**Introduction**

Evolution and ecology are intertwined

Indirect effects may diffuse through communities

Pitcher plant system

Why rotifers

Temperature matters

Temperature is changing

Aims & hypotheses

**Methods**

Field Collection of Rotifers

*Sarracenia purpurea* leaf water samples were collected from 97 plants across three sites in the Apalachicola National Forest in northern Florida in July of 2024. These sites were chosen for the presence of known *S. purpurea* populations. Fifty samples were collected from Site 1 (Crystal Bog), 38 samples were collected from Site 2 (Pleaphase Savanna), and eight samples were collected from Site 3 (Twisted Cyprus Bog). At each site, leaves were chosen haphazardly for sampling with a minimum of ~1 m distance between each leaf. No two leaves were sampled from the same plant. Samples were collected using clean plastic transfer pipettes with ~5 cm of the tip cut away to avoid getting clogged with leaf water detritus. Before collection, the leaf water was mixed to suspend detritus by repeatedly pipetting up and down. Leaf contents were transferred to 50 ml conical tubes. The entire volume of leaf water was collected when possible; if the leaf volume exceeded 50 ml, only 50 ml was collected. Samples were then transported to Forida State University and stored at room temperature.

Within 24 h of collection, samples were visually inspected for the presence of late-instar mosquito larvae (*Wyeomyia smithii*), which if present were removed using a plastic transfer pipette. The excess leaf water discarded during the process of mosquito larvae removal was filtered using a xx µm mesh sieve and frozen for later use as a seed community of bacteria to help mimic the conditions of natural pitcher plant leaf water.

Sample tube lids were sealed with parafilm and transported via commercial flight in a checked bag to California State University, Northridge. Sample tubes were then stored in a growth chamber (growth chamber company) with a 12/12 h light/dark cycle, at 25C during the day and 18C at night. Using custom Palmer cell-style counting slides, 100 µl samples from each tube were scored for the presence of *Habrotrocha rosa*, other species of rotifers, and ciliates. Samples were monitored daily for the presence of developing mosquito larvae, which were removed promptly upon detection.

Rotifer Isolation and Rearing

In order to test for diversity in response to temperature, clonal cultures of *H. rosa* were first established by isolating individuals from the field-collected leaf water samples. To be selected for use in isolations, leaf water samples had to meet two criteria: the absence of an overwhelming abundance of ciliates, and an estimated rotifer density of >2 individuals per 100 µl. Meeting these criteria were seven samples from Site 1 (Crystal Bog), six samples from Site 2 (Pleaphase Savanna), and one sample from Site 3 (Twisted Cyprus Bog).

To isolate rotifers, 0.5 ml of leaf water was transferred into a 12-well tissue culture plate. Individual rotifers were transferred with the smallest volume of leaf water possible using a clean, pulled Pasteur pipette into another well filled with 0.5 ml of sterile spring water. This washing step was repeated a second time into another well of sterile spring water, and a third time into an unoccupied well in a 24-well tissue culture plate that contained 0.3 ml of a modified version of the rotifer media described by Plasota and Plasota, 1980 (1% sodium chloride, 0.06% ammonium chloride, 0.05% dipotassium phosphate, 0.02% monopotassium phosphate, 0.002% magnesium sulfate dissolved in deionized water). To serve as a basal resource for the bacteria carried over during isolation, xx mg of fish food flakes (TetraColor Tropical Fish Flakes, Tetra) were added to each isolation well.

Preliminary experiments using this isolation method show that the likelihood of a haphazardly-chosen individual of *H. rosa* proliferating into a culture of clones is only xx% (unpublished data, Mendelson). This is consistent with expectations, because the reproductive period of *H. rosa* lasts only 5-7 d. With a lifespan of 12-24 d, some individuals have been observed to live for nearly two weeks after losing the ability to reproduce (Ellison and Bledzki, xxxx). Therefore, in order to reach the goal of five clonal cultures per leaf, 32 individuals were isolated from each selected leaf water sample, for a total of 448 isolated individuals of *H. rosa* across 20 24-well tissue culture plates.

At 14 and 28 days after isolation, every well in each 24-well tissue culture plate was observed, and if more than 20 individuals were present, the entire contents of the well was transferred to a sterile 125 ml flask filled with 100 ml of rotifer media and containing one sterile wheat seed as a resource for bacteria. Of the 448 attempted isolations, 85 were successful, representing x leaves from x site, and x leaves from x site. *Habrotrocha rosa* was only present in two leaf water samples from Twisted Cyprus Bog at the time of isolation, and fewer than five isolations from these samples were successful. For these reasons, Twisted Cyprus Bog was excluded from these experiments.

