

RESEARCH PAPER

Ion fluxes across the pitcher walls of three Bornean *Nepenthes* pitcher plant species: flux rates and gland distribution patterns reflect nitrogen sequestration strategies

Jonathan A. Moran^{1,*}, Barbara J. Hawkins², Brent E. Gowen³ and Samantha L. Robbins²

¹ School of Environment and Sustainability, Royal Roads University, 2005 Sooke Road, Victoria, BC, V9B 5Y2 Canada

² Centre for Forest Biology, University of Victoria, PO Box 3020, STN CSC, Victoria, BC, V8W 3N5 Canada

³ Department of Biology, University of Victoria, PO Box 3020, STN CSC, Victoria, BC, V8W 3N5 Canada

* To whom correspondence should be addressed: E-mail: jonathan.moran@royalroads.ca

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Abstract

Nepenthes pitcher plant species differ in their prey capture strategies, prey capture rates, and pitcher longevity. In this study, it is investigated whether or not interspecific differences in nutrient sequestration strategy are reflected in the physiology and microstructure of the pitchers themselves. Using a non-invasive technique (MIFE), ion fluxes in pitchers of *Nepenthes ampullaria* Jack, *Nepenthes bicalcarata* Hook.f., and *Nepenthes rafflesiana* Jack were measured. Scanning electron microscopy was also used to characterize the distribution of glandular and other structures on the inner pitcher walls. The results demonstrate that nutrient sequestration strategy is indeed mirrored in pitcher physiology and microstructure. Species producing long-lived pitchers with low prey capture rates (*N. ampullaria*, *N. bicalcarata*) showed lower rates of NH_4^+ uptake than *N. rafflesiana*, a species producing short-lived pitchers with high capture rates. Crucially, species dependent upon aquatic commensals (*N. ampullaria*, *N. bicalcarata*) actively manipulated H^+ fluxes to maintain less acid pitcher fluid than found in 'typical' species; in addition, these species lacked the lunate cells and epicuticular waxes characteristic of 'typical' insectivorous congeners. An unexpected finding was that ion fluxes occurred in the wax-covered, non-glandular zones in *N. rafflesiana*. The only candidates for active transport of aqueous ions in these zones appear to be the epidermal cells lying beneath the lunate cells, as these are the only sites not visibly coated with epicuticular waxes.

Key words: Digestive glands, H^+ , ion flux, MIFE, *Nepenthes*, NH_4^+ , phytotelm, pitcher plant, scanning electron microscopy.

Introduction

Carnivorous pitcher plants of the palaeotropical genus *Nepenthes* (Nepenthaceae) use fluid-filled traps to sequester nutrients from animal prey (Adamec, 1997; Schulze *et al.*, 1997; Moran and Moran, 1998; Pavlovič *et al.*, 2009). A variety of digestive enzymes are secreted by *Nepenthes* pitchers, including peptidase, aspartic and cysteine proteases, ribonuclease, chitinase, phosphatase, and esterase (Jentsch, 1972; Heslop-Harrison, 1975; An *et al.*, 2002a, b; Athauda *et al.*, 2004; Takahashi *et al.*, 2005; Eilenberg *et al.*, 2006; Plachno *et al.*, 2006; Stephenson and Hogan, 2006; Hatano and Hamada, 2008; Thornhill *et al.*, 2008). Secretion of free radicals into the pitcher fluid may also

facilitate digestion (Chia *et al.*, 2004). Nitrogen (N) from digested prey is transported actively across the *Nepenthes* pitcher wall, and transporters have been identified for NH_4^+ , amino acids and peptides (Schulze *et al.*, 1999; Rischer *et al.*, 2002); conversely, protons (H^+) may be pumped into the pitcher fluid, to optimize pH for enzymatic degradation of prey material (Higashi *et al.*, 1993; An *et al.*, 2001).

The typical *Nepenthes* pitcher shows distinct zonation, with three functional zones (Hooker, 1875; Adams and Smith, 1977; Owen and Lennon, 1999). The 'attractive zone' consists of the pitcher lid and the peristome, a collar-like structure surrounding and overhanging the pitcher mouth.

This zone is rich in nectaries, serving to attract and retain prey. Colour patterns and scent in this zone may also aid in attraction of anthophilous insects (Moran, 1996; Moran *et al.*, 1999; DiGiusto *et al.*, 2008; Bauer *et al.*, 2009). The peristome itself is a wettable, anisotropic structure: when wetted, it becomes slippery, causing invertebrate visitors to lose traction and fall into the pitcher (Bonn and Federle, 2004; Bauer *et al.*, 2008).

The 'conductive zone' comprises the upper inside surface of the pitcher and is often characterized by lunate cells and epicuticular waxes (Juniper and Burras, 1962; Pant and Bhatnagar, 1977; Gaume *et al.*, 2002, 2004; Gorb and Gorb, 2006), both of which function to deny traction and conduct the prey downwards under gravity into the third, or 'digestive' zone. This encompasses the fluid-filled base of the pitcher, the inner walls of which are lined with digestive glands (Owen *et al.*, 1999; Gorb *et al.*, 2004; Plachno *et al.*, 2006; Thornhill *et al.*, 2008). These glands undergo an ontogenetic shift in function: in immature pitchers, they secrete the pitcher fluid, but once the pitcher matures, secretion ceases and the function switches to the absorption of digestion products (Owen and Lennon, 1999; Owen *et al.*, 1999). The pitcher fluid itself possesses viscoelastic qualities, which in species such as *N. rafflesiana*, may contribute to prey retention to a greater degree than does the waxy zone (Gaume and Forterre, 2007; DiGiusto *et al.*, 2008; Gaume and DiGiusto, 2010).

