

Evolution in Response to Direct and Indirect Ecological Effects in Pitcher Plant Inquiline Communities

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ABSTRACT: Ecologists have long recognized the importance of indirect ecological effects on species abundances, coexistence, and diversity. However, the evolutionary consequences of indirect interactions are rarely considered. Here I conduct selection experiments and examine the evolutionary response of *Colpoda* sp., a ciliated protozoan, to other members of the inquiline community of purple pitcher plants (*Sarracenia purpurea*). I measured the evolution of six traits in response to (1) predation by mosquito larvae, (2) competition from other ciliated protozoans, and (3) simultaneous predation and competition. The latter treatment incorporated both direct effects and indirect effects due to interactions between predators and competitors. Population growth rate and cell size evolved in response to direct effects of predators and competitors. However, trait values in the multispecies treatment were similar to those in the monoculture treatment, indicating that direct effects were offset by strong indirect effects on the evolution of traits. For most of the traits measured, indirect effects were opposed to, and often stronger than, direct effects. These indirect effects occurred as a result of behavioral changes of the predator in the presence of competitors and as a result of reduced densities of competitors in the presence of predators. Incorporating indirect effects provides a more realistic description of how species evolve in complex natural communities.

Keywords: *Colpoda*, competition, predation, protozoa, *Sarracenia purpurea*, selection.

Introduction

Although most ecological and evolutionary experiments examine the direct effects of species on one another, species in natural biological systems exist within a network of species interactions and may interact both directly and indirectly with many species simultaneously. Indirect, or higher-order ecological interactions occur when the direct effect of one species on another is affected by the presence of a third species (Vandermeer 1969; Holt 1977; Strauss

1991; Wootton 1994). In more diverse and complex communities, indirect effects are likely to increase in importance. As the number of species in a community increases, the number of possible indirect pathways between species increases exponentially relative to the number of direct effects (Abrams 1992; Menge 1995). Indeed, in some cases, multiple indirect effects can swamp the influence of direct ecological effects (e.g., Stone and Roberts 1991; Abrams 1992; Miller 1994). The effect of indirect interactions on ecological patterns has been well studied and can result in changes in species abundances, coexistence, and community diversity (e.g., Vandermeer 1969; Strauss 1991; Lawler 1993; Miller 1994; Wootton 1994; Menge 1995). Given their ecological importance, it seems likely that indirect effects would also be critical in understanding how species evolve in a community context.

Most experiments in evolutionary biology remove species from a community context and measure selection or evolution in response to abiotic factors or, at most, one other species. For instance, experiments documenting the direct effect of predators on prey evolution are abundant. Selection by predators or herbivores can result in the evolution of various traits, including coloration (Alatalo and Mappes 1996), size (Núñez-Farfán and Dirzo 1994; terHorst et al. 2010), chemical defenses (Mauricio and Rausher 1997; Shonle and Bergelson 2000), and structural defenses (Seeley 1986; Reimchen and Nosil 2002). The evolutionary consequences of competitive interactions have been considered as well, and experiments have demonstrated selection for traits such as growth rate (Schluter 1994; Santos et al. 1997; McGoey and Stinchcombe 2009), body size (Santos et al. 1997; McGoey and Stinchcombe 2009; terHorst 2010), foraging behavior (Sokolowski et al. 1997), and floral traits (Smith and Rausher 2008). The consequences of direct positive species interactions such as facilitation and mutualism on trait evolution have been studied as well (Herre et al. 1999; Fenster et al. 2004). These evolutionary studies of direct species interactions have produced a century's worth of knowledge about how species evolve, but only in controlled experiments with

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very simple systems. They do not incorporate complex interactions such as indirect effects, which may limit their application to evolutionary dynamics in nature.

Understanding evolution in a community context requires quantification of multiple direct species interactions, as well as indirect interactions. The evolutionary consequences of indirect ecological effects were considered by Miller and Travis (1996), whose heuristic model predicted changes in species effect on, or in response to, associate species on the basis of the types of direct and indirect effects. This theoretical work was followed by a modest amount of empirical work that focused on selection in response to indirect ecological effects. Astles et al. (2005) demonstrated significant genetic variation in a species response to indirect effects, indicating that the raw material required for natural selection was present. Two other empirical studies (Irwin 2006; Lau 2008) have examined indirect interactions among species of plants and insects and have inferred significant selection that occurred in response to indirect ecological effects. However, given variability in selection pressures across time and space, it is unclear whether selection in response to indirect effects will ultimately result in the evolution of traits. Selection experiments in controlled environments are required to quantify the effects of both direct and indirect species interactions on the evolution of traits measured in common-garden environments to distinguish evolved genetic differences from the effects of phenotypic plasticity.

Here I use the natural microcosm community that exists within the leaves of carnivorous pitcher plants to conduct the experiments described above and to measure the evolution of a suite of traits in a ciliated protozoan (*Colpoda* sp.). Ecological interactions in the inquiline community found in the water-filled leaves of *Sarracenia purpurea* (purple pitcher plant) have been well described (reviewed in Miller and Kneitel 2005), but more recently this community has served as a model system in which to examine evolution in response to direct species interactions (terHorst 2010; terHorst et al. 2010). The host plant is widely distributed, extending from northern Florida in the United States to the Northwest Territories in Canada, and the community is representative of other types of natural microcosm communities (Srivastava et al. 2004). Purple pitcher plants produce cup-shaped leaves that fill with rainwater and attract insect prey, mostly ants, and primarily to young leaves. The energy in the prey captured by the leaves serves as a food source for a community of bacterial decomposers. These bacteria are consumed by protozoa and rotifers, which are in turn consumed by a specialist mosquito (*Wyeomyia smithii*). There is a strong hierarchy in mosquito larval preference for prey species that is largely based on size. Mosquito larvae prefer large prey, such as rotifers and the protozoan *Colpidium* sp.