Temperature Response Experiment

To assess the effect of increased temperature on growth rate and dry mass, clonal cultures of *Habrotrocha rosa* were left to grow at two temperatures for 39 d. The clones used in this experiment were randomly selected from available clonal stock cultures in such a way that they represented three individuals isolated from each of three leaves across two sites located >5 km apart. These 18 clonal stock cultures represented diversity between sites, among leaves within sites, and among clones within leaves.

To prepare for this experiment, rotifers from clonal stocks were first concentrated and washed. To concentrate the 100 ml stock cultures, between 20 and 60 ml (dependent on initial estimates of stock concentration) was passed through a 40 µm sterile nylon mesh cell strainer (fisherbrand). To wash away excess debris from the stock flasks, 5 ml of sterile media was pipetted into the filter and allowed to drain away. Rotifers captured by the filter remained submerged throughout the duration of these steps (Figure 1).

After being concentrated and washed, rotifers were transferred to 15 ml conical tubes previously coated with 0.1% gelatin to prevent them from sticking to the tube walls. The concentration of rotifers in these tubes was then estimated by averaging the counts of three 0.3 ml samples. Using a simple dilution calculation, the appropriate volume of concentrated and washed rotifers was added into each of six replicate tubes so that each tube began the experiment at a density of 5 rotifers per ml in 10 ml of media. This process was repeated for all 18 clonal stock cultures, however the culture chosen for Leaf 1 from the Crystal Bog site did not meet the minimum required density to start six experimental replicate cultures. Unfortunately, there were no other suitable clonal stock cultures from that leaf, and thus it is only represented by two clones. In total, n = 102 experimental cultures were prepared, n = 6 for each of 17 clones.

Replicates were all inoculated with 50 ul of a natural *S. purpurea* leaf water bacterial community of unmeasured concentration and fed 100 ul of a fish food solution (25 mg ground, sterile fish food flakes dissolved in 25 ml of sterile deionized water).

Evolution Experiment

A similar species of rotifer has a clearance rate of 3.79 ± 2.1 ul ind-1 h-1 at 20 C and 1.65 ± 1.4 ul ind-1 h-1 at 15 C. We assumed *H. rosa* have a slightly higher clearance rate of ~4 ul ind-1 h-1 (Devetter 2007).

*Tetrahymena* sp. has been measured grazing on suspended bacteria at a rate of 1,382 ± 1,029 cells ind-1 h-1 (Eisenmann et al. 1998).

Preliminary Competition Experiment

In the *S. purpurea* leaf water community, many species of protists compete with *H. rosa* for bacteria when resources are limited. An unidentified species of ciliate in the genus *Tetrahymena* exhibits similar logistic growth parameters to those of *H. rosa*, albeit with a much shorter generation time.

Competition Experiment

Rotifers from each evolutionary history of temperature (25C and 30C, n=10) were grown at contemporary temperatures matching their historic temperature or at a novel temperature (for example, rotifers with an evolutionary history of 25C were grown at both 25C and 30C). Each of these evolutionary history and novel temperature combinations were grown with and without the presence of a natural competitor: *Tetrahymena* sp., a bacterivorous ciliate frequently found cooccurring with *H. rosa* in the leaves of *S. purpurea.* Control monocultures of *Tetrahymena* sp. were also grown at 25C and 30C. Experimental cultures were prepared in a total volume of 20 ml of rotifer media (described above) inside of 50 ml conical tubes. The source cultures for each of the ten replicates were counted using Palmer cell style counting slides, and the appropriate volume from each source culture was added to its respective experimental cultures so that each one began the experiment at a density of ~20 rotifers ml-1. The same procedure was performed to add *Tetrahymena* to the relevant cultures at a density of ~40 cells ml-1. All cultures received 5 mg of sterile fish food powder dissolved in DI water. The cultures were then topped off with media to reach a total volume of 20 ml.

Estimating Growth Rate

Rotifers in a 0.3 ml sample were counted using a compound microscope and custom-made counting slides every 3 d during each experiment. Counting continued until ~90% of replicates appeared to reach carrying capacity. These data were fit to a logistic growth model using the R package ‘growthrates’ (R 4.4.3).