Despite recent research undertaken into *Nepenthes* pitcher function, there have been no direct measurements of ion fluxes across the pitcher wall to date. The MIFE ion-selective electrode technique has been used successfully to measure ion fluxes in plant roots, as well as in bacteria and protists (Cuin *et al.*, 2008; Hawkins *et al.*, 2008; Shabala *et al.*, 2009a, b). In this study, its use has been extended to investigate ion fluxes in the trap of a carnivorous plant. The MIFE technique was used to measure net fluxes of NH_4^+ and H^+ across the pitcher walls of three *Nepenthes* species. These differ in their trapping ecology, rates of prey capture, pitcher morphology and longevity (see Discussion). The first aim of the current study was to determine whether or not interspecific differences in ecology are mirrored by differences in nutritional physiology, as quantified by ion fluxes. The second aim was to determine whether or not the microstructure of the inner pitcher wall could also be related to the nutrient sequestration strategy.

Materials and methods

Plant culture and pitcher preparation

Tissue-cultured *Nepenthes* specimens were obtained commercially (Hawaiian Botanicals, Richmond, BC, Canada). Three Bornean species were used: *Nepenthes ampullaria* Jack; *Nepenthes bicalcarata* Hook.f.; and *Nepenthes rafflesiana* Jack. Plants were grown in a terrarium at 25–30 °C and 70% relative humidity under a 12/12 light/dark cycle ($600 \mu\text{mol m}^{-2} \text{s}^{-1}$). A fully-formed pitcher (terrestrial form: see Moran, 1996) was gently removed from the plant, emptied of liquid and sliced longitudinally into sections. The section including the 'winged' portion of the pitcher was discarded,

and the remainder divided into four roughly equal sections. These were tied with sewing thread onto a Perspex® holder, which was placed vertically in a test tube filled with 95 ml aerated measurement solution (500 μM NH_4NO_3 and 200 μM $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$, adjusted to pH 4.0 with 2 M HCl), and positioned under $300 \mu\text{mol m}^{-2} \text{s}^{-1}$ irradiance at 20 °C for a minimum of 30 min. Immediately prior to ion flux measurements, the holder with a pitcher section was removed from the test tube and placed horizontally in a Perspex® container filled with 40 ml fresh measurement solution. Sections remained in the container for at least 5 min before the measurement of net ion fluxes began.

Measurement of ion fluxes

Ion flux measurements were made on the inner surface of pitchers of each species ($n=3$ per species). Fluxes of H^+ and NH_4^+ were measured using a non-invasive microelectrode ion flux measurement system (MIFE, Unitas Consulting, Hobart, Australia), as described in Shabala *et al.* (1997). Electrode blanks were pulled from 1.5 mm borosilicate glass capillaries, dried in an oven at 220 °C for 4 h, and silanized with tributylchlorosilane (Fluka, Seelze, Germany). Cooled microelectrodes were backfilled with 200 mM NH_4Cl for NH_4^+ , and 15 mM NaCl and 40 mM KH_2PO_4 (adjusted to pH 6.0 using 0.1 M NaOH) for H^+ . Electrode tips were then filled with commercially-available ion-selective H^+ or NH_4^+ cocktails (Fluka). Electrodes were calibrated with a set of known standards. The slopes were 54–59 mV pIon⁻¹.

The electrodes were mounted on an electrode holder (MMT-5, Narishige, Tokyo, Japan) providing three-dimensional positioning, and positioned in a line 20 μm above the inner surface of the pitcher with their tips spaced 3–4 μm apart. The chamber was attached to a computer-controlled micromanipulator (PatchMan NP2, Eppendorf AG, Hamburg, Germany). During flux measurements, the MIFE computer gently moved the chamber up and down, providing virtual movement of the electrode tips between two positions, 20 μm and 60 μm above the pitcher surface, in a 10 s square-wave cycle. The concentration of each ion was calculated from its electrochemical potential at each position. The flux of each ion was later calculated from the measurements of the difference in the electrochemical potential between these positions (Shabala *et al.*, 1997). For analysis, the first 1 s of each half-cycle was ignored.

Fluxes were measured at five positions (A–E) on each pitcher section (Fig. 1). Position A was at the base of the pitcher, position C was immediately below the top of the digestive zone, and position B was half way between positions A and C. Position D was approximately 1 cm above the digestive zone in the conductive zone, and position E was approximately 1 cm below the peristome. The digestive zone was recognized by the dark-flecked, glandular appearance of the inner surface of the pitcher. In *N. ampullaria* pitchers, the entire inner surface appears dark-flecked and glandular; therefore, ion flux measurements were performed at the same proportional distances from the base as for the other species (Fig. 1). Ion fluxes were measured for an average of 5 min at each position and all measurements for one pitcher section were usually complete within 80 min. Ion concentrations declined by a maximum of 3 μM (or 0.3 pH units) during measurements.