(phylum Ciliophora), over smaller, flagellated protozoa, such as *Bodo* (phylum Kinetoplasta; Kneitel 2002). These predators show an intermediate preference for intermediate-sized prey, such as *Colpoda*. This suite of protozoa species compete for bacteria, presumably via exploitative competition, with competitive ability that is also largely determined by body size (Kneitel 2002).

Species abundances change over a period of weeks during ecological succession in pitcher-plant leaves. Predation by mosquito larvae on protozoan bacterivores is highest in young leaves, but competition among these protozoans is greatest in older leaves, where resources become limiting (T. E. Miller and C. P. terHorst, unpublished data). Evolution of species traits can occur in response to both predation and competition separately (terHorst 2010; terHorst et al. 2010). Species in intermediately aged leaves experience an extended period of time in which they are exposed to both predation and competition. This study asks whether any of six traits of *Colpoda* sp., a ciliated protozoan, evolve in response to direct effects of predators and competitors. Additionally, do indirect ecological effects alter the evolution of those traits?

Methods

The evolutionary response of traits to predation, competition, and indirect effects were measured in replicate populations of *Colpoda* sp., a unicellular, ciliated protozoan that is intermediate in size, competitive ability, and predator tolerance relative to other bacterivores in the community (Kneitel 2002; Leibold and Miller 2004). Sequence data from the 18S rRNA gene suggests that all individuals used in this study belong to the same previously unsequenced species within the family Colpodidae (phylum Ciliophora, class Colpodea, order Colpodida; terHorst 2010). Because of practical limitations on the number of replicates, the experiment was conducted in two time blocks (April 2009 and June 2009), using unique lines of *Colpoda* collected from the field in each time block. Samples of water within pitcher-plant leaves were collected from haphazard leaves at each of two sites in the Apalachicola National Forest in northern Florida: Crystal Bog and Naczi Borrow Pit. In each time block, *Colpoda* were collected from four leaves to create four independent stocks, which were later mixed to create a pool of genotypes in each replicate.

To initiate selection experiments, ~300 individuals from a mixed-stock culture (along with the associated bacteria) were used to inoculate replicate laboratory microcosms that mimic pitcher-plant leaves (50-mL plastic macrocentrifuge tubes). Protozoa in these laboratory microcosms have similar population dynamics to those in pitcher-plant leaves in the field (Miller et al. 1994). Each microcosm contained 20

mL of sterile well water, plastic beads (2–5 mm in diameter), and 6 mg (± 0.15 mg) of Tetramin fish food (Tetra Holding, Blacksburg, VA) as a food source for the bacteria. Previous experiments have used ants instead of Tetramin as a food source for bacteria, but they produced similar qualitative results (terHorst et al. 2010). The use of Tetramin allowed for a standardized, precise amount of resource to be added to each microcosm. The plastic beads served as a potential refuge from predation and competition for *Colpoda* and mimicked the detritus and frass found at the bottom of natural pitchers (terHorst et al. 2010).

Most reproduction in *Colpoda* is asexual and occurs every 4–8 h, depending on genotype and culture conditions (Lüftnegger et al. 1990; terHorst et al. 2010), although some sexual reproduction may occur infrequently (Dunthorn et al. 2008). I used selection experiments to examine the evolution of a suite of *Colpoda* traits after 20 days (60–120 generations). Replicate populations of *Colpoda* ($n = 4$ per treatment per block, each containing a mix of genotypes) were maintained in four selection regimes with different species interactions: (1) In monocultures, individuals experienced only intraspecific competition. (2) The predation treatment used two third-instar mosquito larvae (*Wyeomyia smithii*) that were sterilized in H_2O_2 for 30 s (terHorst et al. 2010). (3) In the competition treatment, a species of ciliated protozoan (*Pseudocryptolophis alpestris*) was added at twice the initial density of *Colpoda*. *Pseudocryptolophis alpestris* was identified via sequencing of a portion of the 18S rRNA gene, and it belongs to the same order as *Colpoda*. Previous work suggests that *P. alpestris*, which was previously identified as *Colpidium* sp., has large competitive effects on abundance and evolution of traits in *Colpoda* populations (Kneitel 2002; terHorst 2010). (4) The multispecies treatment contained the same initial density of *P. alpestris*, as well as two *W. smithii* larvae. This final treatment allowed for both direct and indirect species interactions to occur. Hereafter, I refer to these four treatments as monoculture, predation, competition, and multispecies, respectively.

The abundance of both mosquito larvae and *P. alpestris* were monitored daily, and all experimental microcosms were reestablished every 3 days to avoid coevolution between competitors, satiation of predators, or the exclusion of *Colpoda* by competition or predation. During each reestablishment, a random sample of 300 *Colpoda* were transferred from the appropriate replicate and new mosquito larvae and *P. alpestris* were added from laboratory stock cultures. Random samples were collected by inverting the tube twice and swirling the contents into a homogenous mixture. It is possible that *Colpoda* cysts were transferred in addition to the 300 cells, but since cysts are usually not produced during the first 3 days in culture, it is likely that relatively few cysts were transferred.

All cultures were maintained in a growth chamber with a diurnal cycle of light (12L : 12D) and temperature (day = 30°C, night = 20°C). At the end of the selection experiment, a random sample of 300 *Colpoda* individuals were isolated and removed from replicates in all treatments and placed into identical environments containing 20 mL of sterile well water and 6 mg of Tetramin. *Colpoda* were grown under these common conditions for 24 h (three to four generations) to minimize the role of environmental plasticity and maternal effects before traits were measured.

