

Experimental evolution of protozoan traits in response to interspecific competition

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Abstract

Decades of experiments have demonstrated the ecological effect of competition, but experimental evidence for competitive effects on trait evolution is rare. I measured the evolution of six protozoan traits in response to competitors from the inquiline community of pitcher plants. Replicate populations of *Colpoda*, a ciliated protozoan, were allowed to evolve in response to intra- and interspecific competition for 20 days (approximately 100 generations), before traits were measured in two common garden environments. Populations that evolved with interspecific competition had smaller cell sizes, produced fewer cysts and had higher population growth rates relative to populations grown in monoculture. The presence of interspecific competitors led to differential lineage sorting, most likely by increasing the strength of selection. These results are the first to demonstrate protozoan evolution in response to competition and may have implications for species coexistence in this system.

Introduction

Competition among species for resources is known to be an important species interaction, with implications for the ecology of species and the evolution of species traits. Hundreds of ecological experiments have documented that interspecific competition limits the distribution and abundance of species in natural communities (Connell, 1983; Schoener, 1983; Goldberg & Barton, 1992; Gurevitch *et al.*, 1992). There is considerably less evidence of the evolutionary implications of such competition. Decades of observations of character displacement invoked competition as the likely mechanism driving the divergence of species traits (Brown & Wilson, 1956; Schluter, 2000). However, character displacement has been often difficult to confirm (e.g. Strong *et al.*, 1979), and character displacement alone does not implicate competition as the driving evolutionary force (Connell, 1980). Demonstration of the evolutionary effect of competition is best accomplished with experiments that compare the evolu-

tion of several traits in competitive environments relative to evolution in control environments.

There are few experimental studies of evolution owing to competition, so a general statement about the evolutionary effect of competition is difficult. Studies of intraspecific competition have found significant evolutionary changes in traits such as size and growth rate (e.g. Santos *et al.*, 1997; Sokolowski *et al.*, 1997; Stanton *et al.*, 2004). The few examples of trait evolution in response to interspecific competition (Taper, 1990; Schluter, 1994; Smith & Rausher, 2008; McGoey & Stinchcombe, 2009) have largely focused on plant taxa. Others have documented significant evolutionary changes in competitive ability, but have not examined the traits that might lead to such a change (e.g. Miller, 1995; Goodnight & Craig, 1996; Joshi & Thompson, 1996). More recently, microcosm studies have proven useful in demonstrating character displacement and evolutionary dynamics among species of bacteria (MacLean *et al.*, 2005; Tyerman *et al.*, 2008).

Historically, protozoa have proven to be useful model systems in ecological competition experiments (e.g. Gause, 1934; Vandermeer, 1969; Fox, 2002), but experimental evolution in these systems has rarely been explored (but see terHorst *et al.*, 2010). Here, I used naturally co-occurring protozoa species from the

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inquiline community of purple pitcher plant (*Sarracenia purpurea*) leaves to examine the evolution of species traits in response to competition. Evolutionary changes of six traits in one species were measured in response to both intra- and interspecific competitions.

Methods

Study system

The inquiline community found in the water-filled leaves of *Sarracenia purpurea* (purple pitcher plant) has been well described elsewhere (reviewed in Miller & Kneitel, 2005) and so will be only briefly described here. 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). Pitcher plants produce cup-shaped leaves that fill with rainwater and attract insect prey – primarily ants. The energy in the prey captured by the leaves serves as a basis for a community of bacterial decomposers, bacterivorous protozoa and rotifers, and a specialist mosquito (*Wyeomyia smithii*) that consumes bacterivores. Previous work has demonstrated that the evolution of a particular suite of protozoan traits evolves in response to predation by mosquito larvae (terHorst *et al.*, 2010).

However, competition also plays a large role in structuring these communities (Kneitel, 2002; Leibold & Miller, 2004). A suite of protozoa, along with rotifers, compete for bacteria, presumably via exploitative competition, and can reduce the densities of other bacterivores by 30–100% (Kneitel, 2002). In particular, *Pseudocyrilophosis alpestris* (sometimes misidentified as *Colpidium* in this system) has strong competitive effects on *Colpoda* and most other bacterivores in the community (Kneitel, 2002).

Selection experiments on *Colpoda* were conducted in three time blocks. To confirm the species identity of *Colpoda* lines used in each experiment, 7 of the original 12 stocks (see Selection Experiments), along with two additional stocks collected from the same sites in August 2009, were used to extract DNA for sequencing of a portion of the 18S rRNA gene (Spears *et al.*, 1992, see Appendix A for methods).

DNA sequences were used to create a phylogenetic tree in PAUP (Version 4.0; Sinauer Associates, Inc., Sutherland, MA, USA), using reference sequences from Genbank at the National Center for Biotechnology Information (see Appendix B for accession numbers) from similar species within the Colpodida (Ciliophora: Colpodea) and three outgroups (*Bryometopus pseudochilodon*, *Cyrtolophosis mucicola* and *Oxytricha nova*), all obtained from the phylogeny of Dunthorn *et al.* (2008). A bootstrap consensus tree was created using parsimony, retaining up to 1000 trees in each of 1000 bootstrap replicates. All new sequences obtained in this study have been uploaded to Genbank (Appendix B).

Selection experiments

The evolutionary effects of competition were measured in replicate populations of *Colpoda* spp., a group of unicellular ciliated protozoa that are intermediate in size, competitive ability and predator tolerance relative to other bacterivores in the community (Kneitel, 2002; Leibold & Miller, 2004). Because of practical limitations on the number of replicates, the experiment was conducted in three time blocks (October 2008, April 2009, and June 2009), using unique lines of *Colpoda* from the field in each 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 Nazi Borrow Pit. In each time block, *Colpoda* were collected from four leaves to create four independent stocks that were later mixed to create a pool of genotypes in each replicate.

In each time block, approximately 300 individuals from a mixed-stock culture were used to initiate a selection experiment by inoculating replicate laboratory microcosms (along with the associated bacteria) that mimic pitcher plants (50-mL plastic macrocentrifuge tubes). Each microcosm contained 20 mL of sterile well water, plastic beads (2–5 mm diameter) and 6 mg (± 0.15 mg) of tetramin (Tetra Holding, Inc., Blacksburg, VA, USA) as a food source for the bacteria. The plastic beads mimicked the detritus and frass found at the bottom of natural pitchers (terHorst *et al.*, 2010). Protozoa in these laboratory microcosms have similar population dynamics to those in pitcher plant leaves in the field (Miller *et al.*, 1994).