Following flux measurements at pH 4.0, pitcher sections and their holders were removed from the Perspex® container, placed in a test tube in 500 μM NH_4NO_3 and 200 μM CaSO_4 solution adjusted to pH 6.0, and put back under lights for 23 h. The next day, fluxes were re-measured in fresh solution at pH 6.0 using the procedure described above. In preliminary trials, fluxes were more consistent if measurements were made first at pH 4.0, followed by a 23 h acclimation to pH 6.0.

pH values of 4 and 6 were chosen as they fall within the naturally-occurring range of values found in pitchers of these species in the wild (mean \pm SE, n : 2.55 \pm 0.17, 20; 3.72 \pm 0.09, 20; 4.33 \pm 0.14, 20 for *N. rafflesiana*, *N. ampullaria*, and *N. bicalcarata*, respectively; Clarke, 1997), and represent a 100-fold difference in

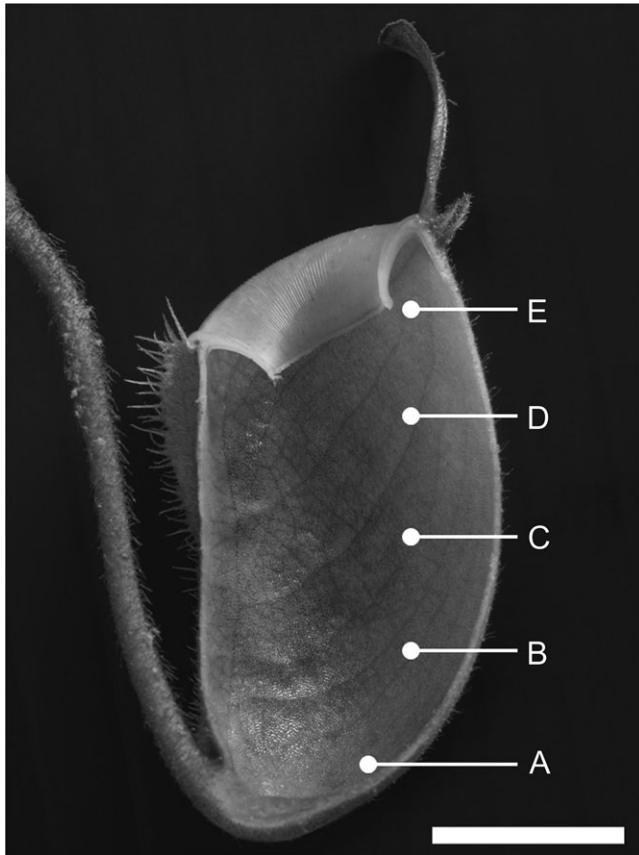


Fig. 1. Longitudinal section of *N. ampullaria* pitcher, showing zones at which ion fluxes were measured (A to E). Bar=1 cm.

proton concentration in the pitcher fluid. This would provide a sufficient range of acidity for us to differentiate between species that actively maintain moderately acidic pitcher conditions and those maintaining more highly acidic conditions.

Scanning electron microscopy (SEM) and image analyses

Pitcher wall samples were fixed in glutaraldehyde (4% in 0.1 M sodium phosphate buffer), then stained with osmium tetroxide, dehydrated via an ethanol dilution series and critical point dried, after which they were sputter coated with gold. Digital images of the specimens were recorded using a Hitachi S-3500 N scanning electron microscope (Hitachi Inc., Toronto, Canada) at 15 kV. Measurements of gland diameter and gland area/area of pitcher wall were recorded from the digital images using ImageJ v.1.38 software (Research Services Branch, National Institutes of Health, USA; <http://rsbweb.nih.gov/ij/index.html>). Mean and SE values were calculated for each species from 1 mm² areas of pitcher wall ($n=5$ per zone, 25 per species). In *N. ampullaria* and *N. bicalcarata*, glands were present in all zones (Fig. 2). By contrast, *N. rafflesiana* lacked glands in zones D and E. Instead, the inner pitcher wall was covered with lunate cells and epicuticular wax crystals (Fig. 2, see inset). However, after it was found that ionic fluxes were occurring in these zones in this species (see Discussion), the width and area of the aperture beneath each lunate cell were measured and entered into the data as 'gland' diameter and 'gland' area, respectively.

Statistical analyses

For the ion flux measurements, three pitchers were used per species; for the gland distribution, five pitchers were used per species. Despite the small sample sizes, the effects of the factors on ion fluxes and gland measurements were found to be sufficiently

