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. Moran1,*, 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

Received 14 October 2009; Revised 7 December 2009; Accepted 4 January 2010

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 NH4+ 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, NH4+, 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 pro- teases, 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 NH4+, 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 overarching the pitcher mouth.
This zone is rich in nectaries, serving to attract and retain prey. Colour patterns and scent in this zone 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 ontogenic 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 µmol m$^{-2}$ 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$^\text{TM}$ holder, which was placed vertically in a test tube filled with 95 ml aerated measurement solution (500 µM NH$_4$NO$_3$ and 200 µM CaSO$_4$.2H$_2$O, adjusted to pH 4.0 with 2 M HCl), and positioned under 300 µmol m$^{-2}$ 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$^\text{TM}$ 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$_4$Cl for NH$_4^+$, and 15 mM NaCl and 40 mM KH$_2$PO$_4$ (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 pH$^{-1}$.

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$^\text{TM}$ container, placed in a test tube in 500 µM NH$_4$NO$_3$ 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 ± SE, n: 2.55 ± 0.17, 20; 3.72 ± 0.09, 20; 4.33 ± 0.14, 20 for *N. rafflesiana*, *N. ampullaria*, and *N. bicalcarata*, respectively; Clarke, 1997), and represent a 100-fold difference in
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 robust to circumvent Type II errors at β=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).

**NH$_4^+$ fluxes**

There was a significant effect of ‘Species’ on NH$_4^+$ 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). NH$_4^+$ 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 NH$_4^+$ 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

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

| Measurement | Source Type III SS | df | $F$ | $P$ |
|-------------|-------------------|----|-----|-----|
| Width       | Species           | 2  | 36.301 | < 0.001 |
|             | Zone              | 4  | 636.833 | < 0.001 |
|             | Species × Zone    | 8  | 46.329  | < 0.001 |
| Area        | Species           | 2  | 134.356 | < 0.001 |
|             | Zone              | 4  | 919.468 | < 0.001 |
|             | Species × Zone    | 8  | 276.498 | < 0.001 |

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

| Species      | Measurement | Zone | Test used |
|--------------|-------------|------|-----------|
| *N. ampullaria* | NH$_4^+$ | A B C D E | n/a $^a$ | Tukey |
| *N. bicalcarata* | H$^+$ | d b c abcd a | | Tukey |
| *N. rafflesiana* | NH$_4^+$ | abcd b c d a | | n/a |
|              | H$^+$ |             | | n/a |

$^a$ 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).
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 3. ANOVA table for NH$_4^+$ and H$^+$ fluxes by ‘Species’, ‘pH’, and ‘Zone’

| Ion     | Source      | Type III SS | df | F     | P      |
|---------|-------------|-------------|----|-------|--------|
| NH$_4^+$| Species     | 651.860     | 2  | 11.851| <0.001 |
|         | pH          | 33.229      | 1  | 0.477 | ns     |
|         | Zone        | 614.287     | 4  | 12.965| <0.001 |
|         | Species x pH| 746.293     | 2  | 5.354 | 0.008  |
|         | Species x Zone| 2 054.347  | 8  | 3.685 | 0.002  |
|         | pH x Zone   | 591.515     | 4  | 2.122 | ns     |
| H$^+$   | Species     | 220.313     | 2  | 1.588 | ns     |
|         | pH          | 1 795.14    | 1  | 17.006| <0.001 |
|         | Zone        | 711.349     | 4  | 2.564 | 0.047  |
|         | Species x pH| 80.726      | 2  | 0.582 | ns     |
|         | Species x Zone| 1 614.789  | 8  | 2.910 | 0.008  |
|         | pH x Zone   | 77.162      | 4  | 0.278 | ns     |

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

**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, NH$_4^+$; 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 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 x Zone |
|-------------|---------|--------------|----------------|---------------------|
|             | F       | P            | F              | P                  |
| *N. ampullaria*| NH$_4^+$| 2.825 | ns$^a$ | 1.248 | ns | 0.852 | ns |
|             | H$^+$   | 23.761 | <0.001 | 5.292 | 0.004 | 0.137 | ns |
| *N. bicalcarata*| NH$_4^+$| 1.170 | ns | 3.858 | 0.018 | 0.445 | ns |
|             | H$^+$   | 3.423 | ns | 2.745 | ns | 3.002 | 0.043 |
| *N. rafflesiana*| 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 anthropophilous insect taxa, allowing exploitation of volant prey (Moran, 1996; Moran et al., 1999; DiGiusto et al., 2008); Nepenthes albonervigata 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 lowi 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 ±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; Verdonchot 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 → Bacterial film on leaf → ‘Climbing’ mosquito larvae → 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).
Ion fluxes and gland distribution in *Nepenthes* | 1371

