The Morphological and Biochemical Effects of Feeding Hooker's Pitcher with *Formica fusca* or *Calliphora vicina* Larvae

(Kesan Morfologi dan Biokimia terhadap Pemberian Makan Periuk Kera Hooker dengan *Formica fusca* atau Larva *Calliphora vicina*)

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ABSTRACT

The aim of this study was to determine if feeding Hooker's pitcher (Nepenthes × hookeriana) *with blowfly larvae* (Calliphora vicina *Rob-Desvoidy) or ants* (Formica fusca *L.) affected the plant's biometric and biochemical parameters. The research included measurements of length and width of the laminae and pitchers, and the total contents of the ash, assimilation pigments, polyphenols, and flavonoids. These parameters were measured 30 days after the last feeding. This study demonstrated that feeding the plants with blowfly larvae resulted in increases in the lengths and widths of both the laminae and pitchers. Moreover, the laminae had reduced carotenoid content after feeding with blowfly larvae and ants whereas the pitchers of Hooker's pitcher had increased chlorophyll and carotenoid content after feeding with blowfly larvae. Feeding Hooker's pitcher plants with blowfly larvae also resulted in a reduction of the total polyphenol content in the laminae whereas the total flavonoid content increased in both the laminae and pitchers of Hooker's pitcher. Feeding* Nepenthes × hookeriana *with insects ensures that their nutritional needs are met and leads to pitchers with good physiological condition.*

Keywords: Assimilation pigments; Hooker's pitcher; laminae; pitchers; polyphenols

ABSTRAK

Tujuan kajian ini adalah untuk menentukan sama ada dengan memberi makan periuk kera Hooker (Nepenthes × hookeriana) *dengan larva langau* (Calliphora vicina *Rob-Desvoidy) atau semut* (Formica fusca *L.) dapat mempengaruhi parameter biometrik dan biokimia tumbuhan tersebut. Penyelidikan ini merangkumi pengukuran panjang dan lebar lamina serta periuk kera dan juga kandungan keseluruhan abu, pigmen asimilasi, polifenol serta flavonoid. Parameter ini diukur 30 hari selepas pemberian makanan terakhir. Kajian ini menunjukkan bahawa pemberian makanan kepada tumbuhan ini dengan larva langau meningkatkan panjang dan lebar kedua-dua lamina dan periuk kera. Tambahan pula, kandungan karotenoid lamina berkurangan setelah diberi larva langau dan semut sedangkan terdapat peningkatan kandungan klorofil dan karotenoid selepas periuk kera Hooker diberi larva langau. Pemberian larva langau kepada periuk kera Hooker mengakibatkan pengurangan jumlah kandungan polifenol dalam lamina manakala jumlah kandungan flavonoid meningkat dalam kedua-dua lamina dan periuk kera Hooker. Pemberian serangga kepada* Nepenthes × hookeriana *dapat memastikan bahawa keperluan pemakanan mereka dipenuhi dan menghasilkan periuk kera dengan keadaan fisiologi yang baik.*

Kata kunci: Lamina; periuk kera; periuk kera Hooker; pigmen asimilasi; polifenol

INTRODUCTION

Nepenthes × hookeriana, or Hooker's pitcher plant, is a common natural hybrid of *N. ampullaria* Jack and *N. rafflesiana* var. *typica* Jack (Rosli et al. 2018). Its natural distribution areas are the tropical forests of Southeast Asia including Borneo, Sumatra, Malaysia, and Singapore, primarily at altitudes of 0 to 500 m above sea level (Yulita & Mansur 2012). All species of the genus *Nepenthes* require climates with high humidity and rich rainfall. Prolonged drought negatively impacts the development and growth of these plants and may even lead to their deaths. Typical habitats for pitcher plants include primary rain forests that are overgrown with *Dipterocarpus* trees, swamp woods, *Erica* forests, mountain forests, the upper and lower stories of cloud forests, steep slopes, saltwater mangrove forests, rocky cliffs, areas of destroyed and recovering secondary vegetation (such as after forest cutting, roadsides, and pastures) and bushes and pine forests subject to seasonal drought (Setiawan et al. 2015).

Insectivorous plants grow in acidic and marshy areas, which have reduced nutrient levels while non-insectivorous plants generally obtain nutrients from the soil (Adamec 1997). Compared to other plants, pitcher plant roots are markedly smaller and thinner, and may constitute 3.4 to 23% of the plant total biomass. The roots are unable to obtain the necessary nutrients from the soil, and so the plants require an alternative method of nutrient absorption (Adlassing et al. 2005; Bechie & Bidochka 2013). Therefore, *Nepenthes* plants have developed pitchers which constitute a perfect mechanism for luring and catching small insects.

