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Mineral nutrient uptake from prey and glandular phosphatase activity as a dual test of carnivory in semi-desert plants with glandular leaves suspected of carnivory

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• *Background and Aims Ibicella lutea* and *Proboscidea parviflora* are two American semi-desert species of glandular sticky plants that are suspected of carnivory as they can catch small insects. The same characteristics might also hold for two semi-desert plants with glandular sticky leaves from Israel, namely *Cleome droserifolia* and *Hyoscyamus desertorum*. The presence of proteases on foliar hairs, either secreted by the plant or commensals, detected using a simple test, has long been considered proof of carnivory. However, this test does not prove whether nutrients are really absorbed from insects by the plant. To determine the extent to which these four species are potentially carnivorous, hair secretion of phosphatases and uptake of N, P, K and Mg from fruit flies as model prey were studied in these species and in *Roridula gorgonias* and *Drosophyllum lusitanicum* for comparison. All species examined possess morphological and anatomical adaptations (hairs or emergences secreting sticky substances) to catch and kill small insects.
• *Methods* The presence of phosphatases on foliar hairs was tested using the enzyme-labelled fluorescence method. Dead fruit flies were applied to glandular sticky leaves of experimental plants and, after 10–15 d, mineral nutrient content in their spent carcasses was compared with initial values in intact flies after mineralization.
• *Key Results* Phosphatase activity was totally absent on *Hyoscyamus* foliar hairs, a certain level of activity was usually found in *Ibicella*, *Proboscidea* and *Cleome*, and a strong response was found in *Drosophyllum. Roridula* exhibited only epidermal activity. However, only *Roridula* and *Drosophyllum* took up nutrients (N, P, K and Mg) from applied fruit flies.
• *Conclusions* Digestion of prey and absorption of their nutrients are the major features of carnivory in plants. Accordingly, *Roridula* and *Drosophyllum* appeared to be fully carnivorous; by contrast, all other species examined are non-carnivorous as they did not meet the above criteria.

Key words: *Roridula gorgonias*, *Drosophyllum lusitanicum*, *Proboscidea parviflora*, *Ibicella lutea*, *Cleome droserifolia*, *Hyoscyamus desertorum*, phosphatase, phosphomonoesters, fruit flies, N, P, K, Mg uptake from prey.

INTRODUCTION

Of the total 300 000 species of vascular plants worldwide, about 650 are considered to be carnivorous (Rice, 2006). Plants have to fulfil several criteria to be considered carnivorous. Unfortunately, these criteria have been variously described (cf. Lloyd, 1942; Juniper *et al.*, 1989; Adamec, 1997). In their concept of the 'carnivorous syndrome', Juniper *et al.* (1989) introduced six principal characters of carnivorous plants including attraction and digestion of prey. After it was shown that attraction of prey might not occur or was unknown in some taxa considered to be carnivorous (e.g. Karlsson *et al.*, 1987) or that other taxa did not provide their own prey digestion (e.g. Jaffe *et al.*, 1992), attention has mainly focused on the essential ecophysiological consequences of carnivory – uptake and utilization of nutrients from prey by plants – as crucial criteria of carnivory (Adamec, 1997 *sensu* Lloyd, 1942). However, in addition to 'direct' carnivory in the majority of carnivorous plants, an 'indirect' means of carnivory, mediated by commensal hemipteran bugs of the genus *Pameridea*, has recently been described and experimentally proven in the genus *Roridula* (Anderson and Midgley, 2003; Anderson, 2005). Although these plants catch prey by using a sticky hydrophobic secretion on the heads of immobile tentacles, they do not digest them. The prey is eaten by hemipteran bugs that climb only on the host plants. However, the faeces of these hemipteran bugs stay on the plant (leaf) surface and the nutrients are absorbed via the porous cuticle of the leaves (Anderson, 2005). In this way, the plant takes up and utilizes mineral nutrients coming from prey animals and is thus also carnivorous. Accordingly, the commensal relationship may be described as symbiotic. Płachno *et al.* (2006) revealed phosphatase activity on the leaves of *Roridula*, suggesting also that direct carnivory may be possible.

