

INVITED REVIEW

## Recent ecophysiological, biochemical and evolutionary insights into plant carnivory

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- **Background** Carnivorous plants are an ecological group of approx. 810 vascular species which capture and digest animal prey, absorb prey-derived nutrients and utilize them to enhance their growth and development. Extant carnivorous plants have evolved in at least ten independent lineages, and their adaptive traits represent an example of structural and functional convergence. Plant carnivory is a result of complex adaptations to mostly nutrient-poor, wet and sunny habitats when the benefits of carnivory exceed the costs. With a boost in interest and extensive research in recent years, many aspects of these adaptations have been clarified (at least partly), but many remain unknown.
- **Scope** We provide some of the most recent insights into substantial ecophysiological, biochemical and evolutionary particulars of plant carnivory from the functional viewpoint. We focus on those processes and traits in carnivorous plants associated with their ecological characterization, mineral nutrition, cost–benefit relationships, functioning of digestive enzymes and regulation of the hunting cycle in traps. We elucidate mechanisms by which uptake of prey-derived nutrients leads to stimulation of photosynthesis and root nutrient uptake.
- **Conclusions** Utilization of prey-derived mineral (mainly N and P) and organic nutrients is highly beneficial for plants and increases the photosynthetic rate in leaves as a prerequisite for faster plant growth. Whole-genome and tandem gene duplications brought gene material for diversification into carnivorous functions and enabled recruitment of defence-related genes. Possible mechanisms for the evolution of digestive enzymes are summarized, and a comprehensive picture on the biochemistry and regulation of prey decomposition and prey-derived nutrient uptake is provided.

**Key words:** Carnivorous plant, *Dionaea*, *Drosera*, evolution of carnivory, terrestrial and aquatic species, co-option, cost–benefit relationships, mineral nutrient economy, *Nepenthes*, digestive enzymes, regulation of enzyme secretion, hunting cycle.

### INTRODUCTION

... we see how little has been made out in comparison with what remains unexplained and unknown.

[Darwin \(1875, p. 223\)](#)

Carnivorous plants (CPs) are a diverse ecological group of flowering plants, usually growing in nutrient-poor and wet or aquatic habitats, and are able to gain a proportion of mineral nutrients from animal carcasses captured by specialized traps to support their growth ([Ellison and Adamec, 2018a](#)). Since [Darwin \(1875\)](#) first described this plant group and its carnivorous habit, thousands of various studies covering all aspects of carnivorous plant biology have been published to date (see [Juniper \*et al.\*, 1989](#); [Ellison and Adamec, 2018a](#)). Immense accumulation of new items of knowledge on CPs led the latter

authors to edit a new monograph covering the many diverse topics of plant carnivory. We now understand all of the basic purposeful adaptations of most genera of CPs relatively well, and this extends to all the possible differences between CPs and ‘normal’, non-carnivorous plants (non-CPs). Botanists have been attracted by the remarkable traits of CPs such as their convergent evolution, the formation of sophisticated traps, digestive enzymes which are mainly co-opted from pathogenesis-related (PR) proteins, the particulars of mineral nutrition and the stimulation of photosynthesis and subsequently of growth and development of CPs using prey-derived nutrients.

Here we review, from a functional perspective, the main ecophysiological traits of CPs associated mainly with mineral nutrition and photosynthesis, the cost–benefit relationships and the biochemistry of prey digestion and nutrient absorption. The origin of digestive enzymes and genomes of CPs are discussed in evolutionary relationships. Additionally, we compare various

traits between terrestrial and aquatic CPs, as this issue is commonly neglected, and between CPs and their non-CP counterparts. This review follows up on several chapters in Ellison and Adamec (2018a) (Adamec, 2018; Adamec and Pavlovič, 2018; Ellison and Adamec, 2018b; Fleischmann *et al.*, 2018; Givnish *et al.*, 2018; Matušková *et al.*, 2018; Renner *et al.*, 2018), but we put an emphasis on reviewing the newest literature not included in the monograph.

