

ON THE REGULATION OF DISSOLVED OXYGEN BY *NEPENTHES* PITCHERS

KADEEM J. GILBERT • Museum of Comparative Zoology • Harvard University • Cambridge  
• Massachusetts • USA • and • Department of Entomology • Pennsylvania State University  
• State College • Pennsylvania • USA • kjpg5649@psu.edu

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Abstract: The digestive fluid of tropical pitcher plants (Nepenthaceae: *Nepenthes* L.) typically harbor communities of symbiotic organisms in nature, an example of a phytotelm ecosystem. While the inhabitants of pitchers (primarily the arthropods) have been described and studied from a community ecology perspective for over a century, many abiotic properties of the fluid environment have not been thoroughly documented. As the abiotic properties of the pitcher fluid micro-environment may have fitness and/or functional consequences on the inhabitants, it is of interest to examine intra- and interspecific variation in fluid properties. Here I report observations of dissolved oxygen levels in *Nepenthes* pitchers. I find evidence that *Nepenthes* pitchers actively modify dissolved oxygen levels in the fluid and that this oxygenation ability may vary ontogenetically, with relatively hypoxic conditions in newly opened pitchers and high oxygen levels maintained in more mature pitchers.

Introduction

Most phytotelmata (plant-held aquatic microhabitats) are ephemeral resources prone to desiccation and hypoxia (Kitching 2001). Communities of heterotrophic organisms living within water-filled tree holes or bromeliad tanks, for example, can easily deplete the oxygen dissolved in the water unless photosynthetic algae are also present, as the host plant tissues lack the ability to actively maintain the dissolved oxygen (DO) of their phytotelm (Laessle 1961). Pitcher plants, on the other hand, can actively maintain DO. Past studies demonstrated that *Sarracenia* pitchers normally maintain high DO even with communities dominated by heterotrophic inquilines (Cameron *et al.* 1977; Bradshaw 1983). *Sarracenia* pitchers contain chloroplasts in the epidermal cells of the pitcher interior, which is unusual as this is non-stomatal tissue that would typically lack chloroplasts in terrestrial plant species (Joel & Gepstein 1985). Bradshaw (1983) demonstrated that *Sarracenia* (Sarraceniaceae) pitchers assimilate dissolved CO<sub>2</sub> in the presence of light while maintaining stable DO without much regard to light. The oxygenation of pitcher fluid by the host plant allows the survival of the highly oxygen-sensitive *Metriocnemus knabi* (Cameron *et al.* 1977), an obligately associated chironomid midge, which benefits the host *Sarracenia purpurea* by aiding in prey digestion (Heard 1994). Thus, fluid oxygenation seems to have an important role in enabling this mutualistic association. On the other hand, dissolved oxygen levels in the pitchers of the convergently evolved Old World tropical pitcher plants (Nepenthaceae: *Nepenthes*) have not been described in published reports, to my knowledge. Studies on the photosynthetic efficiency of *Nepenthes* pitchers and laminae for four species (*N. talangensis*, *N. alata*, *N. mirabilis*, and *N. ventricosa*) showed that pitchers exhibit relatively low net assimilation rates (Pavlovič *et al.* 2007, 2009; Adamec 2010). That the functions of digestion and photosynthesis are carried out by two distinct organs in *Nepenthes* as opposed to the dual role of the pitchers of the Sarraceniaceae presents an interesting complication when trying to draw parallels between what was shown for *Sarracenia* and what may be the case in

*Nepenthes*. Relative to *Sarracenia*, *Nepenthes* pitchers do not have as much photosynthetic tissue, but there are increased chloroplasts in the digestive zone, surrounding the glands (Pavlovič *et al.* 2007). Thus, I sought to determine whether *Nepenthes* pitchers oxygenate their fluid as has been shown for *Sarracenia*. I was interested in whether there might be species and/or ontogenetic differences in pitcher fluid oxygenation. Here I present my observations of oxygen levels in *Nepenthes* pitchers from various natural and experimental contexts.