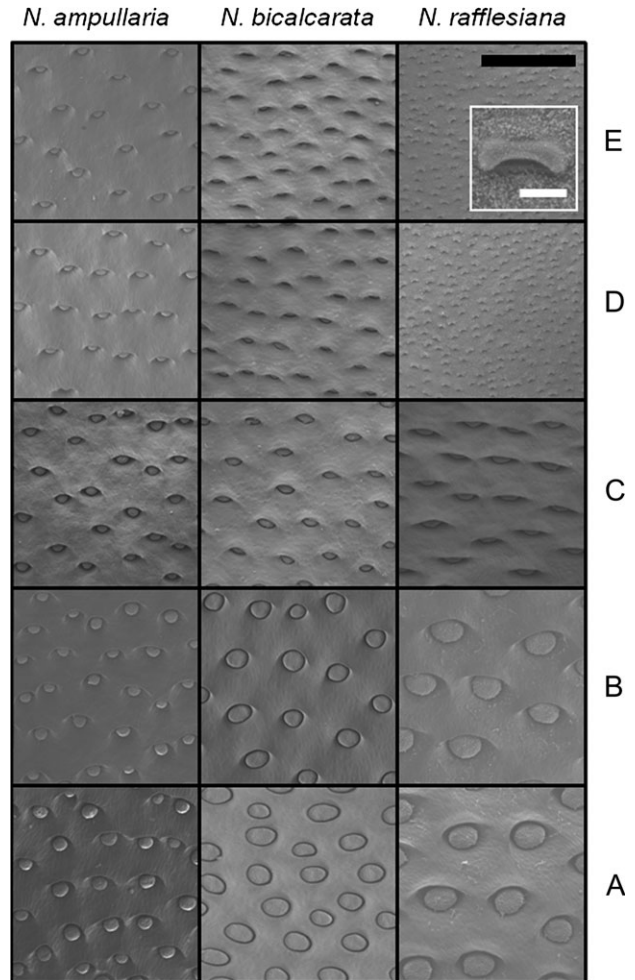


Fig. 2. Scanning electron microscope (SEM) images comparing the inner pitcher surface at zones A through E for *N. ampullaria*, *N. bicalcarata*, and *N. rafflesiana*. Inset outlined in white for *N. rafflesiana* shows individual lunate cell. Note pale epicuticular wax crystals on surface. Black bar=0.5 mm; white bar=25 μm.

robust to circumvent Type II errors at $\beta=0.05$ in the statistical models used. Data were analysed first by ANOVA, using the factors 'Species', 'pH' and 'Zone' for the ion flux data, and 'Species' and 'Zone' for the gland distribution data (SYSTAT v.13; Systat Software Inc., Chicago IL); however, since the interaction term of 'Species' and 'Zone' or 'pH' was usually significant, further analyses were conducted separately by species. Pairwise comparisons between means were determined using the Tukey test. All data were tested for normality and homoscedasticity; data sets violating the assumptions were transformed and re-tested prior to running parametric tests (Sokal and Rohlf, 1981). In cases where transformation failed to resolve the violation of assumptions, the non-parametric Student–Neuman–Keuls test was used instead of the Tukey test.

Results

Gland morphology and distribution

N. rafflesiana possessed lunate cells and epicuticular wax crystals in zones D and E; these structures were absent from *N. ampullaria* and *N. bicalcarata* (Fig. 2, inset). There was

a general trend of decreasing gland diameter from zones A to E (i.e. from pitcher base towards pitcher mouth; $P < 0.001$), although there were noticeable interspecific differences (Figs 2, 3A; Tables 1, 2). *N. ampullaria* differed from the other species in two respects. Firstly, there was the smallest absolute difference in gland size across the zones; secondly, and partly as a result, although *N. ampullaria* had the smallest glands at zone A, it had the largest at zones D and E (Figs 2, 3A). The relative ratio of area of gland to area of wall also decreased from zones A to E ($P < 0.001$; Fig. 3B; Tables 1, 2). As with gland diameter, *N. ampullaria* showed the smallest change in gland area/area of wall with zone (Figs 2, 3B).

NH₄⁺ fluxes

There was a significant effect of ‘Species’ on *NH₄⁺* uptake rate, in the relative order *N. rafflesiana* > *N. bicalcarata* > *N. ampullaria* ($P < 0.001$; Fig. 4; Table 3). There was a general

trend of decreasing uptake rates from zones A to E ($P = 0.018$, $P = 0.002$ for *N. bicalcarata* and *N. rafflesiana*, respectively, no significant effect of ‘Zone’ for *N. ampullaria*; Fig. 4; Table 4). *NH₄⁺* uptake occurred at all zones in all species (Fig. 4), indicating that zones A to E were capable of active transport of this ion, even the wax-covered upper zones (D and E) of *N. rafflesiana*, in which digestive glands were replaced by lunate cells (Fig. 2). There was no significant effect of pH on *NH₄⁺* uptake rate in any of the three species (Table 4).

H⁺ fluxes

pH exerted a significant effect on *H⁺* flux ($P < 0.001$; Table 3; Fig. 4). At pH 6.0, protons were pumped into the lumen side (i.e. into the pitcher fluid in an intact pitcher) in all zones, in the order *N. rafflesiana* > *N. bicalcarata* > *N. ampullaria* (Fig. 4). At pH 4.0, both *N. ampullaria* and *N. bicalcarata* actively pumped protons out of the lumen side (i.e. increasing the pH of the fluid of an intact pitcher) in

