1997		F ratio	<i>P</i>
	Range	$\bar{x} \pm SE$	Range	$\bar{x} \pm SE$		
Temperature (°C)	5.00–16.00	10.19 \pm 1.17	6.75–15.90	11.70 \pm 0.69	2.22	0.148
SRP (mol L ⁻¹)	0.02–0.25	0.09 \pm 0.02	0.00–0.17	0.05 \pm 0.01	5.23	0.031
DIN (mol L ⁻¹)	1.18–11.96	5.86 \pm 0.98	0.18–6.57	1.85 \pm 0.49	17.51	0.000
DIN/TP	1.63–24.08	9.69 \pm 1.66	0.42–15.31	3.37 \pm 1.19	16.33	0.000
Zoopore density (cells ml ⁻¹)	0.00–351.51	154.49 \pm 27.87	11.88–3,166.64	440.03 \pm 201.75	0.96	0.335
<i>D. pulicaria</i> density (ind L ⁻¹)	0.00–16.72	3.96 \pm 1.38	0.00–107.98	15.85 \pm 6.79	6.91	0.014
Prevalence on <i>D. pulicaria</i>	0.00–100.00	85.93 \pm 5.19	8.40–100.00	81.54 \pm 8.38	0.28	0.600
Burden on <i>D. pulicaria</i> *	0.00–3.27	1.72 \pm 0.29	0.08–3.46	1.64 \pm 0.29	0.01	0.910
Adult prevalence on <i>D. pulicaria</i>	0.00–29.00	11.92 \pm 8.88	0.00–99.94	82.24 \pm 9.32	15.19	0.000
Adult burden on <i>D. pulicaria</i>	0.00–0.30	0.12 \pm 0.09	0.00–3.22	1.76 \pm 0.31	16.44	0.000
<i>M. laciniatus</i> density (ind L ⁻¹)	0.00–29.10	13.55 \pm 2.50	35.11–97.32	64.64 \pm 4.72	31.13	0.000
Prevalence on <i>M. laciniatus</i>	0.00–90.99	51.78 \pm 7.90	0.00–94.70	62.71 \pm 8.58	1.10	0.300
Burden on <i>M. laciniatus</i> *	0.00–1.78	0.83 \pm 0.19	0.00–1.97	0.94 \pm 1.64	0.50	0.490

* Note the different burden scale used for *D. pulicaria* and *M. laciniatus* (see text).

pulicaria developed considerably later (Fig. 1a) and under lower temperatures in 1996, compared with 1997 (ANOVA $P < 0.05$ for interannual differences in temperature when daphnids > 0.2 ind L⁻¹).

D. pulicaria showed a markedly patchy distribution in Río Seco lake, with the slope of Taylor's model significantly different from 1, $b = 2.53$ (linear regression, $r^2 = 0.978$, $P < 0.001$) and $b = 2.16$ ($r^2 = 0.945$, $P < 0.001$) for 1996 and 1997, respectively. *M. laciniatus* was also aggregated distributed, although the regression fitted to Taylor's model was much weaker than that for *D. pulicaria* ($b = 1.59$, $r^2 = 0.408$, $P = 0.034$ for 1996 and $b = 4.05$, $r^2 = 0.477$, $P = 0.004$ for 1997).

Phytoplankton community seasonality—The phytoplankton community was very simple (Fig. 1b). The period after the thaw was dominated by *C. nevadensis*, *D. chlorelloides*, and *Ochromonas* sp. Zoospores of *K. gracilipes* appeared on the plankton just after the thaw, increased gradually during

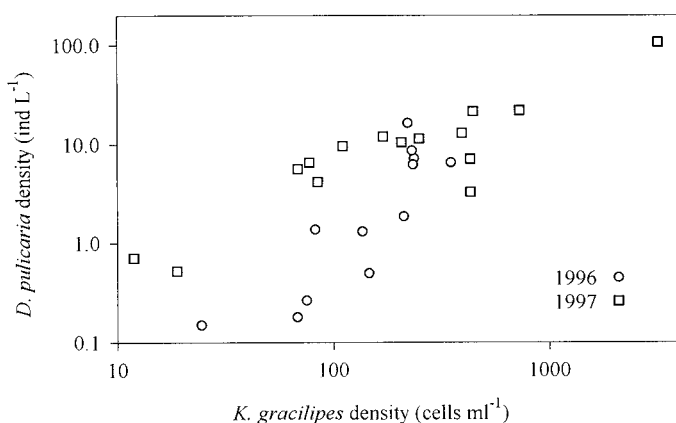


Fig. 2. *D. pulicaria* density versus *K. gracilipes* zoospores density during 1996 and 1997. Values are daily average for five sampling points. Values = 0 are not shown.

