Ecoevolutionary feedbacks of phenotypic plasticity and mono- vs. polyclonal communities in bi-and tritrophic systems

Trophic interactions in aquatic communities are significantly affected by the performance of the interacting partners. Different performance levels can depend on different traits such as edibility, reproductive rate or growth rate can significantly affect population dynamics and community structures. Trait variability in populations may result from either phenotypic plasticity or genetic diversity. A form of phenotypic plasticity are inducible defences which have been shown to protect the prey but additionally to dampen the oscillations of predator-prey population cycles. However, there is a coevolutionary adaptation in the form of inducible offenses in some predators which partly overcome the induced defenses. **We here examined if trait variability and phenotypic plasticity on the consumer level stabilize trophic interactions more than trait variation on two (consumer and predator) trophic levels within a tritrophic system.** For this, we used freshwater ciliates of the genus *Euplotes* feeding on the non-plastic algae *Chlorogonium*. *Euplotes* expresses defensive features by increasing their cell length and width in the presence of the predatory ciliate *Lembadion*. *Lembadion* can counter this kind of prey plasticity, by a plastic reaction to the enlarged prey and gradually adjust peristome size facilitating ingestion of defended prey.
To set up the system in our lab, we first isolated ciliophora from the field and taxonomically described them using visual classification. Despite advancements in molecular and microscopy techniques, ciliate identification methods in ecological and biomonitoring research have largely remained stagnant. Conventional identification primarily utilizes silver staining techniques, where silver salts visualize key features like basal bodies or nuclei. However, the time intensity and steep learning curve make these methods less desirable for non-ciliate specialists. Therefore, in line with identifying our own isolated ciliates, we developed a novel and efficient taxonomic identification approach. Silver-impregnation stainings follow time consuming protocols and using comparatively toxic reagents. We therefore developed a simplified staining methodology with which *Euplotes* and other Ciliophora can easily be identified. In the developed protocol we apply a DNA binding stain, such as DAPI, to visualize the nuclei and the tubulin DM1A (anti-α-tubulin antibody, fig. 1). This protocol is straightforward without using advanced equipment and will serve beneficial for many ecological investigations of Ciliophora.

