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# Comparative anatomy of the assimilatory organs of *Nepenthes* species

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**Abstract.** There is a lack of data on comparative anatomy of the assimilatory organs of the enigmatic carnivorous *Nepenthes* species; the linkages between their leaf tissue anatomy and physico-chemical properties are also rarely considered. We examined the anatomy of the leaf (lamina) and its conjoint pitcher in five *Nepenthes* species (*Nepenthes ampullaria*, *N. bicalcarata*, *N. gracilis*, *N. hemsleyana* and *N. rafflesiana*). A *Nepenthes* leaf displays the usual cuticle–epidermis–hypodermis–palisade–spongy structure with ample stomata distribution for gas exchange. The conjoint pitcher has similar anatomy but lacks a palisade mesophyll layer, and its inner epidermal wall is endowed with digestive glands of three cell layers. A higher level of variation exists in the anatomy of the pitcher relative to the leaf. Both stomata and digestive glands, being similar in origin, display the usual negative log–log relationship between size and density. Across species, the mean size but not density of the glands varied across three readily identified zones of the digestive section of the pitcher. Leaf and pitcher thicknesses correlated ($P < 0.05$) with stomatal and digestive-gland sizes. Organ longevity, lignin content and construction cost negatively correlated with lower cuticle, epidermal and mesophyll dimensions, and positively so with stomatal and digestive-gland densities. In contrast, major nutrients of N, P, K, and total ash had minimal influence on anatomical size dimensions. It is likely that in *Nepenthes* leaf and its conjoint pitcher, both the protective and physiological tissues drive anatomical differences and organ functions. The observed bivariate relationships between the anatomical traits also fit into the worldwide leaf economy spectrum.

**Additional keywords:** Brunei, carnivorous plants, digestive glands, leaf anatomy, South-east Asia, stomata, trait variation.

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## Introduction

*Nepenthes* is a unique, monotypic group of carnivorous plant species, majority of which are evergreen, woody climbers or scrambling shrubs with shallow roots growing in sunny but nutrient-poor habitats. Such plants compensate for the lack of soil nutrients (mainly nitrogen; Osunkoya *et al*. 2007) by using a modified part of their leaf (the pitcher) to catch prey, digest and absorb nutrients from it (Clarke and Moran 2016). *Nepenthes* distribution is confined to the Madagascar–Southeast Asia–Northern Australia region, with 35–40 of the ~140 known species endemic to the Island of Borneo (Clarke and Lee 2004; Clarke and Moran 2016).

The past two decades have seen an upsurge on reported studies on members of this genus, focussing on pitcher microscopy, spectral quality and fluid content and properties (Owen and Lennon 1999; Owen *et al*. 1999; Bazile *et al*. 2015; Kanokratana *et al*. 2016), captured prey identity, rates and mechanisms (Moran 1996; Moran *et al*. 2001; Bohn and Federle 2004; Bauer *et al*. 2008; Bazile *et al*. 2015), pathway of nutrient exchange and uptake (Owen and Lennon 1999; Moran and Clarke 2010; Moran *et al*. 2010; Chen *et al*. 2016), identification of trans-membrane transporters and mode of action within the pitcher (Schulze *et al*. 1999; Moran *et al*. 2010), physiology and assimilation (Pavlovič *et al*. 2007; Ellison and Adamec 2011), and cost–benefit analyses of carnivory (prey capture and digestion) relative to autotrophy, i.e. photosynthesis (Ellison 2006; Osunkoya *et al*. 2007, 2008; Pavlovič and Saganová 2015). However, there is still a dearth of data on comparative anatomy of *Nepenthes* assimilatory organs (leaf vs the pitcher but see Pavlovič *et al*. 2007; Moran *et al*. 2010). This is despite the usefulness of such a study in gaining a better understanding of within- and across-species variation in the eco-physiological–chemical traits highlighted above in relation to organ function and fitness, and, hence, the reason for the present study. In addition, linking the anatomy of the assimilatory organs with previously published data on longevity and physico-chemical properties of the same *Nepenthes* species (Osunkoya *et al*. 2007, 2008) allows us to explore further how this enigmatic carnivorous plant group fits into the worldwide leaf economy spectrum (LES) of Wright *et al*. (2004). The LES

represents a unified axis of leaf-trait variation, e.g. that leaf mass per unit area (LMA) is a central variable of the trait network that is strongly correlated with other traits across species (Sack *et al*. 2013). The LES rarely considers the linkages between leaf internal anatomy, physiological performance, organ life-span or construction cost (Osunkoya *et al*. 2014). By focussing on a single genus and, hence, minimising the influence of phylogeny, the study aimed to address the following questions:

(1) will the pattern of variation in anatomical trait be similar, irrespective of assimilatory organ type, especially given that one (the pitcher) is a derivative of the other (the leaf; Thornhill *et al*. 2008);
(2) what are the patterns of bivariate relationships between anatomical traits (e.g. leaf mesophyll size or stomatal density vs leaf thickness or mass per unit area) within and across species, and are their linkages with *Nepenthes* leaf and pitcher physico-chemical properties and life-span; and
(3) are allocation patterns in *Nepenthes* leaf and pitcher anatomy similar to those exhibited by other plant groups, and, hence, in line with the LES strategy?

## Materials and methods

### *Study species and habitat*

The *Nepenthes* species used in the present study occur sympatrically and abundantly in sunny habitats of lowland heath (‘kerangas’) and peat-swamp as well as at the margins of secondary forests and open scrubland (‘padang’) of Brunei, Borneo. They have also been the focus of past studies (Osunkoya *et al*. 2007, 2008). Samplings were undertaken in the above-mentioned habitats in the Kuala Belait district of Brunei (48340N, 1148250E). The following two organs of assimilation are recognised in this carnivorous plant: (1) the main photosynthetic flat base (henceforth called the leaf); and (2) the modified, non- or limited-photosynthetic, jug-like structure at the end of the leaf tendril (henceforth called the pitcher), which is a passive trap that can catch and digest prey and absorb the breakdown products. The pitcher is a modified epiascidiate leaf blade, in which the adaxial surface curls around and fuses to form the inner wall of the pitcher (Owen and Lennon 1999). *Nepenthes* plants produce two types of pitchers, known as terrestrial (ovoid in shape) and aerial (cylindrical in shape) forms; they differ in their placement relative to the ground but not in their physico-chemical properties (Daud 2004).

A typical *Nepenthes* pitcher shows distinct zonation, with three functional properties, namely, the attractive (lid and the rim (peristome)) zone, the conductive or waxy (upper, inside surface of the cup) zone, and the digestive (fluid-filled base) zone; Owen and Lennon 1999; Moran *et al*. 2010). In the present study, we focussed on the waxy and the digestive zones of the pitcher cup. The ‘conductive or waxy zone’ comprises the upper inside surface of the pitcher and is often characterised by lunate cells and epicuticular waxes (Gorb and Gorb 2011), both of which function to deny traction and conduct the prey downward into the third (digestive) zone (Bauer *et al.* 2008). The digestive zone is the fluid-filled base of the pitcher, the inner walls of which are lined with digestive glands (Owen and Lennon 1999; Gorb *et al*. 2004; Thornhill *et al*. 2008). These glands, arising from the epidermis, undergo an ontogenic shift in function, switching from secretion (e.g. proton ($H^+$) ions) when the pitchers are immature (Schulze *et al*. 1999; An *et al*. 2001) to absorption of digested products from captured preys, using identifiable transporters of $NH_4^+$, amino acids and peptides, once the pitchers are fully formed and open (Owen and Lennon 1999; Owen *et al*. 1999; Schulze *et al*. 1999; Takeuchi *et al*. 2011). The pitcher fluid itself possesses viscoelastic qualities that contribute to prey retention to a greater degree than does the waxy zone (Bauer *et al*. 2008; Bazile *et al*. 2015).