### Materials and Methods

In July-September 2013, I conducted exploratory investigations of oxygenation in *Nepenthes* using 3 small potted individuals each of *N. maxima*, *N. albomarginata*, and *N. ampullaria* (Black Jungle Terrarium Supply), reared in a growth chamber in Cambridge, Massachusetts (12 hour day/night cycle, 80% relative humidity, 30°C). To examine whether pitchers can manipulate oxygen levels in the absence of inquiline communities, I added distilled deionized water (ddH<sub>2</sub>O) to pitchers which I had emptied and washed out (removing any prey items). Drawing up 1 mL water samples into a Luer-Lok syringe, carefully so as to prevent agitation and aeration of the sample, I then measured DO concentration in the samples using the Shriwastav *et al.* (2010) micro-Winkler titration method (110 samples). The Shriwastav *et al.* (2010) method is a modification of a standard technique for measuring DO in limnological studies, which was adapted for measuring small samples. The sample is kept under a layer of n-hexane before fixing in order to prevent atmospheric O<sub>2</sub> contamination. The sample is fixed with 5 µL each of MnSO<sub>4</sub> and alkali-azide-iodate solution; next the resulting precipitate is dissolved with 0.1 mL H<sub>2</sub>SO<sub>4</sub>, and then finally the resulting iodine solution is stained with starch indicator and titrated with sodium thiosulfate until clear. I also measured oxygen levels from ddH<sub>2</sub>O in glass containers with this method (22 samples) and also measured DO in the endogenous fluid from pitchers that had just opened one or two days prior (7 samples). For the majority of measurements in this experiment, I collected and fixed the sample for titration within 24 hours of adding the ddH<sub>2</sub>O, but in a few cases I measured DO in pitchers/glass containers after letting the ddH<sub>2</sub>O sit for ~1 week (10 samples, including 2 from glass containers) or ~1 month (14 samples, 3 from glass) after its addition. For statistical analysis, I conducted linear mixed models in R 3.5.0 (RCoreTeam 2013) using the 'lmer' function in the 'lme4' package (Bates *et al.* 2015) and including plant/pitcher ID as a random effect to account for multiple sampling and correct for pseudoreplication.

In January 2014, I took opportunistic measurements of dissolved oxygen levels in unopened pitchers of wild *Nepenthes gracilis* in Kent Ridge Park, Singapore using the Shriwastav *et al.* (2010) micro-Winkler titration method. I sampled fluid from 10 unopened pitchers, but as many of these had fluid volumes less than 1 mL, I had to pool them, which resulted in 5 samples for titration.

In August 2014, I measured dissolved oxygen concentrations in *Nepenthes* pitchers in a Singapore horticultural glasshouse (HortPark *Nepenthes* nursery; plants sourced from Borneo Exotics Ltd.) with a well-regulated indoor environment (16°C temperature, 80% relative humidity). I used a fiber-optic probe and spectrometer-based sensing system (Ocean Optics, Inc., Dunedin, Florida, USA). The probe was calibrated on-site using saturated and deoxygenated ddH<sub>2</sub>O standards. Pitchers were covered with a dark opaque fabric during measurements to mitigate noisy measurements that could occur due to ambient light. I recorded point measurements taken from the middle of the fluid column; I made sure to keep the probe in the same relative position for each measurement as I had observed possible differences in oxygen level depending on the depth of the probe within individual pitchers.

In February 2016, I collected fluid from several wild *N. gracilis* in Kent Ridge Park in Singapore, homogenized and filtered that fluid in a sterile bottle-top vacuum filter, and added 1.0 mL allotments of this stock fluid to open glass vials (with similar dimensions to *N. gracilis* pitchers) and healthy mature pitchers from potted *N. gracilis* plants that I had emptied out and washed beforehand. Each vial or pitcher also received an equal-sized mealworm segment and each container type was equally divided into four treatments: one mosquito (Diptera: Culicidae, either *Culex* or *Tripteroides*) larva added, one *Endonepenthia* (Diptera: Phoridae) larva added, a combination of one mosquito and one *Endonepenthia* larva added, and no larvae added. I measured oxygen levels with an oxygen optrode (Ocean Optics) after about 18 hours. This experiment was conducted in a greenhouse at the Raffles Institution, with the original goal of observing the effects of inquilines on pitcher ammonia levels, but this experiment incidentally worked well for studying DO.