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 for the presence of acid-intolerant visitors 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 (Schmitz 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 overlapping 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 avatism, 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.
Acknowledgements

We thank Alison Moran and two anonymous referees for significantly improving earlier drafts of the manuscript, Brendan Porter for help with the MIFE measurements, and Jeanie and Jack Wootton at Hawaiian Botanicals for help in obtaining specimens.

References

Adam JH. 1997. Prey spectra of Bornean Nepenthes species (Nepenthaceae) in relation to their habitat. *Pertanika Journal of Tropical Agricultural Science* 20, 121–134.

Adamec L. 1997. Mineral nutrition of carnivorous plants: a review. *Botanical Review* 63, 273–299.

Adams RM, Smith GW. 1977. An SEM survey of the five carnivorous plant genera. *American Journal of Botany* 64, 265–272.

An C-I, Fukusaki E-I, Kobayashi A. 2001. Plasma-membrane H+-ATPases are expressed in pitchers of the carnivorous plant *Nepenthes alata* Blanco. *Planta* 212, 547–555.

An C-I, Fukusaki E-I, Kobayashi A. 2002a. Aspartic proteinases are expressed in pitchers of the carnivorous plant *Nepenthes alata* Blanco. *Planta* 214, 661–667.

An C-I, Takekawa S, Okazawa A, Fukusaki E-I, Kobayashi A. 2002b. Degradation of a peptide in pitcher fluid of the carnivorous plant *Nepenthes alata* Blanco. *Planta* 215, 472–477.

Athauda SBP, Matsumoto K, Rajapakshe S, Kuribayashi M, Kojima M, Kubomura-Yoshida N, Iwamatsu A, Shibata C, Inoue H, Takahashi K. 2004. Enzymic and structural characterization of nepenthesin, a unique member of a novel subfamily of aspartic proteinases. *Biochemical Journal* 381, 295–306.

Bonn HF, Federle W. 2004. Insect aquaplaning: *Nepenthes* pitcher plants capture prey with the peristome, a fully wettable water-lubricated anisotropic surface. *Proceedings of the National Academy of Sciences, USA* 101, 14138–14143.

Bauer U, Bohn HF, Federle W. 2008. Harmless nectar source or deadly trap: *Nepenthes* pitchers are activated by rain, condensation and nectar. *Proceedings of the Royal Society B* 275, 259–265.

Bauer U, Willmes C, Federle W. 2009. Effect of pitcher age on trapping efficiency and natural prey capture in carnivorous *Nepenthes rafflesiana* plants. *Annals of Botany* 103, 1219–1226.

Bradshaw WE, Creelman RA. 1984. Mutualism between the carnivorous purple pitcher plant and its inhabitants. *American Midland Naturalist* 112, 294–304.

Carpenter SR. 1982. Sternum flow chemistry: effects on population dynamics of detritivorous mosquitoes in tree-hole ecosystems. *Oecologia* 53, 1–6.

Chia TF, Aung HH, Osipov AN, Goh NK, Chia LS. 2004. Carnivorous pitcher plant uses free radicals in the digestion of prey. *Redox Report* 9, DOI: 10.1179/135100004225006029.

Clarke CM. 1997. *Nepenthes of Borneo*. Kota Kinabalu, Malaysia: Natural History Publications.

Clarke CM. 1998. A re-examination of geographical variation in *Nepenthes* food webs. *Ecography* 21, 430–436.

Clarke CM, Bauer U, Lee CC, Tuen AA, Rembold K, Moran JA. 2009. Tree shrew lavatories: a novel nitrogen sequestration strategy in a tropical pitcher plant. *Biology Letters* 5, 632–635.