Replicate populations ($n = 4$ per treatment per block) were maintained in (i) monoculture with only intraspecific competition and (ii) two-species cultures with both intra- and interspecific competition from *P. alpestris* (hereafter referred to as monoculture and competition, respectively) for 20 days, which encompassed 60–120 *Colpoda* generations. Asexual reproduction in *Colpoda* occurs every 4–8 h (Lüftnegger *et al.*, 1990), but some sexual reproduction may occur infrequently (Dunthorn *et al.*, 2008). The abundance of *Colpoda* in each treatment was measured to determine the ecological effect of *P. alpestris*. The ecological effect was determined after 24 h to minimize the evolutionary changes that can result in a change in ecological effect size (terHorst *et al.*, 2010).

In the competition treatment, *P. alpestris* was added at twice the density (approximately 600 individuals) of *Colpoda*. Although natural densities of these species vary widely, these densities fall within the range found in natural pitchers. To avoid competitive exclusion of *Colpoda* by *P. alpestris* and to prevent co-evolution between these species, I used serial transfers to reestablish the experimental microcosms in both treatments every 3 days. A random sample of 300 *Colpoda* from each replicate was used to inoculate new microcosms with the same conditions as mentioned previously. New

P. alpestris were added from laboratory stock cultures maintained in the same culture conditions as mentioned previously. Random samples were collected after inverting the microcosm twice and swirling the contents into a homogenous mixture.

All cultures were maintained in a growth chamber with a diurnal cycle (12/12) of light and temperature (day = 30 °C, night = 20 °C). At the end of the selection experiment, approximately 300 *Colpoda* were isolated and removed from replicates in both monoculture and competition treatments and placed in identical environments containing 20 mL of sterile well water and 6 mg of tetramin. *Colpoda* were grown under these common conditions for 24 h (3–6 generations) to diminish the role of phenotypic plasticity, because of environmental or maternal effects, before measuring traits.

Measurement of traits

At the onset of the selection experiment, four traits of *Colpoda* were measured: cell size, cell speed, peak population density and population growth rate. Following 20 days in the selection environments and growth in a common environment for 24 h, two random subsamples of 300 individuals were taken from each replicate in both monoculture and competition treatments and used to measure traits in two common garden environments: monoculture and competition. I refer to the environment in which selection occurred for 20 days as the Selection Environment and the environment in which traits were measured as the Measurement Environment. In each replicate from both selection environments, the four traits that were measured initially were measured in both measurement environments, along with two additional traits: habitat use and cyst production. Traits of individuals are difficult to determine, although individual cell sizes and speeds can be determined. All traits were measured at the population level and reflect the average trait value of individuals within populations.

Digital videos and image analysis (NIH Image J, <http://rsb.info.nih.gov/ij/>) were used to estimate cell area (mm²) and swimming speed (mm/s) of the first 30 individuals encountered in the video. Habitat use was determined by removing two 0.1-mL samples from each microcosm, one in the water column and one in the beads, and counting the number of individuals on a Palmer counting cell. Habitat use was determined as: $\log(\# \text{ in beads} / \# \text{ in water})$. This dimensionless measure of habitat use provides an unbounded index, with a value of 0 indicating equal densities in the beads and water; a value of 1 indicates an order of magnitude higher density in the beads.

Population growth rates were measured by growing each subsample of 300 individuals in microcosms with 20 mL of sterile well water and 6 mg of tetramin. In the competition measurement environment, 600 *P. alpestris*

were added to each microcosm. 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 every 6 h, for at least 48 h, or until populations peaked in abundance. Populations displayed exponential growth up to a peak and then abundance dropped off rapidly. Per capita growth rates per hour were determined by estimating r from the best-fit exponential growth model ($N_t = N_0 e^{rt}$), using abundance data up to the peak. The decline in population abundance in *Colpoda* is followed shortly by the appearance of resting cysts, which are associated with a decline in food resources (Yamaoka *et al.*, 2004; Foissner *et al.*, 2009). These cysts are resistant to desiccation and may allow populations to survive transient aquatic environments (Ekelund *et al.*, 2002). The peak in density (# per 0.1 mL) 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.

Additionally, traits of *P. alpestris* were measured, but only in the measurement environment with competition. Cell size, cell speed, population growth rate and habitat use of *P. alpestris* populations were measured in the same way as *Colpoda* populations previously.

Statistical analyses

All data met the assumptions of parametric tests or were transformed to meet these assumptions, and statistical analyses were performed in JMP version 4.0.4 (SAS Institute Inc., Cary, NC, USA). A two-way mixed model ANOVA examined the ecological effect of competition from *P. alpestris* (fixed effect) and time block (random effect). MANOVA examined the effect of selection environment and measurement environment, testing these mean squares over those of their respective interactions with time block. The effects on individual traits were analysed with a three-way mixed model ANOVA for each of the six traits, using selection environment and measurement environment as fixed factors and time block as a random factor. Maximum likelihoods were calculated for separate linear models with and without all combinations of interactions between main effects. Akaike's information criterion was used to find the best-fit model, which was used for statistical analysis of each trait.

Traits are often not independent of one another and trade-offs among traits can lead to the evolution of one trait in response to selection on another. As only population averages were measured for most traits, the distribution of traits within populations was not available, and normal quantitative genetic approaches were inappropriate (e.g. Lande & Arnold, 1983). Instead, Pearson product-moment correlations were used to determine whether there were any significant relationships between each pairwise combination of traits. To account for multiple comparisons among traits, the data

were bootstrapped 10 000 times in R (The R Development Core Team), maintaining pairs of data during bootstrapping, to obtain mean correlation coefficients and 95% confidence limits. Correlations between traits were considered significant if the 95% confidence interval did not include zero.