July–August, and remained at high levels until the fall. *K. gracilipes* made up ~26% (1996) and 51% (1997) of the total phytoplankton biomass when *Daphnia* was abundant (Fig. 1a,b), although there was no significant difference in its absolute density (Table 1). *K. gracilipes* is usually the dominant taxon during the second half of the ice-free period (Cabrera-Fernández 1996; Morales-Baquero et al. pers. comm.). It is important to note that, in 1996, the phytoplanktonic species densities, except for *K. gracilipes* density, were significantly higher than those normally found (Cabrera-Fernández 1996; Morales-Baquero et al. pers. comm.), probably because the low temperatures of 1996 led to a significant decrease in zooplankton density and, consequently, to a lesser grazing pressure on algal species.

There was a significant correlation between daily zoospore and *D. pulicaria* densities in both years studied (Fig. 2; $r = 0.84$, $P < 0.001$ for 1996; $r = 0.89$, $P < 0.001$ for 1997). However, zoospore density was not related to *M. laciniatus* density in either year ($r = 0.33$, $P = 0.274$ for 1996; $r = 0.12$, $P = 0.661$ for 1997).

Zoospores also showed a patchy distribution in the lake (Taylor's model; $b = 3.42$, $r^2 = 0.778$, $P < 0.001$ for 1996 and $b = 2.17$, $r^2 = 0.879$, $P < 0.001$ for 1997). Their spatial pattern was coincident with the *D. pulicaria* pattern in both years (Mantel test; $t = 1.973$, $P < 0.01$ for 1996 samples when *D. pulicaria* > 1.3 ind L⁻¹ and $t = 2.814$, $P < 0.01$ for 1997), whereas it showed no significant relation with the *M. laciniatus* pattern (Mantel test; $t = 0.250$, $P > 0.05$ for 1996 and $t = 0.195$, $P > 0.05$ for 1997).

Epibiont prevalence and burden—*K. gracilipes* were observed attached to all the zooplankton species present in the lake. However, the prevalence and burden on distinct taxa were highly variable. On the less common species (all except *M. laciniatus* and *D. pulicaria*), we only found chlorangioids, at a prevalence of 0% to 66% and with a low number of epibionts attached.

The prevalence on *M. laciniatus* and *D. pulicaria* in-

creased rapidly at the beginning of the growing period and remained at high levels until the end of the ice-free period (Fig. 1c). The prevalence on *D. pulicaria* was higher than that on *M. laciniatus* in both years (Table 1; ANOVA: $F = 6.76$, $P = 0.015$ for 1996; $F = 6.32$, $P = 0.018$ for 1997).

The prevalence on *D. pulicaria* was significantly related to the density of *D. pulicaria* in both years ($r = 0.84$, $P < 0.001$ for 1996; $r = 0.79$, $P < 0.001$ for 1997). The prevalence on *M. laciniatus* was not related to *M. laciniatus* density in either year ($r = -0.18$, $P = 0.579$ for 1996; $r = 0.04$, $P = 0.877$ for 1997). However, *M. laciniatus* prevalence was significantly related to *D. pulicaria* density ($r = 0.82$, $P < 0.001$ for 1996; $r = 0.80$, $P < 0.001$ for 1997).

D. pulicaria showed the highest epibiont burden in both years (Fig. 1d). It should be noted that we used different scales for the degree of infestation of the two species (see Methods). In fact, the epibiont load on *M. laciniatus* was never higher than level 2 of the *D. pulicaria* scale. The burden on *D. pulicaria* and *M. laciniatus* was also significantly related to daphnid density in both years (*D. pulicaria*: $r = 0.78$, $P = 0.003$ for 1996 and $r = 0.61$, $P = 0.017$ for 1997; *M. laciniatus*: $r = 0.86$, $P < 0.001$ for 1996 and $r = 0.75$, $P < 0.001$ for 1997). In contrast, no relation was found between the burden on *M. laciniatus* and its density ($r = -0.27$, $P = 0.396$ for 1996; $r = 0.02$, $P = 0.959$ for 1997).

In 1996, only 11.9% of daphnids showed attached ankyroids/adults, and, among these, no more than four ankyroids/adults per individual were observed. In contrast, in 1997, 82.2% of daphnids carried a high number of ankyroids/adults attached to their legs (Fig. 1c,d; Table 1) to the degree that the daphnids had an immediately apparent green color. The adult burden on daphnids was significantly related to *D. pulicaria* density in both years ($r = 0.81$, $P = 0.002$ for 1996; $r = 0.59$, $P = 0.022$ for 1997). Ankyroids and adults were rarely observed on *M. laciniatus*, and, from a total of 6,988 individuals of this taxon analyzed for epibionts between 1996 and 1997, only 38 presented ankyroids/adults, and, among these, no more than three individuals per animal were observed.