Measurement of Traits

After growth in a common environment for three to four generations, two random subsamples of 300 individuals from each replicate were used to measure traits in a monoculture “common-garden” environment to discount effects of phenotypic plasticity. The generations in the common garden ensure that any differences in trait values between selection treatments are due to evolved genetic differences. In previous work, traits were measured in multiple common-garden environments (i.e., monoculture environments, environments with predators, and environments with competitors), but analyses revealed no significant interactions between the effects of measurement and selection environment on trait values (terHorst 2010; terHorst et al. 2010). For simplicity, only traits measured in common monoculture environments are presented here. In each replicate from each selection treatment, six traits of *Colpoda* were measured: cell size, cell speed, cyst production, peak population abundance, population growth rate, and refuge use. These traits were measured in terms of population means. This measurement does not account for individual variation within populations, but measuring traits of single cells is difficult because cultures cannot easily be initiated with a single cell.

Cell size in this system is positively correlated with competitive ability and negatively correlated with predator tolerance (Kneitel 2002). An increase in cell speed may increase the ability to escape predators or competitors, or alternately, it might increase encounter rates with these associate species. Digital videos and image analysis (acquired from the National Institutes of Health’s ImageJ, <http://rsb.info.nih.gov/ij/>) were used to estimate cell area (in mm^2) and swimming speed (in $mm\ s^{-1}$) of the first 30 individuals encountered in the video of each replicate.

In *Colpoda* populations, the simplest explanation for an increase in population growth rate is an increase in the rate of asexual propagation (terHorst et al. 2010). Population growth rates were measured by growing each subsample of 300 individuals in a microcosm with 20 mL of sterile well water and 6 mg of Tetramin. Population size was estimated by counting the number of individuals in

a 0.1-mL random subsample of each culture on a Palmer counting cell (Wildlife Supply Company, Buffalo, NY) every 6 h until populations peaked in abundance. Populations displayed exponential growth up to a peak at 48–72 h, and then abundance dropped off rapidly. Growth rates were determined by estimating r from the best-fit exponential growth model ($N = N_0 e^{rt}$), using abundance data up to the peak. The decline in population abundance in *Colpoda* is followed shortly by the appearance of resting cysts (C. P. terHorst, personal observations), which are associated with a decline in food resources (Yamaoka et al. 2004; Foissner et al. 2009). These cysts are resistant to desiccation and allow populations to survive transient aquatic environments (Ekelund et al. 2002). The peak in abundance was used as a proxy measure of the efficiency of resource use. Cyst production was estimated by counting the number of cysts in a 0.1-mL subsample from each microcosm after 6 days.

The refuge provided at the bottom of the microcosm may be important for escaping predation; the lack of a refuge prevents the coexistence of mosquito larvae and *Colpoda* for more than 24 h. The refuge might also be important for escaping competition. The refuge use of *Colpoda* was determined by removing two 0.1-mL samples from each microcosm, one from the water column and one from within the beads at the bottom of the microcosm, and counting the number of individuals on a Palmer counting cell. Refuge use was determined as $\log(\text{number in beads} / \text{number in water})$. This measurement of refuge use provides an unbounded index, with a value of 0 indicating equal densities in the beads and in the water and positive values indicating a heavier use of the refuge. The refuge use of *P. alpestris* was also measured at the same time. A separate experiment examined foraging in the refuge by mosquito larvae. *Colpoda* and two mosquito larvae were placed in microcosms with and without *P. alpestris* ($n = 10$). The location of the larvae within the microcosm was noted two to three times per day for 4 days to estimate the percentage of time spent in the refuge.

Statistical Analyses

All data met the assumptions of parametric tests or were transformed to meet those assumptions. All parametric analyses were conducted in JMP, version 4.0.4 (SAS Institute, Cary, NC). To test for the ecological effects of potential predators and competitors, an unrestricted three-way ANOVA (Quinn and Keough 2003) compared the effect of (1) presence of mosquito larvae, (2) presence of *P. alpestris*, and (3) time block on the abundance of *Colpoda*. The effects of mosquito larvae and *P. alpestris* on population abundance were determined after 24 h to minimize evolutionary effects that can result in changes in

ecological effects (terHorst et al. 2010). In this statistical design, an interaction between predators and competitors indicated a significant indirect effect. Nonsignificant interactions ($P > .25$) were removed from statistical models. A three-way MANOVA tested the effect of the same factors on the evolution of all six traits. The MANOVA was followed by univariate ANOVAs for each of the six traits.

This statistical design tests for the presence of significant direct and indirect effects, but it does not test the direction or strength of those effects. The effect sizes of mosquito larvae and *P. alpestris* on the evolution of each trait were estimated as a percent change by standardizing the difference between the predation or the competition and monoculture selection treatments by the mean trait value in the monoculture selection treatment. For example, the direct effect of mosquito larvae on *Colpoda* trait T can be described as

$$\text{predator effect}_T = \frac{\bar{T}_{\text{predation}} - \bar{T}_{\text{monoculture}}}{\bar{T}_{\text{monoculture}}}. \quad (1)$$

The predicted values in the multispecies treatment based only on the additive effects of predation and competition were calculated for each trait by adding the unstandardized effect sizes of predation and competition to the mean trait value in the monoculture treatment:

$$\begin{aligned} \text{predicted multispecies}_T &= \bar{T}_{\text{monoculture}} \\ &+ (\bar{T}_{\text{predation}} - \bar{T}_{\text{monoculture}}) + (\bar{T}_{\text{competition}} - \bar{T}_{\text{monoculture}}). \end{aligned} \quad (2)$$

In an example with trait values of 10 in the monoculture treatment, 15 in the predation treatment, and 17 in the competition treatment, the predicted multispecies value based on the additive effects of the predation and the competition treatments would be 22. Indirect effects were estimated as the difference between the mean observed trait value in the multispecies selection treatment and the predicted multispecies value, standardized by the monoculture selection treatment:

$$\text{indirect effect}_T = \frac{\bar{T}_{\text{multispecies}} - \bar{T}_{\text{predicted multispecies}}}{\bar{T}_{\text{monoculture}}}. \quad (3)$$