Results

Species identification

The primers used to sequence the 18S rRNA gene captured a region varying in length from 506–530 bp. These sequences resulted in a poorly resolved tree, but still distinguished two distinct species in the October time block. In the April and June time blocks, all of the stock lines belonged to the same monophyletic group and were

considered the same species (Fig. 1). Lines within this clade shared identical sequences, except for an insert at the beginning and end of the sequence in some lines. None of the reference sequences fall within this same group, however, indicating that this portion of this gene in this species has not previously been sequenced. In October, at least one of the stock lines belonged to this same monophyletic group, but two other stock lines do not. One of these lines (Oct1) is likely *Colpoda steinii* (Fig. 1) and differs from the previously unsequenced species by 32 bp. The other October line (Oct2) falls into its own clade (Fig. 1) and differs from the previously unsequenced species by 16 bp.

Prior to the April and June experiments, I recognized two distinct morphotypes among samples and used only a single morphotype thereafter. All stock lines in the previously unsequenced species have similar

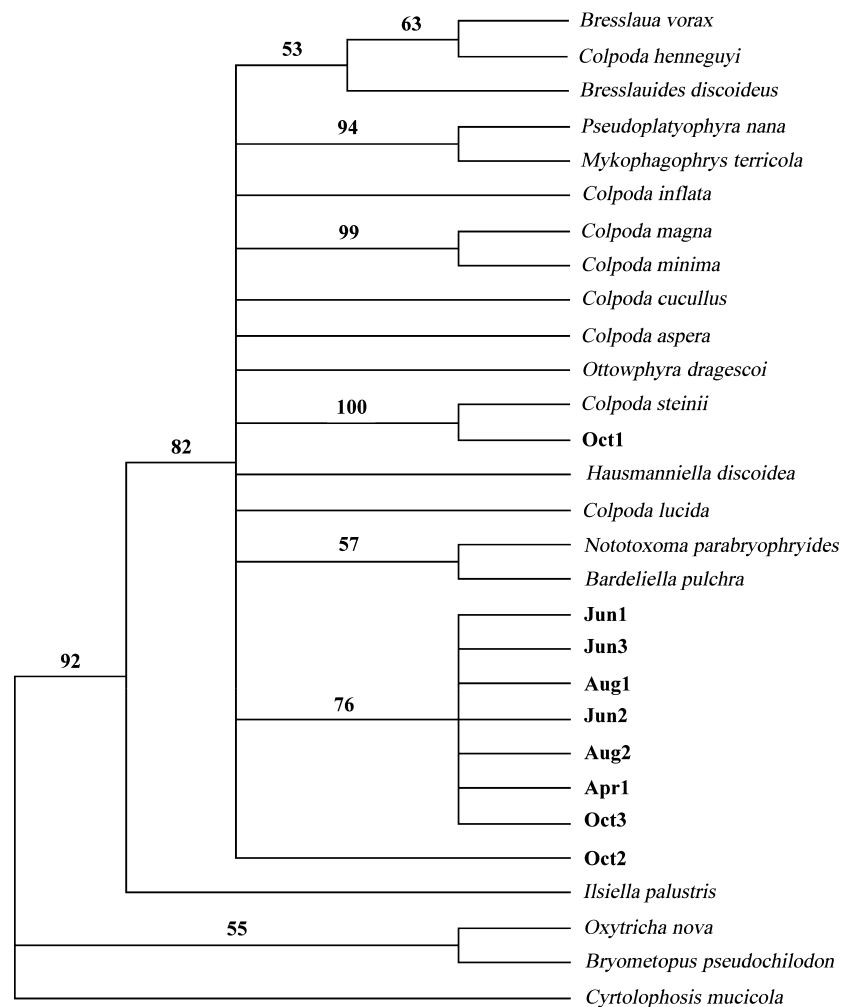


Fig. 1 Bootstrap consensus tree using parsimony generated using nucleotide sequences from a region of the 18S rRNA gene. Samples in bold were sequenced in this study. Oct, Apr and Jun samples represent a portion of the lines from different time blocks; lines from Aug were collected in similar fashion, but not used in any experiment. Reference sequences were obtained from Genbank (Appendix B).

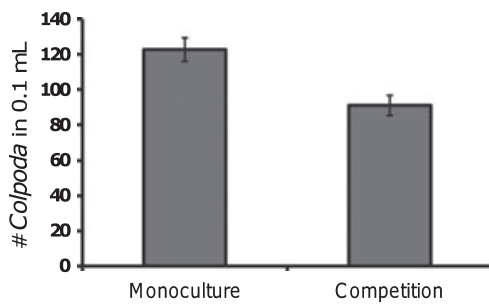


Fig. 2 The significant ecological effect of competition from *P. alpestris* on the abundance of *Colpoda* after 24 h.

morphologies and are conspicuously larger than *Colpoda steinii*, with more obvious colouration and frequently visible vacuoles within the cell. Further, the gliding movement of the unknown species is smoother than the more jerky movement of *Colpoda steinii*. The Oct2 line is composed of both morphotypes, which may explain why it does not group distinctly with either group. Although several stock lines were unable to be recovered for sequencing, the unrecovered lines were morphologically similar to those of the unsequenced species. To account for potential species-level selection in the October time block, I analysed the trait values with and without this block. Results were qualitatively similar, particularly with respect to the selection treatment, and for simplicity, I present the ANOVA results with only a single morphotype (April and June time blocks). The analysis including the October time block is available in Appendix C.

Ecological effects of competition

The abundance of *Colpoda* was reduced significantly by *P. alpestris* after 24 h ($F_{1,13} = 11.8$, $P = 0.004$, Fig. 2). *P. alpestris* reduced abundances by 26%, on average. Time block had no significant effect on *Colpoda* abundance after 24 h ($F_{1,13} = 0.46$, $P = 0.51$), and the block \times treatment interaction was not part of the best-fit model. In the October time block, *P. alpestris* had a similar effect and reduced *Colpoda* abundance by 24%.

Evolutionary effects of competition

Selection environment had a significant effect on the evolution of *Colpoda* traits (MANOVA: $F_{6,6} = 52.1$, $P < 0.001$) and affected the evolution of three of the six traits measured: population growth rate, cell size and cyst production. Measurement environment also had a significant effect on *Colpoda* traits measured (MANOVA $F_{6,6} = 10.6$, $P = 0.006$), largely because of cyst production and refuge use.