Carnivorous plants are typically wetland or aquatic (amphibious) plants growing in nutrient-poor, wet or waterlogged

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soils, generally in sunny habitats (Givnish *et al.*, 1984; Juniper *et al.*, 1989). Under these stressful conditions, as suggested by the above authors, the ecological benefit of carnivory can equal or even exceed the ecological costs (structural and energetic). However, the distinction between carnivorous and non-carnivorous plants is not particularly clear. A speciose group of plants with glandular and sticky shoots are able to trap and sometimes also to digest fine arthropods, organic debris or pollen. Species of this group do not generally inhabit wetland areas. Spomer (1999) showed that many of them (i.e. protocarnivorous) exhibited proteinase activity on their leaves and that some absorbed digestion products of a protein applied to the leaves. Although it is generally accepted that the main evolutionary function of these sticky surfaces is to defend the plants against small arthropod herbivores (e.g. Duffey, 1986), their role in mineral nutrition has not yet been excluded. Plants of the genera *Ibicella* and *Proboscidea* have glandular and sticky shoot surfaces capable of catching fine prey, and they have sometimes been classed as carnivorous although their carnivory has never been tested (Rice, 2008). Most 'historically true' carnivorous plants possess high phosphatase activity in their secretory hairs and emergences (Juniper *et al.*, 1989). Recently, this has been shown clearly in living secretory hairs and glands in such plants using the enzyme-labelled fluorescence (ELF) method (Płachno *et al.*, 2006).

Species of the genus *Drosera* have been shown to be able to take up a substantial proportion not only of total N, but also of P, K and Mg, from fruit flies as model prey (Dixon *et al.*, 1980; Hanslin and Karlsson, 1996; Adamec, 2002). If true carnivorous plants are able to take up N, P, K and Mg efficiently from the carcasses of prey animals and if mineral nutrient uptake from prey is the main ecophysiological consequence and benefit of carnivory, then the test of mineral nutrient uptake from a model prey animal should be the preferred criterion of carnivory over enzyme activity on the shoot surface.

The aim of the present study was to investigate uptake of N, P, K and Mg from fruit flies as model prey in two American species of sticky plants suspected of carnivory, namely *Ibicella lutea* and *Proboscidea parviflora* (Martyniaceae), and in two semi-desert species with glandular sticky leaves, *Cleome droserifolia* (Capparidaceae) and *Hyoscyamus desertorum* (Solanaceae) from Israel. Moreover, the same characteristics were studied for comparison in two carnivorous plant species, *Drosophyllum lusitanicum* (Droseraceae), the only known xeromorphic carnivorous plant, and *Roridula gorgonias* (Roridulaceae), a representative of plants showing 'indirect' carnivory. In parallel, activity of external phosphatase on glands or emergences on leaves was investigated in all these species. The use of these two tests as criteria of plant carnivory is discussed.

MATERIALS AND METHODS

Plant material

Mineral and enzymatic tests of carnivory were conducted in four semi-desert plant species suspected of carnivory. *Ibicella lutea* (Lindl.) Van Eselt. and *Proboscidea parviflora* (Woot.) Woot. & Standl. are annual herbs growing natively in subtropical areas in semi-deserts of North, Central and South America. The surfaces of their leaves and young stems are densely covered by abundant glandular hairs with sticky secretions that are able to capture fine prey. Two other experimental plants of this group, *Cleome droserifolia* (Forssk.) Del., a perennial species, and *Hyoscyamus desertorum* (Aschers. ex Boiss.) Taeckh., an annual species, have glandular, sticky surfaces on leaves and grow natively in semi-desert areas of Israel. The latter two species are also able to catch fine prey on their sticky surface. For comparison as controls, two generally accepted carnivorous plants, *Roridula gorgonias* Planch., native to South Africa, and *Drosophyllum lusitanicum* (L.) Link, native in warm but dry areas of the Iberian peninsula, were studied.