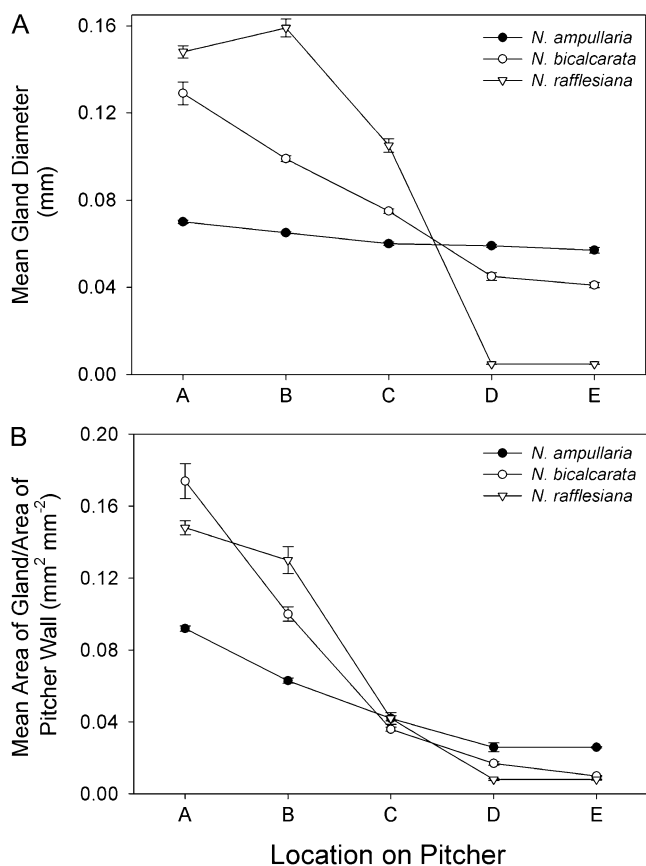


Fig. 3. Gland distribution by zone for pitchers of *N. ampullaria*, *N. bicalcarata*, and *N. rafflesiana*. (A) Mean gland diameter (mm). (B) Mean area of gland/area of pitcher wall (mm² mm⁻²). Mean and SE were calculated for each species from 5 × 1 mm² areas of pitcher wall ($n = 5$ per zone, 25 per species). In *N. rafflesiana*, zones D and E possess lunate cells rather than typical digestive glands. However, the width and area of the aperture beneath each lunate cell are represented here as ‘gland’ diameter and ‘gland’ area, respectively (see Discussion).

Table 1. ANOVA table for gland measurements by ‘Species’ and ‘Zone’

Measurement	Source	Type III SS	df	F	P
Width	Species	0.005	2	36.301	<0.001
	Zone	0.178	4	636.833	<0.001
	Species × Zone	0.026	8	46.329	<0.001
Area	Species	0.006	2	134.356	<0.001
	Zone	0.089	4	919.468	<0.001
	Species × Zone	0.053	8	276.498	<0.001

Table 2. All-pairwise multiple comparisons for effect of ‘Zone’ on ion fluxes and gland measurements

Species	Measurement	Zone ^a					Test used
		A	B	C	D	E	
<i>N. ampullaria</i>	Ions						
	<i>NH₄⁺</i>	-	-	-	-	-	n/a ^b
<i>N. bicalcarata</i>	<i>H⁺</i>	d	b	c	abcd	a	Tukey
	<i>NH₄⁺</i>	a	-	-	-	a	Tukey
<i>N. rafflesiana</i>	<i>H⁺</i>	-	-	-	-	-	n/a
	<i>NH₄⁺</i>	abcd	b	c	d	a	Tukey
<i>N. ampullaria</i>	Glands						
	Width	ab	ac	ad	a	bcd	Tukey
	Area	abc	ad	a	b	cd	SNK ^c
	<i>N. bicalcarata</i>	Width	a	a	a	a	a
<i>N. bicalcarata</i>	Area	ab	ac	ad	a	bcd	SNK
	<i>N. rafflesiana</i>	Width	ab	ac	ad	a	bcd
<i>N. rafflesiana</i>	Area	ab	ac	ad	a	bcd	SNK

^a Zones sharing the same lower case letter are significantly different (i.e. $P < 0.05$).

^b Not applicable, i.e. ANOVA $P > 0.05$ for the effect of ‘Zone’ in this species.

^c Student–Neuman–Keuls test (non-parametric).