Population growth rate of *Colpoda* was significantly higher in populations that had evolved with competi-

tion than those that evolved in monoculture ($F_{1,27} = 6.72$, $P = 0.015$, Fig. 3a). Neither measurement environment ($F_{1,1} = 0.36$, $P = 0.65$, Fig. 3a) nor time block ($F_{1,1} = 11.1$, $P = 0.19$) had a significant effect on *Colpoda* population growth rate. The best-fit model included a nonsignificant interaction between time block and measurement environment ($F_{1,27} = 2.49$, $P = 0.13$). The initial population growth rate of *Colpoda* was lower than both selection environment treatments ($r = 0.118 \pm 0.009$). Population growth rates of *P. alpestris* were more difficult to measure because populations had not peaked within 48 h. Fitting an exponential curve to the available data may overestimate population growth rate, but gave a population growth rate of 0.086 (± 0.006 SE) – considerably lower than the population growth rate of *Colpoda* (Fig. 3a).

Selection environment had a marginally significant effect on cell size ($F_{1,1} = 52.0$, $P = 0.088$). When statistical power was increased by including the October time block, this effect was significant ($P = 0.009$, Appendix C). Individuals that evolved with competition were smaller than those that evolved in monoculture (Fig. 3b). Although measurement environment ($F_{1,1} = 36.2$, $P = 0.105$) and time block ($F_{1,1} = 7.05$, $P = 0.229$) had no significant effect on cell size, they did have a large effect when the October data were included in the analysis (Appendix C). The best-fit model included all two-way interactions. The interaction between measurement and selection environment was one of magnitude and not direction ($F_{1,25} = 69.2$, $P < 0.001$). Cells selected in competition were always smaller than those in monoculture, and cells measured in competition were always larger than those measured in monoculture (Fig. 3b). Similarly, the interactions between selection environment and time block ($F_{1,25} = 15.4$, $P < 0.001$) and measurement environment and time block ($F_{1,25} = 3.81$, $P = 0.062$) did not affect the interpretation of the main effects. The initial cell size of *Colpoda* grown in monoculture was larger than either selection environment treatment ($1.75 \pm 0.1 \times 10^{-3}$ mm). The average cell size of *P. alpestris* (mean cell area = 9.5×10^{-4} mm $\pm 4.6 \times 10^{-5}$) was smaller than *Colpoda* (Fig. 3b).

Selection environment has a significant effect on cyst production ($F_{1,28} = 5.67$, $P = 0.024$), as populations of *Colpoda* that evolved with competition produced significantly fewer cysts than those that evolved in monoculture (Fig. 3c). Measurement environment had a similar effect on cyst production in that populations measured in monoculture produced more cysts than those measured in competition ($F_{1,28} = 6.14$, $P = 0.020$, Fig. 3c). Time block also had a significant effect on cyst production, with more cysts produced in June ($F_{1,28} = 7.89$, $P = 0.009$). The best-fit model did not include any interactions.

Selection environment had no significant effect on *Colpoda* refuge use ($F_{1,28} = 0.65$, $P = 0.426$, Fig. 3d).

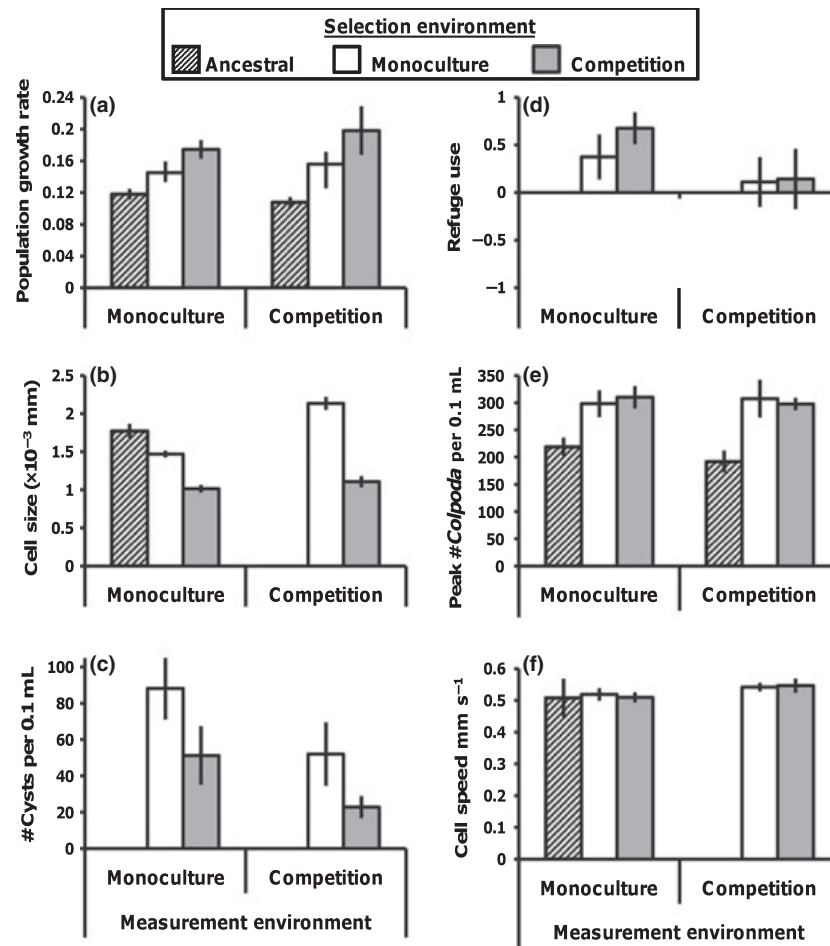


Fig. 3 Averages (\pm SE) of six traits of replicate *Colpoda* populations from two different selection environments (monoculture: white bars; interspecific competition: grey bars) measured in two different common garden conditions. Hatched bars represent the ancestral state of population traits (when available) before the selection experiment. In (a), the units are per capita growth rates per hour. In (d), positive values indicate greater density in the refuge environment; negative values indicate greater density in the water column.

Measurement environment had a marginally significant effect on refuge use ($F_{1,28} = 3.73$, $P = 0.064$). *Colpoda* measured in monoculture used the refuge more heavily than in competition (Fig. 3d). Time block had a significant effect on refuge use ($F_{1,28} = 13.3$, $P = 0.001$), as *Colpoda* used the refuge more heavily in June than in April. The best-fit model included only these main effects. Conversely, *P. alpestris* used the refuge very little and were found in higher densities in the water column relative to the refuge (refuge use = -0.36 ± 0.12).