Cleome and *Hyoscyamus* seeds were collected from natural sites of these species near the Dead Sea in Israel. Seeds of all other experimental species were provided from outdoor or indoor cultures. Germinating seeds of all species were sown in suitable substrates in plastic pots, i.e. a mixture of peat and sand (1:2, v/v) for *Drosophyllum* and *Roridula*, garden loam for *Proboscidea* and *Ibicella*, and a mixture of sand and garden loam (8:1) for *Cleome* and *Hyoscyamus*. The plants in pots were grown in 0·84-m^2 white polypropylene containers 0·4 m high. Rain water was used for bottom watering the plants. Photosynthetically active irradiance on the level of plants was 15–20 % of that in the open area outdoors. The containers with the plants were maintained in a naturally lit greenhouse. Daily temperatures at plant level fluctuated between 20 and 36 °C and relative air humidity (RH) between 60 and 90 % during the day, and between 16 and 22 °C and 80 and 96 % RH at night. The microclimatic conditions were the same for all plants used throughout this study. Immature plants of all six experimental species were used for both types of tests. Plants of *Roridula* were about 1 year old, whereas plants of all other species were about 3 months old.

Mineral nutrient uptake from flies

As a model prey for testing mineral uptake from prey, fruit flies (*Drosophila melanogaster*) were used. This species has been most commonly used in similar studies (Dixon *et al.*, 1980; Adamec, 2002). *D. melanogaster* (strain Oregon R) flies were cultured in a carbohydrate-rich medium. Adult flies (d. wt approx. 0·3 mg) were narcotized with ether and stored frozen at −12 °C in a vial until use. Dead fruit flies were applied to fully expanded young to medium aged leaves of experimental plants between 16 May and 5 July 2007. Two flies were put on each of three leaves of each of eight experimental plants of each species, totalling six flies per plant. The distance between the two flies on the same leaf was at least 8 mm. After 10 d for *Roridula* and 15 d for the other species, the spent carcasses were carefully removed by fine forceps and put in mineralization glass flasks. For each plant, one fly was always used for N analyses, two for P, and three for K and Mg analyses. As a control, intact dead flies were analysed. Eight parallel analyses of fruit flies were performed on each plant species as well as in control flies. Parallel leaves of the same age were used for determination of phosphatase activity.

The flies were digested and mineralized using concentrated acids, diluted, and analysed for N, P, K and Mg content. N and P content were analysed colorimetrically by FIA star 5010 Analyser (Foss Tecator AB, Hoganas, Sweden) and the metal cations by AAS (Varian Inc., Melbourne, Australia). Further sample preparation and analytical details are provided in Adamec (2002). Nutrient levels of intact and spent flies are expressed as the content of a given nutrient per fly. The difference between intact (controls) and spent flies is expressed as the percentage of nutrient content in intact flies as nutrient uptake (positive sign) by a plant species or nutrient release (negative sign). Differences between control and spent flies for each species were tested by one-way ANOVA (Tukey's HSD test).

Determination of phosphatase activity

For determination of phosphatase activity, leaves of experimental plants were hand-sectioned with a razor blade and assayed with ELF®97 phosphatase substrate (ELFP, Molecular Probes, Eugene, OR, USA) following the protocol of Płachno *et al.* (2006). Unstained samples were used to check for possible autofluorescence. For each species, three plants (two different samples per plant) were examined. Data for phosphatase activity of *Roridula* and *Drosophyllum* were taken from Płachno *et al.* (2006).