To complement previous reports on physico-chemical properties of *Nepenthes* assimilatory organs (see Osunkoya *et al*. 2007, 2008), we studied the anatomy of the leaf and its conjoint pitchers of five distinct *Nepenthes* species (*Nepenthes ampullaria* Jack, *N. bicalcarata* Hook. f., *N. gracilis* Korth, *N. rafflesiana* Jack and *N. hemsleyana* Macfari; the last two species were previously considered as part of the *N. rafflesiana* complex, and were then referred to as *N. rafflesiana* Jack var. typical, and var. elongata respectively; Scharmann and Grafe 2013). Detailed description of the collection sites, morphology of the leaf and pitcher samples, and subsequent morphometric measurements taken (i.e. thickness, area, dry mass, mass per unit area, longevity and chemistry (N, P, K and total ash) have been fully reported in Osunkoya *et al*. (2007, 2008). Briefly, plant materials were collected from the sunny heath and peat swamp habitats at four to six localities in Brunei, northern Borneo, in Southeast Asia. The upper assimilatory organs (leaf and its conjoint pitcher cup) were collected from 4–6 individuals per species, with the exception of those of *N. ampullaria*, which were collected from the forest floor as occurrence of aerial pitchers are rare (Clarke and Lee 2004). Three leaves and their conjoint pitchers were sampled per individual plant, and thus the anatomical trait measurements were based on a total of at least $3 \times 4 = 12$ leaves per species.

### *Sample preparation and image analyses*

Leaves and pitchers collected were put in labelled, sealed clear plastic bags that contained damp paper towel and kept in an air-conditioned laboratory overnight to allow for rehydration before processing. Once the samples were rehydrated, the leaf and the attached pitcher were separated by cutting at the middle of the U-shaped part of the tendril. Leaf thickness was measured between the major veins with a digital micro-meter screw gauge in four places per leaf. The pitcher thickness was measured between the major veins (ribs) in six places per pitcher, including three in the waxy zone and three in the digestive zone. Leaves were cut with a scalpel at areas between the major veins and midrib, and then soaked in a Petri dish containing distilled water to prevent drying. These were then sliced into thin strips with a Reichert sliding microtome to produce sections. For the pitchers, the jug-like (cup) part was first sliced into two parts, namely, the waxy zone and the digestive zone (the latter is characterised by the evident glands in the inner surface of the pitcher wall), with the exception of *N. ampullaria* and *N. bicalcarata*, each of which lack a distinctive waxy zone (see also Moran *et al*. 2010). Sections from the digestive zone were further cut into three equal segments, namely, upper, middle and lower digestive zones; thereafter, cutting was at areas between the major veins, and again immediately soaked

in Petri dishes filled with distilled water. Sections were cut with a sliding microtome, then placed on a slide and viewed under a light microscope fitted with a calibrated ocular micrometer for measurement of the thickness of various anatomical tissues. For each image, four measurements were made of the thickness of the entire leaf section, upper and lower cuticle, epidermis and, if present, hypodermis and palisade (leaf only), and spongy mesophyll layers, avoiding the major and minor veins. The ratios of the tissue layers to the total leaf or pitcher thickness, and the ratio of palisade to spongy mesophyll (for leaf only) were also calculated. Three sections of each individual leaf and pitcher anatomy were photographed with a Nikon Eclipse 50i light microscope fitted with Nikon DS-Fi1 camera head (Fig. S1, available as Supplementary material to this paper).

Epidermal impressions of lower leaf surfaces were taken using clear nail polish, which was allowed to set before removal with clear tape and transferred onto a slide. Images were captured using the same equipment mentioned above. Three views were captured of each impression, avoiding midribs and margins. Leaf stomata (under ×400 magnification) and pitcher inner-wall digestive glands (under ×10 magnification) were counted using the quadrat-sampling method and values were converted into density; stomatal guard (leaf) and gland-cell (pitcher) dimensions (length, width, including stomatal pore size and gland depth respectively) were also measured. The length of the guard cells was used to calculate stomatal pore-area index (SPI; Sack *et al*. 2013), using the formula $Sd \times GCL^2$ (Sd being the stomatal density, and GCL being the mean guard-cell length; the latter trait was henceforth referred to as stomatal length, SL). Similar approach was used in estimating digestive gland-area index (DGI) as density × sectional length$^2$ of the gland.

### *Statistical analysis*

Data were analysed using SPSS software (version 22.0, IBM Corporation, USA). The variables used in the study were thickness of the leaves and pitchers and their associated tissue dimensions, including stomatal density and size for the leaf blade, and gland density and size for the pitchers. Data were checked for assumptions underlying parametric tests and normality of the data by using graphical approach (e.g. frequency distribution), and were considered to be satisfactory. A two-way ANOVA using generalised linear model was implemented, with species (5 levels) and organ type (2 levels) as fixed factors, replicate readings within a given organ as random factor, and measured leaf and pitcher traits as the response variables. Within the pitcher organ itself, a two-way ANOVA (species, pitcher cup zone, and their interactions) was also performed to test for

**Table 1. Summary results of two-way ANOVA of effects of species, organ type (leaf vs pitcher cup) and their interactions on anatomical traits measured**

Significant effects are in bold. Where there are significant differences between organs, in all cases, leaf < pitcher dimensions, except for the upper cuticle thickness of the leaf and outer cuticle thickness of the pitcher, in which leaf > pitcher

| Leaf and pitcher trait | Species × organ (leaf vs pitcher) type effect | | | | | |
|---|---|---|---|---|---|---|
| | Species | | Organ | | Species × organ | |
| | *F*-ratio (d.f. = 4, 8) | *P*-value | *F*-ratio (d.f. = 1, 2) | *P*-value | *F*-ratio (d.f. = 4, 8) | *P*-value |
| Organ saturated wet mass | 6.23 | **0.040** | 171.62 | **0.006** | 8.62 | **0.005** |
| Organ thickness | 29.78 | **0.001** | 13.95 | 0.060 | 5.61 | **0.020** |
| Upper or outer cuticle[A] | 2.74 | 0.105 | 35.37 | **0.027** | 2.63 | 0.114 |
| Upper or inner epidermis[A] | 5.78 | **0.017** | 90.42 | **0.001** | 1.62 | 0.250 |
| Upper hypodermis | 1.21 | 0.378 | – | – | – | – |
| Palisade mesophyll | 17.87 | **0.001** | – | – | – | – |
| Spongy mesophyll | 13.75 | **0.001** | – | – | – | – |
| Palisade : spongy ratio | 2.74 | 0.090 | – | – | – | – |
| Total mesophyll | 13.96 | **0.001** | 72.54 | **0.014** | 7.59 | **0.008** |
| Lower hypodermis | 1.81 | 0.249 | – | – | – | – |
| Lower or inner epidermis[A] | 0.821 | 0.547 | 0.95 | 0.433 | 1.14 | 0.410 |
| Lower or inner cuticle[A] | 17.39 | **0.003** | 29.12 | **0.030** | 2.89 | **0.090** |
| Leaf | | | | | | |
| Stomatal density | 4.31 | **0.040** | – | – | – | – |
| Stomatal length | 4.58 | **0.030** | – | – | – | – |
| Stomatal width | 14.52 | **0.001** | – | – | – | – |
| Aperture pore length | 5.89 | **0.016** | – | – | – | – |
| Pitcher | | | | | | |
| Gland density | 11.12 | **0.002** | – | – | – | – |
| Gland length | 18.40 | **0.001** | – | – | – | – |
| Gland depth | 2.15 | 0.165 | – | – | – | – |
| Gland head | 3.53 | 0.450 | – | – | – | – |
| Gland, 2nd layer | 9.987 | **0.015** | – | – | – | – |
| Gland, 3rd layer | 11.24 | **0.001** | – | – | – | – |
| Stomatal and gland-area index | 11.19 | **0.002** | 187.55 | **0.005** | 12.57 | **0.002** |

[A]Leaf and pitcher, respectively.

differences in trait mean values between the zones (e.g. waxy vs digestive sections) of the pitcher cup. Mean values of the trait measured were deemed significantly different if $P \leq 0.05$. Regressions and correlations were performed to determine the relationship between many of the traits measured both within and across species (e.g. stomatal density and dimensions vs leaf thickness, or the relationship between the gland density or dimension and pitcher thickness). Across the five species, we also explored the linkages between anatomical traits and whole leaf and pitcher physico-chemical properties (N, P, K, lignin, total ash, organ construction cost and longevity) that have been reported in earlier publications and of the same composite samples (Osunkoya *et al*. 2007, 2008). Finally, ordination technique of principal component analyses (PCA) was performed on species mean values to generate major axes that will capture and summarise significant variation in the trait dataset, and to see which anatomical traits clearly separate the leaf from the pitcher. The extent of correlations between the generated major PCA axes and organ physico-chemical properties were also explored.