## Results and Discussion

My observations from the *N. maxima*, *N. albomarginata*, and *N. ampullaria* in the growth chamber provide some insight into the possibility that dissolved oxygen in pitchers is influenced by plant physiology, which is seen primarily in age-dependent differences in DO levels (Fig. 1). Pitcher age/condition has a significant effect on the DO of the experimentally added ddH<sub>2</sub>O, specifically, in reference to mature pitchers, newly opened pitchers tend to have lower DO (correlation estimate =

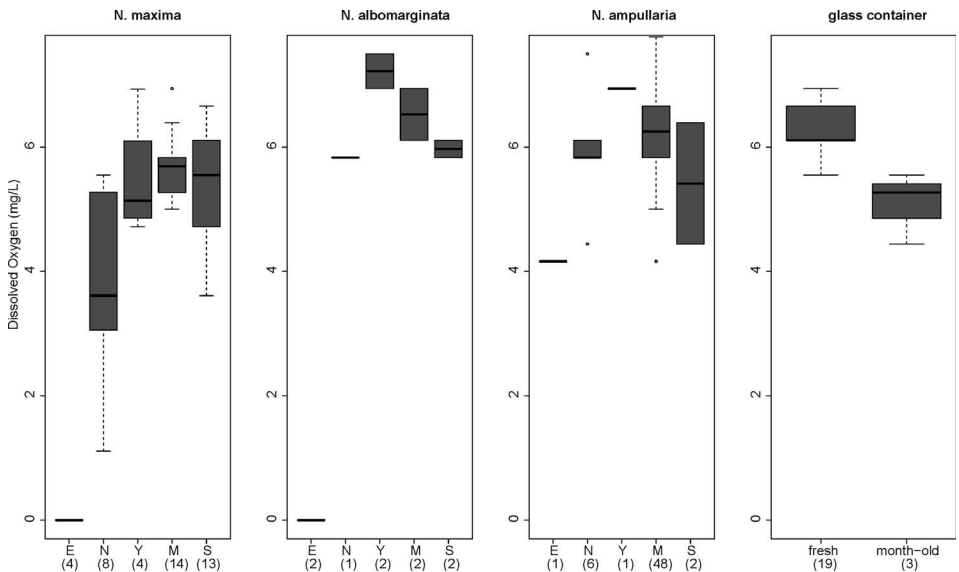


Figure 1: Results of 2013 growth chamber study, dissolved oxygen levels for the three *Nepenthes* species are broken down by pitcher age/condition, where E = endogenously-produced fluid in newly-opened (1-2 days post-opening) pitchers, N = “newly-opened” (<1 week post-opening) pitchers, Y = “young” (~2 weeks post-opening), M = “mature” (>2 weeks post-opening) pitchers, and S = “senescent” (brown/dying/dead) pitchers. For glass containers, “fresh” refers to ddH<sub>2</sub>O DO measured less than one week after addition, and “month-old” refers to ddH<sub>2</sub>O DO measured about one month after addition. Sample sizes for each pitcher condition or treatment is given underneath in parentheses.

-1.14,  $p = 0.0006$ ), and likewise the endogenous fluid of newly opened pitchers has exceptionally low DO (correlation estimate = -5.41,  $p \ll 0.0001$ ); senescent (browning/dead) pitchers have a slight, nonsignificant trend towards lower DO (correlation estimate = -0.53,  $p = 0.08$ ), and young pitchers are not significantly different (correlation estimate = 0.20,  $p = 0.62$ ). On the other hand, variation in DO levels cannot be explained by letting the water sit for different periods of time ( $p = 0.47$  and  $0.85$  for week-long and month-long waiting period, respectively). The observations within these alternative waiting periods still vary according to the pitcher condition: for example, of the week-long waiting period samples, the DO of the 3 mature pitchers average 6.29 mg/L while DO of the 2 newly opened pitchers average 3.47 mg/L. Note that fully saturated water (i.e. water with 21% O<sub>2</sub>) would hold an estimated 7.55 mg/L at 30°C or 8.26 mg/L at room temperature.