The inside of the upper portion of a pitcher is covered with microscopic, waxy plates that are set like tiles which break off under the weight of a standing insect and adhere to its legs, thus reducing its grip level. The insect is unable to stay on the wall and falls down into the trap (Bazile et al. 2015). The plant has glands that secrete a sweet substance that lures insects; these glands are located on the lids and rims of the pitcher openings. The shapes and sizes of the glands vary by the level of the feeding zone. They are smaller in the upper portion, with an oval or elongated shape whereas at the bottom they are noticeably larger and may even attain a wavy shape (Wang et al. 2009a). Liquid (a mixture of rainwater and non-nectar plant secretions) accumulates in the lower portion of the pitcher and on the outside of the lip that encircles the pitcher opening. By attempting to collect the nectar, insects place themselves over the pitcher opening, with each careless movement resulting in the insects slipping further down the pitcher (Moran & Clarke 2010). The bodies of prey are decomposed by digestive enzymes secreted by the glands located in the lower portion of the pitcher (Adlassing et al. 2011; Miguel et al. 2018). Pitchers can produce from 0.2 mL to 1.5 L of the digestive liquid, which is acidic (with pH 3.0 to 5.5), viscous, rich in oxygen and contains digestive enzymes (Ravee et al. 2018). Pitchers primarily obtain nitrogen and phosphorus from insects (Bechie & Bidochka 2013). Typical pitcher preys include beetles, ants, termites, flies, wasps, moths, and cockroaches (Rischer et al. 2002).

Pavlovič et al. (2009) reported that feeding *N. talangensis* with meal worm (*Tenebrio molitor*) resulted in an increase in the chlorophyll and nitrogen contents of the laminae and stimulation of the rate of photosynthesis and utilisation of absorbed light energy for photochemistry in plants. In contrast to the laminae, the pitchers are unable to increase their rates of photosynthesis (A_N) in response to feeding, suggesting that they are highly specialised for nutrient acquisition. The ability of the laminae to increase biomass and photosynthetic efficiency in response to prey capture may provide a competitive advantage for these plants over non-carnivorous plants in sunny and nutrient-poor habitats. Moran and Moran (1998) studied the benefits of carnivory on some parameters of *N. rafflesiana* and showed that resource limitations caused decreases in chlorophyll content and increases in the production of anthocyanins, denoting possible nitrogen or phosphorus limitation.

Miguel et al. (2018) reported that *Nepenthes* species have developed a number of ecological traits that have attracted the attention of plant biologists. The different adaptation strategies that these plants have developed make them a source of inspiration for many applications, from therapeutic treatments to agricultural biocontrol solutions. More recent reports described the use of different species of *Nepenthes* in phytotherapy (Dhamecha et al. 2016). In the folk medicine of India and Southeast Asian countries, they are used in the treatment of leprosy, cholera, night blindness, gastrointestinal discomfort, dysentery, stomachache, and bed-wetting, among other ailments (Sanusi et al. 2017). The phytochemical compounds isolated from *Nepenthes* species include polyphenols, terpenoids, tannins, alkaloids, and steroids (Thanh et al. 2015).

The aim of the study was to determine if feeding Hooker's pitcher with blowfly larvae or ants affected the plant morphological measurements (i.e. the lengths and widths of the laminae and pitchers) and the ash, assimilation pigment, total polyphenol and total flavonoid contents.

MATERIALS AND METHODS

EXPERIMENTAL DESIGN AND SAMPLING

The laboratory experiment was conducted using 18 individual *Nepenthes × hookeriana* Hort. Veitch ex Mast. (*N. ampullaria* Jack × *N. rafflesiana* var. *typica* Jack) plants, which were obtained from Carnisana (Sosnowiec, Poland). Plants grown in greenhouse conditions in pots were placed in trays with small amounts of distilled water to maintain substrate humidity. Plants in the same developmental stages that did not differ morphologically were selected for the experiment. There were four mature pitchers on each plant.