The density of zoospores was significantly related to the *D. pulicaria* prevalence and burden in both years (prevalence: $r = 0.77$, $P = 0.003$ for 1996; $r = 0.84$, $P < 0.001$ for 1997 and $r = 0.82$, $P < 0.001$ for adult prevalence of 1997; burden: $r = 0.61$, $P = 0.033$ for 1996, $r = 0.64$, $P = 0.010$ for 1997 and $r = 0.63$, $P = 0.012$ for adult burden of 1997).

Within each species, chlorangioid prevalence and burden were not significantly different between the years (Table 1). Nevertheless, the total density of chlorangioids was lower in 1996, because the zooplankton density was lower. The adult prevalence and burden on *Daphnia* were very different between the years (Table 1).

Abiotic relationships—The year 1996 was a cold and wet year in the Sierra Nevada mountains, which caused a delay in the thawing of the lake in 1996 (late July), compared with 1997 (early July). Water temperatures increased rapidly after the thaw and reached maximum values in late July in 1997 and in early August in 1996. Temperatures remained at high values (~ 12 – 16°C) through August and declined from early

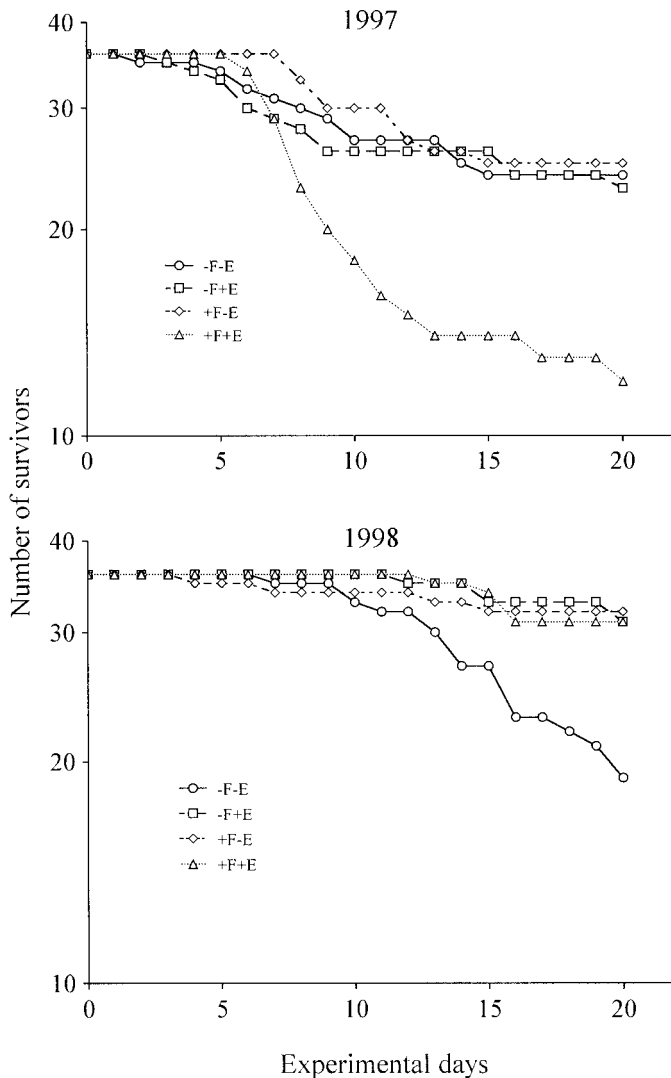


Fig. 3. Number of survivors in the different treatments during the survival experiment. Treatment abbreviations: -F-E, absence of food and absence of epibionts; -F+E, absence of food and presence of attached epibionts; +F-E, addition of food and absence of epibionts; and +F+E, addition of food and presence of attached epibionts.

September to the fall. Zoospore and *D. pulicaria* densities and daily prevalence and burden on *D. pulicaria* showed a significant negative relation with temperature values in 1996 (Pearson's correlation, all $P < 0.05$), whereas no significant relationship was found in 1997.

Nutrient conditions were significantly different between the years, with a higher DIN, SRP, and DIN:TP ratio in 1996 with respect to 1997 (Table 1). The nutrient conditions were not related to epibiont abundance, except for the positive relationship between zoospore density and SRP, DIN, and DIN:TP in 1997 (Pearson's correlation, all $P < 0.05$).