There was no effect of selection environment ($F_{1,26} = 0.002$, $P = 0.970$, Fig. 3e), measurement environment ($F_{1,1} = 0.002$, $P = 0.972$, Fig. 3e) or time block ($F_{1,1} = 0.005$, $P = 0.955$) on the peak population density. The best-fit model included two interactions (measurement \times selection environment and block \times measurement environment), but neither were significant ($P > 0.60$). Selection environment had no effect on the speed of

Colpoda (Fig. 3f, $F_{1,27} = 0.024$, $P = 0.878$). Neither measurement environment ($F_{1,1} = 4.55$, $P = 0.279$, Fig. 3f) nor time block ($F_{1,1} = 19.9$, $P = 0.140$) had a significant effect on cell speed. The best-fit model included a nonsignificant block \times measurement environment interaction ($F_{1,27} = 0.014$, $P = 0.906$). The initial speed of *Colpoda* prior to selection was slightly slower than either selection environment treatment ($0.508 \pm 0.060 \text{ mm s}^{-1}$). The speed of *P. alpestris* ($0.20 \pm 0.007 \text{ mm s}^{-1}$) was much slower than the average speed of *Colpoda* (Fig. 3f).

Most traits were significantly correlated with other traits, although the nature and strength of the correlation was dependent on the specific traits under consideration (Table 1). When data were subdivided into different measurement and selection environments, correlations were qualitatively similar to one another. As such, the values in Table 1 reflect pooled data with increased power. The one exception to this case was the correlation

Table 1 Average correlation coefficients among six traits after bootstrapping data pairs. Asterisks indicate values for which the 95% confidence interval does not include zero.

	Pop. growth rate	Peak abundance	Cell area	Swimming speed	Cyst production	Refuge use
Pop. growth rate	1.0	0.36*	-0.56*	-0.32*	0.25*	0.29*
Peak abundance		1.0	-0.28*	-0.37*	0.29*	0.15
Cell area			1.0	0.36*	-0.17	-0.21*
Swimming speed				1.0	-0.34*	-0.22*
Cyst production					1.0	0.18
refuge use						1.0

between cell area and refuge use. The overall correlation with pooled data was significantly negative, but this was largely because of a strong negative correlation of individuals from the monoculture selection environment grown in the monoculture measurement environment ($r = -0.45$, $P < 0.05$). In all other selection and measurement environments, the correlation between cell size and refuge use was nonsignificant (range of $r = -0.04$ – 0.16 , $P > 0.05$).

Discussion

Protozoa have been used successfully as a model system of resource competition for decades (e.g. Gause, 1934; Vandermeer, 1969; Fox, 2002), but this is the first study to measure the evolution of protozoan traits in response to competition. Populations grown in interspecific competition evolved faster population growth rates and smaller cell sizes and produced fewer cysts relative to populations that evolved with only intraspecific competition (Fig. 3). Population growth rates and cell sizes measured before the start of the experiment suggest that these traits evolved to some extent in both selection treatments, but that populations in interspecific competition evolved to a greater extent (Fig. 3). The difference between treatments could be a result of increased competition for bacterial resources in the interspecific competition treatment, increasing the strength of selection. Initial densities of interspecific competitors were twice as high as intraspecific competitors, resulting in three times the total density of organisms. Regardless of mechanism, competition has a significant effect on the evolution of these traits.

Genetic variation in these experiments may have come from three sources of biological organization: among species (only in the October time block), among lineages within species and new variance within lineages created by mutations during the experiment. Assuming that reproduction was entirely asexual in *Colpoda*, lineage sorting at any of these levels is the most likely mechanism of evolution. However, sexual reproduction (Dunthorn *et al.*, 2008) and non-Mendelian inheritance of traits, as observed in other protozoa taxa (Nowacki *et al.*, 2008), may affect the heritability of traits within lineages and may play an important role in evolutionary dynamics

beyond simple lineage sorting. However, given that the inclusion of the October time block in the ANOVA analyses produced qualitatively similar results (Appendix C), lineage sorting within species, rather than species sorting, is the most likely mechanism of evolution in all three time blocks. Further, although significant time block effects in this latter analysis (Appendix C) could have been because of differences in species identity (i.e. October different from April or June), Tukey post hoc tests suggest that when time block effects did occur, they were largely because of differences in traits in the June time block. Time block effects may have occurred as a result of seasonal shifts in the bacterial community associated with pitcher plant inquiline communities through time. Development of microsatellite markers for these species for use in future experiments will allow for distinguishing between different mechanisms of evolution and whether different traits evolve via alternate mechanisms.

Both an increase in cell division rate and cyst production may have allowed these populations to tolerate intra- and interspecific competitions. Genetic lines that increased resource consumption via asexual cell proliferation would have been better equipped to monopolize resources and gain a numerical competitive advantage over other lineages and species. An increase in cell division rate would increase fitness during the 3-day bouts of competition within a microcosm. The faster dividing lineages also encysted more frequently (Table 1), allowing them to tolerate conditions when resources were scarce, but also increasing fitness in a new environment (i.e. a new microcosm or a newly opened leaf). The production of cysts is density dependent and thought to be related to the availability of bacteria in the environment (Yamaoka *et al.*, 2004; Foissner *et al.*, 2009), which may be a reliable cue for the imminent demise of transient aquatic habitats (Ekelund *et al.*, 2002). Such dynamics occur in several invertebrate taxa, where resting cysts allow for the tolerance of poor environmental conditions (e.g. Fenchel *et al.*, 1997; Caceres, 1998; Dumont *et al.*, 2002). Interestingly, cyst production decreased in *Colpoda* populations that evolved with competition, suggesting that encystment may be triggered by the abundance of conspecifics.

Other traits may have contributed to fitness by increasing survival in environments with competitors.

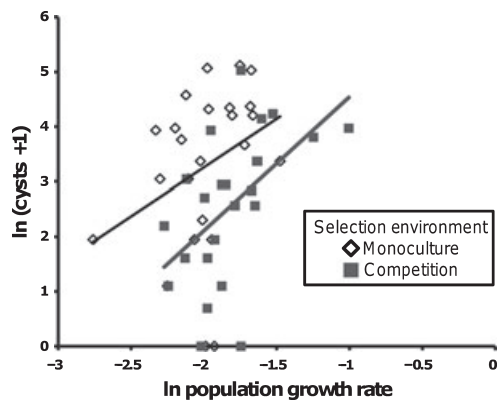


Fig. 4 Correlations between population growth rate and cyst production from two selection environments. The two traits are positively correlated, but selection is not constrained by the correlation. Evolution in competition results in decreased cyst production and increased growth rate, but the correlation is maintained in both treatments.