For glass containers, while not statistically significant (two sample t-test,  $t = 3.33$ ,  $p = 0.06$ ), month-old ddH<sub>2</sub>O samples clearly reach lower DO levels than those taken from more freshly added ddH<sub>2</sub>O examined no longer than a week after addition. The fresh ddH<sub>2</sub>O in glass containers serves as a baseline for unmanipulated ddH<sub>2</sub>O DO levels and the observations of month-old ddH<sub>2</sub>O in glass shows that it takes at least a month for neutral processes (e.g. evaporation, microbial respiration) to drive down the DO in this context, and even then, it did not reach any lower than 4.44 mg/L. Thus, it is especially surprising that in less than 24 hours, ddH<sub>2</sub>O added to certain pitchers can reach much lower levels than that (as low as 4.27 and 1.11 mg/L in senescent and new pitchers, respectively).

What I had expected prior to this study was that living pitchers would oxygenate their fluid and have higher DO than senescent pitchers, but what I found instead is that the youngest pitchers seem capable of rapidly deoxygenating their fluid, while older pitchers (whether healthy or senescent) stay within the normal DO levels seen in the glass controls during the short waiting period scheme. As pitchers in this experimental design were void of any insect inquilines and likely void of substantial microbial communities that could drive down DO with respiration, it is not possible to discern from this whether fluid oxygenation switches on in young/mature pitchers and then turns off in senescent ones—of note however, the sole month-old ddH<sub>2</sub>O sample from a senescent pitcher reached the markedly low DO level of 3.61 mg/L while the month-old samples from healthy mature pitchers averaged 5.97 mg/L. While not conclusive due to small sample sizes, this is consistent with senescent pitchers acting more like inert containers and healthy pitchers actively maintaining higher DO levels over time. More conclusive here is the occurrence of active deoxygenation in newly opened pitchers. The pattern is best seen in *N. maxima*, which had the most even sampling, but the pattern of relatively lower DO in new pitchers can still be seen in the other two species (Fig. 1).

The most striking finding, alongside the hypoxic conditions in newly opened pitchers, is that the endogenous fluid within newly opened pitchers appears to be anoxic (0 mg/L) in all observations from *N. maxima* (4 samples) and *N. albomarginata* (2 samples). My one observation of endogenous fluid from a newly opened pitcher of *N. ampullaria* still followed the pattern of endogenous fluid having the relatively lowest DO within a species, but in this case, it was merely hypoxic at 4.16 mg/L.

Overall, DO did not vary significantly by species in this study (linear mixed model,  $p > 0.4$  for each species). However, when comparing just the mature pitchers of *N. ampullaria* and *N. maxima* (no significant differences between either species and *N. albomarginata*, which has a very low sample size), *N. ampullaria* is found to have significantly higher mean DO here (t-test,  $t = 3.06$ ,  $p = 0.005$ ): 6.26 mg/L compared to *N. maxima*'s 5.69 mg/L. While I cannot firmly conclude from this that pitcher oxygenation is a trait that varies interspecifically across the genus, it could make sense for species that evolved specialized dietary strategies like *N. ampullaria* (Moran *et al.* 2003) to regulate their fluid properties in different ways. For instance, *N. ampullaria* is known to maintain milder pH levels compared to other species, which is believed to create a more permissive environ-

ment for the diverse community of insect inquilines that may help break down captured leaf litter (Moran *et al.* 2010). Similarly, maintaining oxygen-rich fluid could help sustain more inquilines. Perhaps the higher DO in the endogenous pitcher fluid of newly opened *N. ampullaria* compared to the anoxic fluids of the other two species indicates that *N. ampullaria* oxygenates more at all stages (as a caveat: it was more difficult to catch the exact moment of pitcher opening due to the uniquely narrow lid of this species, so that pitcher may have been open relatively longer before sampling than the other species).

Looking further into the potential of interspecific variation in pitcher oxygenation, I obtained data from 12 *Nepenthes* species and hybrids in the HortPark glasshouse in 2014: *N. chaniana* × *veitchii*, *N. clipeata* × *eymae*, *N. copelandii*, *N. densiflora*, *N. fusca*, *N. inermis* × *bongso*, *N. jamban*, *N. khasiana*, *N. muluensis* × *lowii*, *N. sanguinea*, *N. spectabilis* × *talangensis*, and *N. tentaculata*—though sample sizes within each species were generally low (Fig. 2). I did not find significant differences in dissolved oxygen concentrations by species (Kruskal-Wallis chi-squared = 14.124,  $p = 0.23$ ), but there was notable variation across all pitchers, ranging from ~5% to ~30% oxygen (where atmospheric oxygen in the greenhouse was 21%).