Daphnia survival and reproduction—Figure 3 shows the number of survivors in the four treatments for 1997 and 1998. Survival analysis revealed significant differences in the curves between treatments in both years (chi square =

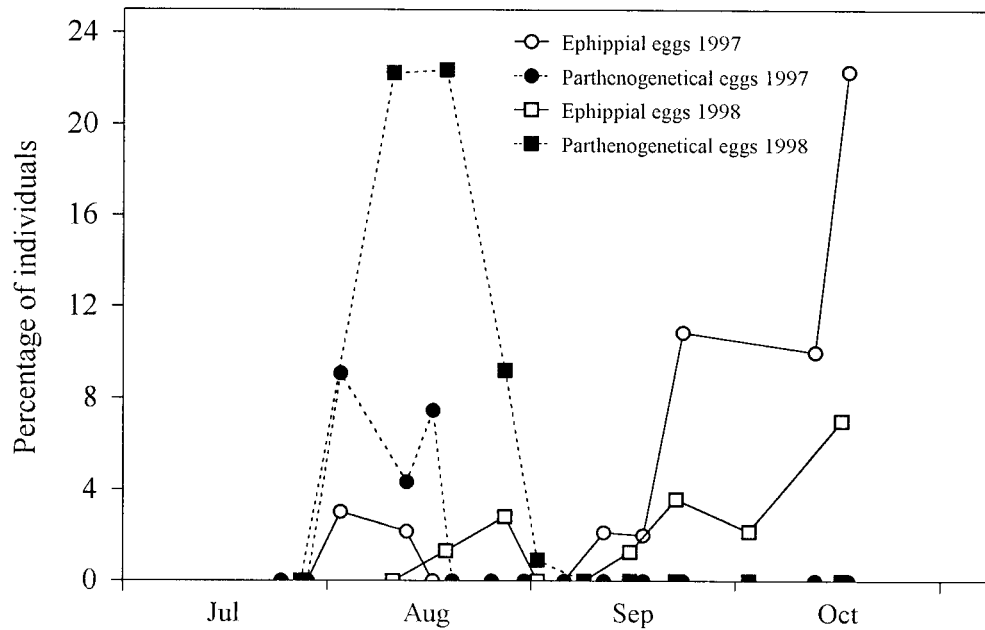


Fig. 4. Percentage of individuals with ehippial and parthenogenetic eggs of the field samples in 1997 and 1998.

12.60, $P < 0.01$ and chi square = 18.09, $P < 0.01$ for 1997 and 1998, respectively). Survival was significantly lower for the treatment with food and with epibionts (+F+E) with respect to the other treatments (log-rank test, all $P < 0.05$) in 1997, whereas, in 1998, survival was significantly lower for the treatment without food and without epibionts (-F-E; log-rank test, all $P < 0.01$).

Parthenogenetic eggs were not produced in any experiment. Only ehippial eggs were found. *D. pulicaria* in Río Seco lake produce mainly ehippial eggs, whereas production of parthenogenetic eggs in the lake was low and was restricted to the beginning of the population development (Fig. 4). It proved impossible to perform the experiments at the time when parthenogenetic eggs were observed in the lake (i.e., early-middle August) because of the low *D. pulicaria* density and low epibiont infection level during this period.

The total and mean number of ehippia produced showed clear differences between the treatments in the two experi-

Table 2. Total number of ehippia produced by daphnids and mean number ($\bar{x} \pm SE$) of ehippia produced per individual ($n = 36$) in the reproduction experiments.

	1997		1998	
	Total No.	$\bar{x} \pm SE$	Total No.	$\bar{x} \pm SE$
-F-E	0	—	0	—
-F+E	16	0.44 \pm 0.50	24	0.67 \pm 0.48
+F-E	6	0.17 \pm 0.38	30	0.83 \pm 0.45
+F+E	21	0.58 \pm 0.55	49	1.36 \pm 0.59

Treatment abbreviations: -F-E, absence of food and absence of epibionts; -F+E, absence of food and presence of attached epibionts; +F-E, addition of food and absence of epibionts; and +F+E, addition of food and presence of attached epibionts.

ments (Table 2). The most obvious result was the absence of ehippia production by the uninfected and starving animals (-F-E) in both experiments, in contrast to the variable number of ehippia produced in the remaining treatments. Therefore, statistical analysis was performed on the rest of the treatments. The mean number of ehippia produced per animal showed significant differences between treatments (Kruskall-Wallis ANOVA; $H [2, n = 108] = 12.30$, $P < 0.005$ for 1997, $H [2, n = 108] = 26.98$, $P < 0.001$ for 1998). Comparisons between pairs of treatments showed a higher production of ehippia by fed and infected animals (+F+E) than by fed and uninfected animals (+F-E) in both experiments (Mann-Whitney test $U = 393.00$, $P = 0.004$ for 1997 and $U = 357.50$, $P < 0.001$ for 1998). Fed and infected animals (+F+E) had a higher production of ehippia, compared with unfed and infected ones (-F+E) in the 1998 experiment ($U = 288.00$, $P < 0.001$), whereas no significant differences between these treatments were found in the 1997 experiment ($U = 568.00$, $P = 0.367$).