In the above example, a trait value of 12 in the multispecies treatment would yield an indirect effect of -10 . A one-way ANOVA using time block as a factor was used to account for any structure in the traits generated by time blocks. The residuals of this analysis were used as trait values in the equations above. Direct and indirect effect sizes were bootstrapped 10,000 times using the R statistical environment (R Core Development Team, Vienna), and

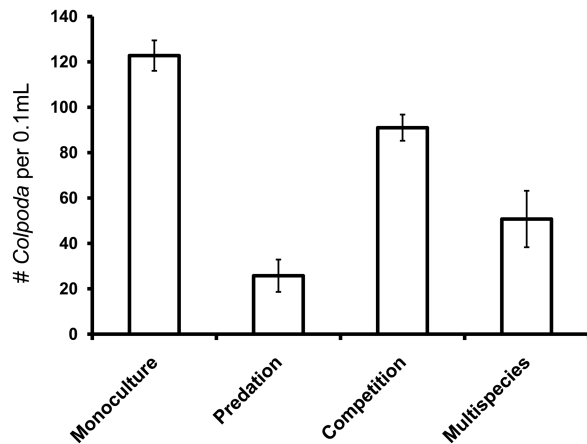


Figure 1: Ecological effect of each selection environment on the mean abundance of *Colpoda* (\pm SE) after 24 h in each environment.

95% confidence limits were estimated to determine whether bootstrapped means significantly differed from 0. This estimation of indirect effects assumes that quantification of indirect effects can be accurately calculated from a linear combination of direct effects. This method is of some concern when direct and indirect effects occur in the same direction, but that is not the case in this study.

Results

Ecological Effects

Both the mosquito larvae and *Pseudocryptolophosis alpestris* had significant effects on the abundance of *Colpoda* after 24 h in the selection experiment (fig. 1), but a significant statistical interaction ($F_{1,24} = 19.0, P < .001$) indicated significant indirect ecological effects as well. The indirect interaction did not vary significantly between time blocks ($F_{1,24} = 0.50, P = .48$). In the absence of competitors, mosquito larvae had a significant effect on the abundance of *Colpoda* ($F_{1,12} = 125.9, P < .001$), reducing populations by an average of 79% (fig. 1). Mosquito larvae also had a significant effect on the abundance of *Colpoda* when competitors were present ($F_{1,12} = 17.0$), but they reduced populations by only 59% on average (fig. 1). The presence of *P. alpestris* had a significant negative effect on *Colpoda* populations in the absence of predators ($F_{1,12} = 11.8, P = .005$), reducing *Colpoda* abundance by an average of 26% (fig. 1). *Pseudocryptolophosis alpestris* had a positive effect on the abundance of *Colpoda* in the presence of predators ($F_{1,12} = 7.40, P = .019$). Although the direct ecological effects of predation and competition were strongly negative (-0.80 and -0.26 , respectively), the indirect ecological ef-

fect ($+0.52$, calculated using eq. [3]) was positive and intermediate in strength to the direct effects.

The presence of mosquito larvae also had a significant effect on the abundance of *P. alpestris* ($F_{1,12} = 46.9, P < .001$). In the multispecies treatment, which contained both *P. alpestris* and mosquito larvae, *P. alpestris* densities were reduced by an average of 76% (fig. 2). There was no effect of time block on *P. alpestris* density ($F_{1,12} = 0.08, P = .79$), nor did treatments differ among time blocks ($F_{1,12} = 3.16, P = .10$). *Pseudocryptolophosis alpestris* tended to avoid the refuge, and it used the water column more often (refuge use = -0.45 ± 0.10) relative to *Colpoda* (fig. 3C). The presence of *P. alpestris* had a significant effect on the location of mosquito larvae ($t = 2.77, df = 18, P = .013$). Mosquito larvae were found in the refuge 71% ($\pm 4.8\%$) of the time when only *Colpoda* were present. When *P. alpestris* were also present, mosquito larvae were found in the refuge only 55% ($\pm 3.9\%$) of the time.

Evolutionary Effects

MANOVA indicated that indirect effects between mosquito larvae and *P. alpestris* had a significant effect on the evolution of traits in *Colpoda* populations ($F_{6,19} = 155.4, P < .001$). The strength of this interaction varied between time blocks ($F_{6,19} = 6.60, P < .001$), but the indirect effect occurred in the same direction in both time blocks. Mosquito larvae had a significant effect on the evolution of traits, both in the presence ($F_{6,7} = 210.0, P < .001$) and the absence ($F_{6,7} = 47.3, P < .001$) of *P. alpestris*. *Pseudocryptolophosis alpestris* also had a significant effect on the evolution of traits, both in the presence ($F_{6,7} = 123.4, P < .001$) and in the absence ($F_{6,7} = 55.0, P < .001$) of mosquito larvae. Time block also had an effect on the evolution of traits ($F_{6,19} = 50.5, P < .001$), but interactions

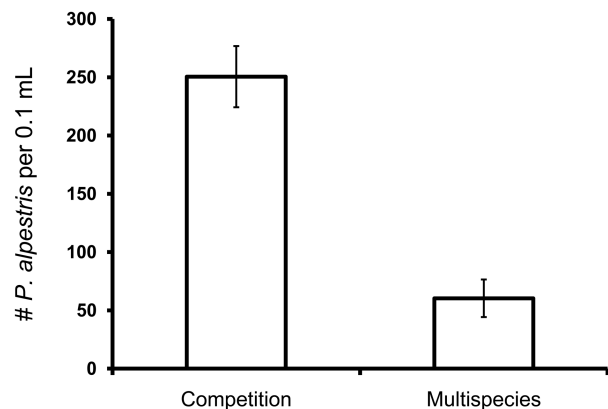


Figure 2: Mean (\pm SE) *Pseudocryptolophosis alpestris* density in two selection treatments.