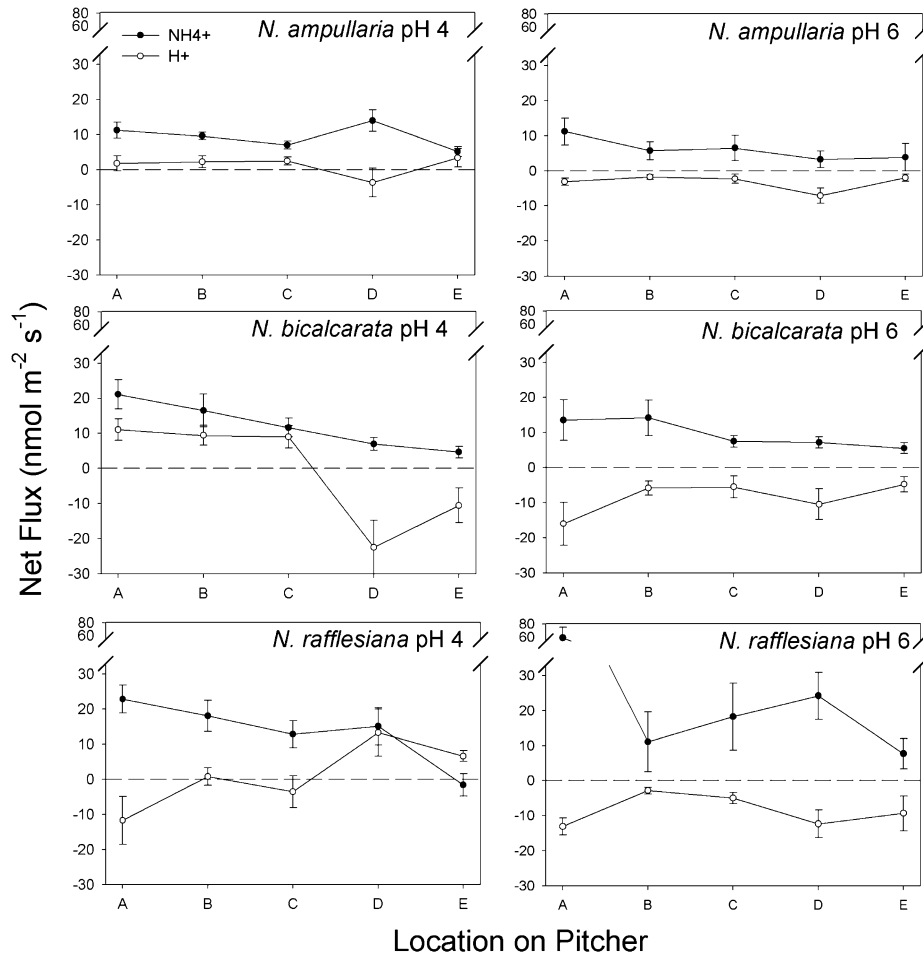


Fig. 4. Mean net ion fluxes (nmol m⁻² s⁻¹) by zone and pH for pitchers of *N. ampullaria*, *N. bicalcarata*, and *N. rafflesiana*. Closed circles, NH₄⁺; open circles, H⁺. Positive values indicate net influx from lumen into pitcher tissue; negative values indicate net efflux into lumen from pitcher tissue. Bars indicate 1 SE (*n*=3 per species).

Table 3. ANOVA table for NH₄⁺ and H⁺ fluxes by 'Species', 'pH', and 'Zone'

Ion	Source	Type III SS	df	F	P
NH ₄ ⁺	Species	1 651.860	2	11.851	<0.001
	pH	33.229	1	0.477	ns ^a
	Zone	3 614.287	4	12.965	<0.001
	Species×pH	746.293	2	5.354	0.008
	Species×Zone	2 054.347	8	3.685	0.002
	pH×Zone	591.515	4	2.122	ns
H ⁺	Species	220.313	2	1.588	ns
	pH	1 179.514	1	17.006	<0.001
	Zone	711.349	4	2.564	0.047
	Species×pH	80.726	2	0.582	ns
	Species×Zone	1 614.789	8	2.910	0.008
	pH×Zone	77.162	4	0.278	ns

^a Not significant, i.e. *P* > 0.05.

zones A to C. In *N. rafflesiana*, protons were pumped into the lumen side (i.e. decreasing the pH of the fluid of an intact pitcher) in zones A and C; the opposite occurred in zones B, D, and E (Fig. 4).

Table 4. ANOVA summary results for ion fluxes by 'pH' and 'Zone', 'Species' treated separately

Species	Ion	Effect of pH		Effect of Zone		Effect of pH×Zone	
		F	P	F	P	F	P
<i>N. ampullaria</i>	NH ₄ ⁺	2.825	ns ^a	1.248	ns	0.852	ns
	H ⁺	23.761	<0.001	5.292	0.004	0.137	ns
<i>N. bicalcarata</i>	NH ₄ ⁺	1.170	ns	3.858	0.018	0.445	ns
	H ⁺	3.423	ns	2.745	ns	3.002	0.043
<i>N. rafflesiana</i>	NH ₄ ⁺	4.196	ns	7.348	0.002	2.910	ns
	H ⁺	8.947	0.007	2.639	ns	2.560	ns

^a Not significant, i.e. *P* > 0.05.

Discussion

Of the ~100 species of *Nepenthes* identified to date, the ecology of perhaps less than a dozen has been studied in any great detail. Nonetheless, it is apparent that there exists a wide variety of N sequestration strategies employed by the Nepenthaceae. For example, in addition to producing scent, *N. rafflesiana* pitchers generate colour contrast signals that

are ‘tuned’ to the visual sensitivity maxima of many anthophilous insect taxa, allowing exploitation of volant prey (Moran, 1996; Moran et al., 1999; DiGiusto et al., 2008); *Nepenthes albomarginata* T. Lobb ex Lindl. pitchers produce a lichen-mimicking tissue to attract termites (Moran et al., 2001; Merbach et al., 2002); in *N. ampullaria*, a significant proportion of foliar N is derived from leaf litter (Cresswell, 1998; Moran et al., 2003); and *Nepenthes lowii* Hook. f. deploys funnel-shaped pitchers to capture and utilize vertebrate faeces (Clarke et al., 2009).

There are also interspecific differences in pitcher longevity and prey capture rates. While some species employ a high prey capture rate/high pitcher turnover strategy, others produce longer-lived pitchers that harvest a slow but steady ‘trickle’ of prey. *N. rafflesiana* falls into the first category: although its pitchers are relatively short-lived at ≤ 3 months (Osunkoya et al., 2008; Bauer et al., 2009), its prey capture rate is high (Moran, 1996; Adam, 1997; Bauer et al., 2009). By contrast, *N. ampullaria* pitchers are long-lived at ≥ 8 months (Clarke, 1997; Osunkoya et al., 2008) and exhibit a prey capture rate an order of magnitude less than that of *N. rafflesiana* (Adam, 1997). *N. bicalcarata* is another species characterized by long-lived pitchers (≥ 7 months; Clarke, 1997; Osunkoya et al., 2008) and a low prey capture rate, comparable to that of *N. ampullaria* (Adam, 1997).