Although not significant, *Colpoda* populations that evolved with competitors tended to use the refuge more heavily, possibly to avoid competition with *P. alpestris* in the water column (Fig. 3d). Plate counts of bacteria from each habitat suggest that bacterial densities were two orders of magnitude higher in the refuge ($9.7 \pm 49 \times 10^8$ cells mL⁻¹) than in the water column ($8.6 \pm 72 \times 10^6$ cells mL⁻¹). Use of the refuge by *Colpoda* was size dependent, with smaller cells using the refuge more heavily than larger cells that tend to remain in the water column (Table 1, C.P. terHorst, unpublished data). Since *P. alpestris* used the water column the most, the larger *Colpoda* cells were most likely to experience competition, resulting in selection for decreased cell size (Fig. 3b) as a function of refuge use. Although cell size is positively correlated with competitive effect among species of bacterivores in the pitcher plant inquiline community (Kneitel, 2002; Leibold & Miller, 2004), this is not true of all protozoan species (Fox, 2002). It is impossible to determine whether cell size evolved as a result of direct selection on cell size or as a consequence of direct selection on refuge use, coupled with the correlation between refuge use and cell size.

The possibility of indirect selection on cell size occurs when traits are correlated with one another and selection on one trait results in the evolution of correlated traits. However, correlations between traits do not necessarily impose a constraint on selection, but rather must be considered relative to the strength of selection on each trait. For instance, population growth rate and cyst production are positively correlated with each other (Table 1). Yet, evolution in competition resulted in increased growth rates and decreased cyst production (Fig. 3). Had selection on cyst production been weak, selection for increased growth rates probably would have

resulted in increased cyst production, owing to the positive correlation between these traits (Fig. 4). The reduction in cyst production suggests that selection on this trait was stronger than that on population growth rate and able to overcome the correlation between traits. The effect size of selection environment on these traits suggests that indeed selection for cysts may have been stronger than that on growth rate (Fig. 4).

Traditional niche-based theory dictates that competitors' traits should diverge through evolutionary time (MacArthur & Levins, 1967; May & MacArthur, 1972; Chase & Leibold, 2003), although more recent theory predicts both convergence and divergence (Fox & Vasseur, 2008; terHorst & Miller, in review). Although there are many documented cases of character divergence (Schluter, 2000), there are considerably fewer, if any, cases of convergence in the literature. In this experiment, different traits converged or diverged from those of the competitor. Population growth rates of *Colpoda* diverged from *P. alpestris* traits, but cell sizes of these two competitors converged (Fig. 3). Most theory on character convergence and divergence examines a single trait (e.g. resource use or competitive ability), but these data highlight the need to examine independently evolving traits that may each evolve differently in response to competition.

Evolution is predicted to ultimately alter species interactions, particularly if evolution occurs on an ecological time scale (Carroll *et al.*, 2007; Strauss *et al.*, 2008; terHorst *et al.*, 2010). Evolution in this system was rapid relative to the lifespan of other species in this community (e.g. predatory mosquito larvae) and the lifespan of the leaf itself (approximately 40 weeks). However, analysis of competitive response of *Colpoda* to *P. alpestris* over the course of this experiment reveals no significant change in ecological effect (C.P. terHorst, unpublished data). Coevolution between these competitors might maintain ecological effects, but is unlikely since *P. alpestris* was continually restocked from a stock culture. *Colpoda* traits that display significant plasticity, such as refuge use or cyst production, may also affect competitive interactions with *P. alpestris* and obscure changes in ecological interactions caused by evolutionary changes in traits. Ongoing work in this community is attempting to determine whether evolution of traits in natural pitchers affects competitive interactions among a suite of competitors and ultimately affects species coexistence.