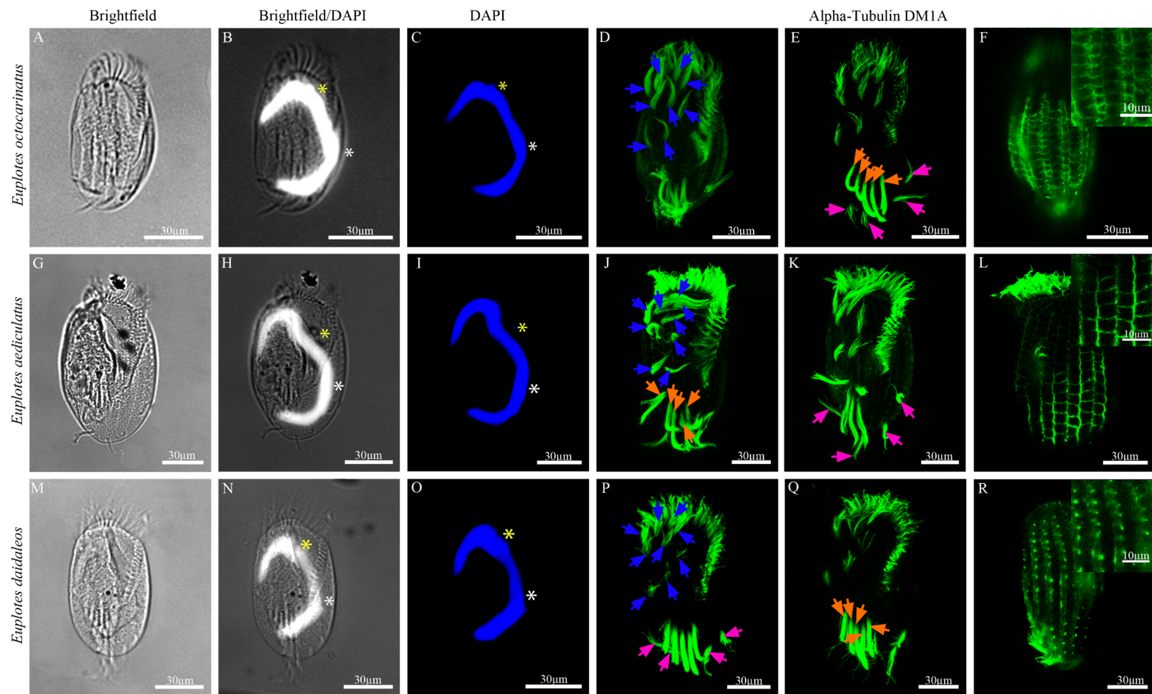

Figure 1: Brightfield and confocal immunofluorescence microscopic images of three different *Euplotes* species were recorded after staining with antibodies directed against α-tubulin. *Euplotes octocarinatus* (A-F), *Euplotes aediculatus* (G-L) and *Euplotes daidaleos* (M-R) are illustrated, using brightfield microscopy (A, G, M), brightfield microscopy in combination with fluorescent microscopy (B, H, N) and fluorescent microscopy (C, D, E, F, I, J, K, L, O, P, Q, R). DAPI stained the differently shaped dimorphic nuclei (B, C, H, I, N, O). The most common form is a simple inverted C-shape seen in *E. octocarinatus* (B, C) and *E. daidaleos* (N, O). The macronucleus of *E. aediculatus* (H, I) shows a modified 3-shape. White star = macronucleus, yellow star = micronucleus. DM1A allowed the targeted detection and analysis of cilia (D, E, J, K, P, Q): frontoventral, transversal and caudal cirri (D, E, J, K, P, Q) and the specific tubulin network, the so called silverline-system (F, L, R). Fig. 1 F, L, R additionally contain enlarged images of the specific tubulin network. Groups of cirri: blue arrows = frontoventral cirri; orange arrows = transversal cirri; pink arrows = caudal cirri. Additional detection of species-specific different pattern of silverline-systems is possible (F, L, R): F and R = double-patella type (E; *Euplotes octocarinatus* and R; *Euplotes daidaleos*), L = double-eurystomus type (L; *Euplotes aediculatus*), scale bar = 30µm.

In the next phase of the experiment, we measured individual trait variability in the reaction norms in isolated strains of the validated species: *E. octocarinatus*, *E. daidaleos* and *E. aediculatus* and the predator *Lembadion* (fig. 2 a). This was done by co-culturing them with different densities of the plastic predator *Lembadion* and the non-plastic predator *Stenostomum sphagnetorum* (Turbellaria) under unlimited food conditions. In response to both predators, the smallest strain *E. octocarinatus* EO1 showed the strongest defence (fig. 2a). The medium sized *E. daidaleos* AS3 showed an intermediate increase in cell size and the largest *E. aediculatus* LV7, showed the smallest size increase (fig. 2a). The increase of cell size of all three prey species is predator dependent and the presence of the small strain *Lembadion* clone L1 induces smaller traits than the large strain L2 (fig. 2a). Trait variability of size adjustments in *Lembadion* were also measured by culturing it with above mentioned-prey that

were selected due to size differences (*E. octocarinatus* (EO1) as small prey, *E. daidaleos* (AS3) as medium prey and *E. aediculatus* (LV7) as large prey (fig. 2b).