Ion fluxes

Are interspecific differences in pitcher ion fluxes related to nutrient sequestration strategy? We will take each species in turn. Before doing so, however, it is important to note that, from a functional viewpoint, zones A to C are the most important in terms of ion fluxes, as they comprise the digestive zone of the pitcher (see Introduction). Pitchers of *N. rafflesiana* and *N. bicalcarata* are almost never completely filled with fluid, and only zones A to C are usually below the fluid level. Interpretation of Fig. 4 should therefore be undertaken with this in mind. By contrast, *N. ampullaria* pitchers are often filled with fluid up to zone D or even occasionally E (J Moran, personal observation), the implications of which will be dealt with in the next section.

As outlined above, *N. rafflesiana* is a ‘typical’ invertebrate-trapping species that deploys relatively short-lived pitchers to trap and utilize large numbers of prey over a brief period of time. It would be expected that a high rate of prey input to the pitcher might be reflected in a correspondingly high rate of absorption of the nitrogenous products of digestion. This strategy is confirmed by the observed rates of NH_4^+ uptake, which are the highest among the species in this study (Fig. 4). At both pH 4 and 6, *N. rafflesiana* actively pumps H^+ into the pitcher fluid at zones A to C, presumably to maintain optimally acidic conditions for enzymatic activity. This corresponds to the finding of Clarke (1997): in its natural habitat, *N. rafflesiana* maintains a significantly lower pH in its pitchers than do *N. ampullaria* or *N. bicalcarata* (mean \pm SE, n : 2.55 ± 0.17 , 20; 3.72 ± 0.09 , 20; 4.33 ± 0.14 , 20, respectively. $P < 0.001$, $F = 43.3$, ANOVA).

N. ampullaria is unique among the Nepenthaceae in that, although it catches invertebrate prey (albeit at a much slower rate than ‘typical’ species; Adam, 1997), it derives a significant amount of its N (*c.* 35%) from abscised leaves that have fallen into its pitchers from the forest canopy (Moran et al., 2003). *N. ampullaria* pitchers possess a suite of morphological adaptations to this unusual mode of nutrition (Moran et al., 2003); in addition, they are home to a rich assemblage of aquatic organisms: more than a dozen such species have been described, the majority of them dipteran larvae, mosquitoes in particular (Mogi and Yong, 1992; Clarke and Kitching, 1993; Clarke, 1998; Cresswell, 1998). Moran et al. (2003) hypothesized that *N. ampullaria* relies on these aquatic organisms to break down the litter and release assimilable nitrogen species such as NH_4^+ , based on processes occurring in tree-holes, aquatic habitats analogous to *Nepenthes* pitchers (Carpenter, 1982; Fish and Carpenter, 1982; Bradshaw and Creelman, 1984; Yanoviak, 1999; Paradise, 2004; Verdonschot et al., 2008). A simple putative pathway from organically bound N in leaf litter to assimilable NH_4^+ within the *N. ampullaria* pitcher is as follows:

Leaf litter \rightarrow Bacterial film on leaf \rightarrow ‘Grazing’ mosquito larvae \rightarrow Excreted NH_4^+

This pathway is comparable to that described for the unrelated North American pitcher plant *Sarracenia purpurea* L. (Sarraceniaceae), in which aquatic invertebrates, bacteria, and fungi mineralize organically bound N which is then assimilated by the plant (Bradshaw and Creelman, 1984; Mouquet et al., 2008). The results of the current study confirm that *N. ampullaria* is capable of the active uptake of NH_4^+ , albeit at a slower rate than *N. rafflesiana*. Such a slow rate of uptake is not unexpected, given the slow but steady ‘trickle’ of abscised leaves into the pitcher, the increased time required for plant cell contents to be made available via bacterial breakdown of the cellulose cell wall, and the longevity of the pitchers themselves. The *N. ampullaria* pitcher appears to function to some extent as a tree-hole analogue, complete with leaf litter inputs from which it ultimately derives mineralized N. Although phytotelms such as tree holes (and *N. ampullaria* pitchers) are typically acidic environments, too low a pH can have negative effects on bacterial degradation of leaf litter (Kok and Van der Velde, 1994). Invertebrate pitcher inhabitants are also sensitive to very low pH: increased acidity in phytotelms has been shown to discourage egg-laying by some diptera, as well as having negative impacts on the survival of mosquito larvae and other aquatic invertebrates (Carpenter, 1982; Harrison, 2001; Kitching, 2001). Therefore, if *N. ampullaria* is dependent upon bacterial- and invertebrate-mediated release of N from leaf litter inputs, the plant would be expected to exert control on the pH of the pitcher fluid in order to prevent hyperacidic conditions from developing. Our results confirm this: at pH 6, *N. ampullaria* showed the lowest H^+ efflux rate, i.e. the lowest degree of pitcher fluid acidification compared to *N. bicalcarata* and *N. rafflesiana*. At pH 4, *N. ampullaria* demonstrated H^+ uptake in most zones, i.e. the pitchers were actively reducing acidity levels (Fig. 4).

It is also important to bear in mind that, in addition to leaf-litter inputs, a significant proportion of *N. ampullaria* foliar N is animal-derived (Moran *et al.*, 2003). It is therefore likely that *N. ampullaria* must perform a balancing act between creating optimal conditions for micro-organism-derived N mineralization on the one hand, and digestion of animal-derived N species on the other.