Model fitting, method of least squares using the Levenberg-Marquardt algorithm

Estimating Dry Mass

For each replicate, a total of 80 ul was transferred to a custom counting slide (20 ul in each corner to ensure an even distribution of rotifers). Videos of each replicate were then recorded using a compound microscope and a Sony a7ii mirrorless camera. To avoid imaging the same individual twice, these videos traced the path of the maze-like pattern on the counting slide (inspired by [type of counting plate thing]). When possible, at least 20 images from each video featuring a unique and fully extended rotifer were exported using Adobe Premier Pro. Rotifer images were downscaled by 50% and converted to 8-bit before batch processing using a custom trained pixel classifier in Ilastik to produce segmented images differentiating rotifers from the background (Ilastik). Segmented images were converted to binary and further separated into individual regions of interest (ROIs) using the particle analysis features in Fiji, a distribution of ImageJ. The resulting ROI masks that met a minimum aspect ratio of 2.5, that is, at least 2.5x longer than they were wide, were further analyzed using a custom Python script: First, the raw masks were rescaled back to their original resolution and cleaned (gaps and holes filled). Then, a midline was estimated using the skeletonize function in the skimage library. This midline was then compared to the original binary mask to produce width estimates along the length of the rotifer. These width estimates were integrated over the length of the midline, and using the sum of resulting frustum segments, an estimate of volume was derived. Consistent with [paper that talks about vol -> mass], volume was converted to dry mass.

**Results**

Evolutionary response to temperature

The maximum population growth rate of rotifers did not differ between two evolutionary histories of temperature (F1,72 = 1.481, p = 0.228). Evolutionary history did affect the carrying capacity of rotifers independent of current temperature and the presence of a competitor (F1,72 = 4.254, p = 0.043). The carrying capacity of rotifers that evolved at 25C was 9% higher than those that evolved at 30C (estimate = 5.48 ± 2.66, t72 = 2.062, p = 0.0428).

Competitive response of rotifers to protists

The effect of competition on rotifer growth rate was dependent on current temperature (F1,72 = 6.854, p = 0.011). Back-transformed growth rate was 60% higher in the absence of a competitor at a current temperature of 30C (estimate = 0.686 ± 0.14 SE, t72 = 4.899, p < 0.001), but competition had no effect on growth rate at 25C (estimate = 0.167 ± 0.14 SE, t72 = 1.197, p = 0.235). Competition affected rotifer carrying capacity independent of current temperature and evolved temperature (F1,72 = 44.051, p = <0.001). The carrying capacity of rotifers in the absence of a competitor was 26% higher (estimate = 17.6 ± 2.66, t72 = 6.637, p = <0.001).

Competitive effect of rotifers on protists

Protist growth rate was affected by competition, but the effect was dependent on current temperature (F1,54 = 4.533, p = 0.015). Post-hoc comparisons of competition indicate protist growth rate was further dependent on the evolutionary history of their rotifer competitors. At a current temperature of 30C, protist growth rate was 30% lower in the presence of rotifers, but only if the rotifers evolved at 30C (estimate = 0.577 ± 0.207 SE, t54 = 2.789, p = 0.0196). At the same current temperature of 30C, rotifers with an evolutionary history of 25C did not have an effect on protist growth rate (estimate = 0.404 ± 0.207 SE, t54 = 1.953, p = 0.1339). Protist growth rate was unaffected by the presence of competitors when the current temperature was 25C (all p > 0.05).

Competition also affected the carrying capacity of protists, but the effect was again dependent on current temperature (F1,54 = 17.1554, p = <0.001). Protist carrying capacity was 36% lower in the presence of rotifer competitors, but only at a current temperature of 25C (estimate = -395.9 ± 59.2, t54 = -6.681, p = <0.001). The carrying capacity of protists was unaffected by the evolutionary history of their rotifer competitors (p > 0.05).

**Discussion**

**References**

***Notes:***

Lau 2012. Evolutionary indirect effects of biological invasions

There are two types of evolutionary indirect effects: indirect ecological effects can acting as selective pressures that lead to evolution, and evolution itself as the driver of indirect ecological effects (evolution-mediated indirect effects). Lau (2012) discusses these interactions in the context of biological invasion.

Can populations recover from indirect ecological effects by balancing effects? If so, is that always the case? Are there examples of this happening but recovery not occurring in examples with ecological effects only, and would we expect the same thing to happen when evolution is the mediator of the indirect effect? Does evolution have some increased capability of maintaining a balance through natural selection that ecological effects lack, making potentially harmful evolution-mediated indirect effects less unstable?

Rich in examples of trade-offs leading to unexpected indirect effects on species interactions.

Bowers and Puttick 1988.

Rausher and Simms 1989.

Laukau 2007.

Strauss and Irwin 2004.

Leimu and Koricheva 2006.

Lankau and Strauss 2008.

Lankau 2009.

Pelletier et al. 2009.

Lau and terHorst 2020. Evolutionary responses to global change in species-rich communities.

Devetter 2007. Clearance rates of the bdelloid rotifer, *Habrotrocha thienemanni*, a tree-hole inhabitant.

Eisenmann et al. 1998. Grazing of a *Tetrahymena* sp. on adhered bacteria in percolated columns monitored by in situ hybridization with fluorescent oligonucleotide probes.