## CARNIVOROUS PLANTS: DIVERSITY AND GENERAL FEATURES

### *Carnivorous syndrome and atypical cases*

All currently known approx. 810 CP species exhibit at least some or all of the substantial functional processes which enable the CPs to capture prey and obtain a proportion of their nutrients from this (Ellison and Adamec, 2018b). They are: rapid trap movements that are (in Droseraceae) regulated electrophysiologically; trap secretion of hydrolytic enzymes that digest organic macromolecular compounds and solubilize them; foliar (trap) uptake of digested nutrients; stimulation of root nutrient uptake by foliar nutrient uptake; and stimulation of plant growth by foliar nutrient uptake. To what extent are these processes exclusive to CPs? All these processes can also occur in non-CPs, but usually singly, and frequently have dissimilar or marginal functions. In CPs, these processes are normally coupled in series, forming a co-ordinated, purposeful cluster of traits called the ‘carnivorous syndrome’ (Juniper *et al.*, 1989; Adamec, 1997; Ellison and Adamec, 2018b). The proportion of each trait is variable among CP genera and species. As the uptake of growth-limiting mineral nutrients from prey is evidently the main and direct physiological benefit of carnivory, in line with the literature (cf. Lloyd, 1942; Givnish, 1989; Juniper *et al.*, 1989; Adamec, 1997, 2011; Rice, 2011; Pavlovič and Saganová, 2015), Ellison and Adamec (2018b) have recently formulated five essential traits of the carnivorous syndrome: (1) prey capture in specialized traps; (2) killing the captured prey; (3) digesting the prey; (4) absorption of nutrients from the killed and digested prey; and (5) utilization of these nutrients for plant growth and development.

All five of these traits must occur in all terrestrial and aquatic CP species. However, why is ‘attraction of prey’ not on the list, as this process has also been stated by many authors (e.g. Givnish, 1989; Horner *et al.*, 2018)? Recently, it has been considered that only roughly half of CP species (all with pitcher traps, *Drosera* spp., *Dionaea* and *Drosophyllum*) do attract their prey, while prey attraction has not been confirmed or is unknown in others (e.g. *Catopsis*, *Paepalanthus*, *Genlisea* and *Utricularia*) – it is thus neither universal nor essential, but only amplifies prey capture. Why is ‘digesting of prey’ considered a syndrome trait even though many CP species do not secrete their own digestive enzymes? In fact, CPs form a gradient or continual series of digestive enzyme secretion: from species secreting (fully or partly) their own digestive enzymes (‘holocarnivorous’, genera *Aldrovanda*, *Byblis*, *Dionaea*, *Drosera*, *Drosophyllum*, *Cephalotus*, *Nepenthes*, *Philcoxia*, *Pinguicula*, *Sarracenia* and *Utricularia*) or none

(‘hemicarnivorous’ or ‘paracarnivorous’, *Brocchinia*, *Catopsis*, *Darlingtonia*, *Heliophora*, *Paepalanthus* and *Roridula*; see Ellison and Adamec, 2018a, b). However, it is impossible to simply class the carnivorous genera within this gradient as some genera (typically *Sarracenia*) contain various species differing in their enzyme secretion; in others, enzyme secretion is insufficiently known (Ellison and Adamec, 2018b; Miller *et al.*, 2018). Furthermore, digestive enzymes could have evolved in carnivorous plants partly by appropriating the digestive enzymes of other organisms (Wheeler and Carstens, 2018). Yet the digestive fluid of all CP species always contains commensal micro-organisms (e.g. bacteria, fungi and ciliates) and/or also other organisms (e.g. insects) which contribute to or even fully perform prey digestion as more or less specialized digestion mutualists (Bittleston, 2018; Cross *et al.*, 2018; Miller *et al.*, 2018; Moran *et al.*, 2018; Sirová *et al.*, 2018a). Ellison and Adamec (2018b) have also mentioned an entirely underestimated process of prey autolysis in prey digestion. Therefore, whatever the source of digestive enzymes is, the captured prey must be digested in the traps before the nutrients are absorbed. A partial exception may be carnivory in two *Roridula* species in which the captured prey is consumed by specific hemipteran bugs as digestive mutualists which defecate on the leaves: the pre-digested nutrients from the faeces are absorbed through specialized cuticular gaps (Cross *et al.*, 2018; Moran *et al.*, 2018). Moreover, several *Nepenthes* species are known to partly lose some carnivorous characteristics as they gain their nutrients partly from the faeces or urine of mutualistic tree shrews or bats (‘coprophagy’) or from leaf detritus and litter (‘detritivory’); however, the plants still secrete their own digestive enzymes (Adamec and Pavlovič, 2018; Kocáb *et al.*, 2021; Zulkapli *et al.*, 2021; see below). Thus, the dominant majority of CP species can obtain nutrients directly from prey using digesting enzymes, while several other species also benefit indirectly from plant debris, or the faeces or urine of digestive mutualists living inside (e.g. bacteria) or outside (e.g. mammals) the traps.