The experimental plants were divided into three groups (six individuals in each group) where the first group of Hooker's pitcher received only water, the second was watered and fed with blowfly larvae (*Calliphora vicina* Rob-Desvoidy, *Diptera*), and the third group was watered and fed with ants (*Formica fusca* L., Hymenoptera). The insects used in the experiment were obtained from our own breeding in the Department of Bioengineering of West Pomeranian University of Technology in Szczecin, Poland. The plants were fed three times, at the beginning of the experiment (day 0), day 10 and day 20. For feeding the plants, one blowfly larva or a weight-equivalent number of ants were added to each pitcher. The blowfly larvae were

selected such that each plant received a similarly-sized larva. Before being added to the pitchers, the larvae were weighed on a Petri dish. The average larval weight was 20 ± 2 mg. The ants were also weighed on a Petri dish and also weighed, on average at 20 ± 2 mg. There were 3 to 4 ants per blowfly larva. The average number of introduced insects was 23 ± 2 mg per plant over 20 days. Throughout the experiment, growth conditions were maintained at ideal conditions for Hooker's pitcher. Thirty days after the last feeding, the biometrical measurements of the laminae and pitchers, as well as their assimilation pigment, total polyphenol, total flavonoid and ash contents were measured.

BIOMETRIC MEASUREMENTS

For biometric measurements, digital recordings were performed using a photographic camera GH5 (UK and Ireland). To standardise the results, a ruler was placed within the frame of view while taking pictures. The lengths and widths of the laminae and pitchers were measured with the aid of an image analysis program (ImageJ). For each measurement, the distance measurement was calibrated according to the ImageJ instruction manual. The lengths were measured without including any tendrils. The width measurement was based on the widest region. The lengths and widths of the laminae and pitchers were given as the percentage change (PCh) calculated according to the formula in (1):

$$PCh = \frac{A_F - A_0}{A_F} \cdot 100\% \qquad (1)$$

where A_F is the final length or width, and A_0 is the starting length or width. The results of the changes in lengths and widths were presented in the form of percentage changes, which allowed for a better comparison and eliminated possible differences at the beginning of the experiment.

DETERMINATION OF ASSIMILATION PIGMENT AND ASH CONTENTS

Photosynthetic pigment contents were determined according to the method reported by Arnon et al. (1956), as modified by Lichtenthaler and Welburn (1983). Accordingly, for each plant, 100 mg of laminae and 50 mg of pitchers were homogenised in 10 mL of 80% acetone using a mortar and pestle, and the homogenates were collected in a centrifuge tube. The samples were centrifuged at 1,500 rpm for 10 min using a MicroCL 21R centrifuge (Thermo). The supernatants were used for the determination of pigment contents. The chlorophyll a (Chl$a$), chlorophyll b (Chl$b$) and carotenoid (Car) contents were determined by measuring the absorbance at 440, 645, and 663 nm, respectively, using a MarcelMini spectrophotometer (Zielonka, Poland). For each

plant, the assimilation pigments were measured in quadruplicate. The amount of assimilation pigments present was expressed as mg/kg fw.

In order to assess the total amount of minerals, the total ash content was determined. The method for determining the total ash involved incinerating the analytical sample at 550 °C in a muffle furnace until the organic substances were completely burnt, after which the weight determination of the residues was performed (AOAC 2000).

DETERMINATION OF TOTAL POLYPHENOL AND TOTAL FLAVONOID CONTENTS

Total polyphenol and total flavonoid contents were determined by the methods of Yu et al. (2002) and Kumaran and Karunakaran (2007), respectively. Accordingly, for each plant, 100 mg of the plant material was homogenised in 10 mL of 80% methanol using a mortar and pestle and the homogenate was collected in a centrifuge tube. The samples were centrifuged for 5 min at 14,800 rpm using a MicroCL 21R centrifuge (Thermo). The supernatants were used for the determination of the total polyphenol and flavonoid contents. For each plant, the extracts were prepared in quadruplicate.

To determine the total polyphenol content, 0.1 mL of supernatant was mixed with 0.5 mL of the Folin-Ciocalteu reagent and 1.5 mL of 20% sodium carbonate. The mixture was shaken thoroughly and the solution volume was increased to 10 mL using distilled water. Afterwards, the samples were incubated for 2 h at 25 °C. Then, the absorbance at 765 nm was determined. These data were used to estimate the total polyphenol content using a standard curve obtained from various concentrations of gallic acid.

To determine the total flavonoid content, 1 mL of supernatant was mixed with 1 mL of 2% aluminum chloride and a drop of glacial acetic acid. The samples were shaken at room temperature for 40 min. After this time, each sample volume was increased to 25 mL with methanol and the absorbance was determined against a reagent blank at wavelength, λ, of 415 nm. These data were used to estimate the total flavonoid content using a standard curve obtained from various concentrations of quercetin. A UV-1800 spectrophotometer (Shimadzu, Japan) was used to determine the total polyphenol and total flavonoid contents.