The timing of ehippia production was different in 1997 and 1998. In 1997, animals reproduced in the first instar after the start of the experiment, whereas in 1998 they reproduced in the second instar. Intermolt time was affected by experimental temperature, and values of 9.77 ± 0.18 d ($\bar{x} \pm SE$) and 6.03 ± 0.11 d were found in 1997 and 1998, respectively. Within the year, the mean intermolt time of daphnids was affected by the treatment conditions (Kruskall-Wallis ANOVA; $H [3, n = 144] = 33.70$, $P < 0.001$ for 1997, $H [3, n = 144] = 81.28$, $P < 0.001$ for 1998). The longest and shortest intermolt times were shown by -F-E and +F+E treatments, respectively (Table 3).

Daphnia sinking rate—The sinking rates of infected and uninfected daphnids were $0.500 \text{ cm s}^{-1} \pm 0.008$ ($\bar{x} \pm SE$; n

Table 3. Mean intermolt time (days) of the daphnids in the reproduction experiments.

Treatment	1997	1998
	$\bar{x} \pm \text{SE}$	$\bar{x} \pm \text{SE}$
-F-E	12.48 \pm 0.36	7.43 \pm 0.26
-F+E	9.00 \pm 0.11	6.32 \pm 0.09
+F-E	9.15 \pm 0.14	5.36 \pm 0.11
+F+E	8.52 \pm 0.18	5.05 \pm 0.08

Treatment abbreviations: -F-E, absence of food and absence of epibionts; -F+E, absence of food and presence of attached epibionts; +F-E, addition of food and absence of epibionts; and +F+E, addition of food and presence of attached epibionts.

= 68) and $0.426 \text{ cm s}^{-1} \pm 0.007$ ($\bar{x} \pm \text{SE}$; $n = 119$), respectively. Infected daphnids weighed $50.70 \mu\text{g dw} \pm 1.20$ ($\bar{x} \pm \text{SE}$; $n = 68$), whereas the weight of noninfected ones was $37.16 \mu\text{g dw} \pm 0.97$ ($\bar{x} \pm \text{SE}$; $n = 119$). ANCOVA showed that the sinking rates and weight of infected animals were significantly higher than those of uninfected animals (Fig. 5; sinking rate results: $\text{df} = 1$, $F = 21.03$, $\text{df error} = 184$, $P < 0.001$; test of parallelism $P = 0.904$; weight results: $\text{df} = 1$, $F = 55.48$, $\text{df error} = 184$, $P < 0.001$; test of parallelism $P = 0.961$).

Discussion

Epibiont substrate species preference and seasonality—*K. gracilipes* is not host specific, since this epibiont was observed on most of the zooplankton taxa of Río Seco lake. However, epibiont prevalence and burden differed widely between the host species in the 2 yr studied. Our results clearly show that *K. gracilipes* has a preference for *D. pulicaria* in Río Seco lake, and the abundance of this taxon strongly influenced the abundance and the seasonality of the epibiont. These conclusions are supported by several findings:

(1) The reproductive stages of *K. gracilipes* were almost exclusively observed on *D. pulicaria*. This suggests a narrow dependence of the epibiont on this taxon, given that the adult stage is responsible for the formation of zoospores (dispersal phase) and of cysts (overwintering resistance cells). *D. pulicaria* is therefore a key species for the intraannual development and interannual permanence of *K. gracilipes* in Río Seco lake.