Is the boundary between CPs and non-CPs clear cut? Thousands of vascular plants (e.g. potato and *Salvia glutinosa*) are able to trap small insects by their sticky, glandular leaves, stems or flowers (Fig. 1). They were called ‘protocarnivorous’, but this term is confounding. Many of them possess proteinase activity on their surface and some even exhibit uptake of organic nutrients (Spomer, 1999; Darnowski *et al.*, 2006). However, sticky organs with proteinase activity have apparently originated as a surface defence against small herbivores or microbial pathogens (Fig. 1; Matušková *et al.*, 2018). In line with this, Fleischmann *et al.* (2018) suggest that the common ancestor of CPs of the order Caryophyllales (see below) had glandular hairs or multicellular, vascularized glands, which represented a first step (exaptation) in the evolution of carnivory. Płachno *et al.* (2009) distinguished true CP species from four species with glandular leaves suspected of carnivory on the basis of high uptake of N, P, K and Mg from fruit flies and denoted this mineral uptake as a main physiological criterion of carnivory. Nutritionally, the latter authors suggested the basic criterion that prey capture in CPs should be ecologically important, which evidently does not apply in protocarnivorous plants. For example, *Stylidium* species only have a sticky glandular



FIG. 1. Two sides of the same coin. Sticky organs in plants from the same order Lamiales. (A) The inflorescence of *Salvia glutinosa* is covered by sticky trichomes, which capture insects for purely defensive purposes, and is not considered carnivorous, whereas (B) sticky leaves of the carnivorous butterwort *Pinguicula grandiflora* clearly digest captured prey. Such defensive structures might have been a prerequisite for evolution of botanical carnivory.

inflorescence to potentially capture fine insects only during the flowering period. Nge and Lambers (2018) verified this concept in eight field-growing *Stylidium* species in Western Australia. They estimated  $\delta^{15}\text{N}$  signatures that resembled more closely those in co-occurring non-CPs than in two *Drosera* species, which supports the view that *Stylidium* species do not rely on prey capture as an N source.

#### Evolutional lineages

At least 810 species of CPs have been reported in the most recent list (Ellison and Adamec, 2018a; Fleischmann et al., 2018), but some new species are found and described every year. However, <0.3 % of all vascular plants are carnivorous. These species are currently classed with 19 genera, 12 plant families and five orders (Fig. 2; Table 1). Evidently, CPs represent a polyphyletic grouping as they originated at least ten times independently of each other (Ellison and Gotelli, 2009; Fleischmann et al., 2018; Hedrich and Fukushima, 2021). They possess many structural and physiological trap adaptations and also expression of specific genes. Five structural types of foliar traps have evolved in CPs: adhesive ('flypaper') traps with a sticky glandular surface; pitfall ('pitcher') traps forming a central cavity or small tanks; mobile snap-traps with rapidly closing lobes; suction ('bladder') traps actively forming negative pressure inside; and specialized eel ('lobster-pot' and 'cork-screw') traps formed by screwed, tubular leaves with a narrow cavity lined with retrorse hairs (Fig. 2). Remarkably, these trap types reflect convergent, parallel or even divergent evolution of the structural traits associated with carnivory and represent an advantageous model of plant trait evolution (Ellison and Gotelli, 2009): e.g. the adhesive traps of various structure have evolved independently by convergent evolution in at least five recent lineages and this is also the case with the pitfall traps of modified structure. Convergent evolution of functional traits of carnivory (e.g. modes of prey capture and

prey digestion) is even more striking. In contrast to the initially adhesive traps in the ancestors of Droseraceae and *Nepenthes*, these have diverged to respective snap and pitcher traps. The ancestors of Lentibulariaceae have diverged to eel and suction traps between closely related genera. Some *Drosera* species have combined adhesive traps with rapidly moving external tentacles (Poppinga et al., 2012).