STATISTICAL ANALYSIS

All the biometric and biochemical parameter data were analysed using STATISTICA 13.1. The data were analysed using a two-way ANOVA test followed by Tukey's HSD post hoc test. Differences were considered significant at the $P < 0.05$ level. All results were expressed

as mean ± standard deviation. Moreover, Pearson's correlation coefficients were calculated among the assayed biochemical parameters. *P*-values were corrected for multiple testing using the Bonferroni correction (Ramette 2007).

RESULTS

INFLUENCE OF FEEDING HOOKER'S PITCHER WITH INSECTS ON THE BIOMETRIC MEASUREMENTS OF LAMINAE AND PITCHERS

Watering the plants caused mean increases in the widths of laminae and pitchers of 9.21 and 13.18%, respectively. After feeding the plants with blowfly larvae, the mean width increases in the laminae and pitchers were 33.33

and 24.91%, respectively, whereas after feeding the plants with ants, the mean width increases in the laminae and pitchers were 12.09 and 15.32%, respectively (Figure 1(a)). The statistical analysis showed significant differences only between the widths of laminae fed with blowfly larvae and the laminae of watered plants.

The mean percentage changes in the lengths of the laminae and pitchers of watered-only Hooker's pitcher were 9.30 and 11.14%, respectively. The mean percentage changes in the lengths of the laminae and pitchers of plants fed with blowfly larvae were 20.32 and 18.56%, respectively, and those of plants fed with ants were 15.52 and 14.89%, respectively (Figure 1(b)). The statistical analysis showed significant differences only between the lengths of laminae fed with blowfly larvae and the laminae of watered plants.
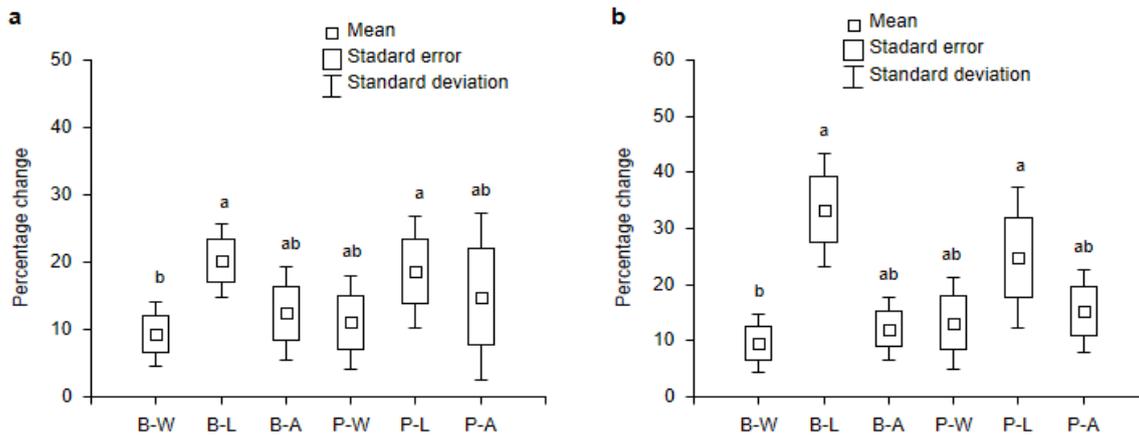

FIGURE 1. Percentage changes in (a) width, and (b) length of Hooker's pitcher laminae (B) and pitchers (P); W – plants that only received water, L – plants fed with blowfly larvae, and A – plants fed with ants. Values are mean values ± SD (n=6), different letters show significant differences at $P < 0.05$ (post hoc Tukey's HSD test)

INFLUENCE OF FEEDING HOOKER'S PITCHER WITH INSECTS ON THE ASSIMILATION PIGMENT CONTENTS IN LAMINAE AND PITCHERS

The Chl*a* and Chl*b* contents in the laminae of Hooker's pitcher did not change significantly after feeding the plants with blowfly larvae or ants, whereas the Car content was reduced after feeding the plants with blowfly larvae or ants by 21.23 or 26.70%, respectively, compared to watered-only plants. However, in the pitchers of Hooker's pitcher fed with blowfly larvae, the contents of Chl*a*, Chl*b,* and Car increased by 91.33, 80.27, and

89.85%, respectively, in comparison to watered-only plants (Table 1). In laminae, the Chl*a*/Chl*b* ratio did not change in either feeding regimen, whereas in pitchers, there was a 32.88% increase in this ratio after feeding with ants, compared to watered-only plants. There were interesting changes in the (Chl*a*+Chl*b*)/Car ratio in laminae. In plants fed with blowfly larvae or ants, this ratio increased by 29.11 or 32.49%, respectively, whereas for pitchers, a statistically significant increase in the (Chl*a*+Chl*b*)/Car ratio was observed only after feeding with ants (25.58%, compared to watered-only plants).