(2) The density of the epibiont (zoospores and attached stages on *M. laciniatus* and *D. pulicaria*) was highly and exclusively related to *D. pulicaria* density in both years, indicating that *D. pulicaria* density regulates the development of the epibiont population. Several authors found a positive relationship between epibiont infestation level and host density (Prasad et al. 1989; Mohlenberg and Kaas 1990; Chiavelli et al. 1993; Willey and Threlkeld 1993). Chiavelli et al. (1993) also found a relationship between *Daphnia* density and the prevalence of three phototrophic epibionts, despite the fact that cyclopoids rather than *Daphnia* showed the highest prevalence for one of the epibionts. They concluded that *Daphnia* appears to regulate the abundance of the three epibionts. In contrast, many other studies show no relationship between epibiont infection and prevalence (see table 1

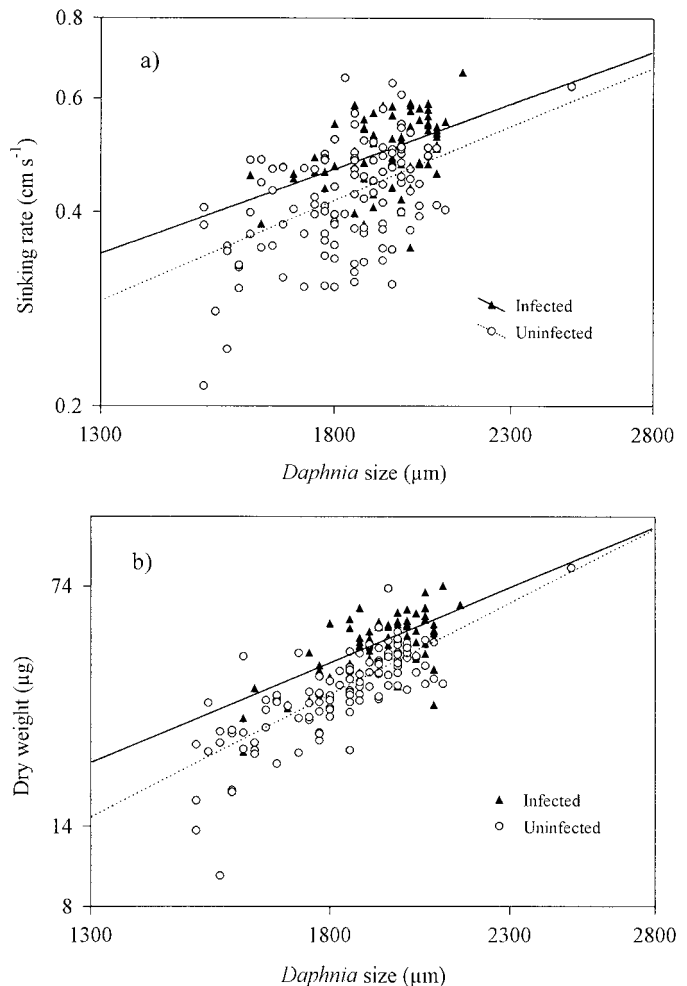


Fig. 5. (a) Sinking rate and (b) dry weight of infected and uninfected *D. pulicaria* of different length. ANCOVA showed sinking rates and weight of infected animals were significantly higher than those of uninfected animals (see Results).

of Threlkeld et al. 1993). Threlkeld et al. (1993) suggested that the variation in this relationship supports the idea of the existence of source and sink substrate habitats for the epibiont (Pulliam 1988). Thus, in our study, *Daphnia* would be a reproductive source habitat and *M. laciniatus* a reproductive sink habitat. We think that this consideration depends on the timescale analyzed. Both species would act as source habitats during the ice-free period, because they allow epibiont maintenance and growth. Epibionts could benefit from *M. laciniatus* as a secondary substrate for growth and dispersal and mainly by attachment to this taxon during the early growth season, when daphnids are absent. However, *M. laciniatus* would be a sink habitat for epibiont interannual persistence because epibiont cysts are not produced on it.

(3) As well as the temporal correlation, there is a spatial coincidence between zoospores and daphnids within the lake, whereas no relationship was found with *M. laciniatus*. Again, the density of *D. pulicaria* seems to determine the density of zoospores. The spatial pattern of substrate organisms may also be a determinant factor in epibiont colonization (Threlkeld and Willey 1993). In Río Seco lake, the

strong aggregative behavior of *Daphnia* could favor epibiont dispersal and maintenance. The positive spatial correlation between zoospore density and *D. pulicaria* density indicates that any individual within a *Daphnia* patch is more likely to be colonized than is a randomly collected individual of the plankton. Thus, the probability of zoospore colonization should increase as *Daphnia* density increases, since a "cloud" of zoospores would exist within the *Daphnia* patch.