I noticed a possible trend of upper pitchers having smaller DO values than lower pitchers (Fig. 3). Most species did not have multiple pitcher morphs available, and in most cases, there either was not a significant difference or enough replication to be meaningful. However, I obtained several

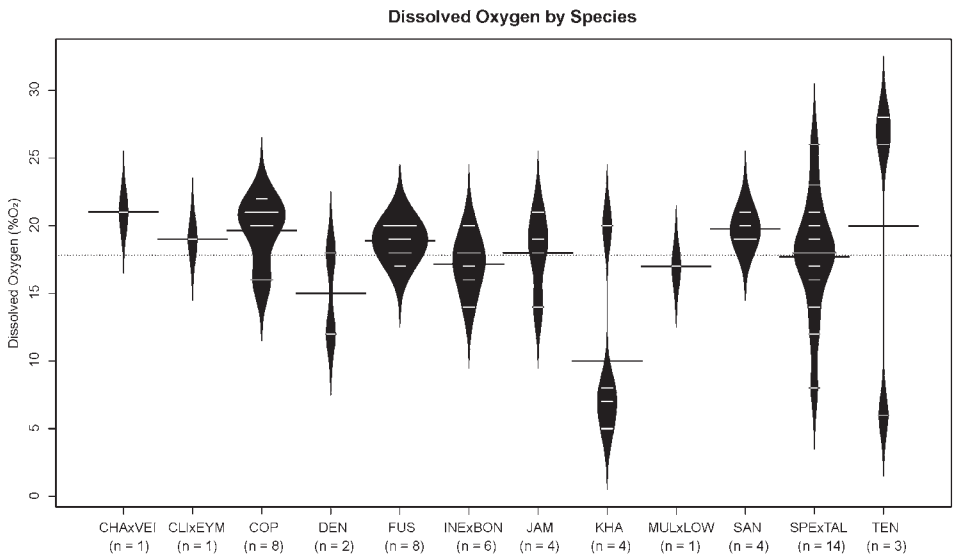


Figure 2: Beanplot showing results of oxygen measurements in 12 *Nepenthes* species and hybrids in the HortPark glasshouse. CHAxVEI = *N. chaniana* × *veitchii*, CLIXEYM = *N. clipeata* × *eymae*, COP = *N. copelandii*, DEN = *N. densiflora*, FUS = *N. fusca*, INEXBON = *N. inermis* × *bongso*, JAM = *N. jamban*, KHA = *N. khasiana*, MULxLOW = *N. muluensis* × *lowii*, SAN = *N. sanguinea*, SPEXTAL = *N. spectabilis* × *talangensis*, TEN = *N. tentaculata*. Please note that names are based on 2014 taxonomic classifications (e.g. *N. fusca* is likely now *N. dactylifera*). Sample sizes for each species given below in parentheses. In the beanplot, thin white lines represent individual samples (length proportional to number of samples at a given DO) and thick black bar represents mean DO for that species. Dotted line indicates overall mean.

