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Community ecology

Dipteran larvae and microbes facilitate nutrient sequestration in the *Nepenthes gracilis* pitcher plant host

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The fluid-containing traps of *Nepenthes* carnivorous pitcher plants (Nepenthaceae) are often inhabited by organisms known as inquilines. Dipteran larvae are key components of such communities and are thought to facilitate pitcher nitrogen sequestration by converting prey protein into inorganic nitrogen, although this has never been demonstrated in *Nepenthes*. Pitcher fluids are also inhabited by microbes, although the relationship(s) between these and the plant is still unclear. In this study, we examined the hypothesis of digestive mutualism between *N. gracilis* pitchers and both dipteran larvae and fluid microbes. Using dipteran larvae, prey and fluid volumes mimicking *in situ* pitcher conditions, we conducted *in vitro* experiments and measured changes in available fluid nitrogen in response to dipteran larvae and microbe presence. We showed that the presence of dipteran larvae resulted in significantly higher and faster releases of ammonium and soluble protein into fluids in artificial pitchers, and that the presence of fluid microbes did likewise for ammonium. We showed also that niche segregation occurs between phorid and culicid larvae, with the former fragmenting prey carcasses and the latter suppressing fluid microbe levels. These results clarify the relationships between several key pitcher-dwelling organisms, and show that pitcher communities facilitate nutrient sequestration in their host.

1. Introduction

Carnivorous pitcher plants of the genus *Nepenthes* produce modified, water-holding leaf structures that capture and digest animal prey to supplement their nutrient requirements [1]. Some organisms have adapted to living in the aquatic habitats provided by pitchers, and may even be entirely dependent on *Nepenthes* for their survival [2]. Dipteran larvae are key components of such communities and are thought to facilitate pitcher nitrogen sequestration by converting insoluble, protease-inaccessible prey protein into inorganic nitrogen which is then absorbed by pitcher glands [3,4]. The idea of digestive mutualism is certainly not restricted to the genus *Nepenthes*, and has been demonstrated variously across many unrelated carnivorous plant taxa [5]. Dipteran larvae inhabiting pitchers of the functionally similar North American *Sarracenia purpurea* facilitate the conversion of protein into inorganic nitrogen which is then absorbed by the plant, providing an energetically efficient nitrogen sequestration mechanism for their host [6,7]. Scharmann *et al*. [8], however, suggested that the predatory ant-mutualist, *Camponotus schmitzi* suppresses populations of dipteran larvae which would otherwise deprive *N. bicalcarata* pitchers of a significant proportion of prey nitrogen. In balance, it is possible that the plant–dipteran mutualism becomes exploitative at higher larval densities or at low prey levels. Such conditional outcomes of mutualism are common [9], and have been shown in the

Table 1. Combinations of dipteran larvae, prey and fluid types used for the (*a*) digestion experiments, and (*b*) interaction experiments.

condition	dipteran larvae	prey	fluids
(*a*) *digestion experiments* ($n = 6$)			
PREY + DIP	3 × *E. schuitemakeri* + 10 × *Tripteroides* spp.	10 × *O. smaragdina*	coarse-filtered
PREY ONLY	none	10 × *O. smaragdina*	coarse-filtered
PREY + AMPICILLIN	none	10 × *O. smaragdina*	microbe-free
CONTROL	none	none	coarse-filtered
(*b*) *interaction experiments* ($n = 3$)			
CUL + PHOR	5 × *E. schuitemakeri* + 5 × *Tripteroides* spp.	10 × *O. smaragdina*	coarse-filtered
CULICIDS	10 × *Tripteroides* spp.	10 × *O. smaragdina*	coarse-filtered
PHORIDS	10 × *E. schuitemakeri*	10 × *O. smaragdina*	coarse-filtered
PREY ONLY	none	10 × *O. smaragdina*	coarse-filtered
CONTROL	none	none	coarse-filtered

sticky carnivorous plant, *Roridula dentata* and *Pameridea* sp. bugs [10]. With the exception of a unique bat–plant mutualism in *N. hemsleyana* [11], digestive mutualism remains largely unexamined in pitchers of *Nepenthes*.