How many times in plant evolution has carnivory evolved and how old are CPs? On the basis of molecular–taxonomic studies, Fleischmann et al. (2018) were able to conclude that plant carnivory probably evolved independently at least ten (or 11) times: once in Oxalidales, once (or twice according to Palfalvi et al., 2020) in the non-core Caryophyllales (i.e. Nepenthales), twice in the Ericales (families Sarraceniaceae and Roridulaceae) and three times each in the Lamiales (families Byblidaceae, Lentibulariaceae and Plantaginaceae) and Poales (family Eriocaulaceae and genera *Brocchinia* and *Catopsis* within Bromeliaceae) (see Table 1). The repeated and independent origin of CPs is mirrored in that CP families are scattered among other non-carnivorous taxa, in both monocots and dicots. Moreover, no plant order is entirely carnivorous, but carnivory dominates in families: of 12 families including CPs, eight are exclusively carnivorous (Droseraceae, Nepenthaceae, Drosophyllaceae, Sarraceniaceae, Roridulaceae, Cephalotaceae, Byblidaceae and Lentibulariaceae), while non-carnivorous genera or species predominate in the other four families (Bromeliaceae, Eriocaulaceae, Dioncophyllaceae and Plantaginaceae; Table 1). Carnivorous species in these four families are relatively young (estimated phylogenetic age of 1.9–19.3 Mya; Fleischmann et al., 2018). Moreover, in the formerly carnivorous family Dioncophyllaceae, a conspicuous retreat from carnivory has evolved due to an adaptation to living in the rain forest: only *Triphyophyllum peltatum* has remained as a part-time carnivorous plant, while its two sister genera (*Dioncophyllum* and *Habropetalum*) have completely lost their carnivory (for a discussion, see Fleischmann et al., 2018). If the origin of the first flowering plants (angiosperms) may be dated to approx. 194 Mya, molecular clock estimations have revealed the stem age of carnivorous Caryophyllales as the oldest lineage 83–95.1 Mya and the divergence time from Droseraceae at 76.8–84.8 Mya (see Fleischmann et al., 2018). On the generic level, the phylogenetic age of *Nepenthes* is 84.8 Mya, of *Drosera*, *Dionaea* and *Aldrovanda* 48–53.4 Mya, and of *Pinguicula*, *Genlisea* and *Utricularia* 31–33.5 Mya. Therefore, the phylogenetic tree of CPs with molecular clock estimations indicate that CPs originated relatively early in the phylogeny of flowering plants and that their origin was supported by frequent radiation of flowering plant taxa during the Tertiary period. Curiously, the oldest known fossil of CPs (*Roridula* from Baltic Sea amber) is 'only' 35–47 Mya old (Sadowski et al., 2015).

#### Genome size and gene number

During the last two decades, rapid and cheap DNA sequencing as well as the development of long-read technologies have contributed to elucidate the principles of genomic changes which preceded or accompanied the evolution of CPs. One can raise the following questions. What has the genomic evolution