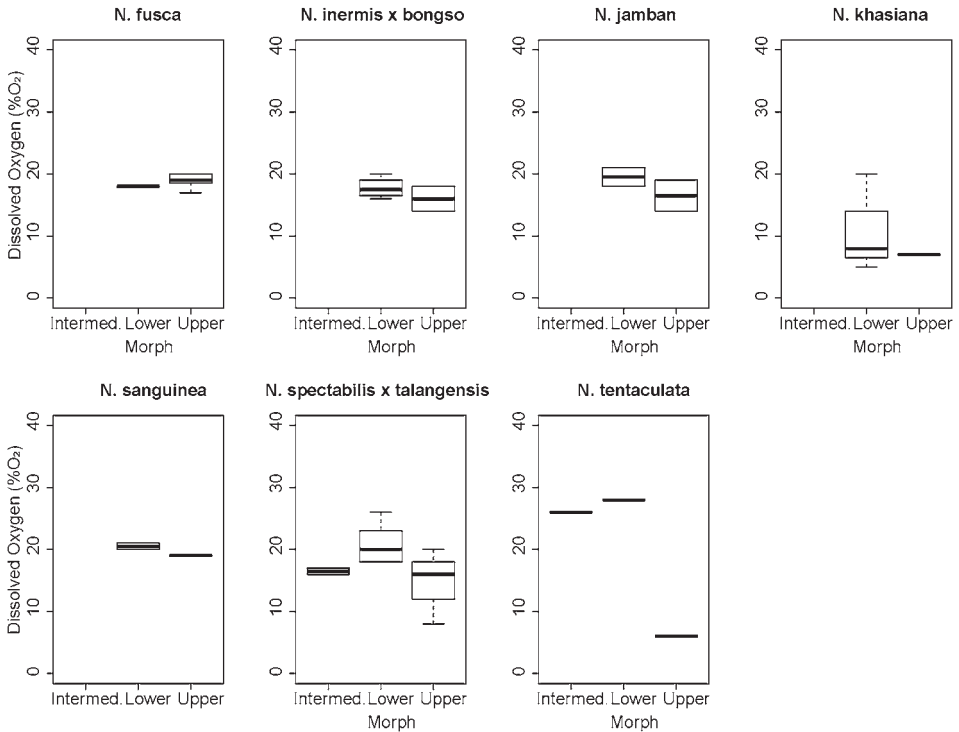


Figure 3: Boxplots of data from the 2014 HortPark glasshouse study, broken down by pitcher morph (upper, lower, or intermediate) for applicable species/hybrids.

datapoints for multiple *N. spectabilis* × *talangensis* plants containing upper, lower, and intermediate pitcher morphs, and the difference in means between upper and lower pitchers was significant in this case (t-test,  $t = -2.58$ ,  $p = 0.03$ ). While I only had one sample of each morph from *N. tentaculata*, the same trend can be found in that species as well (Fig. 3)—in both cases, lower pitchers have greater DO levels than upper pitchers, and the intermediate morphs notably fall somewhere in between. Note that most of the points that fall below 15% in Figure 2 are from upper pitchers (except one from *N. densiflora* and two from *N. khasiana*). Upper pitchers are necessarily younger than lower pitchers on the same plant, so I believe this trend may again indicate that more newly opened pitchers decrease their fluid DO.

Measuring DO in fluid from unopened pitchers of wild *N. gracilis* provided further support for the idea that younger pitchers normally have decreased DO levels. All five fluid samples were fairly hypoxic: 2.88 mg/L, 4.33 mg/L, 4.33 mg/L, 3.11 mg/L, and 3.77 mg/L, for a mean of 3.68 mg/L. This unopened *N. gracilis* fluid was not anoxic like I had previously observed in newly-opened *N. maxima* and *N. albomarginata* in the growth chamber, but still comparably hypoxic to the newly-opened *N. ampullaria*. Perhaps *Nepenthes* pitcher fluid in unopened/newly-opened pitchers is generally hypoxic, but different species vary in their minimum levels.

A possible mechanism that can explain how pitchers in their earliest stages of development deoxygenate their fluid is due to new pitchers releasing large amounts of gaseous carbon dioxide (Baby *et al.* 2017), this would flush oxygen out of the fluid; this process may cease as the pitchers mature, possibly giving way to increased input of oxygen from photosynthesis.