## Results

### *Gross anatomy*

The leaf anatomy of *Nepenthes* consists of the cuticle, upper epidermis (with one or two hypodermal cell layers containing crystals), two or three layers of columnar palisade mesophyll cells, about four to six layers of scattered spongy mesophyll cells, a lower hypodermis and epidermis, and the cuticle (Fig. S1). Cells of the conjoint pitcher organ were less differentiated than were those observed in their leaves. The basic components of the pitcher wall are cuticle (inner and outer), epidermis (inner and outer) and an undifferentiated mesophyll layer that are often lacking in air spaces. The two vertical zones (the waxy and the digestive sections) of the pitcher cup are distinctive to the naked eye, except for *N. ampullaria* and *N. bicalcarata*. Many parts of the inner walls of the digestive zones are lined with secretory–digestive glands, which can be seen as outgrowth of the epidermis. The digestive glands are seen as ovoid-spherical structure of three cell layers, protected by hooded ridges (Fig. S1). The first layer of a typical gland (called the glandular head; *sensu* Owen *et al*. 1999) consists of columnar cells characterised by thick outer wall (cutin); the second and the third layers varied in shape with relatively thinner walls (Fig. S1; see Gorb *et al*. 2004 for greater details on the gland structure and properties).

### Nepenthes *leaf versus pitcher*

Mean values of the anatomical traits are presented in Table S1, available as Supplementary material to this paper. Majority of the leaf and pitcher anatomical traits examined varied significantly ($P < 0.05$) among the five *Nepenthes* species investigated, except for thickness of the cuticle (upper (for the leaf) and outer (for the pitcher)), and upper and lower hypodermis of the leaf (Table 1). Leaf stomatal traits varied across species more in terms of guard-cell width ($F_{2,4} = 14.52$, $P < 0.001$) than stomatal aperture ($F_{2,4} = 5.89$, $P < 0.02$), length ($F_{2,4} = 4.58$, $P < 0.03$) or density ($F_{2,4} = 4.31$, $P < 0.04$). For the pitcher digestive glands, significant variation across species occurred both for the density ($F_{2,4} = 11.22$, $P < 0.002$) and the length ($F_{2,4} = 18.40$, $P < 0.001$), whereas depth remained unchanged ($F_{2,4} = 2.15$, $P = 0.165$; range 0.044–0.082 mm). The dimensionless indices of SPI and DGI varied among species (especially for the pitcher, where highest values were attained in *N. bicalcarata* (0.811), moderate values for *N. rafflesiana* (0.462) and *N. hemsleyana* (0.455), and lowest values for *N. ampullaria* (0.287) and *N. gracilis* (0.267)).

Standardising and converting the size measurements to ratios, the allocation patterns of the tissue anatomy remained fairly constant across species for both the leaf and pitcher, with the exception of outer cuticle thickness of the pitcher ($F_{4,8} = 3.81$, $P < 0.05$), in which *N. bicalcarata* exhibited the highest investment (0.73%). For the leaf, the average proportional anatomical dimensions were as follows: upper cuticle (1.8%), upper epidermis (2.3%), upper hypodermis

**Table 2. Summary results of two-way ANOVA of effects of species, pitcher zone (digestive vs waxy) position and their interactions on anatomical traits measured**

Significant effects are in bold

| Pitcher trait | Species × pitcher cup (waxy vs digestive) zone effect | | | | | |
|---|---|---|---|---|---|---|
| | Species | | Pitcher cup zone | | Species × pitcher cup zone | |
| | *F*-ratio | *P*-value | *F*-ratio | *P*-value | *F*-ratio | *P*-value |
| Pitcher cup | | | | | | |
| Total thickness | 14.82 | **0.001** | 38.73 | **0.025** | 5.41 | **0.021** |
| Outer cuticle | 1.42 | 0.310 | 2.17 | 0.279 | 0.82 | 0.547 |
| Outer epidermis | 6.31 | **0.015** | 2.92 | 0.230 | 0.66 | 0.728 |
| Mesophyll | 12.18 | **0.002** | 430.64 | **0.002** | 3.59 | 0.059 |
| Inner epidermis | 2.96 | 0.090 | 6.52 | 0.125 | 1.34 | 0.336 |
| Inner cuticle | 8.33 | **0.006** | 31.71 | **0.030** | 80.18 | **0.001** |
| Gland density | 11.22 | **0.002** | 3.55 | 0.130 | 17.77 | **0.001** |
| Gland length | 18.40 | **0.001** | 90.35 | **0.001** | 5.74 | **0.001** |

**Fig. 1.** Box plot (indicating 50% of the observation and the median line) of changes in size and density of digestive glands along three digestive zones of the pitcher cup in five *Nepenthes* species: (*a*, *g*) overall mean values across the five species investigated; (*b–f*, *h–l*) individual species data. Significant zonation trends are indicated: ***$P < 0.001$; **$P < 0.02$; NS, not significant.