Many pitcher inquilines feed on microbes inhabiting pitcher fluids [12], which are diverse and ubiquitous in pitchers [13]. The literature is however largely divided over the ecological interpretation of the plant–microbe relationship(s). Many researchers believe that microbes benefit the pitcher by assisting in prey decomposition and nitrogen sequestration [3,13], or even prevent prey escape by reducing the surface tension of pitcher fluid [14]. Although indirect evidence for the former are available for *Nepenthes* [15,16], these studies are not without criticism, and do not demonstrate unequivocally the benefit derived from microbial colonization of its pitchers. By contrast, a growing number of studies in more recent years have shown that pitcher secretions are anti-microbial, an observation that seems to point in the direction of an antagonistic relationship between plant and fluid microbes [17–19].

In this study, we examined the hypothesis of digestive mutualism between *N. gracilis* pitchers and both dipteran larvae and microbes. Using dipteran larvae, prey and fluid volumes mimicking *in situ N. gracilis* pitcher conditions, we conducted *in vitro* digestion experiments and measured changes in available fluid nitrogen in response to dipteran larvae and microbe presence. *In vitro* experiments eliminate the interference of pitchers from the system, preventing their absorption of fluid nutrients, suppression of microbe populations, or adjustment of hydrolytic enzyme levels.

2. Material and methods

(a) *In situ* observations

Observations were made at three different localities in Singapore at which *Nepenthes gracilis* are found growing naturally. The dipteran larvae of 91 randomly sampled *N. gracilis* pitchers were sorted and counted according to the methodology described in the electronic supplementary material: supplementary methods. *Endonepenthia schuitemakeri* (Phoridae) larvae occurred in 44 of these pitchers (48%), and culicid larvae in 73 (80%) (electronic supplementary material, figure S1). In general, culicid larvae were more abundant (mean: 5.3) than those of *E. schuitemakeri* (mean: 3.6).

(b) Digestion experiments

Digestion experiments were performed in 15 ml plastic Cellstar® centrifuge tubes (18 mm diameter; similar to the diameter of typical *N. gracilis* pitchers) in the laboratory. Prey, dipteran larvae and 3 ml aliquots of filtered pitcher fluids were transferred into each tube according to table 1*a*. Numbers of larvae selected for this experiment represented the 80th and 75th percentiles of culicid and *E. schuitemakeri in situ* abundances, respectively. These values were chosen so as to test the digestive mutualism hypothesis at a level where it is less likely to hold, while still well within the natural range of dipteran larva densities. Soluble protein, ammonium concentration and fluid turbidity were measured on the fourth, eighth and 12th day of the experiment. Methodological details are contained in the electronic supplementary material, supplementary methods and figures S2–S3).

(c) Interaction experiments

A further set of experiments was conducted to investigate potential differences in feeding activities and nitrogen metabolism rates between culicids and phorids. Experimental set-ups were similar to those of the digestion experiments, with dipteran larva and prey combinations varied according to table 1*b*. Ammonium and microbe concentrations were examined as had been done in the digestion experiments, on day 6 of the experiment.

3. Results

(a) Digestion experiments

Turbidity (ΔOD_{600}) increased with time in *PREY ONLY* set-ups, but not in *PREY + DIP* and *PREY + AMPICILLIN* set-ups, where filter-feeding *Tripteroides* larvae and antibiotic ampicillin largely suppressed microbe populations (figure 1*a*).

Ammonium concentrations (ΔNH_4) rose quickly in *PREY + DIP* set-ups, and fell only after day 8, when large proportions of both *E. schuitemakeri* and *Tripteroides* larvae had transitioned into metabolically inactive pupal stages (figure 1*b*).