Scanning electron microscopy (SEM)

The procedures for preparing samples for SEM were as described by Płachno *et al.* (2005*a*, *b*). Briefly, traps were hand-sectioned with a razor blade and fixed in 2·5 % formaldehyde and 2·5 % glutaraldehyde in 0·05 M cacodylate buffer (pH 7·0) or in 70 % ethanol with 1 % glycerine. Material was later dehydrated in an ethanol and acetone series, and critical-point dried using liquid CO_2. Dried tissues were sputter-coated with gold and viewed with an Hitachi S-4700 microscope (Scanning Microscopy Laboratory of Biological and Geological Sciences, Jagiellonian University).

RESULTS

All species examined had sticky glands on the leaves and stem surface (Fig. 1A–H) where mainly small dipterans were trapped and killed (Fig. 1F). There was some diversity in gland structure among the plant species (Table 1). No phosphatase activity was found in *Hyoscyamus desertorum* (Fig. 1D) and *Proboscidea parviflora* hairs (Fig. 1H). In the other two semi-desert species, phosphatase activity was found (Fig. 1B, F) but was variable (Table 2). A sweet scent was only produced by leaves of *H. desertorum*.

Application of model prey on the leaves revealed two distinct groups of plants based on uptake of mineral nutrients from prey (Table 3). *Roridula* and *Drosophyllum* were found to take up substantial amounts of both N (33–47 % of the total N content), P (62–75 %), K (44–86 %) and Mg (33–39 %) from fruit flies. Except for N uptake in *Drosophyllum*, which showed considerable variability, uptake of all nutrients from flies was statistically significant in these two species. By contrast, usually very weak release of N (7–18 % of the total N content), P (1–3 %), K (4–19 %) and Mg (4–21 %) to flies, rather than nutrient uptake from flies, was found in the four remaining plant species, *Proboscidea*, *Ibicella*, *Cleome* and *Hyoscyamus*. However, this nutrient release to flies was not statistically significant.

DISCUSSION

All species examined possess morphological and anatomical adaptations (hairs or emergences secreting sticky substances) enabling them to catch and kill small insects, especially flies (Table 1, Fig. 1). Phosphatase activity was absent on *Hyoscyamus* and *Proboscidea* foliar hairs whereas low and variable activity was usually found in the two other semi-desert taxa examined (Table 2, Fig. 1). This activity was very low compared with that of other typical carnivorous plants (cf. Płachno *et al.*, 2006). Among true carnivores, *Roridula* has strong phosphatase activity only in epidermis but none in emergences while *Drosophyllum* has strong activity in leaf digestive emergences (Płachno *et al.*, 2006). However, strong external phosphatase activity was also found in tissues in non-carnivorous plants: in active secretory structures such as nectaries or salt glands (Fahn, 1979). External enzyme activity may have a different role, for example to slow microbial activity or release nutrients (P) from organic debris, dust or pollen retained on the leaves (see Adamec, 1997). Spomer (1999) showed that many sticky plants exhibit proteinase activity on their leaves (species of the genera *Geranium*, *Cerastium*, *Stellaria*, *Potentilla*, *Ribes*, *Rosa*, *Solanum*, *Gilia*, *Heuchera*, *Mimulus* and *Penstemon*). Moreover, *Geranium viscosisimum* and *Potentilla arguta* absorbed digestion products of a protein applied to the leaves. Similarly, Darnowski *et al.* (2006) showed strong evidence of protocarnivory in *Stylidium*. It appears that the primary evolutionary function of sticky surfaces in *Proboscidea*, *Ibicella* and *Cleome* is to defend the plants against herbivorous animals (mammals as well as insects). Moreover, species of the Martyniaceae produce a scent (repellent effect) that is unpleasant to some animals, in contrast to *Drosophyllum* which has a honey-like, sweet scent that acts as an attractant to prey (Juniper *et al.*, 1989). A sweet scent is also produced by *Hyoscyamus*, although its role remains unclear.