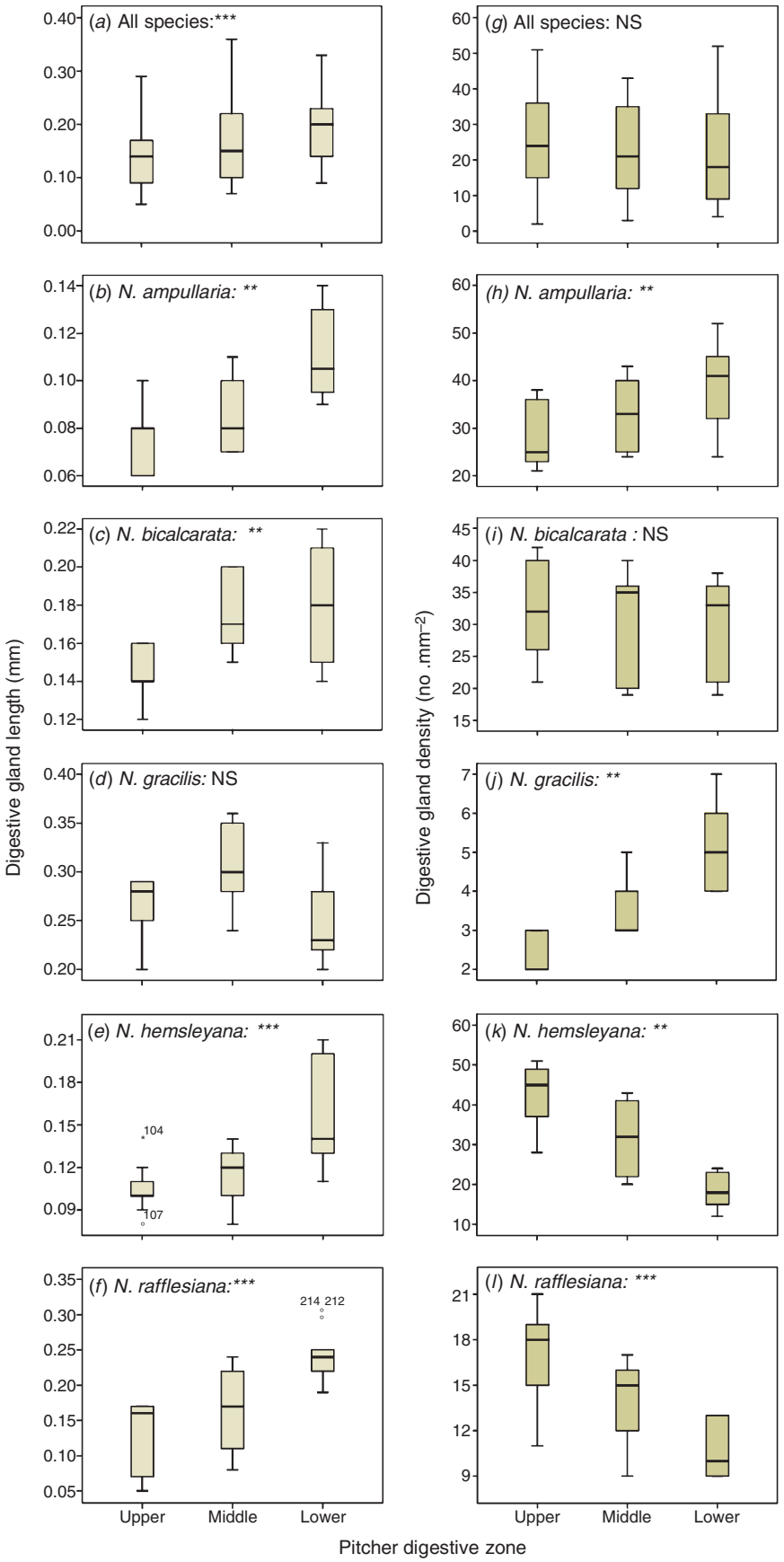


**Fig. 1.** (*continued*)

(9.6%), palisade mesophyll (24.9%), spongy mesophyll (49.5%), lower hypodermis (7.9%) and lower epidermis (3.2%). For the pitcher, the average proportions were as follows: outer cuticle (0.5%), outer epidermis (2.8%), spongy mesophyll (92.4%), inner epidermis (3.3%) and inner cuticle (1.3%). Thus, across species, 86–94% of the pitcher dimension constitutes the mesophyll section, but only 30–60% for the leaf. The leaf and pitcher are significantly different in all anatomical dimensions, except for lower epidermis of the leaf versus inner epidermis of their conjoint pitcher ($F_{1,2}=0.95$, $P=0.433$; Table 1). In all cases where significant trait differences were detected between organs, the leaf values were always lower than those of the pitcher, except for the leaf upper epidermal layer and the pitcher inner epidermal layer in which the pitcher value > the leaf value.

### *Pitcher waxy and digestive zones*

Within the pitcher and after discounting species differences, the thickness of the waxy versus digestive zones in terms of dimensions of their outer cuticle and epidermal layers (outer and inner) were not significantly different (Table 2). A significant difference was detected for their mesophyll layers ($F_{1,2}=430.64$, $P=0.002$), with the digestive zone having a higher mesophyll thickness (0.58 µmm ± 0.14) than that of the waxy layer (0.410 µmm ± 0.12). This mesophyll size difference between the waxy and digestive zones was, in a decreasing order, as follows: *N. rafflesiana* > *N. hemsleyana* >> *N. gracilis* (pitchers of *N. ampullaria* and *N. bicalcarata* lack distinctive waxy zone, and, hence, digestive versus waxy comparison could not be made).

The digestive-gland dimensions varied among species and their cell layers (Table 2), with the gland head having the largest dimension (mean ±s.e.; head length: 19.12 ± 1.52 µmm; second layer: 13.02 ± 0.81 µmm; 3rd layer: 9.04 ± 0.91 µmm). Among species, gland size was, in a decreasing order, as follows: *N. gracilis* > *N. rafflesiana* > *N. bicalcarata* ≥ *N. hemsleyana* > *N. ampullaria* (Figs 1, 2*a*), whereas gland density was highest in *N. hemsleyana*, *N. bicalcarata* and *N. ampullaria* (mean value: 30.78–33.33 mm$^{-2}$; no significant difference was detected in gland density among these three species), intermediate in *N. rafflesiana* (mean: 13.89 mm$^{-2}$) and lowest in *N. gracilis* (mean: 3.63 mm$^{-2}$).

Overall, moving from the upper to the lower portion of the pitcher digestive zone, we found a significant increase in gland size ($F_{2,4}=90.35$, $P<0.0001$), but not in gland density ($F_{2,4}=3.56$, $P=0.13$; Fig. 1). Gland size increased significantly and linearly from the upper to the lower portion of the pitcher digestive zones for all species tested, except for *N. gracilis.* The interactions between species and digestive-zone position for these traits were also significant (gland density: $F_{8,16}=17.74$, $P<0.0001$; gland size: $F_{8,16}=5.74$, $P=0.001$), suggesting that patterns were not consistent across species, especially for gland density (Fig. 1). Gland density of *N. bicalcarata* was fairly consistent along the pitcher digestive zones (Fig. 1*i*). For *N. ampullaria* and *N. gracilis*, gland density showed an increasing value from the upper to the lower portion of the pitcher (Fig. 1*h*, *j*), whereas the opposite trend was observed for *N. hemsleyana* and *N. rafflesiana* (Fig. 1*k*, *l*). These inconsistencies in the direction of trends across the five species studied contributed to an overall lack of digestive-zone effect on gland density.

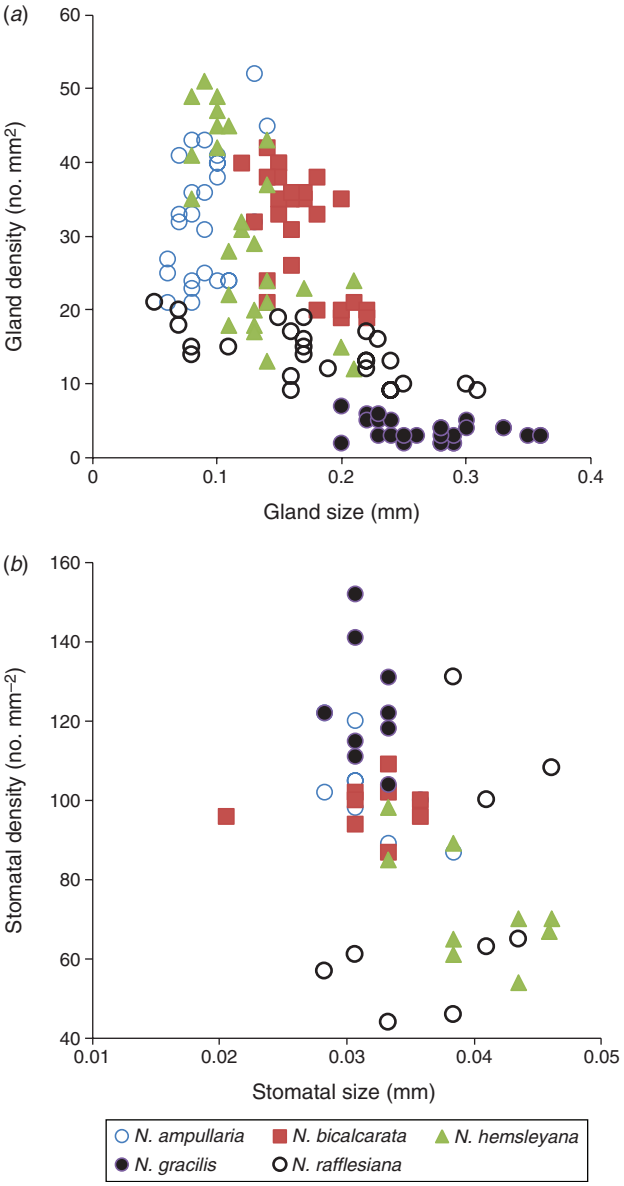


**Fig. 2.** Relationship between size and density for (*a*) digestive glands and (*b*) stomata in five *Nepenthes* species.