TABLE 1. Content of assimilation pigments (mg/kg fw) in Hooker's pitcher; values are mean values ± SD, different letters show significant differences at $P < 0.05$ (post hoc Tukey's HSD test)

Part of plants	Feeding	Chl$a$	Chl$b$	Car	Chl$a$/Chl$b$	(Chl$a$+Chl$b$)/ Car
Laminae	W	738.15 ± 48.63[a]	304.28 ± 18.34[a]	440.38 ± 40.28[a]	2.43 ± 0.07[a]	2.37 ± 0.07[d]
	L	745.69 ± 18.82[a]	288.69 ± 9.29[a]	346.89 ± 17.85[b]	2.58 ± 0.02[a]	3.06 ± 0.26[c]
	A	721.84 ± 43.15[a]	287.60 ± 30.03[a]	322.79 ± 32.05[b]	2.52 ± 0.11[a]	3.14 ± 0.18[b]
Pitchers	W	115.59 ± 24.58[c]	79.98 ± 19.74[c]	65.05 ± 8.89[d]	1.46 ± 0.09[c]	3.01 ± 0.27[c]
	L	221.16 ± 37.49[b]	144.18 ± 17.81[b]	123.50 ± 5.02[c]	1.52 ± 0.12[c]	3.05 ± 0.18[c]
	A	128.26 ± 19.80[c]	65.66 ± 13.11[c]	51.72 ± 2.47[d]	1.94 ± 0.10[b]	3.78 ± 0.21[a]

Chl$a$ chlorophyll a, Chb chlorophyll b, Car carotenoids, W watered-only plants, L plants fed with blowfly larvae, A plants fed with ants

INFLUENCE OF FEEDING HOOKER'S PITCHER WITH INSECTS ON THE CONTENT OF POLYPHENOLIC COMPOUNDS IN LAMINAE AND PITCHERS

The total polyphenol content in the laminae of Hooker's pitcher was significantly reduced after feeding the plants with blowfly larvae (18.19%, compared to watered-only plants), but this was not significantly affected by feeding the plants with ants. No significant change in total polyphenol content was observed in the pitchers after either feeding regime (Figure 2(a)).
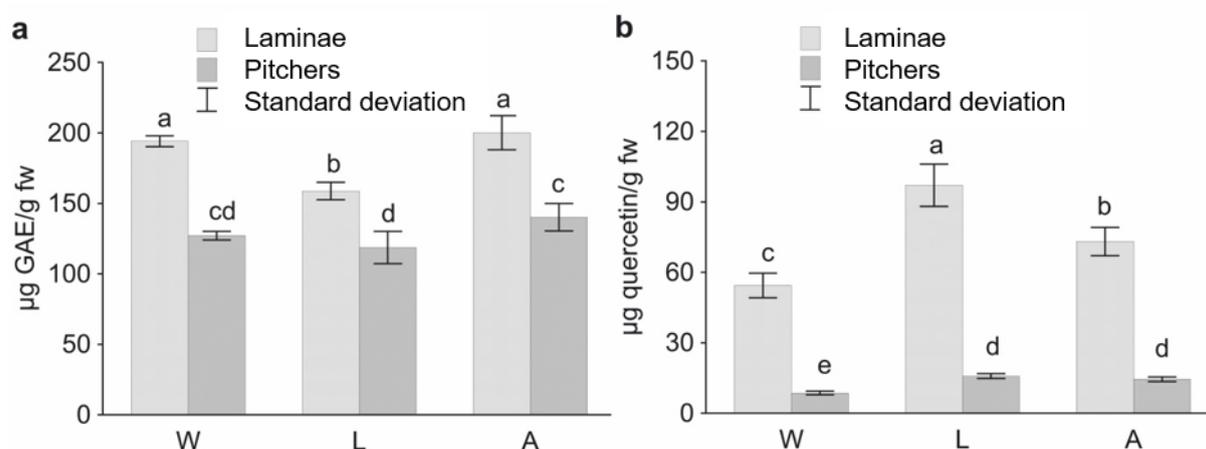

FIGURE 2. Content of (a) total polyphenols, and (b) total flavonoids in Hooker's pitcher laminae and pitchers; W – plants that only received water, L – plants fed with blowfly larvae, A – plants fed with ants, GAE – gallic acid equivalents. Values are mean values ± SD, different letters show significant differences at $P < 0.05$ (post hoc Tukey's HSD test)

However, the total flavonoid content increased in both the laminae and pitchers of Hooker's pitcher after the plants were fed with the insects. In the laminae, feeding with blowfly larvae or ants led to increases of 78.34 or 34.28%, respectively, compared to watered-only plants, whereas pitchers fed with blowfly larvae or ants showed increases of 83.33 or 68.29%, respectively (Figure 2(b)).