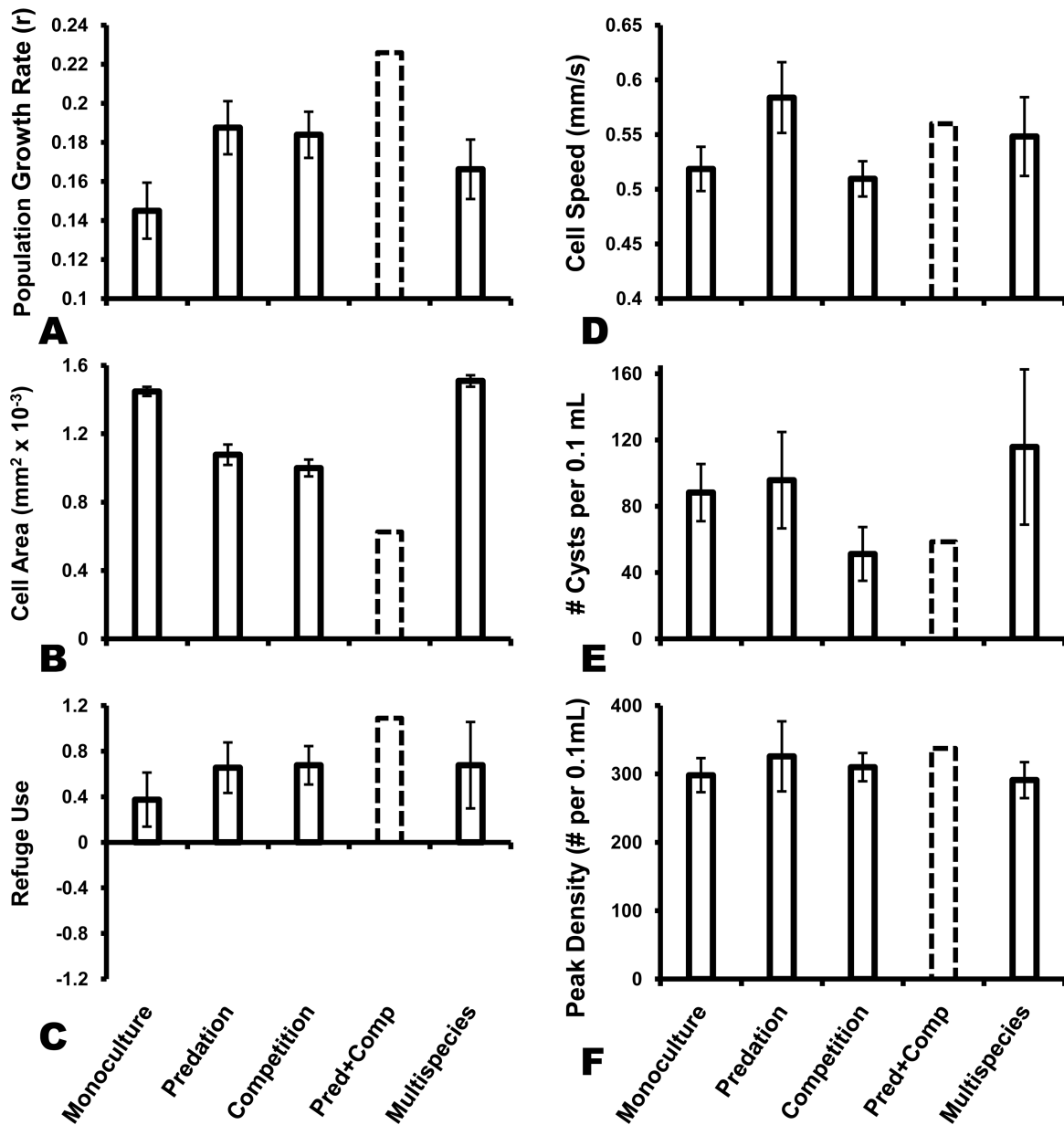


Figure 3: Mean values (\pm SE) of six traits from four different selection treatments, measured in a monoculture common garden. The dashed bar represents the predicted trait value on the basis of the additive effects of predation and competition.

between time block and predator or competitor presence were not significant (block \times predator: $F_{6,19} = 1.66$, $P = .18$; block \times competitor: $F_{6,19} = 0.248$, $P = .95$). Since different traits responded differently to main effects, univariate ANOVAs were used to determine which traits evolved in response to selection treatments.

The effects of mosquito larvae and *P. alpestris* on the evolution of population growth rates of *Colpoda* populations were context dependent (predator \times competitor:

$F_{1,27} = 6.79$, $P = .015$; fig. 3A), demonstrating a significant indirect effect on the evolution of population growth rate. There was no difference in this interaction among time blocks ($P > .25$). In the absence of competitors, *Colpoda* populations evolved significantly higher population growth rates in the presence of mosquito larvae ($F_{1,13} = 6.14$, $P = .028$; fig. 3A). When competitors were present, mosquito larvae had no significant effect on the evolution of population growth rate ($F_{1,13} = 1.22$, $P = .29$). In the

presence of *P. alpestris* but the absence of predators, *Colpoda* populations evolved marginally significantly higher population growth rates ($F_{1,13} = 4.01$, $P = .066$). There was no significant effect of *P. alpestris* on population growth rate in the presence of mosquito larvae ($F_{1,13} = 2.72$, $P = .12$; fig. 3A). The effect sizes of predation, competition, and indirect interactions on population growth rate were significantly different from 0 (fig. 4A). Indirect interactions were nearly as strong as the sum of direct interactions, but they had an effect of decreasing rather than increasing population growth rate (fig. 4A). Time block also had a significant effect on population growth rate ($F_{1,27} = 12.0$, $P = .002$), but interactions with other main effects were nonsignificant ($P > .35$).