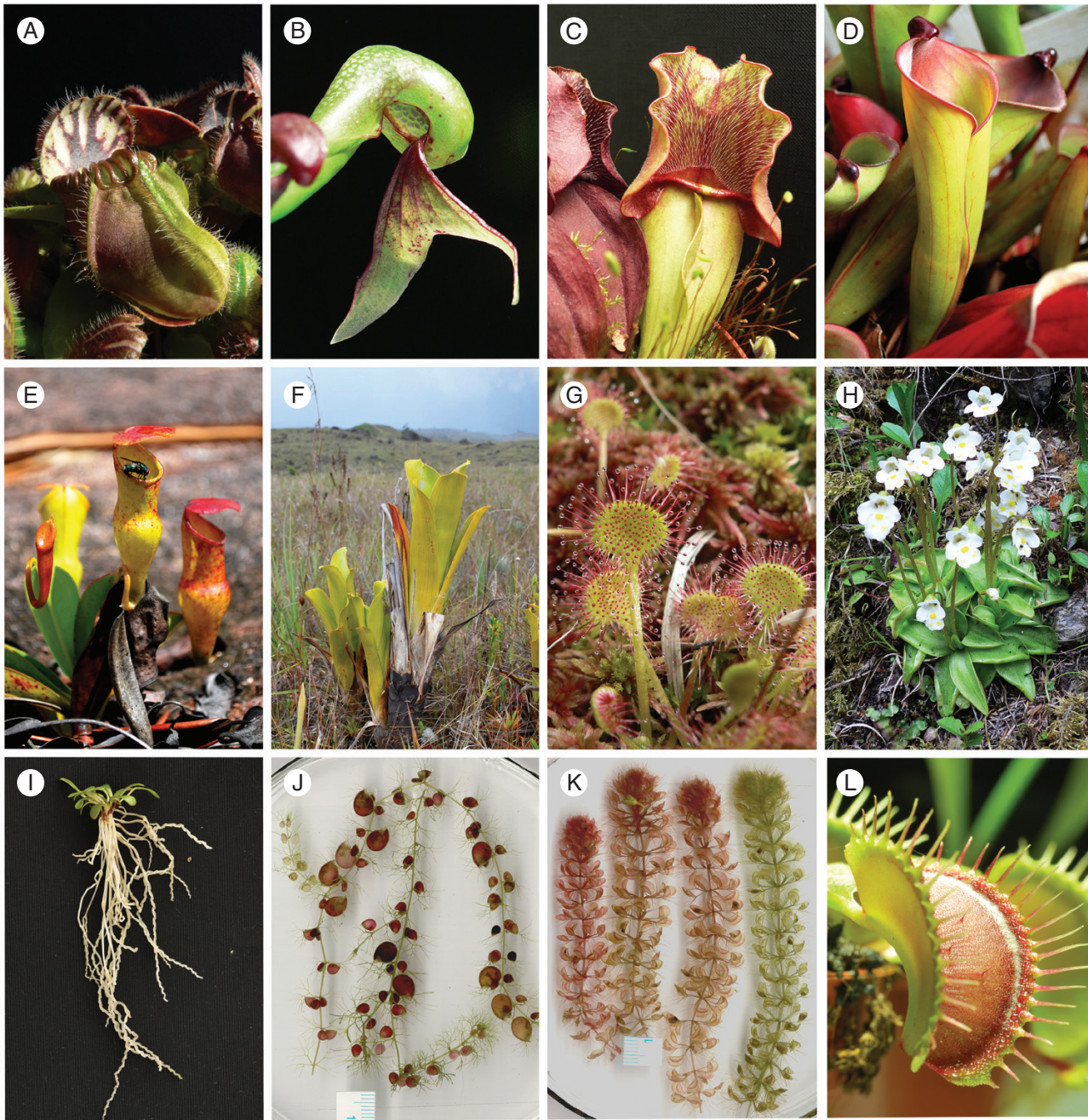


FIG. 2. The carnivorous plants capturing their prey by different trapping mechanisms. Pitcher traps: (A) Albany pitcher plant, *Cephalotus follicularis* (Cephalotaceae), (B) California pitcher plant, *Darlingtonia californica* (Sarraceniaceae), (C) North American pitcher plant, *Sarracenia purpurea* ssp. *venosa* (Sarraceniaceae), (D) marsh pitcher plant, *Heliamphora folliculata* (Sarraceniaceae), (E) tropical pitcher plant, *Nepenthes pervillei* (Nepenthaceae), (F) bromelia, *Brocchinia hectioides* (Bromeliaceae); sticky traps: (G) sundew, *Drosera rotundifolia* (Droseraceae), (H) butterwort, *Pinguicula alpina* (Lentibulariaceae); eel traps: (I) corkscrew plant, *Genlisea hispida* (Lentibulariaceae); suction traps: (J) bladderwort, *Utricularia reflexa* (Lentibulariaceae); snap traps: (K) waterwheel plant, *Aldrovanda vesiculosa* (Droseraceae), (L) Venus flytrap, *Dionaea muscipula* (Droseraceae).

achieved? What is the genome size and number of genes in extant CPs? Do they reflect carnivory? Since the pioneering study by Greilhuber *et al.* (2006), genome size has been measured in >200 CP species of nearly all genera (e.g. Ibarra-Laclette *et al.*, 2013; Fleischmann *et al.*, 2014; Veleba *et al.*, 2014, 2017, 2020; Lan *et al.*, 2017; Renner *et al.*, 2018; Palfalvi *et al.*, 2020). Surprisingly, all estimated nuclear genome sizes (1C) varied by at least two orders of magnitude: from 61 Mbp in *Genlisea*

*tuberosa* to 10 417 Mbp in *Drosophyllum lusitanicum*, but varied by 20× also within families, e.g. from 61 to 1510 Mbp within three genera of Lentibulariaceae and from 244 to 5464 Mbp within three genera of Droseraceae. Even within a single genus, a 25-fold range of genome sizes was found in *Genlisea* (Fleischmann *et al.*, 2014; Vu *et al.*, 2015). On the other hand, species of *Drosera* within evolutionary lineages had uniform genome size, but young *Drosera* lineages had either very small



TABLE 1. Ten clades of carnivorous plants of independent origin after Fleischmann et al. (2018)