True carnivorous plants possess not only anatomical modifications to trap animals and external enzyme activity in traps, but crucially show sufficient mineral nutrient absorption from prey carcasses to support plant growth and fitness in competition with non-carnivorous plants (Juniper *et al.*, 1989; Adamec, 1997). Of all six species tested, only *Roridula* and *Drosophyllum* have been found to take up nutrients (N, P, K, Mg) from applied fruit flies (Table 3; cf. Adamec, 2002). Despite the absence of phosphatase activity on *Roridula* emergences, the efficiency of P uptake and of other mineral nutrients in *Roridula* was very high. Here, the applied fruit flies were evidently not in direct contact with foliar epidermis containing high phosphatase activity (Płachno *et al.*, 2006) which would have enabled direct nutrient uptake through cuticular pores (Anderson, 2005). As these nutrients from the applied prey carcasses could not have been lost beyond the plants in any way, this suggests that external digestive plant enzymes may not be essential for efficient mineral nutrient uptake

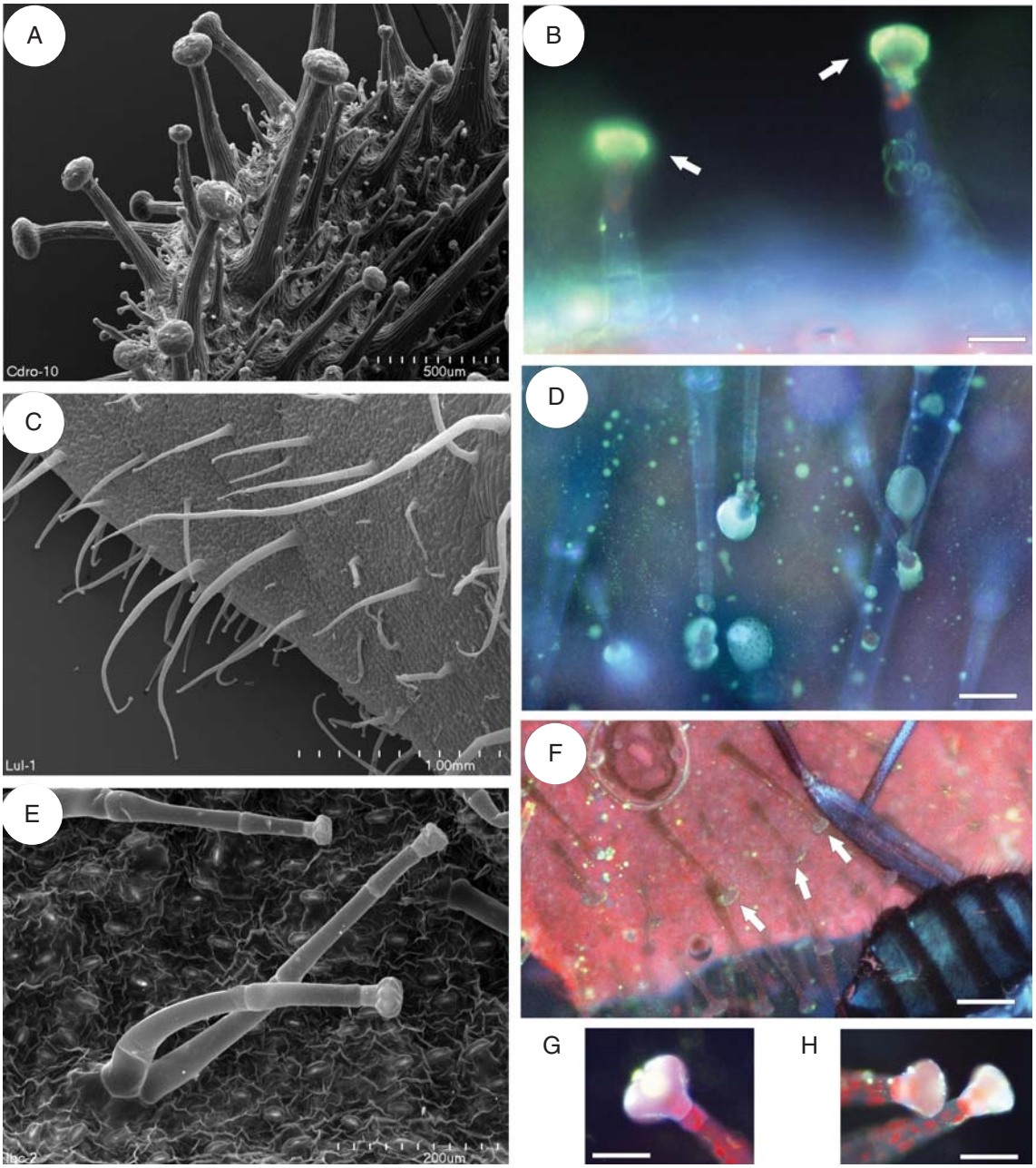

FIG. 1. Sticky hairs of experimental plants. (A) Part of a *Cleome droserifolia* leaf covered with sticky hairs. (B) Positive phosphatase activity in secretory cells (arrows) of hairs of *Cleome droserifolia.* Scale bar = 42 μm. (C) Part of a *Hyoscyamus desertorum* leaf covered with sticky hairs. (D) No phosphatase activity in secretory cells of hairs of *Hyoscyamus desertorum.* Scale bar = 70 μm. (E) Sticky hairs of *Ibicella lutea*. (F) Positive phosphatase activity in secretory cells (arrows) of hairs of *Ibicella lutea*. Note trapped small dipterans. Scale bar = 282 μm. (G) Enlarged head of hairs of *Ibicella lutea.* Scale bar = 56 μm. (H) No phosphatase activity in secretory cells of hairs of *Proboscidea parviflora.* Scale bar = 56 μm.

TABLE 1. *Comparison of features of glands in semi-desert plants*