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References

- Brown, W.L. & Wilson, E.O. 1956. Character displacement. *Syst. Zool.* **5**: 49–65.
- Caceres, C.E. 1998. Interspecific variation in the abundance, production and emergence of *Daphnia* diapausing eggs. *Ecology* **79**: 1699–1710.
- Carroll, S.P., Hendry, A.P., Reznick, D.N. & Fox, C.W. 2007. Evolution on ecological time scales. *Funct. Ecol.* **21**: 387–393.
- Chase, J.M. & Leibold, M.A. 2003. *Ecological Niches: Linking Classical and Contemporary Approaches*. University of Chicago Press, Chicago, Illinois.
- Connell, J.H. 1980. Diversity and the coevolution of competitors, or the ghost of competition past. *Oikos* **35**: 131–138.
- Connell, J.H. 1983. On the prevalence and relative importance of interspecific competition: evidence from field experiments. *Am. Nat.* **5**: 661–696.
- Dumont, H.J., Nandini, S. & Sarma, S.S.S. 2002. Cyst ornamentation in aquatic invertebrates: a defence against egg predation. *Hydrobiologia* **486**: 161–167.
- Dunthorn, M., Foissner, W. & Katz, L.A. 2008. Molecular phylogenetic analysis of class Colpodea (phylum Ciliophora) using broad taxon sampling. *Mol. Phylogenet. Evol.* **6**: 316–327.
- Ekelund, F., Frederiksen, H.B. & Ronn, R. 2002. Population dynamics of active and total ciliate populations in arable soil amended with wheat. *Appl. Environ. Microbiol.* **68**: 1096–1101.
- Fenchel, T., Esteban, G.F. & Finlay, B.J. 1997. Local versus global diversity of microorganisms: cryptic diversity of ciliated protozoa. *Oikos* **80**: 220–225.
- Foissner, W., Weissenbacher, B., Krautgartner, W.D. & Lutz-Meindl, U. 2009. A cover of glass: first report of biomineralized silicon in a ciliate, *Maryna umbrellata* (Ciliophora: Colpodea). *J. Eukaryot. Microbiol.* **56**: 519–530.
- Fox, J.W. 2002. Testing a simple rule for dominance in resource competition. *Am. Nat.* **159**: 305–319.
- Fox, J.W. & Vasseur, D.A. 2008. Character convergence under competition for nutritionally essential resources. *Am. Nat.* **172**: 667–680.
- Gause, G.F. 1934. Experimental studies on the struggle for existence I. Mixed population of two species of yeast. *J. Exp. Biol.* **9**: 389–402.
- Goldberg, D.E. & Barton, A.M. 1992. Patterns and consequences of interspecific competition in natural communities: a review of field experiments with plants. *Am. Nat.* **139**: 771–801.
- Goodnight, C.J. & Craig, D.M. 1996. Effect of coexistence on competitive outcome in *Tribolium castaneum* and *Tribolium confusum*. *Evolution* **50**: 1241–1250.
- Gurevitch, J., Morrow, L.L., Wallace, A. & Walsh, J.S. 1992. A meta-analysis of competition in field experiments. *Am. Nat.* **140**: 539–572.
- terHorst, C.P., Miller, T.E. & Levitan, D.R. 2010. Evolution of prey in ecological time reduces the effect size of predators in experimental microcosms. *Ecology* **91**: 629–636.
- Joshi, A. & Thompson, J.N. 1996. Evolution of broad and specific competitive ability in novel versus competitive environments in *Drosophila* species. *Evolution* **50**: 188–194.
- Kneitel, J.M. 2002. Species diversity and trade-offs in pitcher plant (*Sarracenia purpurea*) inquiline communities. Ph.D. Dissertation, Florida State University.
- Lande, R. & Arnold, S.J. 1983. The measurement of selection on correlated characters. *Evolution* **37**: 1210–1226.
- Leibold, M.A. & Miller, T.E. 2004. From metapopulations to metacommunities. In: *Ecology, Genetics, and Evolution of Metapopulations* (I. Hanski & O. Gaggiotti, eds), pp. 133–150. Academic Press, San Diego, California.
- Lüftnegger, G., Foissner, W. & Adam, H. 1990. r- and K-selection in soil ciliates: a field and experimental approach. *Oecologia* **66**: 574–579.
- MacArthur, R. & Levins, R. 1967. The limiting similarity, convergence, and divergence of coexisting species. *Am. Nat.* **101**: 377–385.
- MacLean, R.C., Dickson, A. & Bell, G. 2005. Resource competition and adaptive radiation in a microbial microcosm. *Ecol. Lett.* **8**: 38–46.
- May, R.M. & MacArthur, R.H. 1972. Niche overlap as a function of environmental variability. *Proc. Natl. Acad. Sci. USA* **69**: 1109–1113.
- McGoey, B.V. & Stinchcombe, J.R. 2009. Interspecific competition alters natural selection on shade avoidance phenotypes in *Impatiens capensis*. *New Phytol.* **183**: 880–891.
- Miller, T.E. 1995. Evolution of *Brassica rapa* L. (Cruciferae) populations in intra- and interspecific competition. *Evolution* **49**: 1125–1133.
- Miller, T.E. & Kneitel, J.M. 2005. Inquiline communities in pitcher plants as prototypical metacommunities. In: *Metacommunities: Spatial Dynamics and Ecological Communities* (M. Holyoak, M.A. Leibold & R.D. Holt, eds), pp. 122–145. University of Chicago Press, Chicago, Illinois.
- Miller, T., Cassill, D., Johnson, C., Kindell, C., Leips, J., McInnes, D., Bevis, T., Mehlman, D. & Richard, B. 1994. Intraspecific and interspecific competition of *Wyeomyia smithii* (COQ) (Culicidae) in pitcher plant communities. *Am. Midl. Nat.* **131**: 136–145.
- Nowacki, M., Vijayan, V., Zhou, Y., Schotanus, K. & Doak, T.G. 2008. RNA-mediated epigenetic programming of a genome-rearrangement pathway. *Nature* **451**: 153–158.
- Santos, M., Borash, D.J., Joshi, A., Bounluyat, N. & Mueller, L.D. 1997. Density-dependent natural selection in *Drosophila*: evolution of growth rate and body size. *Evolution* **51**: 420–432.
- Schluter, D. 1994. Experimental evidence that competition promotes divergence in adaptive radiation. *Science* **266**: 798–801.
- Schluter, D. 2000. *The Ecology of Adaptive Radiation*. Oxford University Press, Oxford.
- Schoener, T.W. 1983. Field experiments on interspecific competition. *Am. Nat.* **122**: 240–285.
- Smith, R.A. & Rausher, M.D. 2008. Selection for character displacement is constrained by the genetic architecture of floral traits in the ivyleaf morning glory. *Evolution* **62**: 2829–2841.
- Sokolowski, M.D., Pereira, H.S. & Hughes, K. 1997. Evolution of foraging behavior in *Drosophila* by density-dependent selection. *Proc. Natl. Acad. Sci. USA* **94**: 7373–7377.

- Spears, T., Abele, L.G. & Kim, W. 1992. The monophyly of Brachyuran crabs: a phylogenetic study based on 18S rRNA. *Syst. Biol.* **41**: 446–461.
- Srivastava, D.S., Kolasa, J., Bengtsson, J., Gonzalez, A., Lawler, S.P., Miller, T.E., Munguia, P., Romanuk, T., Schneider, D.C. & Trzcinski, M.K. 2004. Are natural microcosms useful model systems for ecology? *Trends Ecol. Evol.* **19**: 379–384.
- Stanton, M.L., Thiede, D.A. & Roy, B.A. 2004. Consequences of intraspecific competition and environmental variation for selection in the mustard *Sinapsis arvensis*: contrasting ecological and evolutionary perspectives. *Am. Nat.* **164**: 736–752.
- Strauss, S.Y., Lau, J.A., Schoener, T.W. & Tiffin, P. 2008. Evolution in ecological field experiments: implications for effect size. *Ecol. Lett.* **11**: 199–207.
- Strong, D.R., Szyska, L.A. & Simberloff, D.S. 1979. Tests of community-wide character displacement against null hypotheses. *Evolution* **33**: 897–913.
- Taper, M.L. 1990. Experimental character displacement in the adzuki bean weevil, *Callosobruchus chinensis*. In: *Bruchids and Legumes: Economics, Ecology and Coevolution* (K. Fujii, A.M.R. Gatehouse, C.D. Johnson, R. Mitchell & T. Yoshida, eds), pp. 289–301. Kluwer Academic, The Netherlands.
- Tyerman, J.G., Bertrand, T., Spencer, C.C. & Doebeli, M. 2008. Experimental demonstration of ecological character displacement. *BMC Evol. Biol.* **8**: 34.
- Vandermeer, J.H. 1969. Competitive structure of communities: an experimental approach with protozoa. *Ecology* **50**: 362–371.
- Yamaoka, M., Watoh, T. & Matsuoka, T. 2004. Effects of salt concentration and bacteria on encystment induction in ciliated protozoan *Colpoda* sp. *Acta Protozool.* **43**: 93–98.