The effects of mosquito larvae and *P. alpestris* on the evolution of *Colpoda* cell size were also context dependent (predator \times competitor: $F_{1,24} = 784.8$, $P < .001$; fig. 3B), demonstrating a significant indirect effect on the evolution of cell size. Although the magnitude of this interaction varied among time blocks ($F_{1,24} = 31.8$, $P < .001$), the rank order of selection treatments was the same in all time blocks, indicating a difference in the magnitude but not the direction of indirect effects between time blocks. When mosquito larvae but not competitors were present, significantly smaller cell sizes evolved in *Colpoda* populations ($F_{1,12} = 219.8$, $P < .001$; fig. 3B). When competitors were present, *Colpoda* populations evolved significantly larger cell sizes in the presence of mosquito larvae ($F_{1,12} = 706.5$, $P < .001$). When *P. alpestris* but not predators were present, *Colpoda* populations evolved significantly smaller cell sizes ($F_{1,12} = 402$, $P < .001$; fig. 3B). When predators were present, *Colpoda* populations evolved significantly larger cell sizes in the presence of *P. alpestris* ($F_{1,12} = 383.3$, $P < .001$). The effect sizes of predation, competition, and indirect interactions on cell size were significantly different from 0 (fig. 4B). As with population growth rate, although indirect and direct effects were nearly equal in magnitude, they had opposite effects on cell size (fig. 4B). Time block also had a significant effect on cell size ($F_{1,24} = 284.8$, $P < .001$), and interactions with main effects were nonsignificant (block \times predator: $P = .055$; block \times competitor: $P = .95$).

The evolution of refuge use was not significantly affected by the presence of mosquito larvae ($F_{1,24} = 0.47$, $P = .50$; fig. 3C) or *P. alpestris* ($F_{1,24} = 0.62$, $P = .44$; fig. 3C). There was no significant interaction between the effects of mosquito larvae and *P. alpestris* on refuge use ($F_{1,24} = 0.47$, $P = .50$; fig. 3C). Accordingly, the effect sizes of mosquito larvae and *P. alpestris* on refuge use were positive but not significantly different from 0 (fig. 4C). The indirect effect size was negative and large relative to the direct effects, but it was not significantly different from 0 (fig. 4C). Time block had a significant effect on refuge

use ($F_{1,24} = 16.9$, $P < .001$), but interactions with other main effects were nonsignificant ($P > .41$).

Mosquito larvae had a significantly positive effect on the evolution of cell speed in *Colpoda* populations ($F_{1,26} = 7.66$, $P = .010$; fig. 3D). *Pseudocyrtolephosis alpestris* had no significant effect on the evolution of cell speed ($F_{1,26} = 1.42$, $P = .24$; fig. 3D). There was no significant interaction between the effects of mosquito larvae and *P. alpestris* on cell speed ($F_{1,26} = 0.50$, $P = .49$; fig. 3D). Direct and indirect effects were in opposite directions, but effect sizes were not significantly different from 0 (fig. 4D). Time block had a significant effect on cell speed ($F_{1,26} = 30.0$, $P < .001$), but interactions with other main effects were not significant (block \times predator: $P = .067$; block \times competitor: $P = .78$).

Finally, the evolution of cyst production and peak population abundance were not significantly affected by either mosquito larvae (cysts: $F_{1,27} = 0.24$, $P = .63$; fig. 3E; peak: $F_{1,27} = 0.02$, $P = .90$; fig. 3F) or *P. alpestris* (cysts: $F_{1,27} = 0.68$, $P = .42$; fig. 3E; peak: $F_{1,27} = 0.12$, $P = .74$; fig. 3F). There was no significant interaction between the effects of mosquito larvae and *P. alpestris* on either of these traits (cysts: $F_{1,27} = 0.44$, $P = .51$; fig. 3E; peak: $F_{1,27} = 0.48$, $P = .50$; fig. 3F). Although the direct and indirect effects on cyst production and peak population abundance were in opposite directions, effect sizes were not significantly different from 0 (fig. 4E, 4F). Time block had no significant effect on either cyst production or peak population abundance (cysts: $F_{1,27} = 0.21$, $P = .65$; peak: $F_{1,27} = 0.04$, $P = .85$), and all interactions with time block were nonsignificant ($P > .30$).

Discussion

The consequences of indirect ecological effects have long been appreciated in the ecological literature, but they also play a large role in how species evolve in a community context. These experiments confirm that evolution in *Colpoda* populations occurs in response to the direct effects of predation by mosquito larvae and competition by *Pseudocyrtolephosis alpestris* (fig. 3; terHorst 2010; terHorst et al. 2010). However, the novel result is that indirect effects also had significant and strong effects on the evolution of traits in *Colpoda* (figs. 3, 4). In all traits measured, indirect effects had the opposite effect on trait values than direct effects (fig. 4). Furthermore, indirect effects were often stronger than direct effects (fig. 4) and resulted in little net evolution of some traits in multispecies populations that experienced both direct and indirect effects (fig. 3).