Structure	Parameter	*Hyoscamus*	*Cleome*	*Ibicella* and *Proboscidea*
Stalk	No. of cells	A few stalk cells and one middle (barrier) cell	A few stalk cells or multicellular stalk	A few stalk cells and one middle (barrier) cell
	Chloroplasts	Present	Present	Present
Head	No. of cells	One or a few	A few or multicellular	Few
	Shape	Club-shaped	Discoid	Head-shaped

TABLE 2. *Comparison of phosphatase activity in glands of experimental plants*

Phosphatase activity	*Hyoscyamus*	*Cleome*	*Ibicella* and *Proboscidea*	*Roridula* and *Drosophyllum*
Presence	Absent, strong autofluorescence of secretory products	Present or absent	Generally absent, low surface activity in *Ibicella*	Present in *Drosophyllum*, absent in *Roridula*
Localization	–	Outer cells of the head, surface activity of head cells	Low surface activity of head cells	*Drosophyllum*, digestive glands; *Roridula*, only epidermis

Data for *Roridula* and *Drosophyllum* are taken from Płachno *et al.* (2006).

TABLE 3. *Comparison of nutrient content in* Drosophila *flies after exposure on plant leaves for 10–15 d*

Species	Nutrient content in one spent fly [μg (% of nutrient uptake)]			
	N	P	K	Mg
Control flies	18·6 ± 1·0	2·49 ± 0·06	2·96 ± 0·11	0·241 ± 0·006
Roridula gorgonias	9·9 ± 1·0** (46·6)	0·62 ± 0·16** (74·9)	0·42 ± 0·14** (85·8)	0·147 ± 0·015** (39·0)
Drosophyllum lusitanicum	$14·8 \pm 3·1^{n.s.}$ (32·5)	0·96 ± 0·27** (61·5)	1·65 ± 0·17** (44·2)	0·162 ± 0·034* (32·9)
Proboscidea parviflora	$20·0 \pm 1·0^{n.s.}$ (−7·9)	$2·56 \pm 0·13^{n.s.}$ (−2·7)	$3·17 \pm 0·23^{n.s.}$ (−7·0)	$0·268 \pm 0·013^{n.s.}$ (−11·2)
Ibicella lutea	$21·9 \pm 0·6^{n.s.}$ (−18·0)	$2·39 \pm 0·09^{n.s.}$ (4·0)	$3·53 \pm 0·10^{n.s.}$ (−19·2)	$0·290 \pm 0·012^{n.s.}$ (−20·6)
Cleome droserifolia	$20·9 \pm 0·6^{n.s.}$ (−11·3)	$2·52 \pm 0·11^{n.s.}$ (−1·2)	$3·08 \pm 0·10^{n.s.}$ (−4·4)	$0·250 \pm 0·015^{n.s.}$ (−3·7)
Hyoscyamus desertorum	$19·8 \pm 1·3^{n.s.}$ (−6·8)	$2·52 \pm 0·10^{n.s.}$ (−1·2)	$2·95 \pm 0·21^{n.s.}$ (−0·3)	$0·262 \pm 0·012^{n.s.}$ (−8·8)