### *Correlations of anatomical traits*

Across-species matrix of correlation values among measured anatomical traits can be found in Table S2, available as Supplementary material to this paper; many of the bivariate relationships are not addressed here because these trends have been fully explored in the literature (Edwards *et al.* 2000; Brodribb *et al.* 2013; Sack *et al.* 2013; Kröber *et al.* 2015). Rather, we concentrated on exploring relationships and linkages between stomata and/or digestive glands and other anatomical traits in the leaf and its conjoint pitcher. A negative

relationship existed overall, and within four of five species, between gland density and size (Fig. 2*a*), with *N. ampullaria* being the exception. The same negative trend was observed across species for stomatal density versus size (however, within species, only two of five species showed the trend (*N. ampullaria* and *N. hemsleyana*), and one species (*N. rafflesiana*) indicated even a positive relationship; Fig. 2*b*). Thus, overall the dynamics of this negative relationship (log–log) was tighter for digestive glands ($R^2 = 0.567$, $P < 0.0001$; $n = 135$) than for stomata ($R^2 = 0.18$, $P = 0.002$; $n = 60$).

A significant positive relationship was detected between leaf thickness and stomatal size, whereas an opposite (inverse) trend occurred for leaf thickness versus stomatal density (Fig. 3*a*, *b*). Similar significant trends were difficult to detect for digestive-gland dimensions or density versus pitcher thickness, primarily because of the dynamics of the size dimensions within *N. gracilis* (see Fig. 3*c*, *d*). For the negative relationship between stomatal density versus leaf thickness, partial correlation and regression analyses (Tables 3, 4) indicated that the main drivers ($P < 0.05$) were the upper and lower cuticle thicknesses

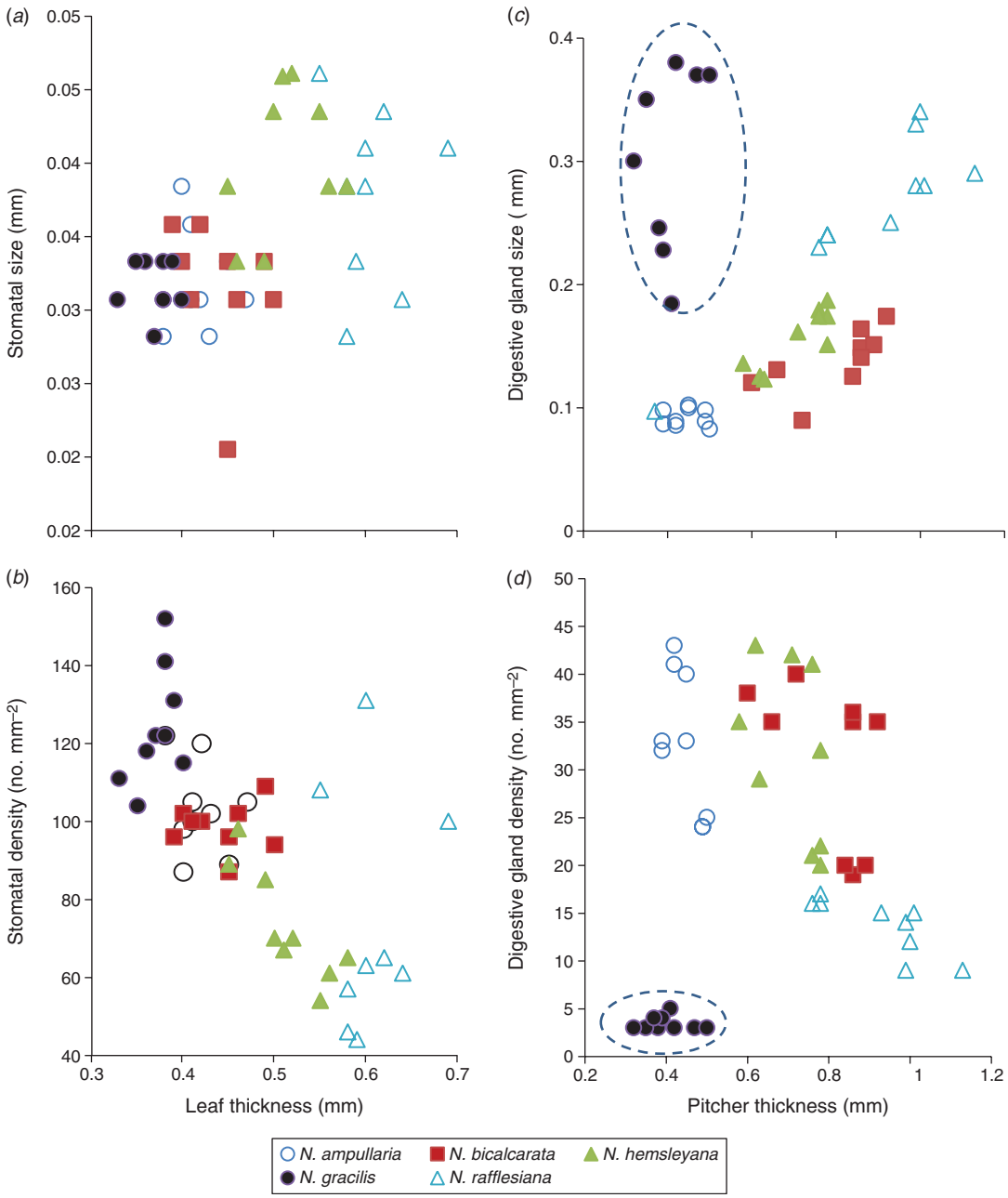


**Fig. 3.** Relationship across five *Nepenthes* species between (*a*) leaf thickness and size of stomata, (*b*) leaf thickness and density of stomata, (*c*) pitcher cup thickness and size of digestive glands and (*d*) pitcher cup thickness and density of digestive glands. Note that the data for *N. gracilis* digestive glands (faint broken loops) are not fitting the general trend.

**Table 3. Summary results of partial correlation and regression analyses to account for drivers of the significant relationship between leaf thickness and stomatal traits**

Intraspecific data were used (with $n = 36–45$), and the same trend occurred at the interspecific level. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; n.s., not significant

| Parameter | Stomatal relationship with leaf thickness | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Stomatal density vs leaf thickness | | | Stomatal size vs leaf thickness | | | Stomatal index vs leaf thickness | | |
| | $r$ | Change in $R^2$ (%) | Significance of change in $R^2$ | $r$ | Change in $R^2$ (%) | Significance of change in $R^2$ | $r$ | Change in $R^2$ value (%) | Significance of change in $R^2$ |
| Initial model fit | –0.658 | – | ** | 0.493 | – | ** | 0.042 | – | n.s. |
| Trait effect removed | | | | | | | | | |
| Upper cuticle | –0.345 | 31.30 | *** | 0.544 | 5.10 | * | 0.411 | 36.90 | *** |
| Upper epidermis | –0.664 | –0.60 | n.s. | 0.499 | 0.60 | n.s. | –0.069 | –11.10 | n.s. |
| Upper hypodermis | –0.650 | 0.80 | n.s. | 0.496 | 0.30 | n.s. | –0.089 | –13.10 | n.s. |
| Palisade mesophyll | –0.628 | 3.00 | n.s. | 0.376 | –11.70 | *** | –0.385 | –42.70 | ** |
| Spongy mesophyll | –0.601 | 5.70 | * | 0.458 | –3.50 | n.s. | 0.243 | 20.10 | n.s. |
| Lower hypodermis | –0.658 | 0.00 | n.s. | 0.493 | 0.00 | n.s. | –0.042 | –8.40 | n.s. |
| Lower epidermis | –0.659 | –0.10 | n.s. | 0.492 | –0.10 | n.s. | –0.107 | –14.90 | n.s. |
| Lower cuticle | –0.577 | 8.10 | ** | 0.426 | –6.70 | ** | 0.149 | 10.70 | n.s. |

**Table 4. Summary results of partial correlation and regression analyses to account for drivers of the significant relationship between leaf thickness and digestive-gland traits**