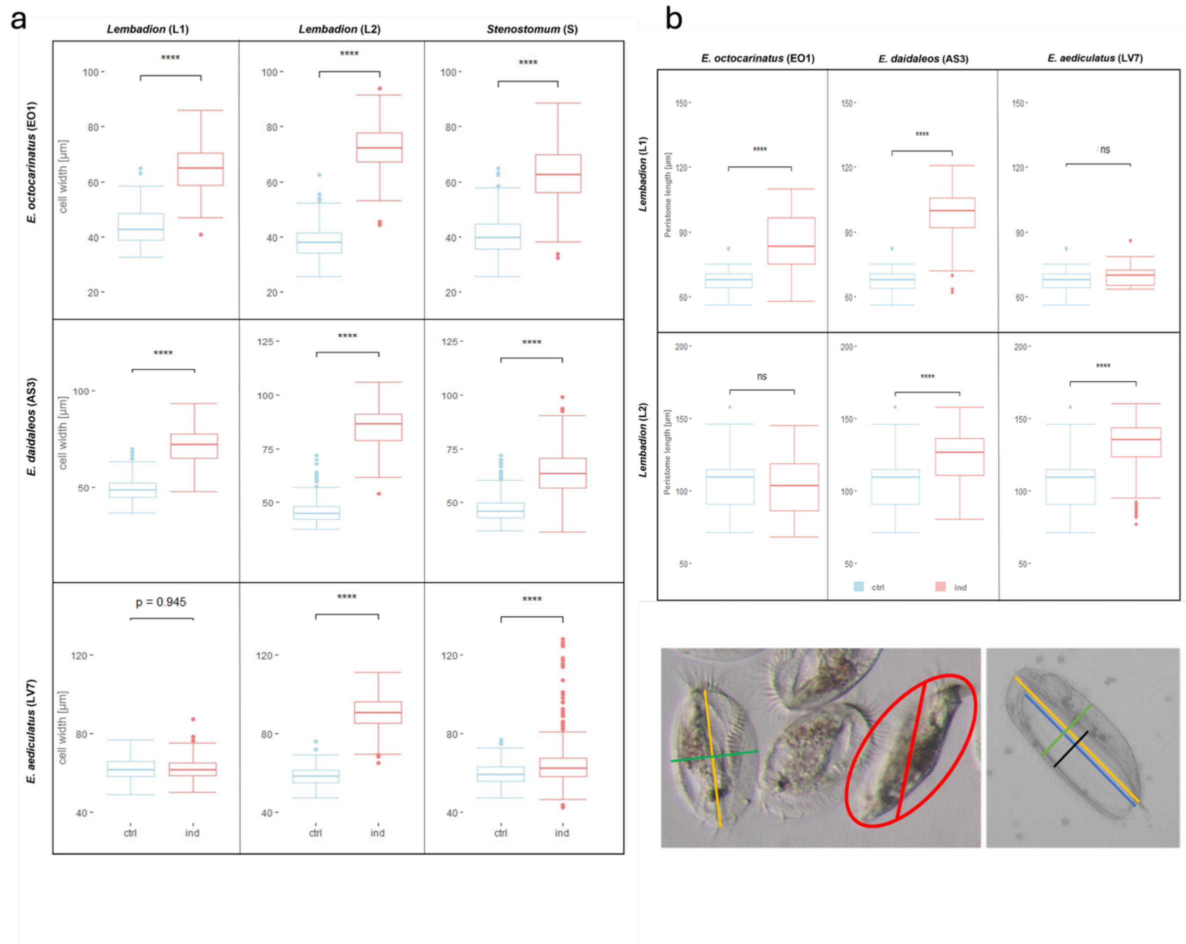

Figure 2: **a)** **Reaction norms of three *Euplotes* species** (*E. octocarinatus, E. daidaleos, E. aediculatus*) to their predator *Lembadion* (L1/L2) and *Stenostomum* during long term experiments about 51 days. **b)** Reaction of the plastic predator *Lembadion* (L1/L2) to different sized and defended *Euplotes* species (*E. octocarinatus, E. daidaleos, E. aediculatus*) during long term experiments about 51 days. **c)** Display of measured ciliates Euplotes left and Lembadion (right).

We then wanted to investigate the effect of prey's trait variability on population dynamics in mono- and polyclonal experiments. But, species distinction and therefore population density determination is the limiting factor in these experiments. We therefore implemented a faster molecular analytical method (fig. 3a). By targeting a species-specific DNA sequence, we can now determine this unicellular organism's cell numbers using quantitative PCR in comparison to a given standard curve (fig. 3b). In lab strain specific and in mixed strain cultures we can now determine the number of individual per species with high accuracy (fig. 3 c, d).