Although *N. bicalcarata* is 'typical' in that its pitchers catch only invertebrate prey, it is also unique in that it has a mutualistic association with a species of swimming ant, *Camponotus schmitzi* Stärke. In return for the provision of a domatium, the ants benefit the plant by repelling pitcher-damaging weevils (Merbach *et al.*, 2007), and entering the pitcher fluid to remove overly-large prey items, preventing putrefaction and consequent pitcher death (Clarke and Kitching, 1995). Like *N. ampullaria*, *N. bicalcarata* actively pumps H^+ from the pitcher fluid under highly acidic conditions (pH 4; Fig. 4D), as might be expected from a species dependent upon a commensal that spends a significant portion of its time in the pitcher fluid (Harrison, 2001). Thus, both *N. ampullaria* and *N. bicalcarata* actively maintain less acidic pitcher conditions than sympatric congeners such as *N. rafflesiana*. This finding may help to explain the presence of acid-intolerant visitors. For example, in Brunei, Northwest Borneo, it is not uncommon to find terrestrial crabs (*Geosesarma* sp.; Ng and Lim, 1987) in *N. ampullaria* pitchers (J Moran, personal observation); whether the crabs are feeding on the detritus in the pitchers is unknown at present. Similarly, tree frogs (*Philautus* sp.; Dring, 1987) lay eggs in pitchers of *N. bicalcarata* (J Moran, personal observation). Both groups of animals are known to be intolerant of hyperacidic conditions (Diesel, 1992; Vatnick *et al.*, 2006).

Gland distribution and morphology

With regard to gland distribution and characteristics, the species investigated fall into two categories (Figs 2, 3). The first comprises *N. rafflesiana*, which possesses a pattern typical of many invertebrate-trapping species. Zones A to C are lined with digestive glands that have the dual functions of secretion and absorption (Owen and Lennon, 1999; Owen *et al.*, 1999). Zones D and E, lying above the fluid level in intact pitchers, possess large numbers of lunate cells and are coated with epicuticular wax crystals (Fig. 2). The lunate cells are modified stomatal guard cells, the orientation of which produces an anisotropic surface that denies traction to insects attempting to ascend the pitcher wall (Fig. 2; Adams and Smith, 1977; Gaume *et al.*, 2002, 2004; Thornhill *et al.*, 2008). In addition, the epicuticular wax crystals clog claws, and the waxes themselves create a surface of low free surface energy, preventing traction via capillarity for insects with hairy pulvillae (Juniper and Burras, 1962; Gaume *et al.*, 2004; Gorb *et al.*, 2004).

The second category comprises *N. ampullaria* and *N. bicalcarata*, from which lunate cells and epicuticular waxes are absent. Digestive glands line the entire inner surface of

the pitcher (Fig. 2). Why do these two species lack structures with demonstrated roles in the capture and retention of prey? In *N. ampullaria*, the pitcher lid (which prevents entrance of rainwater in other species) is reflexed away from the mouth, and the pitchers are often filled with fluid slightly below or even up to the level of the peristome (J Moran, personal observation). This appears to be a strategy to maximize the volume of fluid, and thus the effective size of the aquatic habitat available for colonization by commensals: the larger the volume of a phytotelm, the more organisms can be supported (Schmidl *et al.*, 2008). Since almost the entire inner wall of the pitcher is submerged, the lunate cells and epicuticular waxes would be of little use, and the entire surface has been turned over to the uptake of nutrients via the digestive glands. This is demonstrated by the fact that there is no significant effect of Zone on NH_4^+ uptake rates in this species (Table 4). *N. bicalcarata* also relies on mutualism, in this case with *C. schmitzi*, which often takes up station inside the pitcher, beneath the overhanging peristome. The ant frequently enters the pitcher fluid to hunt dipteran larvae and also to remove oversized prey items (Clarke and Kitching, 1995; Clarke, 1997). A conductive zone possessing lunate cells and epicuticular waxes would possibly prevent easy movement of *C. schmitzi* around the inner surface of the pitcher.

A surprising finding of the study is that in *N. rafflesiana*, zones D and E exhibit ion fluxes (NH_4^+ and H^+ ; Fig. 4). Active transport of NH_4^+ was also found to occur across wax-covered zones D and E in *Nepenthes fusca* Danser pitchers (data not presented). At first glance, this finding is highly counterintuitive, as these zones do not possess the multicellular glands typical of the lower zones. Even more problematically, they are covered in epicuticular waxes, primarily very long chain aldehydes (Riedel *et al.*, 2003, 2007) that present a surface of low free surface energy, and are thus effectively unwettable (Gorb *et al.*, 2004; Gorb and Gorb, 2006). The only possible portal for fluxes of aquatic ions must be the epidermal cell that lies within the depression beneath each overhanging lunate cell, as there is no pore analogous to a stoma (Pant and Bhatnagar, 1977; Owen and Lennon, 1999) and all other surfaces possess epicuticular waxes (Fig. 2, inset). There is evidence that these epidermal cells are capable of active transport: MacFarlane (1893) reported exudation of fluid from the wax-covered conductive zone and identified the depressions beneath the lunate cells as the source of these secretions. Therefore, it appears that, although the conductive zone is rarely submerged beneath the pitcher fluid, it is nonetheless capable of active transport of aqueous ions. This raises the intriguing possibility that the capability of ion exchange in the epidermal cells beneath the lunate cells represents an atavism, and that such structures may represent a primitive form of the digestive glands, which are known to be of epidermal origin (Pant and Bhatnagar, 1977; Owen and Lennon, 1999; Thornhill *et al.*, 2008). However, exploring this possibility is beyond the scope of the current study.

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