Appendix A: DNA extraction, amplification, purification, and sequencing

Genomic DNA was extracted from 100 microlitres (μL) of stock cultures varying in density, from 400 to 11 000 cells mL^{-1} , using SprintPrep[®] magnetic beads (Beckman Coulter, Brea, CA, USA). Each sample was loaded into a 96-well round-bottom plate, mixed with 80 μL of 100% isopropanol and 10 μL of SprintPrep beads and then placed on a SPRIP 96R[®] ring magnetic plate (Beckman Coulter) for 5 min. After the supernatant was drained, samples remained on the magnetic plate through five rinses (30 s each) with cold 70% ethanol. Samples were allowed to dry for 30 min to ensure evaporation of the ethanol. The round-bottom plate was removed from the magnetic plate, and 15 μL of ddH₂O was added to each sample, and the plate was placed on a shaker for 1 h to unbind the DNA from the magnetic beads. Samples were again placed on the magnetic plate for 5 min to separate the beads from the DNA in the supernatant. All the supernatants were removed, and DNA concentration was measured using

a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

A portion of the 18S rRNA gene was obtained using polymerase chain reaction (PCR) with protocols modified from Spears *et al.* (1992). One hundred microlitre reactions were composed of 53.5 μL ddH₂O, 16 μL dNTPs (10 μM), 8 μL MgSO₄ (25 mM), 10 μL PlatinumTaq buffer, 0.5 μL DNA Polymerase High Fidelity PlatinumTaq (Invitrogen, Carlsbad, CA, USA), 5 μL 329 forward primer (20 μM), 5 μL HI reverse primer (20 μM) and 2 μL extracted DNA (5 ng μL^{-1}). Primers were amongst those previously used by Spears *et al.* (1992): 329 forward primer: 5': TAA-TGA-TCC-TTC-CGC-AGG-TT:3'; HI reverse primer: 5':GTG-CAT-GGC-CGT-TCT-TAG-TTG:3'. PCR was carried out on a Veriti 96-well Thermal Cycler (Applied Biosystems, Foster City, CA, USA) using the following profile: 5 min @ 95 °C; 35 cycles of 40 s @ 95 °C, 25 s @ 52 °C, 3 min @ 72 °C and a final extension of 10 min @ 72 °C. PCR product was purified using the same magnetic bead protocol used for extraction. PCR products were sequenced at the DNA Sequencing Laboratory in the Biological Science Department at Florida State University using a 3130 × 1 Genetic Analyzer with Capillary Electrophoresis (Applied Biosystems).

Appendix B: Genbank accession numbers for previous sequences used to create the phylogeny (left column) and new sequences from this study (right column)

Species name	Genbank accession number	Sample name	Genbank accession number
<i>Bardeliella pulchra</i>	EU039884	Oct1	HM215507
<i>Bresslaura vorax</i>	AF060453	Oct2	HM215506
<i>Bresslaurides discoideus</i>	EU039885	Oct3	HM215505
<i>Bryometopus pseudochilodon</i>	EU039888	Apr1	HM215508
<i>Colpoda aspera</i>	EU039892	Jun1	HM215509
<i>Colpoda cucullus</i>	EU039893	Jun2	HM215510
<i>Colpoda henneguyi</i>	EU039894	Jun3	HM215511
<i>Colpoda inflata</i>	M97908	Aug1	HM215512
<i>Colpoda lucida</i>	EU039895	Aug2	HM215513
<i>Colpoda magna</i>	EU039896		
<i>Colpoda minima</i>	EU039897		
<i>Colpoda steinii</i>	DQ388599		
<i>Cyrtolophosis mucicola</i> (Brazil)	EU039898		
<i>Hausmanniella discoidea</i>	EU039900		
<i>Ilsiella palustris</i>	EU039901		
<i>Mykophagophrys terricola</i>	EU039902		
<i>Notoxoma parabyrophyrides</i>	EU039903		
<i>Ottowphyra dragescoi</i>	EU039904		
<i>Oxytricha nova</i>	X03948		
<i>Pseudoplatyophrya nana</i>	AF060452		

Appendix C:

	Population growth rate			Cell size			Cyst production		
	d.f.	F	P	d.f.	F	P	d.f.	F	P
Selection Env.	1,41	10.2	0.003	1,2	113	0.009	1,41	6.46	0.015
Measure Env.	1,2	0.029	0.88	1,2	41.7	0.023	1,2	0.46	0.57
Time block	2,2	4.3	0.19	2,2	29.5	0.033	2,2	10	0.09
Selection × Measure				1,2	8.1	0.104			
Selection × Time				2,2	0.48	0.678			
Measure × Time	2,41	3.84	0.03	2,2	0.18	0.849	2,41	1.47	0.24
Sel × Meas × Time				2,36	7.75	0.002			

	Refuge use			Population peak			Cell speed		
	d.f.	F	P	d.f.	F	P	d.f.	F	P
Selection Env.	1,43	2.33	0.13	1,40	0.093	0.76	1,2	0.23	0.68
Measure Env.	1,43	3.05	0.09	1,2	0.52	0.39	1,41	3.6	0.064

Appendix C: Continued

	Population growth rate			Cell size			Cyst production		
	d.f.	F	P	d.f.	F	P	d.f.	F	P
Time Block	2,43	7.32	0.002	2,2	1.91	0.34	2,2	11.5	0.08
Selection × Measure				1,4	0.25	0.616			
Selection × Time									
Measure × Time				2,40	0.58	0.566	2,41	0.65	0.53
Sel × Meas × Time									

ANOVA results using all three time blocks (October, April and June). For each trait, the best-fit model was determined by the lowest Akaike's information criterion value. Empty cells indicate that the factor was not part of the best-fit model. Significant effects are indicated in bold. Results are qualitatively similar to those using only April and June time blocks, but because of increased power, there are more significant effects here.

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