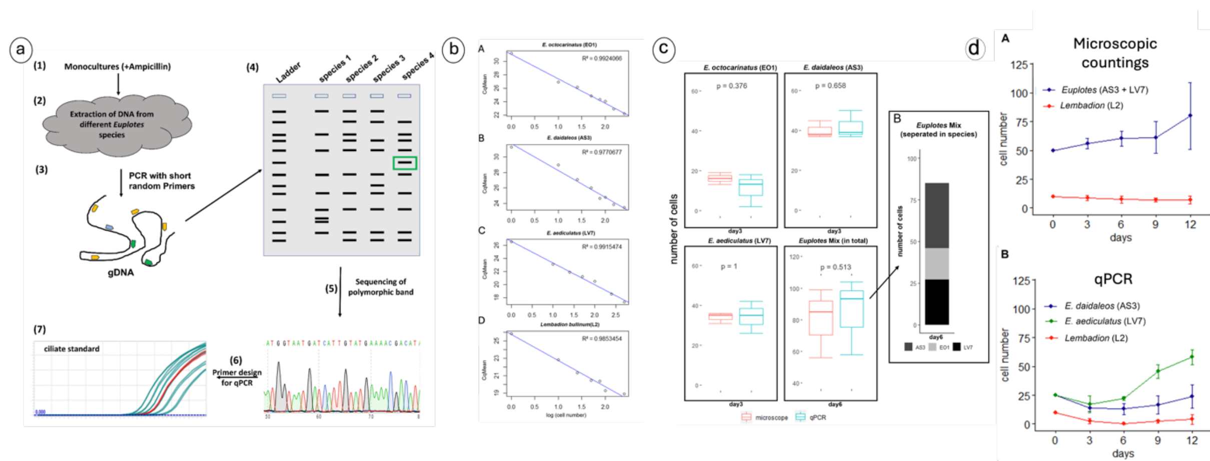

Figure 3: a.) Setup for genetic marker identification. (1) Ciliates were cultivated in SMB with ampicillin [1µg/ml] without a wheat grain to avoid bacterial contamination. (2) DNA of monoclonal cultures of *E. octocarinatus* (EO1), *E. aediculatus* (LV7) and *E. daidaleos* (AS3) were extracted. (3) PCR was performed with random primers (Kusch et. al, 2000) and (4) PCR products were analysed by electrophoresis in 2% agarose gels. (5) polymorphic fragments were purified and sequenced. (6) qPCR Primer were designed using Geneious V.11.1. (7) Creating a standard of defined numbers of ciliate cells with qPCR **b.) Standard curves of the three different *Euplotes* species** (*E. aediculatus* (LV7)*, E. daidaleos* (AS3)*, E. octocarinatus* (EO1)) and their predator *Lembadion* (L2). (A-D) 1/10/25/50/75/100/150/250/500 cells of each species were counted under the microscope and transferred to a 1.5 ml tube for DNA extraction. The standard of each species was generated with species-specific primers using qPCR. This resulted in a correlation coefficient for (A) EO1 with $R^2 = 0.9924$, (B) AS3 with $R^2 = 0.9767$, (C) LV7 with $R^2 = 0.9915$ and (D) L2 with $R^2 = 0.9856$. The mixed sample was prepared from the respective standard of each species with 100 ciliates and diluted in a ratio of 1:4. Calculated cell number was - 0.5 (EO1), - 0.7 (AS3), - 7.2 (LV7) and + 6.6 (L2) cells compared to defined cell number of 25 cells/species counted under the microscope. **c.) Microscopic validation of qPCR results:** *E. octocarinatus* (EO1), *E. daidaleos* (AS3), *E. aediculatus* (LV7) were tested in a monoclonal (1000 *Euplotes* / 10 ml SMB; n=3) and in a mix approach (333 Euplotes / 10 ml SMB; n=3). After 3 and 6 days 500 µl were counted under the microscope and 500 µl were prepared for DNA extraction and cell numbers were determined via qPCR using the created standard curve. Counted numbers were tested against measured cell numbers using a Mann-Whitney U test. (A) *E. octocarinatus* (EO1) (day 3: w = 6.5; p = 0.3758) *E. daidaleos* (AS3) (day 3: w = 3.5; p = 0.6579), and *E. aediculatus* (LV7) (day 3: w = 6.5; p = 0.3758) were tested in a monoclonal and in a mix approach (*Euplotes* Mix) (day 6: w = 3; p = 0.5127). (B) *Euplotes* Mix strain specific composition. **d.) Measurement of cell numbers of prey (*Euplotes*) and their predator (*Lembadion*) in a mix approach**. *E. daidaleos* (AS3), *E. aediculatus* (LV7) cultivated for 12 days with their predator *Lembadion* (L2) with a predator/prey ratio 1:5 (n=3). Samples were taken at day 3, 6, 9 and 12 and counted under the microscope and prepared for measurement via qPCR. The number of cells was calculated using the created standard curve. (A) Cell number counted under the microscope (without deviation). (B) Determined cell numbers using specific primer for *Euplotes* species and the predator *Lembadion* in qPCR, which allows to distinguish the two *Euplotes* species *E. daidaleos* (AS3) and *E. aediculatus* (LV7). Counted numbers were tested against measured cell numbers (in total) using a Mann-Whitney U test. *Euplotes* (day 3: w = 6; p = 0.5127; day 6: w = 5; p = 0.8273; day 9: w = 0; p = 0.0495; day 12: w = 1; p = 0,1266). *Lembadion* (day 3: w = 9; p = 0.0431; day 6: w = 9; p = 0.0463; day 9: w = 9; p = 0.0463; day 12: w = 7; p = 0.2683)