INFLUENCE OF FEEDING HOOKER'S PITCHER WITH
INSECTS ON ASH CONTENT IN LAMINAE AND PITCHERS
OF HOOKER'S PITCHER

The ash content in the laminae and pitchers of Hooker's pitcher did not change after feeding with blowfly larvae, compared to watered-only plants. However, feeding with ants resulted in statistically significant reductions in the ash contents of both the laminae and pitchers, which amounted to 53.33 and 45.54%, respectively, when compared to watered-only plants (Figure 3).
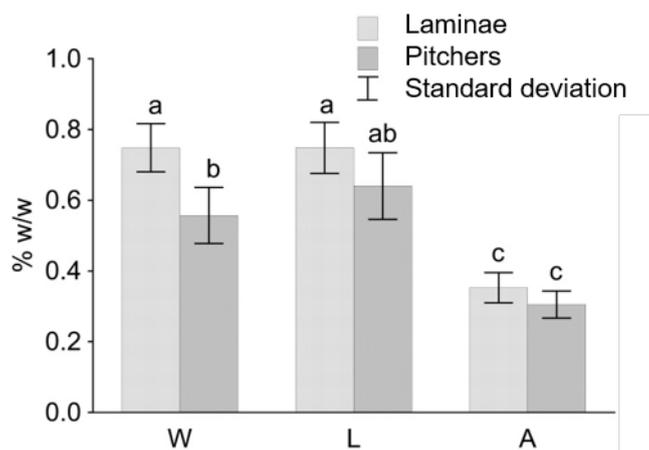

FIGURE 3. Content of ash in Hooker's pitcher laminae and pitchers; W – plants that only received water, L – plants fed with blowfly larvae, A – plants fed with ants. Values are mean values ± SD of four replicates, different letters show significant differences at $P < 0.05$ (post hoc Tukey's HSD test)

RELATIONSHIP BETWEEN BIOCHEMICAL PARAMETERS
OF LAMINAE AND PITCHERS OF HOOKER'S PITCHER

Based on the calculated Pearson's correlation coefficients with the Bonferroni correction, a positive relationship was found between the majority of the determined parameters in the laminae and pitchers of Hooker's pitcher. The highest correlation coefficient (0.987) was recorded between the Chl$a$ and Chl$b$ content (Table 2). However, no significant relationship was observed between the (Chl$a$+Chl$b$)/Car ratio and the majority of the parameters determined.

TABLE 2. Pearson's correlation coefficients at the Bonferroni-corrected level among assayed parameters in laminae and pitchers of Hooker's pitcher

Parameter	Chl$a$	Chl$b$	Car	Chl$a$/Chl$b$	(Chl$a$+Chl$b$)/Car	Phe	Flav	Ash
Chl$a$		0.000	0.000	0.000	0.498	0.000	0.000	0.000
Chl$b$	0.987*		0.000	0.000	0.356	0.000	0.000	0.000
Car	0.955*	0.959*		0.000	0.107	0.000	0.000	0.000
Chl$a$/Chl$b$	0.903*	0.831*	0.815*		0.734	0.000	0.000	0.002
(Chl$a$+Chl$b$)/Car	-0.298	-0.339	-0.494	-0.066		0.473	0.617	0.314
Phe	0.824*	0.790*	0.791*	0.806*	-0.257		0.011	0.019
Flav	0.906*	0.866*	0.773*	0.872*	-0.115	0.675		0.006
Ash	0.865*	0.896*	0.887*	0.711	-0.307	0.556	0.736	

P-values of correlations are shown in the upper right part of the table; *denotes significant coefficients at the Bonferroni-corrected level ($p \leq 0.000521$) which are additionally shown in bold for better visualization; Chl chlorophyll, Car carotenoids, Phe total polyphenols, Flav total flavonoids

DISCUSSION

This study examined the effects of feeding Hooker's pitcher with blowfly larvae (*Calliphora vicina* Rob-Desvoidy) or ants (*Formica. fusca* L.) based on the biometric and biochemical parameters of the laminae and pitchers.