Clade	Family	Genus	Trap type	No. of species
gen. <i>Brocchinia</i>	Bromeliaceae	<i>Brocchinia</i>	Pitfall	2
gen. <i>Catopsis</i>	Bromeliaceae	<i>Catopsis</i>	Pitfall	1
gen. <i>Paepalanthus</i>	Eriocaulaceae	<i>Paepalanthus</i>	Pitfall	1
ord. Nepenthales*	<b>Droseraceae</b>	<i>Drosera</i>	Adhesive	Approx. 250
		<i>Dionaea</i>	Snap	1
		<i>Aldrovanda</i>	Snap	1
	<b>Nepenthaceae</b>	<i>Nepenthes</i>	Pitfall	130–160
	<b>Drosophyllaceae</b>	<i>Drosophyllum</i>	Adhesive	1
	Dioncophyllaceae	<i>Triphyophyllum</i>	Adhesive	1
fam. Sarraceniaceae	<b>Sarraceniaceae</b>	<i>Sarracenia</i>	Pitfall (eel)	11
		<i>Darlingtonia</i>	Pitfall	1
		<i>Heliamphora</i>	Pitfall	23
fam. Roridulaceae	<b>Roridulaceae</b>	<i>Roridula</i>	Adhesive	2
fam. Cephalotaceae	<b>Cephalotaceae</b>	<i>Cephalotus</i>	Pitfall	1
fam. Byblidaceae	<b>Byblidaceae</b>	<i>Byblis</i>	Adhesive	8
fam. Lentibulariaceae	<b>Lentibulariaceae</b>	<i>Pinguicula</i>	Adhesive	Approx. 96
		<i>Genlisea</i>	Eel	30
		<i>Utricularia</i>	Suction	Approx. 240
gen. <i>Philcoxia</i>	Plantaginaceae	<i>Philcoxia</i>	Adhesive	7

For each genus, the number of species is shown; gen., genus; fam., family; ord., order. Exclusively carnivorous families are labelled in bold

\*Palfalvi et al. (2020) suggested that carnivory arose independently in Nepenthaceae and Droseraceae

or very large genomes (Veleva et al., 2017). Uniform genome size was also found in species within the genera *Sarracenia* and *Heliamphora* (Veleva et al., 2020). The smallest genomes (61–102 Mbp) estimated in some *Genlisea* and aquatic *Utricularia* species are comparable with bacterial genomes and represent absolutely the smallest genomes known in flowering plants, much smaller than in the model plant *Arabidopsis thaliana* (157 Mbp; Greilhuber et al., 2006). Evidently, these could become an excellent model for studying the structural–functional traits of plant genomes. Such miniature genomes of CPs have been hypothesized to reflect N and P resource saving (Veleva et al., 2014), or to be a result of an oxidation damage of DNA caused by a unique cytochrome *c* oxidase (COX) mutation (Albert et al., 2010). Recent findings, however, have refuted the role of genome size in plant carnivory (Veleva et al., 2020); small genomes have been found to be operative under sustained selection for rapid cell division and, thus, plant growth in nutritionally limited environments in non-CPs (Hessen et al., 2009).

Although large-scale whole-genome duplication (WGD) and small-scale tandem gene duplications occurred during the evolution of different carnivorous lineages (Droseraceae, Lentibulariaceae and Cephalotaceae), the number of nuclear coding genes in extant CP species is comparable with or usually lower than approx. 28 000 in the model *A. thaliana*: 18 111 in *Drosera spatulata*, 21 135 in *Dionaea muscipula*, 25 123 in *Aldrovanda vesiculosa*, 31 511 in *Utricularia gibba* and 36 503 in *Cephalotus follicularis* (Ibarra-Laclette et al., 2013; Lan et al., 2017; Fukushima et al., 2017; Renner et al., 2018; Palfalvi et al., 2020). Moreover, *U. gibba* with its miniature genome of approx. 100.7 Mbp maintains a typical number of genes for a plant, but the compressed genome architecture reflects a drastic reduction in non-genic DNA (Ibarra-Laclette et al., 2013). The current view on the contribution of WGD and/or tandem gene duplications to the evolution of botanical carnivory slightly differs among the different evolutionary lineages of CPs. Although WGD probably did not contribute to the origins of carnivory-related genes in *Utricularia* and *Cephalotus* (Fukushima et al., 2017;