(4) There is no relation between abiotic conditions and *K. gracilipes* seasonality in Río Seco lake, indicating that these factors did not determine epibiont abundance. Annual variations in average nutrient levels were not consistent with the annual variation of average epibiont concentration, since the epibiont concentration (attached stages) was lower in 1996 than in 1997, whereas the SRP and DIN amounts were higher. Chiavelli et al. (1993) found similar results in a field study on three epibiont taxa. Algal epibionts on zooplankton would be less nutrient-limited than are other free-swimming algae, since they have a good nutrient supply from the constant flow created by their host and from the host excreta (Ikeda 1977; Gibson 1979; Wahl 1989; Mohlenberg and Kaas 1990). In fact, the specific attachment localization of ankyroids and adults on the *Daphnia* legs, where the water currents pass directly over them, may greatly increase their access to nutrients. Furthermore, the nutrient supply from host excreta could be one reason for the epibiont preference for *Daphnia*, because cladocerans have higher excretion rates than do copepods (Lehman 1980). In addition, and because algae are probably N-limited in Río Seco lake (Morales et al. 1999), *K. gracilipes* could take advantage of the higher N/P excretion rate of *Daphnia* with respect to copepods (Elser et al. 1988; Urabe 1993). However, no clear effect of DIN:TP on epibiont abundance can be concluded from our results.

Similar to Chiavelli et al. (1993) and López et al. (1998), we found no relationship between temperature and epibiont development. The negative relationship between epibiont development and temperature in 1996 is not conclusive, since the *Daphnia* density also negatively correlated with temperature. The highest zoospore density and burden coincided with the highest density of *D. pulicaria* (Fig. 1a,b,d) and seemed to be relatively independent of temperature. In contrast, other authors have reported a relationship between temperature and epibiont growth that is mainly derived from the effect of temperature on substrate instar duration, since epibiont reproduction occurs while they are attached to the substrate (Allen et al. 1993; Gaiser and Bachmann 1993; Willey and Threlkeld 1993). *M. laciniatus* has a longer intermolt period (copepodites and adults, abundant from middle August) than *D. pulicaria*. However, epibiont growth on the copepod was low and appeared to depend on *D. pulicaria* density. The higher molting frequency of daphnids with respect to copepods could possibly facilitate epibiont population growth instead of hindering it. Because the production of cysts and a massive number of zoospores occurs precisely when daphnids molts, the relatively frequent molting of *D. pulicaria* may help produce a significant increase in the number of zoospores for dispersal and colonization. Furthermore, it might guarantee the production of a sufficient number of cysts.

Interannual temperature differences could explain the scarcity of ankyroids/adults in 1996, compared with 1997 (Table 1). The lower temperatures and shorter daily light period during the presence of the *D. pulicaria* population in 1996 could have affected the development of ankyroid/adults. Our previous observations on Río Seco lake (Sánchez-Castillo 1987; Cabrera-Fernández 1996) had shown that *D. pulicaria* usually present numerous ankyroids/adults attached to their legs.

Effect of epibiont on Daphnia survival, reproduction, and sinking rates—Our results on *Daphnia* reproduction experiments clearly show that the presence of attached epibionts enhanced the production of ephippia by *D. pulicaria* (Table 2). It is important to note that the production of ephippia should be regarded as a positive effect in Río Seco lake, where the production of overwintering forms is essential for interannual persistence. The production of subitaneous eggs in Río Seco lake at the end of the summer and autumn, when the experimental animals were collected, has no reproductive value because newborn individuals would have little chance of reaching adulthood and reproducing before the lake freezes in early autumn. The positive impact of epibionts on the reproduction of *D. pulicaria* can be explained by the hypothesis that *D. pulicaria* feeds on *K. gracilipes*. Three findings indicate that *K. gracilipes* is a food source for *D. pulicaria*: (1) the enhancement of the ephippial production by infected and starving animals (−F+E) and uninfected and fed animals (+F−E), compared with uninfected and starving animals (−F−E) (Table 2); (2) the presence of green-colored guts in fed animals (+F−E and +F+E) and infected and unfed animals (−F+E), compared with the brown-colored guts of the uninfected and unfed animals (−F−E); and (3) the reduction of the instar duration by epibiont presence and/or food availability (Table 3).

Experimentally infected animals could mainly feed on the *K. gracilipes* zoospores produced by the adult epibionts attached to the *D. pulicaria* legs and also on the zoospores detached from the *D. pulicaria* body. Infected animals had >1,000 adult epibionts, implying a minimum production of 209,387 zoospores per *Daphnia* in each molt. This number of zoospores represents 2.89 $\mu\text{g C}$ (0.2 $\text{pg C } \mu\text{m}^{-3}$; Rocha and Duncan 1985) available for *Daphnia* feeding during each intermolt period, a food concentration of 0.0137 $\mu\text{g C h}^{-1}$ and 0.021 $\mu\text{g C h}^{-1}$ for 1997 (8.77 days intermolt period for −F+E and +F+E) and 1998 (5.69 days intermolt period for −F+E and +F+E), respectively. The estimated food threshold for *D. pulicaria* reared at 20°C is 0.017 $\mu\text{g C h}^{-1}$ (Gliwicz 1990), but this quantity should be lower for our experimental animals, which were reared at a lower temperature. Thus, zoospore production in the experiments should be close to the food threshold for *Daphnia* and presumably higher, because unfed and infected animals (−F+E) were allowed to survive and reproduce.