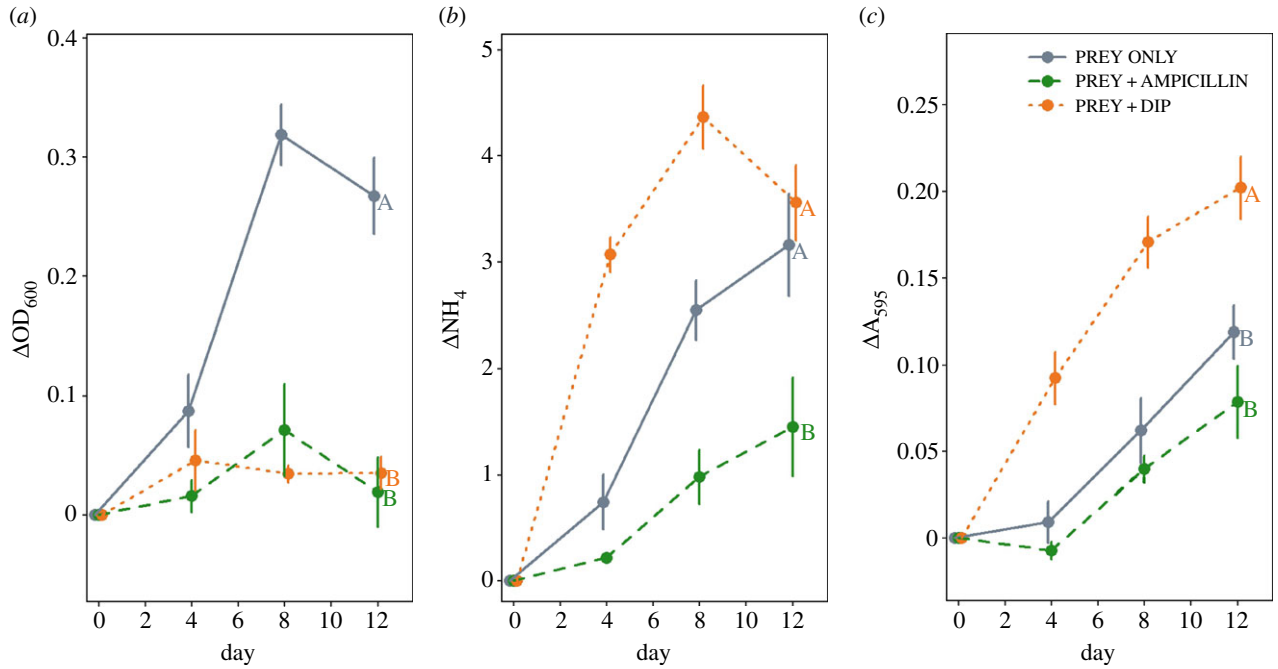

Figure 1. Changes in fluid chemistry in digestion experiment set-ups over time (day): (*a*) turbidity (ΔOD_{600}), a proxy for microbe concentration; (*b*) ammonium concentration (ΔNH_4); and (*c*) soluble protein (ΔA_{595}). Points with the same letter are not significantly different at $p < 0.05$ (Tukey's *post hoc* *t*-test). Data are represented by means ± s.e. (error bars). $n = 6$. (Online version in colour.)

Ammonium concentrations in *PREY ONLY* set-ups also increased steadily with time, although at a slower rate than in *PREY + DIP* set-ups. This increase was likely attributable to microbial activity, since *PREY + AMPICILLIN* set-ups yielded the lowest increments in ammonium.

Soluble protein (ΔA_{595}) accumulated in pitcher fluids with time in all treatments, perhaps due to the gradual release of oligopeptides by the action of fluid enzymes on insoluble prey proteins (figure 1*c*). This occurred fastest in the presence of dipteran larvae, with the most rapid protein accumulation taking place in the early phase of the experiment, possibly because *E. schuitemakeri* greatly fragmented prey carcasses while feeding, increasing the exposed surface areas and exoskeleton breakages in prey carcasses for the access of fluid proteolytic enzymes. Differences caused by microbial action, as compared between *PREY ONLY* and *PREY + AMPICILLIN* set-ups, were not statistically significant.

(b) Interaction experiments

Two-way ANOVA tests were significant for both ΔNH_4 ($F_{3,8} = 7.063$; p-value = 0.012) and ΔOD_{600} ($F_{3,8} = 17.87$, p-value < 0.001), hence they were followed by Tukey *post hoc* pairwise comparisons. ΔNH_4 in all three dipteran larva set-ups were significantly higher than *PREY ONLY* (figure 2). ΔOD_{600} was highest in *PREY ONLY*, and lowest in *CULICIDS* (figure 2).