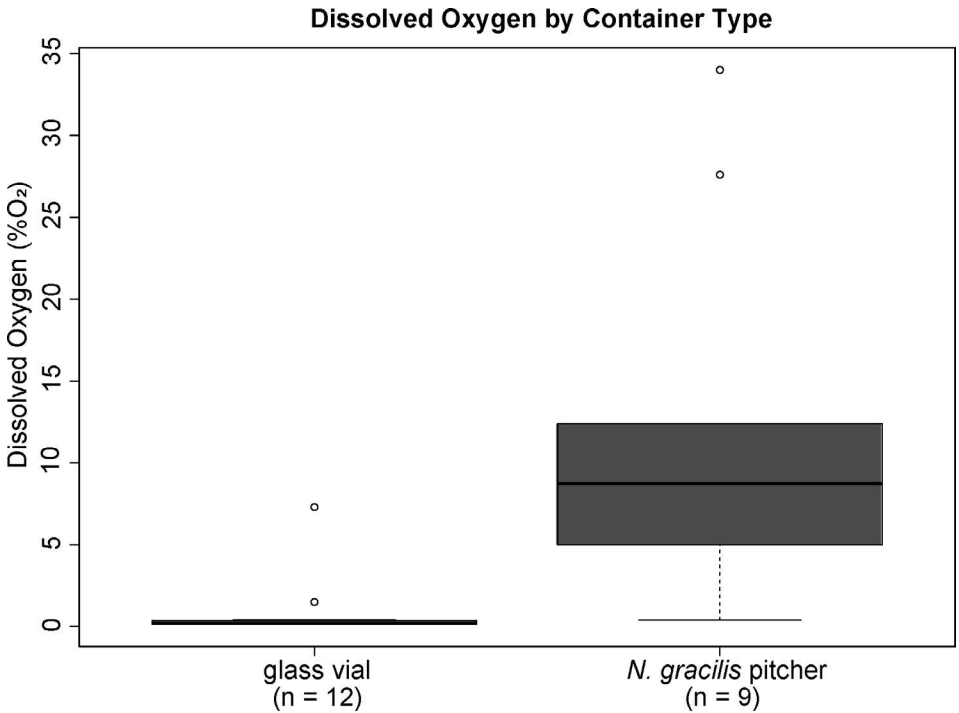


Figure 4: Results of the 2016 Raffles Institute greenhouse experiment, dissolved oxygen levels of pitcher fluid in glass vials and pitchers 18 hours after adding mealworm segment and insect inquiline treatments.

My most direct evidence of active oxygenation in healthy mature pitchers comes from the 2016 greenhouse experiment, where I had natural pitcher fluids with prey and inquilines (Fig. 4). I found significantly higher DO levels in pitchers than in glass vials (Kruskal-Wallis chi-squared = 12.16,  $p = 0.0005$ ) showing both how quickly inquiline respiration can lead to severely hypoxic pitcher fluid in physiologically inactive containers (final  $O_2$  levels falling mostly between 0.11% and 0.42%, where atmospheric oxygen in the greenhouse was 22.8%), and suggesting that the plants keep fluid oxygenated with photosynthesis that outpaces inquiline respiration. There were no significant differences in DO levels between the different insect inquiline treatments (Kruskal-Wallis test,  $p > 0.05$ ), suggesting that the bulk of the oxygen consumption that occurred was due more to microbial respiration rather than insect respiration (though evaporation may also have played some role in oxygen depletion).

The near-anoxic conditions in the glass vials were sufficient to lead to death of the larvae, so pitcher oxygenation likely has fitness consequences for inquilines in the wild, allowing larvae to occupy fluids with substantial microbial populations that would be otherwise uninhabitable if not for oxygenation by the host plant. On the other hand, while hypoxic conditions in pitchers prior to opening may not be of consequence to inquilines when entry is impossible anyway, the persistent removal of oxygen that continues during the first few days after opening would be consequential to pitcher inhabitants. Perhaps these hypoxic conditions might benefit the plant by discouraging insect colonization too early on. Larval inquilines can act either as mutualists or parasites (Scharmann *et al.* 2013; Leong *et al.* 2018, 2019); perhaps larvae can be more beneficial when pitchers are mature



and full of prey, but when the pitcher is still new with limited prey, a growing larva could more easily steal more nutrients than it releases.

## Conclusions

*Nepenthes* appear to be capable of actively oxygenating the fluid within their pitchers, analogous to what was already known for *Sarracenia*. Unexpectedly, I also found that unopened pitchers and pitchers in the earliest stages post-opening may actively decrease their DO levels (reaching total anoxia, at least in *N. maxima* and *N. albormarginata*). Oxygen regulation possibly varies by species, but the difference between pitchers of different ages seems more pronounced than interspecific differences. Understanding how fluid properties like DO are regulated by the plants has implications for understanding how the plant interacts with its symbionts (Adlassnig *et al.* 2011; Bittleston 2018; Gaume *et al.* 2019; Gilbert *et al.* 2020). Interspecific differences in DO regulation could very well contribute to the ability of pitchers to act as filters to shape what insects and microbes live within them.

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