Intraspecific data were used (with $n = 36–45$), and the same trend occurred at the interspecific level. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; n.s., not significant

| Parameter | Digestive-gland relationship with pitcher thickness | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Gland density vs pitcher thickness | | | Gland size vs pitcher thickness | | | Gland index vs pitcher thickness | | |
| | $r$ | Change in $R^2$ (%) | Significance of change in $R^2$ | $r$ | Change in $R^2$ (%) | Significance of change in $R^2$ | $r$ | Change in $R^2$ (%) | Significance of change in $R^2$ |
| Initial model fit | –0.68 | – | ** | 0.826 | – | *** | 0.292 | – | * |
| Trait effect removed | | | | | | | | | |
| Outer cuticle | –0.662 | 1.80 | n.s. | 0.821 | –0.50 | n.s. | –0.496 | –78.80 | ** |
| Outer epidermis | –0.648 | 3.20 | n.s. | 0.827 | 0.10 | n.s. | 0.317 | 2.50 | n.s. |
| Spongy mesophyll | –0.483 | 19.70 | *** | 0.624 | –20.20 | *** | 0.319 | 2.70 | n.s. |
| Inner epidermis | –0.575 | 10.50 | *** | 0.800 | –2.60 | n.s. | 0.596 | 30.40 | ** |
| Inner cuticle | –0.650 | 3.00 | n.s. | 0.833 | 0.70 | n.s. | 0.299 | 0.70 | n.s. |

(correlation coefficients reduced from $r = –0.66$ to $r = –0.345$ and $r = –0.577$ respectively), whereas the positive trend of stomatal length with leaf thickness was chiefly driven by the leaf palisade thickness (correlation coefficient reduced from $r = 0.49$ to $r = 0.38$) and, to a lesser extent, by the cuticle thicknesses. The negative trend in the conjoint pitcher between gland density and cup thickness (removing *N. gracilis* from the dataset) was driven solely by size of the mesophyll and inner epidermis (correlation coefficient reduced from $r = –0.68$ to $r = –0.48$ and $r = –0.58$ respectively); the positive trend of the gland size–pitcher cup thickness relationship was again primarily driven by mesophyll size (correlation coefficient reduced from $r = 0.83$ to $r = 0.62$; Table 4). No trend was discernible between the dimensionless index of SPI and leaf thickness, but the relationship became significantly positive or negative once the effects of upper cuticle or palisade size respectively, were discounted (Table 3). For the dimensionless index of DGI, a positive relationship existed with pitcher thickness ($r = –0.29$, $P = 0.05$; $n = 42$), with the main drivers of the trend being outer cuticle and inner epidermal sizes. Performing the analyses by using mean values for each species resulted in the same trends.

Table 5 shows correlation coefficients between anatomical and species-level physico-chemical traits reported in Osunkoya *et al*. (2007, 2008). It is interesting to note that across species, lignin content, construction cost (mass based) and organ longevity are mostly negatively correlated ($P < 0.05$) with epidermal dimensions and mesophyll thickness. None of the major nutrients of N, P and K or total ash correlated with size dimensions of the anatomical traits, although K concentration varied negatively with whole-organ dimension and palisade size. LMA was also a poor predictor of tissue dimensions. Gland and stomatal density, but not size dimension, correlated positively with organ lignin concentration, longevity and construction $cost_{mass\text{-}based}$ (Table 5, Fig. 4*a*, *c*, *d*). The dimensionless indexes of SPI and DGI correlated negatively only with lignin concentration ($r = –0.63$, $P = 0.05$; $n = 10$; Table 5, Fig. 4*b*).

### *Ordination of species and assimilatory organs*

Ordination using PCA confirmed most of the trends observed with univariate analyses, and indicated that, indeed, the leaves and their conjoint pitchers are different in measured anatomical traits, with a greater variation in the pitcher organ (Fig. 5). Axes I and II of the ordination captured 67.5% and 16% respectively, of the variation in the dataset, with Axis I separating the two assimilatory organs. The main drivers on Axis I, in a decreasing

**Table 5. Across species bivariate (Pearson) correlation values between physico-chemical (reported in Osunkoya *et al.* 2007, 2008) and anatomical traits of *Nepenthes* leaf and pitcher cup**

N, total nitrogen; P, total phosphorus; K, total potassium. LMA, leaf mass per unit area; $CC_{mass}$, organ (leaf or pitcher) construction cost per unit mass; $CC_{area}$, organ construction cost on a unit area; SDI, stomatal pore area index; GDI, digestive gland-area index. Significant trends are highlighted in bold. $n = 10$ for each case, made up of data for five species × two organ types, except (i) organ longevity in which $n = 8$ because of lack of data for *N. rafflesiana* and (ii) palisade and hypodermal layers which are lacking in the pitcher organ, and, hence, $n = 5$ for bivariate relationships of these traits. *$P < 0.05$; **$P < 0.02$

| Leaf and pitcher anatomical trait | Leaf and pitcher physico-chemical traits | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | N | P | K | Total ash | Lignin | LMA | $CC_{mass}$ | $CC_{area}$ | Organ longevity | Leaf area | SDI and GDI |
| Organ thickness | –0.052 | 0.477 | **–0.641*** | 0.293 | –0.589 | 0.448 | **–0.683*** | 0.279 | –0.282 | 0.256 | **0.643*** |
| Upper cuticle | 0.290 | 0.084 | –0.515 | –0.550 | 0.536 | 0.625 | 0.436 | **0.657*** | 0.655 | 0.083 | –0.489 |
| Upper epidermis | 0.036 | 0.380 | –0.304 | 0.548 | **–0.764*** | –0.270 | **–0.852**** | –0.423 | –0.562 | –0.047 | 0.282 |
| Upper hypodermis | –0.150 | 0.408 | –0.588 | –0.019 | –0.337 | 0.718 | –0.261 | 0.619 | –0.583 | 0.476 | **–0.950*** |
| Palisade mesophyll | 0.278 | 0.708 | **–0.907*** | –0.096 | –0.252 | 0.553 | **–0.966**** | 0.245 | –0.045 | –0.225 | –0.044 |
| Spongy mesophyll | –0.287 | 0.584 | –0.217 | 0.416 | **–0.777**** | 0.093 | **–0.739*** | 0.356 | –0.591 | 0.072 | **–0.877**** |
| Total mesophyll | –0.124 | 0.431 | –0.490 | 0.365 | **–0.670*** | 0.312 | **–0.725*** | 0.148 | –0.396 | 0.287 | **0.766**** |
| Lower hypodermis | –0.196 | 0.360 | –0.713 | –0.627 | –0.832 | 0.488 | –0.616 | 0.352 | –0.736 | 0.131 | –0.084 |
| Lower epidermis | 0.066 | 0.368 | –0.531 | 0.363 | –0.220 | 0.478 | –0.329 | 0.357 | 0.129 | 0.212 | 0.413 |
| Lower cuticle | –0.622 | –0.136 | 0.284 | 0.299 | **–0.836**** | –0.107 | –0.522 | –0.172 | **–0.726*** | 0.384 | **0.646*** |
| Stomatal and gland density | 0.325 | –0.173 | –0.171 | –0.366 | **0.666*** | 0.427 | **0.634*** | 0.497 | **0.898**** | 0.021 | –0.505 |
| Stomatal and gland width | –0.181 | 0.139 | 0.248 | 0.252 | –0.318 | –0.386 | –0.354 | –0.417 | –0.596 | –0.067 | 0.516 |
| Stomatal and gland length | –0.163 | 0.163 | 0.268 | 0.292 | –0.349 | –0.413 | –0.377 | –0.449 | –0.619 | –0.050 | 0.546 |
| SDI and GDI | –0.556 | –0.087 | 0.130 | 0.276 | **–0.636*** | 0.199 | –0.434 | 0.115 | –0.456 | 0.288 | 1 |

order, were thicknesses of the mesophyll, hypodermis and epidermis (upper and lower), cuticle (upper), palisade, and gland and stomatal density. Test of correlation of Axes I and II with the organ physico-chemical properties confirmed that Axis I is a good predictor of organ longevity ($r = 0.84$, $P < 0.01$; $n = 8$), lignin concentration ($r = 0.68$, $P < 0.03$; $n = 10$) and marginally so for organ construction $cost_{mass\text{-}based}$ ($r = 0.60$, $P < 0.07$; $n = 10$), but has poor explanatory powers on leaf and pitcher major-nutrient concentrations of N, P and K and total ash ($P > 0.05$).