Lan et al., 2017; Renner et al., 2018), a recent study indicates that ancient WGD brought gene material for diversification into carnivorous functions in Droseraceae (Palfalvi et al., 2020). Although more recent WGD is probably not involved in the origins of carnivory-related genes in any of the CP lineages, the adaptive role of small-scale gene duplication events is more obvious (Renner et al., 2018). Among expanded gene families are, for example, genes encoding digestive enzymes, membrane receptors and numerous nutrient transporters (Carretero-Paulet et al., 2015; Lan et al., 2017; Palfalvi et al., 2020). Generally, the genes ensuring capture and digestion of prey and nutrient absorption in traps of extant CPs have been adapted from those involved in responses to biotic and abiotic stresses, including pathogen and herbivore attack, i.e. from plant defence mechanisms (see below). In particular, the recruitment of genes responsible for root nutrient uptake to the traps is considered the major mechanism in the evolution of carnivory (Palfalvi et al., 2020). The co-option of similar genes in several independent origins of CPs involved carnivory-specific similar selective pressures and resulted in convergent amino acid substitution (Fukushima et al., 2017).

On the other hand, a partial carnivorous nutrition mitigated the selective pressure on genes involved in non-carnivorous habits; this step led to abundant gene losses. In rootless aquatic *Aldrovanda*, a marked loss of genes associated with root structure and functioning occurred (Palfalvi et al., 2020). A similar scenario was also confirmed in two aquatic rootless *Utricularia* species (Bárta et al., 2015). Very similar root gene losses in *U. gibba* (Ibarra-Laclette et al., 2013) suggest that they occurred as early as in their common ancestor. Moreover, a profound convergent loss of many plastid genes was also confirmed in all three genera of Droseraceae (Nevill et al., 2019) and in *Nepenthes* (Gruzdev et al., 2019). Curiously, these gene losses in both families are similar to those occurring in hemiparasitic and mycoheterotrophic plants, and suggest that they have evolved by the similar switch from autotrophy to mixo- or heterotrophy.

## ECOPHYSIOLOGY AND FUNCTIONAL ECOLOGY

*Ecological conditions at CP sites and principal ecophysiological traits of CPs*

No meta-analysis of the available mineral nutrient content in soils inhabited by most terrestrial CPs or those inhabited by only non-CP species has been conducted so far. It follows, though, from many specific studies that the available N, P, K (and in acidic, non-calcareous soils also Ca and Mg) soil content (per dry weight) in typical soils inhabited by CPs is usually around 5–100× lower than in those dominated by non-CPs (cf. Juniper *et al.*, 1989; Adamec, 1997, 2011; van der Ent *et al.*, 2015). However, the total nutrient content in highly organic soils hosting CPs is commonly higher than this by 1–3 orders of magnitude and equals that in soils hosting typical non-CPs. Terrestrial CPs grow in organic, clayish, sandy or rocky soils (but also as epiphytes), which are wet or even waterlogged at least during their growing season. These wet soils are hypoxic or entirely anoxic, possess a low redox potential, may contain some toxic substances, and some essential micronutrients may become unavailable – all these factors are considered stressful for non-wetland plants (e.g. Crawford, 1989). Of all species, there is only one strict exception to the above rule – the xerophytic *D. lusitanicum* (Adlassnig *et al.*, 2006).

It is evident that carnivory represents an ecophysiological adaptation to the combination of both unfavourable factors – nutrient-poor and wet soils. We can ask: which of these two factors is more growth-limiting for CPs? This is an admission that our knowledge of CP adaptation to soil anoxia and waterlogging is still insufficient. Generally, short, weakly branched roots usually represent only from 3.4 to 23 % of the total plant biomass, and CPs can easily recover lost roots (Adamec, 1997, 2011). In a pilot study in a nutrient-poor, wet pine savanna in Mississippi, Brewer *et al.* (2011) compared the root systems of six rooted CP species with those of 48 non-CP species. The mean maximal length of CP roots was only 58 % of that of co-occurring non-CPs, and zero root porosity (i.e. air spaces) was found in all CPs, as opposed to highly variable porosity values ranging between 0 and 70 % in non-CPs (mean 22 %). Yet CPs were four times more indicative of wet habitats than co-occurring non-CPs, were positively associated with the wettest microsites and their abundance was significantly reduced in drier substrates. The results support the hypothesis that carnivory is advantageous in wet habitats but disadvantageous in drier habitats and is more indicative of wet conditions than of nutrient-poor soils alone. Furthermore, in eutrophic wetlands, the growth of CPs with absent or poorly developed root aerenchyma is inhibited by high concentrations of soil toxins. In line with this, the poor survival of *Sarracenia alata* in a nutrient-rich marsh correlated with very low soil redox potential, but not with competition (Abbott and Brewer, 2016). In these habitats, the growth of CPs could also be inhibited by high concentrations of  $\text{NH}_4^+$  (*sensu* Cao *et al.*, 2009). In conclusion, terrestrial wet soils can differ greatly in their available N and P content (e.g. peat vs. lake sediment), but CPs prefer and tolerate only barren wet soils (see below).