Interaction effects of food and epibiont on reproduction are unclear from our results. It seems that there is an enhancement of reproduction when both food and epibiont are present, probably because of an increase in animal energy intake. Absence of this synergic effect in 1997 might be attributed to the high mortality rate of fed and infected an-

imals. In contrast, the synergic effect on instar duration is clear, because the shortest period was found in fed and infected animals of both years (Table 3).

Survival results show differences between the experiments, which might be explained by the differences in experimental temperatures. The higher mortality rate of fed and infected animals (+F+E) in 1997 appears to be related to reproduction. Undoubtedly the production of ephippia represented an energy cost for *D. pulicaria* individuals, which could imply a decrease of energy allocation to survival (Threlkeld 1987). This cost could have been lower for the 1998 animals, which were reared at higher temperature. On the other hand, unfed and uninfected animals in 1997 showed a considerably higher survival rate than did those in 1998 (Fig. 3). Animals growing at a low temperature (8°C) had a low metabolic rate and presumably did not exhaust their energy reserves during the experimental period, whereas animals growing at 13°C (1997) exhausted their reserves in a shorter period.

Consistent with our results, Herman and Mihursky (1964) and Allen et al. (1993) found higher sinking rates for infected animals than for uninfected ones. The increase in the *D. pulicaria* sinking rate in our experiments must be attributed to the significant increase in weight of the epibionts. This may imply a physiological cost affecting host fitness, and Allen et al. (1993) attributed increased host mortality to the added stress of carrying epibionts, although they found no effect on reproduction. Nevertheless, our experimental data showed no negative effect on host fitness from carrying epibionts. On the contrary, host reproduction was enhanced by epibiont infection, and survivorship was not significantly affected. The possible negative effect that carrying epibionts has on survivorship and reproduction could be counteracted by the extra energy intake from feeding on the zoospores. However, although several authors have suggested that algal epibionts could be edible by their hosts (Threlkeld and Willey 1993; Al-Dhaheri and Willey 1996), no field or experimental studies on zooplankton–algal epibiont relationships have examined this possible benefit, except for a recent paper by Bartlett and Willey (1998). These authors found that *Daphnia laevis* fed on the *Colacium* dispersal stage and enhanced its fitness by extended survivorship and increased size of neonates. An additional case of host feeding on its epibiont is the recently demonstrated feeding of the thermal vent shrimp on its epibiotic bacteria (Polz et al. 1998).

K. gracilipes–*D. pulicaria* relationship—The balance between recruitment and losses appears to be positive for *K. gracilipes* living on *Daphnia*, because their density increased with *Daphnia* density and they successfully colonized Río Seco lake. Likewise, the positive effects of feeding on zoospores might counteract the negative effects of carrying epibionts for *Daphnia*. This finding could also be valid for the field system where zoospores are an important food source for *Daphnia*.

Thus, the *D. pulicaria*–*K. gracilipes* relationship could be considered as a mutualism, where both species obtain a net benefit. This interaction could be similar to the farming of fungi by beetles, where the beetle feed on the fungi but carry spores and inocula of the fungi, thus facilitating their dis-

persal (Begon et al. 1986). In our case, *K. gracilipes* serves as food for *Daphnia* growth and reproduction but depends on *Daphnia* for dispersal in time.

There are no *Daphnia* predators in Río Seco lake. Burns (1989) indicated that in systems with very few or no visual predators, epibiont infestations may be very important in structuring zooplankton dynamics, because their role is not masked by predation. Thus, epibiosis may play an important role in population and community regulation in Río Seco lake through the differential benefits and costs for filtrators.

The outcome of the cost-benefit balance for *K. gracilipes* and *D. pulicaria* must evidently be dynamic and a function of different variables, such as the density, physiological conditions, genetic composition or age of both partners, food availability, or climatic conditions (Bronstein 1994). The close association of the *K. gracilipes* life cycle to *D. pulicaria* and the spatial and temporal fitting of both species suggest the possibility of a coevolved relationship. In Río Seco lake, with its short growing season and hard climatic conditions, natural selection must act strongly on the species and organisms to rapidly colonize the system and produce overwintering forms to persist in the lake. Thus, this interaction should always be analyzed in its ecological context.

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