## Discussion

The pitcher of *Nepenthes*, a modified part of the leaf, has internal anatomies that are somewhat similar in tissue identity to those of the lamina; for example, presence of protective (epidermis and cuticle layers) and physiological (mesophyll) tissues was readily identified in the two organs. However, the size allocation of these tissues differed and there was a conspicuous lack of columnar palisade layer (with chloroplast-filled cells required for carbon gain in the leaf) in the mesophyll of the conjoint pitcher. This supported the assertion that a *Nepenthes* pitcher, although may possess a marginal number of stomata on its outer epidermal surface but without a palisade tissue, is limited in its capacity for photosynthesis (Pavlovič *et al*. 2007; Pavlovič and Saganová 2015). Consequently, its main function is chiefly to trap, digest and transport aqueous mineral ions to other parts of the plant (Owen *et al*. 1999; Thornhill *et al*. 2008). The pitcher digestive gland, although unique in structure, is quite similar to the leaf stomata in its epidermal origin (Gorb *et al*. 2004; Thornhill *et al*. 2008) and in its dimension–density relationship, exhibiting the familiar negative log–log trend between size and density (Brodribb *et al*. 2013). The negative relationship between size and density of stomata reflects optimal allocation of leaf epidermal-surface area for gas exchange (Osunkoya *et al*. 2014; de Boer *et al*. 2016). By extension of similarity in developmental origin, the gland dimension versus density relationship could also be deduced to have evolved for efficient transfer of digestive aqueous products from the pitcher lumen, through its mesophyll vascular-bundle compartment to the leaf, and, subsequently, to other parts of the *Nepenthes* plant. An increase in stomatal (or gland) density without concurrent size reduction will place these organs closer together and can lead to interference between the diffusion shells of neighbouring stomata (or gland) tissues (Lehmann and Or 2015).

The average stomatal density and size observed in the present study is similar to reported values for *Nepenthes* species (Pavlovič *et al*. 2007) and other angiosperm species (Hodgson *et al*. 2010; Osunkoya *et al*. 2014; de Boer *et al*. 2016). A higher stomatal density rather than size usually favours better gas exchange because of shorter diffusion path, higher carbon gain and water-use efficiency (Franks and Beerling 2009; Osunkoya *et al*. 2014). Hence, *N. gracilis*, with the highest stomatal density and thinnest leaf (Fig. 3), can be expected to attain this accolade among the species investigated. This could account and offer some explanation for the much wider distribution of *N. gracilis* (Clarke and Moran 2016; Schwallier *et al*. 2016), as well as be suggestive of a reduced need for carnivory, as reflected in its extremely low gland density (3–5 per $mm^2$) and lowest digestive gland–stomatal-area index (DGI : SPI) ratio. Bazile *et al*. (2015) reported that the pitchers of *N. gracilis* (and those of *N. ampullaria*) were comparatively limited in their capability to capture and retain flies and ants compared with those of two other species (*N. hemsleyana* and *N. rafflesiana*) they investigated.

Our observed trends between organ thickness and stomatal density (negative) and size (positive) are well documented in the literature (Beerling and Kelly 1996; Osunkoya *et al*. 2014; Zhang *et al*. 2014). The partial correlation analyses showed that leaf thickness, through its palisade and cuticle components, influences, or is coordinated with, stomatal size and density

for optimal photosynthesis and water-use efficiency (Brodribb *et al*. 2013; Griffith *et al*. 2016). For the conjoint pitcher, main driver of the trend in pitcher thickness–gland density relationship appeared to be the spongy-mesophyll size, and, to a limited extent, the inner epidermis. Thornhill *et al*. (2008) observed that the base of each digestive gland is abutted by vascular bundle within the mesophyll layer (for efficient translocation of digestive products). This vascular bundle–digestive gland coupling is probably constrained by economy of epidermal space, and might be responsible for the observed significant influence of the spongy-mesophyll layer on the pitcher thickness–gland density relationship.

Digestive glands and stomata, being specialised cells, are energetic and expensive to produce and maintain (Franks and Beerling 2009; Vico *et al*. 2011), with variation in density being under greater selection than is size (Brodribb *et al*. 2013). Hence, a cost will be associated with increasing the fractional leaf and pitcher epidermal layers with such structures. This cost (production, operation and maintenance) has been reflected in the present work as the product of density and size$^2$ (SPI and DGI; Franks and Beerling 2009; de Boer *et al*. 2016). This index was higher in the pitcher than in the leaf (Fig. 4*b*), perhaps accounting for a higher cost or need for resource acquisition via carnivory than via autotrophy. The negative trend of the SPI : DGI ratio and lignin concentration might also reflect a trade-off between resource acquisition and organ defence. The positive relationships between stomatal, gland density and lignin concentration, construction cost or organ longevity (Fig. 4) suggested that as *Nepenthes* organ gets loaded with an increasing density of these specialised cells (to improve carbon

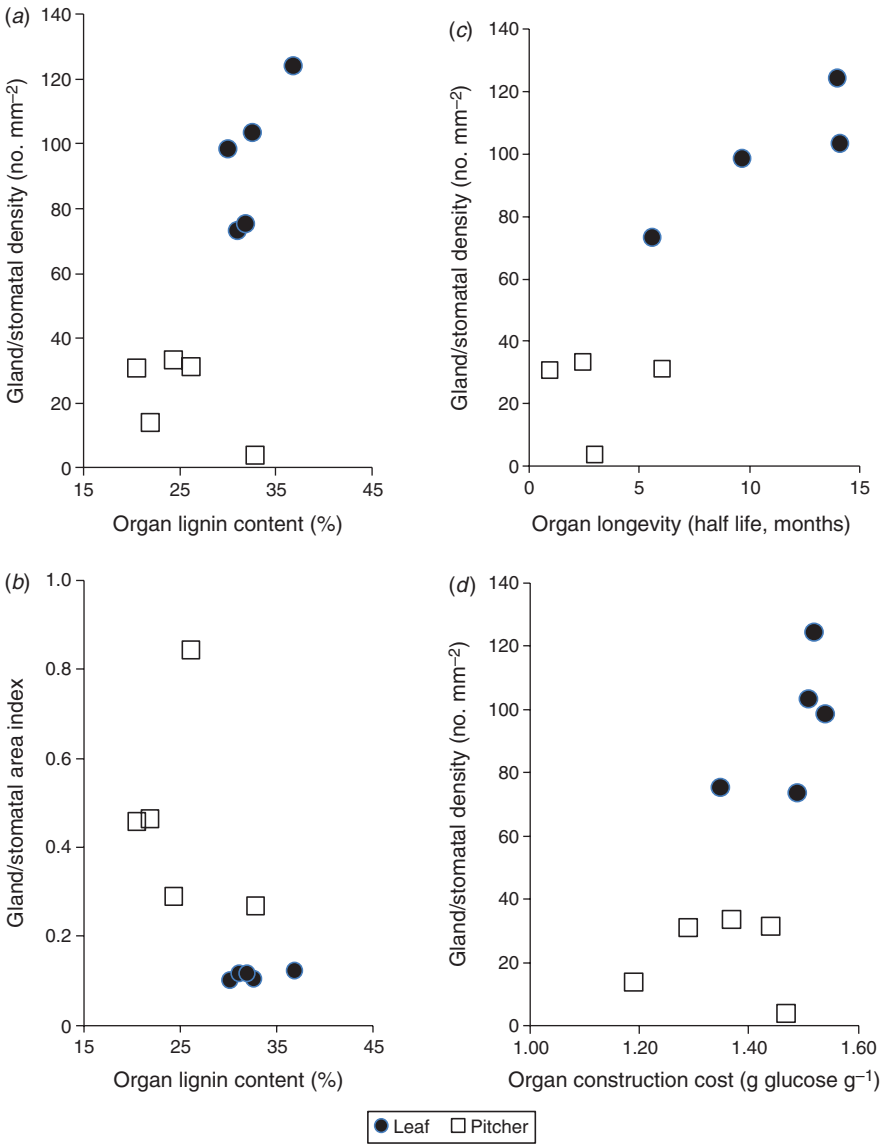


**Fig. 4.** Relationships of (*a*) density of digestive glands and density of stomata versus organ lignin concentration, (*b*) gland area index and stomatal area index versus organ lignin concentration, (*c*) density of digestive glands and density of stomata versus longevity (life-span) and (*d*) density of digestive glands and density of stomata versus organ construction costs across five *Nepenthes* species. Each data point is a species mean value.