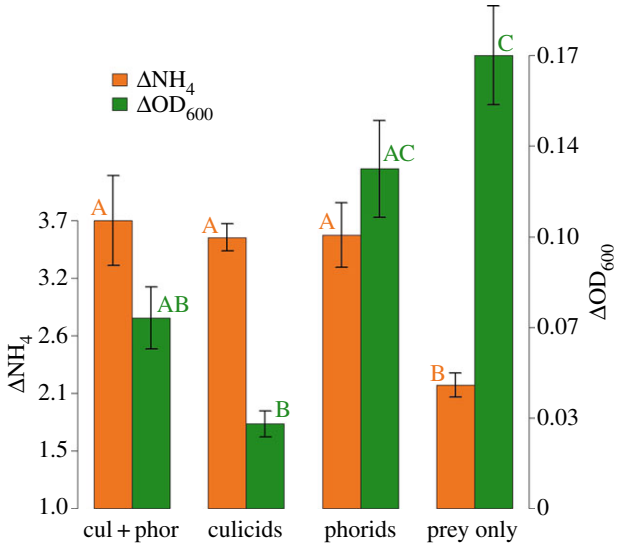

Figure 2. Changes in fluid ammonium concentration (ΔNH_4) (left vertical axis) and turbidity (ΔOD_{600}) (right vertical axis) in the four conditions tested in interaction experiments. Bars annotated with the same letter are not significantly different at $p < 0.05$ (Tukey's *post hoc* *t*-test). Data are represented by means ± s.e. (error bars). $n = 3$. (Online version in colour.)

4. Discussion

We showed that the presence of dipteran larvae resulted in significantly higher and faster releases of ammonium and soluble protein into the fluids of the artificial pitchers, and that the presence of fluid microbes did likewise for ammonium, suggesting that both dipteran larvae and microbes facilitate nutrient sequestration in *N. gracilis* pitchers. Our experiments suggest that a large proportion of prey nitrogen, at least from large-bodied prey species such as *O. smaragdina*, can remain locked away from pitchers in the absence of dipteran larvae or microbes.

While it is possible that the plant–dipteran larva mutualism breaks down at higher larval densities, we demonstrated here using the 75–80th percentile of *in situ* dipteran larva numbers that the facilitative relationship between *N. gracilis* and its most common inhabitants is likely to hold most of the time for the plant–dipteran larva relationship. It might also appear that dipteran larva-assisted digestion results in the conversion of prey protein into ammonium at the expense of an organic-nitrogen pathway (as suggested by Karagatzides *et al.* [7] in *Sarracenia purpurea*). But we found no evidence for this in

N. gracilis, showing that fluid protein levels were even higher in the presence of dipteran larvae, than in their absence.

We also found evidence for niche segregation between culicid and phorid larvae. Larvae of the phorid, *E. schuitemakeri* were observed to fragment prey carcasses extensively while feeding on them, but they did not appear to consume fluid microbes (figure 2). *Tripteroides* larvae, on the other hand, appeared only to graze on the surfaces of prey carcasses, but strongly suppressed fluid microbe levels (figure 2). The messy feeding habit of *E. schuitemakeri* may make resources available to *Tripteroides* larvae and other pitcher inhabitants in more readily accessible forms. Such a relationship would be analogous to that of the larvae of the midge, *Metriocnemus knabi* and mosquito, *Wyeomyia smithii* in *S. purpurea*—a relationship known as processing-chain commensalism [20].

From the perspective of nitrogen sequestration, it appears that the net benefit of microbe proliferation in pitcher fluids is also beneficial to *N. gracilis*. However, our study could not assess the potentially negative by-products of fluid microbial proliferation, such as pathogenic infection of plant tissue. We suggest that future studies investigate the taxon-specificity of antimicrobial pitcher secretions, and clarify the trade-offs between microbe-facilitated nutrient sequestration and the potentially harmful side effects of fluid microbe proliferation.

Ethics. Our work complies with the laws of the Republic of Singapore, which does not regulate the use of invertebrate animals in research. Fieldwork was carried out under the National Parks Board Permit No. NP/RP13-008-2.

Data accessibility. Data supporting this article are available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.j7d50 [21].

Authors' contributions. W.N.L., H.T.W.T. and G.S.A. conceived the study. W.N.L. carried out the fieldwork and experiments. W.N.L. and K.Y.C. analysed the data. W.N.L. wrote the first manuscript draft and all authors contributed to revisions. All authors approved of the final version of the manuscript, and agreed to be held accountable for all aspects of the work presented here.

Competing interests. We declare we have no competing interests.

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