Clarke CM, Kitching RL. 1993. The metazoan food webs from six Bornean *Nepenthes* species. *Ecological Entomology* 18, 7–16.

Clarke CM, Kitching RL. 1995. Swimming ants and pitcher plants: a unique ant–plant interaction from Borneo. *Journal of Tropical Ecology* 11, 589–602.

Cresswell JE. 1998. Morphological correlates of necromass accumulation in the traps of an Eastern tropical pitcher plant, *Nepenthes ampullaria* Jack, and observations on the pitcher infauna and its reconstitution following experimental removal. *Oecologia* 113, 383–390.

Cuin TA, Betts SA, Chalmandrier R, Shabala S. 2008. A root’s ability to retain K+ correlates with salt tolerance in wheat. *Journal of Experimental Botany* 59, 2697–2706.

Diesel R. 1992. Managing the offspring environment: brood care in the bromeliad crab, *Metapaullias depressus*. *Behavioural Ecology and Sociobiology* 30, 125–134.

DiGiusto B, Grosbois V, Fargeas E, Marshall DJ, Gaume L. 2008. Contribution of pitcher fragrance and fluid viscosity to high prey diversity in a *Nepenthes* carnivorous plant from Borneo. *Journal of Biosciences* 33, 121–136.

Dring JCM. 1987. Bornean treefrogs of the genus *Philautus* (Rhacophoridae). *Amphibia-Reptilia* 8, 19–47.

Eilenberg H, Pnini-Cohen S, Schuster S, Movtchan A, Zilberstein A. 2006. Isolation and characterization of chitinase genes from pitchers of the carnivorous plant *Nepenthes khasiana*. *Journal of Experimental Botany* 57, 2775–2784.

Fish D, Carpenter SR. 1982. Leaf litter and larval mosquito dynamics in tree-hole ecosystems. *Ecology* 63, 283–288.

Gaume L, DiGiusto B. 2010. Adaptive significance and ontogenetic variability of the waxy zone in *Nepenthes rafflesiana*. *Annals of Botany* doi: 10.1093/aob/mcp238.

Gaume L, Forthery Y. 2007. A viscoelastic deadly fluid in carnivorous pitcher plants. *PLoS ONE* 11, e1185. doi: 10.1371/journal.pone.0001185.

Gaume L, Gorb S, Rowe N. 2002. Function of epidermal surfaces in the trapping efficiency of *Nepenthes alata* pitchers. *New Phytologist* 156, 479–489.

Gaume L, Perret P, Gorb E, Gorb S, Labat J-J, Rowe N. 2004. How do plant waxes cause flies to slide? Experimental tests of wax-based trapping mechanisms in three pitfall carnivorous plants. *Arthropod Structure and Development* 33, 103–111.

Gorb EV, Gorb SN. 2006. Physicochemical properties of functional surfaces in pitchers of the carnivorous plant *Nepenthes alata* Blanco (Nepenthaceae). *Plant Biology* 8, 841–848.

Gorb E, Kastner V, Peressadko A, Arzt E, Gaume L, Rowe N, Gorb S. 2004. Structure and properties of the glandular surface in the digestive zone of the pitcher in the carnivorous plant *Nepenthes ventrata* and its role in insect trapping and retention. *Journal of Experimental Biology* 207, 2947–2963.

Harrison JF. 2001. Insect acid-base physiology. *Annual Review of Entomology* 46, 221–250.
Hatano N, Hamada T. 2008. Proteome analysis of pitcher fluid of the carnivorous plant Nepenthes alata. Journal of Proteome Research 7, 809–816.

Hawkins BJ, Bouckim H, Plassard C. 2008. A comparison of ammonium, nitrate and proton net fluxes along seedling roots of Douglas-fir and lodgepole pine grown and measured with different inorganic nitrogen sources. Plant, Cell and Environment 31, 278–287.

Heslop-Harrison Y. 1975. Enzyme release in carnivorous plants. Frontiers in Biology 43, 525–578.

Higashi S, Nakashima A, Ozaki H, Abe M, Uchiumi T. 1993. Analysis of feeding mechanism in a pitcher of Nepenthes hybrida. Journal of Plant Research 106, 47–54.

Hooker JD. 1875. Address to the Department of Zoology and Botany. Report to the British Association for the Advancement of Science: Report of the Forty-Fourth Meeting, Belfast, 1874, 102–116.