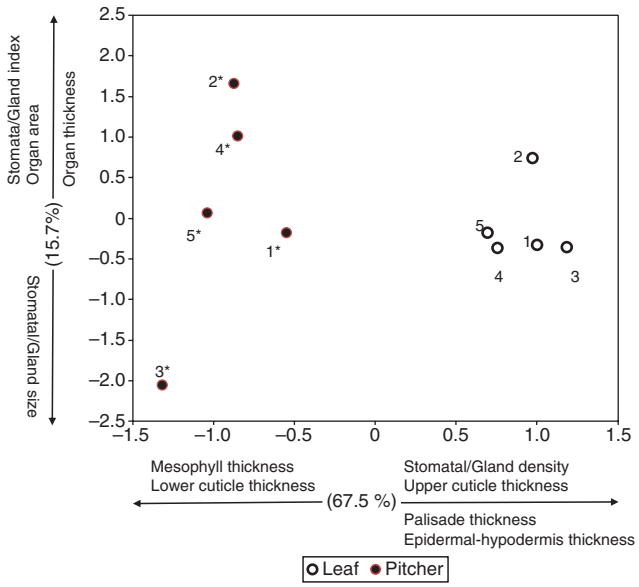


**Fig. 5.** Ordination using principal component analyses and based on mean anatomical trait values of the leaf and its conjoint pitcher in five species of *Nepenthes*. Percentage variation captured and the directions of major drivers of the trend are also indicated on the axes. Number indicates a given species (leaf and its pitcher (*)). 1, *N. ampullaria*; 2, *N. bicalcarata*; 3, *N. gracilis*; 4, *N. hemsleyana*; and 5, *N. rafflesiana*.

gain in the leaf, and inorganic nutrient uptake in the pitcher), it calls for reinforcement in terms of defence compounds such as lignin, which in turn results in a higher construction cost (Osunkoya *et al*. 2008); consequently, it pays to hold onto these organs much longer to ensure optimum payback on their investments.

The high stomatal density linked with longevity might also explain why the pitcher of *N. gracilis*, despite its comparatively smaller organ size and volume (Daud 2004; Bazile *et al*. 2015), has, among the species tested, one of the highest longevity (half-life of 3 months), and its leaf life span is even longer (half-life of 14 months, see Osunkoya *et al*. 2008). Nonetheless, we are still at a loss for a parsimonious explanation of the functional significance of *N. gracilis* digestive gland having a low density and a somewhat larger size (with potentially poor diffusion or osmotic gradient; Vassilyev and Muravnik 1988), as well as the species' poor fit to the gland size–density relationship. *Nepenthes gracilis* has the lowest ratio of pitcher digestive gland to leaf stomatal index (i.e. DGI : SPI). Thus, and as explained earlier, there is a possibility of a limited role of the *N. gracilis* pitcher (compared with other *Nepenthes* species studied) in nutrient sequestration and transfer (see also Bazile *et al*. 2015). A comprehensive comparative and cross-species study of the photosynthetic efficiency of *Nepenthes* leaves (and in some cases of the pitchers; Pavlovič *et al*. 2007) is more than overdue to complement what we know of the prey capture and nutrient sequestration capability of pitchers (Bazile *et al*. 2015; Kanokratana *et al*. 2016).

For most species (3 of 5), the general trend is for increasing gland size and density towards the bottom of the pitcher cup, as has been documented elsewhere (Owen and Lennon 1999; Gorb *et al*. 2004; Moran *et al*. 2010). In contrast, two species (*N. rafflesiana* and *N. hemsleyana*) showed the opposite pattern for gland density (see also Gorb and Gorb 2009), which resulted in overall (i.e. across species) lack of zonation effect on gland density along their pitcher walls. The consequence of such an observation for the above two species will be similarity of ion fluxes (an indicator of aqueous solution transfer), irrespective of position along the digestive zones. Interestingly, Moran *et al*. (2010) first alluded to this observation, and reported that the upper part of *N. rafflesiana* pitcher digestive (and the waxy) zones, despite lack of glands (in their studies), exhibited ion fluxes ($NH4^+$ and $H^+$). They linked such an activity to possible capability of the epidermal cells of these species to engage in active transport of aqueous ions. In contrast, our observations showed a higher density of glands in the upper part of the digestive zone in these two species. How do we explain this discrepancy? This anomaly could be due to differences in the position of measurement of Moran *et al*. (2010) and that of the present study. In our study, we divided the digestive section into three equal parts, whereas the zonation and, hence, measurement position of Moran *et al*. (2010) was based on relative distances from the bottom of the pitcher and the peristome; thus, whereas our lower and upper digestive zones coincided with Zones A and B of Moran *et al*. (2010), their Zone C might not align perfectly with the upper-part zone in the present study. Also, Moran *et al*. (2010) studied pitchers that were tissue-cultured, whereas ours were field-obtained. Another possible explanation is that there is significant phenotypic variation, especially in pitcher traits, within these two species (see Scharmann and Grafe 2013). More studies examining digestive-gland properties along the pitcher walls of *Nepenthes* species, and as influenced by varying environmental resources (e.g. light, water and soil nutrient concentrations), could help shed greater insight on the phenomenon.

## Conclusions

In the present study, many anatomical and physico-chemical traits are highly correlated among themselves, and with the adaptive trait of leaf longevity. In this respect, many of the bivariate relationships of *Nepenthes* assimilatory organs fit into the worldwide LES strategy. However, correlation does not necessarily imply causation and hence we cannot be certain of the direct influence of one measured trait over the other, but traits frequently causally related or associated through trade-offs, especially if they contribute to a common adaptive function (Edwards *et al*. 2000; Shipley and Lechowicz 2000; Sack *et al*. 2013). To disentangle the extent (magnitude and direction) to which each performance trait (e.g. gland and stomatal density, palisade thickness, or LMA) contributes to fitness component (e.g. organ longevity or plant growth) will require taking a path analytical (structural equation modelling) approach, during which alternative path models should be evaluated to gauge direct and indirect effects and relative contributions of traits. Such studies, although increasingly common in other plant groups (e.g. forest plants; Kitajima and Poorter 2010; Sack *et al*. 2013; and winter annuals; Blonder *et al*. 2015), have rarely been conducted on carnivorous plant (but see Jennings *et al*. 2010; for Sundews (*Drosera capillaris*)). Path-analytical approach will definitely improve

our understanding of trait correlation and coordination, as well as provide insight into trait contribution to organ function within the *Nepenthes* genus as a microcosm group.

We have shown that although tissue presence is almost identical, size-allocation patterns of these anatomical tissues are different for *Nepenthes* leaf and its conjoint pitcher, especially in their mesophyll content (Fig. 5). At the species-scale level, major nutrients of N, P, K and total ash (unlike organ construction cost, longevity and lignin content) were not correlated nor coordinated with organ anatomy. The lack of fit for N, P and K with anatomy, although similar to some findings (e.g. Wang *et al*. 2011 for *Leymus chiniensis* (Poaceae)), is in contrast to findings for many other plant groups (see Wright *et al*. 2004; Reich 2014); presumably because of *Nepenthes* evolutionary adaptation to habitats with low soil nutrient and the need to source some portion of these essential nutrients via carnivory to complement autotrophy (Pavlovič *et al*. 2007). Leaf stomata and pitcher digestive glands are derivatives of the epidermal layers and they showed similarity in their size–density relationship. Digestive-gland traits of one species, *N. gracilis*, did not fit this paradigm. We have attempted to ascribe reason for the anomaly (i.e. low level of carnivory); however, no doubt, other proximate explanations remain to be unravelled.

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