Population growth rate evolved in *Colpoda* populations in response to the direct effects of predation and competition. *Colpoda* populations measured in monoculture had higher population growth rates if they had evolved

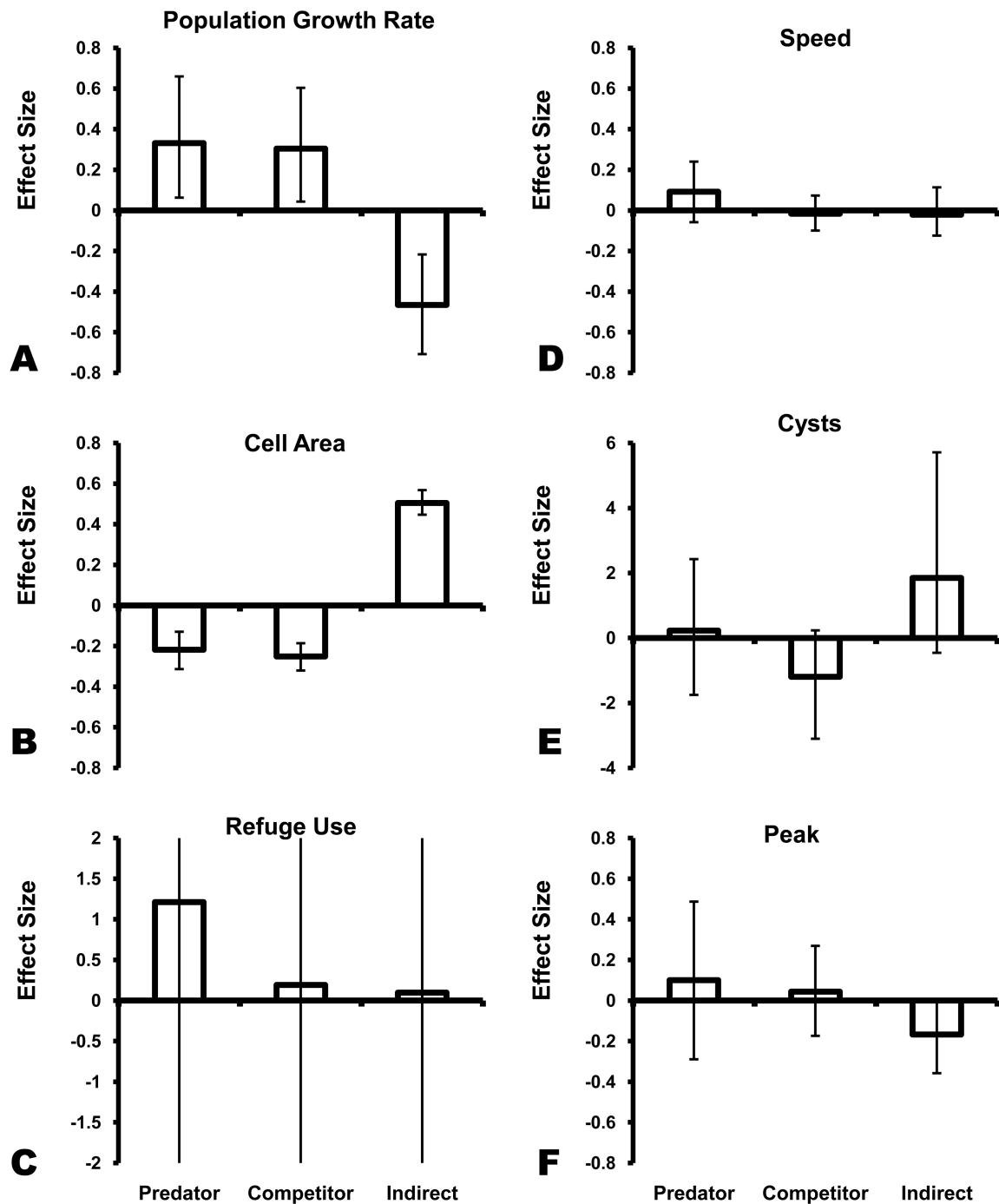


Figure 4: Effect sizes of mosquito larvae (predator), *Pseudocrytolphosis alpestris* (competitor), and indirect effects between these species on the evolution of six traits in *Colpoda* populations. Effect sizes and 95% confidence intervals were calculated on the basis of bootstrapped means. Note the different Y-axis scales in C and E. In C, the error bars greatly exceed the scale of the Y-axis. For each trait, if the confidence interval did not include 0, the effect size was determined to be significantly different from 0. The indirect effect of every trait is in the opposite direction of the direct effect, and it is often comparable to or stronger than the direct effect.

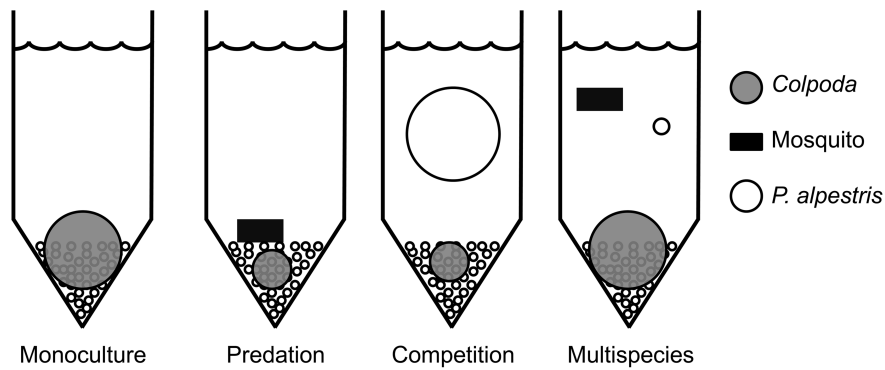


Figure 5: Hypothesized direct and indirect effects of mosquito larval predators and *Pseudocyrtolephosis alpestris* competitors on *Colpoda* populations in experimental microcosms. The beads at the bottom of each microcosm serve as a refuge and are similar to refuges found in natural pitcher plants. The size of the symbol reflects the abundance of each species, and the location reflects use of different parts of a microcosm. In the multispecies treatment, mosquito foraging behavior is altered because of the presence of *P. alpestris*. This change in foraging behavior reduces the abundance of *P. alpestris*, resulting in both behavior- and density-mediated indirect effects on *Colpoda* populations. A color version of this figure appears in the online edition of the *American Naturalist*.

with mosquito larvae or *P. alpestris* (fig. 3A, 3B). Although many traits play a role in determining population growth rate, an increase in *Colpoda* growth rate is, in large part, explained by an increase in the rate of production of daughter cells (terHorst et al. 2010). This trait serves as a tolerance strategy in the presence of mosquito larvae, as cells reproduce at a sufficient rate to keep pace with the rate of predation (terHorst et al. 2010). An increase in population growth rate may also serve as a tolerance strategy when faced with competition, or it may be a means to increase competitive ability. Genetic lines that increase resource consumption via asexual cell proliferation may monopolize resources and gain a competitive advantage.