Jentsch J. 1972. Enzymes from carnivorous plants (Nepenthes): isolation of the protease nepenthacin. FEBS Letters 21, 273–276.

Juniper BE, Burras J. 1962. How pitcher plants trap insects. New Scientist 13, 75–77.

Kitching RL. 2001. Food webs in phytotelma: ‘bottom-up’ and ‘top-down’ explanations for community structure. Annual Review of Entomology 46, 729–760.

Kok CJ, Van der Velde G. 1994. Decomposition and macroinvertebrate colonization of aquatic and terrestrial leaf material in alkaline and acid still water. Freshwater Biology 31, 65–75.

MacFarlane JM. 1893. Observations on pitchered insectivorous plants (part II). Annals of Botany 7, 403–441.

Merbach MA, Merbach DJ, Maschwitz U, Booth WE, Fiala B, Zizka G. 2002. Mass march of termites into the deadly trap. Nature 415, 37.

Merbach MA, Zizka G, Fiala B, Merbach D, Booth WE, Maschwitz U. 2007. Why a carnivorous plant cooperates with an ant-selective defense against pitcher-destructing weevils in the myrmecophytic pitcher plant Nepenthes bicalcarata Hook.f. Ecotropica 13, 45–56.

Mogi M, Yong HS. 1992. Aquatic arthropod communities in Nepenthes pitchers: the role of niche differentiation, aggregation, predation and competition in community organization. Oecologia 90, 172–184.

Moran JA. 1996. Pitcher dimorphism, prey composition and the mechanisms of prey attraction in the pitcher plant Nepenthes rafflesiana in Borneo. Journal of Ecology 84, 515–525.

Moran JA, Booth WE, Charles JK. 1999. Aspects of pitcher morphology and spectral characteristics of six Bornean Nepenthes pitcher plant species: implications for prey capture. Annals of Botany 83, 521–528.

Moran JA, Clarke CM, Hawkins BJ. 2003. From carnivore to detritivore? Isotopic evidence for leaf litter utilization by the tropical pitcher plant Nepenthes ampullaria. International Journal of Plant Sciences 164, 635–639.

Moran JA, Merbach MA, Livingston NJ, Clarke CM, Booth WE. 2001. Termite prey specialization in the pitcher plant Nepenthes albormarginata: evidence from stable isotope analysis. Annals of Botany 88, 307–311.

Moran JA, Moran AJ. 1998. Foliar reflectance and vector analysis reveal nutrient stress in prey-deprived pitcher plants (Nepenthes rafflesiana). International Journal of Plant Sciences 159, 996–1001.

Mouquet N, Daufresne T, Gray SM, Miller TE. 2008. Modelling the relationship between a pitcher plant (Sarracenia purpurea) and its phytotelma community: mutualism or parasitism? Functional Ecology 22, 728–737.

Ng PKL, Lim RP. 1987. The taxonomy and biology of the nepenthophilous freshwater sesamid crab, Geosesarma malayanum Ng and Lim, 1986 (Crustacea, Decapoda, Brachyura, Grapsidae) from Peninsular Malaysia. Malayan Nature Journal 41, 393–402.

Osukoya OO, Daud SD, Wimmer FL. 2008. Longevity, lignin content and construction cost of the assimilatory organs of Nepenthes species. Annals of Botany 102, 845–853.

Owen TP, Lennon KA, Santo MJ, Anderson AN. 1999. Pathways for nutrient transport in the pitchers of the carnivorous plant Nepenthes alata. Annals of Botany 4, 459–466.

Owen TP, Lennon KA. 1999. Structure and development of the pitchers from the carnivorous plant Nepenthes alata (Nepenthaceae). American Journal of Botany 86, 1382–1390.

Pant DD, Bhatnagar S. 1977. Morphological studies in Nepenthes (Nepenthaceae). Phytomorphology 27, 13–34.

Paradise CJ. 2004. Relationship of water and leaf litter variability to insects inhabiting treeholes. Journal of the North American Bearcubological Society 23, 793–805.

Pavlovič Ą, Singerová L, Demko V, Hudák J. 2009. Feeding enhances photosynthetic efficiency in the carnivorous pitcher plant Nepenthes tangalensis. Annals of Botany 104, 307–314.