The results demonstrated that feeding the plants with blowfly larvae resulted in increases in the lengths and widths of laminae and pitchers, whereas feeding the plants with ants had minor effects on the biometric characteristics of laminae and pitchers. The available literature does not provide information on the influence of feeding Hooker's pitcher on the plants' morphological parameters. However, Moran et al. (1999) and Osunkoya et al. (2007) who compared various *Nepenthes* species demonstrated that the *N. mirabilis* species was characterised by the longest and the widest pitchers. Moreover, Marina et al. (2018) reported that of the 901 total fauna trapped in the pitchers of *Nepenthes* spp. grown in Bukit Setiam Forest, Malaysia, 58.82% belonged to the order *Hymenoptera* (primarily ant species) and 15.78% belonged to the order *Diptera* (15.87%). Some studies have also demonstrated that selected ant species inhabit the outside of pitcher plants (Bazile et al. 2012; Grafe & Kohout 2013; Thornham et al. 2012). Therefore, we chose ants and blowfly larvae for our research, the largest quantities of which were found in the pitchers.

In this study, laminae were found to have reduced Car content as a result of feeding with blowfly larvae and ants, whereas the pitchers of Hooker's pitcher had increased chlorophyll content, as well as carotenoid content, after feeding with blowfly larvae. Pavlovič et al. (2009) demonstrated that feeding *N. talangensis* with *Tenebrio molitor* larvae for 2 months resulted in an increased photosynthesis rate and optimal efficiency of PSII (F_v/F_m) in both the leaves and pitchers. The optimal efficiency of PSII (F_v/F_m) is an important indicator of photochemical efficiency in plants (Maxwell & Johnson 2000; Tušek et al. 2016). Feeding pitchers with insect larvae increased the rate of photosynthesis (A_N) in leaf laminae due to increased nutrient acquisition, with a strong correlation with nitrogen concentration, but the A_N of pitchers did not increase despite the increased nitrogen concentrations in their tissue. Increasing the A_N improved growth and reproduction and was likely to increase the competitive advantage of carnivorous over non-carnivorous plants in nutrient-poor habitats (Pavlovič et al. 2009). Increased A_N after feeding was also found by Farnsworth and Ellison (2008) in the genus *Sarracenia*. Moreover, He and Zain (2012) showed that maximal light-saturated photosynthetic rates (P_{max}) were higher in leaves than in pitchers. Moran and Moran (1998) reported a reduction in chlorophyll content and increased production of anthocyanins in the leaves of *N. rafflesiana* plants that were not deprived of their ability to develop in their natural habitat. The rate of photosynthesis was not increased as a result of prey capture in the carnivorous butterwort *Pinguicula vulgaris* (Méndez & Karlsson 1999). However, supplementary feeding increased both rosette size and reproduction through an increase in flowering frequency (Thorén & Karlsson 1998).

The relationships between the chlorophyll and carotenoid contents, namely the Chl*a*/Chl*b* ratio and (Chl*a*+Chl*b*)/Car ratio, constitute very important markers of physiological changes (Biczak et al. 2017; Netto et al. 2005). Changes in the Chl*a*/Chl*b* value clearly indicate the occurrence of oxidative stress in plants and indicate the activation of the plants' defenses (Pawłowska et al. 2017). On the other hand, a significant decrease in Chl*a* content may indicate considerable damage to photosystems (Chen et al. 2014; Wang et al. 2009b). This study demonstrated that significant increases in the Chl*a*/Chl*b* ratio occurred solely in pitchers after feeding with ants. Similar results were found by Pavlovič et al. (2009) in fed and unfed *N. talangensis* plants. However, the observed changes in the (Chl*a*+Chl*b*)/Car ratio were interesting for laminae where the increase was observed after feeding plants with blowfly larvae as well as after feeding them ants, but this parameter was increased in pitchers only after feeding the plants with ants. Carotenoids are necessary for photoprotection of photosynthesis and they play an important role as a precursor in signaling during plant development under abiotic/biotic stress (Ashraf & Harris 2013). They have a significant potential to enhance nutritional quality and plant yield. Nowadays, enhanced carotenoid contents in plants are strongly considered for breeding as well as genetic engineering in different plants (Li et al. 2008). Carotenoids are also present in plant cellular membranes. They protect the membranes from light-dependent oxidative damage. The role of carotenoids in scavenging reactive oxygen species has been well studied (Davison et al. 2002; Pawłowska et al. 2017; Verma & Mishra 2005).