With this information, we first performed monoclonal tritrophic long term experiments with the top predators *Lembadion spp.* (plastic) and *Stenostomum* (non-plastic), and the three different *Euplotes* species as differently sized prey (fig. 4). *Euplotes* was fed with the algae *Chlorogonium elongatum.* Over 50 days we measured the abundance of predator, prey and algae to study population dynamics. At the same time, we measured morphological traits for predator and prey. Population dynamics of *E. octocarinatus* (EO1 small) and E. *daidaleos* (AS3 medium) and the plastic predator *Lembadion* L1 showed predator prey oscillations with small amplitudes. When *Lembadion* L1 was co-cultured with *E. aediculatus* (LV7-large) it died out, as the induced size of *E. aediculatus* outgrows *Lembadion's* peristome thereby hampering food ingestion.

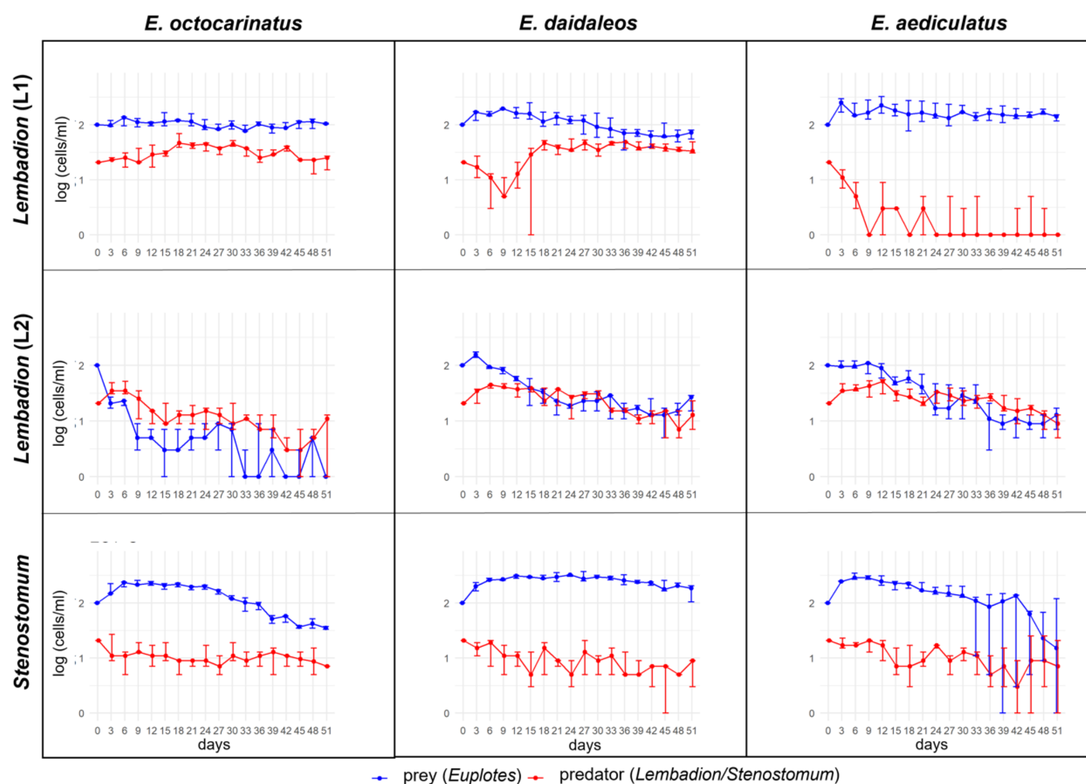

Figure 4: Monoclonal tritrophic experiments of three different *Euplotes* species with their predator *Lembadion* clone L1/L2 and the non-plastic predator *Stenostomum*. 100 individuals of one *Euplotes* species (*E. aediculatus*, *E. octocarinatus* or *E. daidaleos*) and 20 individuals of their predator *Lembadion* or *Stenostomum* (predator:prey ratio 1:5) were kept for 51 days in SMB medium. As a food source for *Euplotes* the algae *Chlorogonium elongatum* (OD_{454}= 0.1) was mixed into the system. Experiments were performed in 6-well-plates with a total volume of 10 ml SMB medium. Samples were taken every 3 days and cell numbers were determined via counting cells under the microscope. Incubation of individual *Euplotes* species with the small predator clone *Lembadion* L1, showed slight population fluctuations but no major abnormalities with the species *E. octocarinatus* and *E. daidaleos*. When the largest *Euplotes* species (E. *aediculatus*) is incubated with *Lembadion* L1*,* the predator got extinct. Experiments with the larger *Lembadion* clone L2, stronger fluctuations can be recognized in all three *Euplotes* species, whereas the smallest species (*E. octocarinatus*) almost got extinct. In comparison to the plastic predator *Lembadion* (clone L1 & L2) population dynamics of the smaller *Euplotes* species *E. octocarinatus* and *E.*

daidaleos show only weak oscillations. When providing *Stenostomum* with *E. aediculatus*, *Euplotes* almost died out.

When co-cultured with the larger and more plastic predator *Lembadion* L2, predator prey oscillations increased in amplitude. The small prey is heavily predated, and defenses do not protect from *Lembadion* L2 predation as population size is significantly reduced but, in our experiments, does not crash. In comparison to the plastic predator *Lembadion* (clone L1 and L2) *Euplotes* species also defend against the non-plastic predator *Stenostomum*. Population dynamics of the small *Euplotes* species EO1 and AS3 show only weak oscillations. When provided with large size prey *E. aediculatus* LV7, *Stenostomum* apparently foraged stronger and LV7 almost died out (fig. 4).

Publications

- Trogant, S., Becker, K., Schweinsberg, M., Ioannidou, I., Tollrian, R., Weiss, L.C., 2020. Simple morphology-based species identification in Euplotes spp. Fundam. Appl. Limnol. 193, 205–211.
- Bamberger V, Marks, A., Weiss, LC, Tollrian, R. (In prep.). Trait variability and phenotypic plasticity on one or two trophic levels affect population dynamics in a tritrophic system.
- Bamberger V, Tollrian, R., Weiss, LC, (In prep.). Monitoring ciliate population dynamics using genetic markers and cell number standard curves for quantitative PCR – a fast and efficient cell quantification system