Plachno BJ, Adamec L, Lichsteidl IK, Peroutka M, Adlassnig W, Vrba J. 2006. Fluorescence labelling of phosphatase activity in digestive glands of carnivorous plants. Plant Biology 8, 813–820.

Riedel M, Eichner A, Jetter R. 2003. Slippery surfaces of carnivorous plants: composition of epicuticular wax crystals in Nepenthes alata Blanco pitchers. Planta 218, 87–97.

Riedel M, Eichner A, Meimberg H, Jetter R. 2007. Chemical composition of epicuticular wax crystals on the slippery zone in pitchers of five Nepenthes species and hybrids. Planta 225, 1517–1534.

Rischer H, Hamm A, Bringmann G. 2002. Nepenthes insignis uses a C₃-portion of the carbon skeleton of L-alanine acquired via its carnivorous organs, to build up the allelochemical plumbagin. Phytochemistry 59, 603–609.

Schmidl J, Sulzer P, Kitching RL. 2008. The insect assemblage in water-filled tree-holes in a European temperate deciduous forest: community composition reflects structural, trophic and physicochemical factors. Hydrobiologia 596, 285–303.

Schulze W, Frommer WB, Ward JM. 1999. Transporters for ammonium, amino acids and peptides are expressed in pitchers of the carnivorous plant Nepenthes. The Plant Journal 17, 637–646.

Schulze W, Schulze ED, Pate JS, Gillson AN. 1997. The nitrogen supply from soils and insects during growth of the pitcher plants Nepenthes mirabilis, Cephalotus follicularis and Darlingtonia californica. Oecologia 112, 464–471.

Shabala L, Bowman J, Brown J, Ross T, McMeekin T, Shabala S. 2009a. Ion transport and osmotic adjustment in Nepenthes alata. Journal of Proteome Research 7, 809–816.
Escherichia coli in response to ionic and non-ionic osmotica. *Environmental Microbiology* **11**, 137–148.

Shabala L, McMeekin T, Shabala S. 2009b. Osmotic adjustment and requirement for sodium in marine protist thraustochytrid. *Environmental Microbiology* **11**, 1835–1843.

Shabala SN, Newman IA, Morris J. 1997. Oscillations in H⁺ and Ca²⁺ion fluxes around the elongation region of corn roots and effects of external pH. *Plant Physiology* **113**, 111–118.

Sokal RR, Rohlf FJ. 1981. *Biometry: the principles and practice of statistics in biological research*, 2nd edn. New York, USA: WH Freeman and Company.

Stephenson P, Hogan J. 2006. Cloning and characterization of a ribonuclease, a cysteine proteinase, and an aspartic proteinase from pitchers of the carnivorous plant *Nepenthes ventricosa* Blanco. *International Journal of Plant Sciences* **167**, 239–248.

Takahashi K, Athauda SBP, Matsumoto K, Rajapakshe S, Kuribayashi M, Kojima M, Kubomura-Yoshida N, Iwamatsu A, Shibata C, Inoue H. 2005. Nepenthesin, a unique member of a novel subfamily of aspartic proteinases: enzymatic and structural characteristics. *Current Protein and Peptide Science* **6**, 513–525.

Thornhill AH, Harper IS, Hallam ND. 2008. The development of the digestive glands and enzymes in the pitchers of three *Nepenthes* species: *N. alata*, *N. tobaica*, and *N. ventricosa* (Nepenthaceae). *International Journal of Plant Sciences* **169**, 615–624.

Vatnick I, Andrews J, Colombo M, Madhoun H, Rameswaran M, Brodkin MA. 2006. Acid exposure is an immune disruptor in adult *Rana pipiens*. *Environmental Toxicology and Chemistry* **25**, 199–202.

Verdonschot RCM, Febria CM, Williams DD. 2008. Fluxes of dissolved organic carbon, other nutrients and microbial communities in a water-filled treehole ecosystem. *Hydrobiologia* **596**, 17–30.

Yanoviak SP. 1999. Effects of leaf litter species on macroinvertebrate community properties and mosquito yield in neotropical tree hole microcosms. *Oecologia* **120**, 147–155.