This study has demonstrated that feeding Hooker's pitcher with blowfly larvae resulted in a reduction of the total polyphenol content in the laminae, whereas the total polyphenol content in both the laminae and pitchers of Hooker's pitcher increased after the plants were fed with ants. Carnivorous plants are characterised by the synthesis of secondary metabolites in the insect-trap tissues, which are used for self-defense (Rischer et al. 2002). Eilenberg et al. (2010) reported that diverse bioactive allelochemicals including alkaloids, terpenes, and phenolics, were found in many representatives of different families of carnivorous plants. The synthesis of protective secondary metabolites is very common in many plants and occurs in response to chemical, biotic or physical stresses (Bringmann & Feineis 2001; Rischer et al. 2002).

The ash content in the laminae was higher than in the pitchers, especially in unfed Hooker's plants. This

was consistent with the results obtained by Osunkoya et al. (2007). Pavlovič et al. (2009) showed the increase in the dry matter of laminae of *N. talangensis* fed with insect larvae. However, in our study, the ash content in Hooker's plants did not differ significantly between those fed blowfly larvae and those who were only watered. Moreover, feeding with ants resulted in statistically significant decreases in the ash contents of both the laminae and pitchers. This probably occurred because ants have chitin armor. From a similar mass of digested prey bodies, more is extracted from blowfly larvae and thus more ash is formed.

Based on the calculated Pearson's correlation coefficients, a positive relationship was found between the majority of the determined parameters in the laminae and pitchers of Hooker's pitcher. Tušek et al. (2016) found that variations in the photochemical efficiency and content of photosynthetic pigments and phenolic compounds correlated with the function of the pitcher of *Sarracenia*. The red-colored operculum and upper pitcher part which has the role of luring and catching prey, contains more phenolic compounds especially anthocyanins which help to attract insects. Moreover, a lower photochemical efficiency has often been observed in carnivorous plants (Bruzzese et al. 2010) and has been correlated with the costs of carnivory (Adamec 2010) as well as with low foliar nitrogen content and the slow growth of carnivorous plants (Ellison & Adamec 2011; Pavlovič & Saganová 2015). Karagatzides and Ellison (2009) measured mass-based construction costs and photosynthesis for the traps, leaves, roots, and rhizomes of 15 carnivorous plant species grown under greenhouse conditions. They found that the mean value construction costs (CC) mass of carnivorous traps was significantly lower than the CC mass of leaves of 267 non-carnivorous plant species. Moreover, Osunkoya et al. (2007) reported that the CC of the assimilatory organs for the pitcher was in agreement with the concept of low marginal cost for carnivory, relative to conventional autotrophy. The poor explanatory power of N, P or the N:P ratio with CC suggests that factors other than the production of expensive photosynthetic machinery (which calls for a high N input) including concentrations of lignin, wax/lipids or osmoregulatory ions like K^+, may give a better explanation of the CC variations across *Nepenthes* species.

Feeding *Nepenthes* plants with insects in various forms ensures that their nutritional needs are met and leads to pitchers with good physiological conditions (physiological activity), which was confirmed by the results of our studies. Providing plants with additional minerals during the growth period from insect digestion prevents, among other things, the appearance of physiological diseases on laminae (chlorosis, necrosis) and on pitchers (necrosis) caused by a deficiency of essential minerals for cultivation without feeding. Therefore, the knowledge presented here regarding the biochemical and physiological responses of Hooker's pitcher to insect feeding may be useful in the breeding of this species.

Conclusion

The available literature contains little information on how feeding insectivorous plants affects the plant morphological and biochemical parameters. This study demonstrated that feeding plants *C. vicina* larvae resulted in increases in the lengths and widths of laminae and pitchers, whereas feeding the plants *F. fusca* had minor effects on the biometric characteristics of the laminae and pitchers. Moreover, the laminae had reduced carotenoid content after feeding on *C. vicina* larvae and *F. fusca*, whereas the pitchers of Hooker's pitcher had increased chlorophyll and carotenoid contents after feeding on *C. vicina* larvae. Furthermore, feeding Hooker's pitcher *C. vicina* larvae resulted in a reduction of the total polyphenol content in laminae, whereas the total flavonoid contents in laminae and pitchers increased after feeding the plants with either *F. fusca* or *C. vicina* larvae. Analysis of the Pearson's correlation coefficients demonstrated a positive relationship between the majority of the parameters in the laminae and pitchers of Hooker's pitcher.

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