Uptake of nutrients from flies as a percentage of control unspent flies (in parentheses) is shown. Statistically significant uptake (positive sign) or release (negative sign) of nutrients (one-way ANOVA): ** $P < 0·01$, * $P < 0·05$; n.s., non-significant at $P > 0·05$; $n = 8$.

from prey in carnivorous plants. Instead, autolysis of prey tissues and release of digestive enzymes by microbial commensals may occur. By analogy, the same has been observed in aquatic *Utricularia* traps, i.e. the absence of aminoproteases from trap fluid (Sirová *et al.*, 2003) but evidently efficient N and P uptake from prey (Friday and Quarmby, 1994; for the genus *Heliamphora* see also Jaffe *et al.*, 1992). The relatively high variance in mineral nutrient uptake found in *Drosophyllum* (Table 3) could be due to the very narrow leaves in these immature plants, the applied flies being in contact with a minimum number of digestive glands.

The fact that Spomer (1999) found proteinase activity in the great majority of examined plant species with glandular sticky leaves indicates that this phenomenon is common in higher plants. However, the author was not able to distinguish whether the enzyme activity was of plant or commensal origin. Moreover, the ability of leaves of two plant species tested to take up substantial amounts of a protein applied onto the leaves suggests that the ability of leaves to absorb organic substances is also common in higher plants. The results provided by Spomer (1999) show clearly that many plants with glandular sticky leaves (protocarnivorous plants) are able to take up mineral and organic nutrients from captured prey carcasses, as for true carnivorous plants. What, therefore is the main difference between carnivorous and non-carnivorous (inclusing protocarnivorous) plants? It appears that the difference is of a quantitative rather than qualitative nature.

In conclusion, the principal criterion distinguishing between carnivorous and all other non-carnivorous (including protocarnivorous) plants, regardless of leaf (trap) architecture and function, should be based on whether prey capture on leaves and subsequent nutrient uptake from prey is or is not ecologically important for the plant under natural conditions, i.e. provides a substantial proportion of its seasonal N and P gain. In protocarnivorous plants with glandular sticky leaves, the carnivorous nutritional function is presumably negligible, while other functions (defence against herbivores, retention of detritus and pollen, protection against excessive irradiance) prevail. Therefore, digestive enzyme secretion and the capability for foliar nutrient uptake, although commonly accepted as criteria of plant carnivory and major features of the carnivorous syndrome, are not sufficient in themselves to confirm that such a relationship is potentially ecologically important. The simple test of mineral nutrient uptake from model prey (Dixon *et al.*, 1980; Hanslin and Karlsson, 1996; Adamec, 2002; present study) is more suitable than enzyme secretion alone to confirm plant carnivory, although the crucial and final criterion should be ecological. Based on this mineral uptake test and together with great capacity for prey capture (L. Adamec, unpubl. res.), *Roridula* and *Drosophyllum* are considered to be carnivorous and *Ibicella*, *Proboscidea Cleome* and *Hyoscyamus* non-carnivorous.

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