Among bacterivorous species in pitcher-plant communities, predator tolerance is negatively correlated with cell size, while competitive ability is correlated with increasing cell size (Kneitel 2002; Leibold and Miller 2004). A similar within-species trade-off would have resulted in divergent cell sizes in predation and competition selection treatments. Instead, smaller cell sizes evolved in both treatments. Smaller individuals may be harder to capture by the filter-feeding mosquito larvae, and they might also gain a competitive advantage by using resources more efficiently. Alternately, cell size might have evolved as a result of indirect selection via selection on population growth rate. Cell size and population growth rate were significantly correlated among populations ($r = -0.51$, $P = .003$). Selection for rapidly dividing genotypes may have resulted in populations with smaller cell sizes.

Indirect effects can occur through density-mediated interactions, in which the abundance of one species is reduced by the presence of another, or through trait-mediated interactions, in which the behavior of one species

is altered in the presence of another (Miller and Kerfoot 1987; Preisser et al. 2005; Trussell et al. 2006). In this experiment, both density- and trait-mediated interactions are likely to have occurred. Trait-mediated effects occurred via a habitat shift by mosquito larvae (fig. 5). *Pseudocyrtolephosis alpestris* populations remained primarily in the water column and were infrequently found in the refuge at the bottom of the microcosm. When *P. alpestris* was present, mosquito larvae were more likely to forage in the water column. In the absence of *P. alpestris*, mosquito larvae were forced to forage for *Colpoda* in the refuge, where their feeding efficiency was likely reduced due to limitations on their mobility there (C. P. terHorst, personal observation). Density-mediated indirect interactions occurred when the pressure of competition on *Colpoda* populations was alleviated by a reduction in *P. alpestris* densities in the presence of mosquito larvae (fig. 2).

As a result of these indirect effects, *Colpoda* experienced much less selection pressure from either predators or competitors when both species were present (fig. 5). The density- and trait-mediated indirect interactions together resulted in indirect effects on trait evolution, whose magnitude was similar to the sum of the direct effects on those traits (fig. 4A, 4B). The individual contribution of each type of indirect effect is difficult to quantify, however. Previous studies of indirect ecological effects have used “indirect effect only” treatments to separate direct and indirect effects of predators (Schmitz et al. 2008). For example, nonconsumptive effects of predators can be measured using caged predators that induce behavioral changes in another species but that do not directly affect abundance (Blaustein 1997; Nelson et al. 2004; Trussell et al. 2006). Such treatments would be difficult in this system,

in part because of the size of the organisms used but also because multiple indirect effects would have to be simulated. Although reducing *P. alpestris* densities might be possible, it would be more difficult to manipulate the behavior of mosquito larvae.

Miller and Travis (1996) made predictions about how the traits of a focal species should evolve in response to the direct and indirect effects of a focal species. In cases where direct effects are negative and indirect effects are positive, as is the case with predator and competitor effects on *Colpoda*, a decreased evolutionary response is predicted (Miller and Travis 1996). A change in response constitutes a trait change that has little effect on the associate species, and thus it maintains a positive indirect effect but reduces the net interaction between associate and focal species. With respect to mosquito larvae as an associate species, the evolution of population growth rate and cell size in *Colpoda* populations are consistent with this prediction. Changes in these traits likely have little effect on the population dynamics of mosquitoes in the multispecies treatment (as the larvae still have a large source of food), and thus it maintains the positive indirect effects of mosquitoes (i.e., eating competitors) while reducing interactions between *Colpoda* and mosquitoes. With respect to interactions with *P. alpestris*, a match to the prediction is less clear. The evolution of cell size may reduce interactions between *Colpoda* and *P. alpestris*, but the evolution of population growth rate may increase competitive interactions between these species.

There was a significant effect of time block on several traits. The time block effect may be a result of properties of different stock lines used in each time block. The four stock lines in each time block may have had different initial trait values. Also, the genetic variance of populations within and among leaves is unknown: one leaf could contain a single genotype or thousands of genotypes. The genetic variance in the different four-population mixtures may have affected the evolvability of populations and resulted in faster rates of evolution in one time block. Population growth rate and cell size (but not the remaining four traits) were measured before the selection experiment. Initial population growth rates were similar in the April and June time blocks and did not differ significantly from the monoculture treatment after selection. Initial cell sizes in April were smaller than they were in June, and traits in all four selection environments tended to evolve more in June, suggesting that evolvability was higher in June than in April. Finally, differences in traits between time blocks may have been due to seasonal differences in the bacterial flora collected from pitcher-plant leaves along with *Colpoda*.

Considering evolution in a community context provides a more realistic depiction of how species evolve in natural

systems. An indirect effect is an emergent property that occurs only at the community level. Consideration of the importance of indirect effects bridges the gap between evolutionary experiments with one or two species and evolutionary dynamics in natural communities. Although the complexity in the community used in this study was greater than that of most evolutionary studies, this system was still very simple compared with complex webs observed in natural communities. Certainly, more diverse communities, where the number of indirect effects dwarfs the number of direct effects, may provide even more complex dynamics. Incorporating additional direct and indirect effects in future experiments will better evaluate the evolutionary forces experienced by species in natural biological communities.

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