The occurrence of terrestrial CPs in eutrophic wetlands is primarily limited physiologically by abiotic stress rather than by competition by non-CPs with highly porous roots (for a

discussion, see Brewer and Schlauer, 2018). Important supportive arguments for this conclusion follow from growing CPs in *in vitro* cultures in which high, non-ecological macronutrient concentrations usually exceed the natural concentrations by two orders of magnitude. Surprisingly, most CP species in sterile cultures can grow well in these concentrated mineral media with sucrose (see Legendre and Darnowski, 2018), which proves that even non-ecologically high macronutrient concentrations in the rooting medium are tolerated if the medium is aerobic. Thus, anoxic stress conditions limit the occurrence of CPs in eutrophic wetlands, while the high levels of macronutrients alone are tolerated. On the other hand, the occurrence of CPs is impossible in nutrient-poor but drier habitats due to the water relations of CPs (Adamec, 2005). However, despite their low proportion and the apparently lesser importance for nutrient uptake, the roots of CPs are physiologically very active: aerobic respiration rate and water exudation rate per unit biomass were comparable with or greater than those reported for non-CPs (Adamec, 2005). This trait is supported anatomically by finding high proportions of central cylinder area to the total root cross-section area (23–47 % in three CP species; Adamec *et al.*, 2006) as opposed to mostly only 3–8 % in many wetland non-CP species (Justin and Armstrong, 1987). This high proportion of central cylinder with vascular bundles in CP roots confirms their important role for the transport of mineral nutrients and water to shoots. Overall, all terrestrial CPs can be classed as ‘stress (S)-strategists’ mainly for their slow growth and low net rate of photosynthesis ( $A_N$ ) (Ellison and Adamec, 2011; Pavlovič and Saganová, 2015; Adamec and Pavlovič, 2018). It is often ignored that their slow growth is also an important adaptive trait and a ‘prerequisite’ for carnivory meeting stressful soil conditions.

How do terrestrial CPs differ from their aquatic counterparts? Submerged aquatic or amphibious CPs comprise the monotypic *Aldrovanda vesiculosa* and around 60 species of *Utricularia*. They are strictly rootless, freely suspended or weakly attached to loose sediments, and absorb all nutrients from the ambient water and captured prey by their shoots and foliar traps. Most species have a linear, modular shoot structure, with leaf nodes bearing filamentous leaves with traps and tubular internodes (Adamec, 2011, 2018; Ellison and Adamec, 2011), but the boundary between terrestrial and amphibious CPs is not strict. Typical habitats of aquatic CPs are shallow standing or slowly running humic, oligomesotrophic waters, and a partly decomposed, nutrient-poor litter or brown peat usually accumulates in their hypoxic sediments. The waters are usually undersaturated with dissolved  $\text{O}_2$ , but highly oversaturated with free  $\text{CO}_2$  (global median 0.30 mM, interquartile range 0.14–0.92 mM), mildly acidic (global median pH 6.3, interquartile range 5.7–7.0), and their global median of total concentrations of humic acids + tannins is 11 mg L<sup>-1</sup> (Adamec, 2012). Rootless aquatic CPs are significantly better adapted to growing in dystrophic waters than co-occurring rooted submerged non-CP species: the mean potential species pool of the former group exceeded that of the latter group by 5× at Central European sites (Adamec, 2012). Generally, due to crucial differences between terrestrial and aquatic habitat factors, key ecophysiological traits and processes greatly differ between terrestrial and aquatic plants regardless of their carnivory (Adamec, 2011, 2018;

Ellison and Adamec, 2011). However, aquatic CPs differ relatively little in  $A_N$ , relative growth rates (RGR) and shoot/foiar N and P contents from their aquatic non-CP counterparts, while large differences in all these traits apply between terrestrial CPs and non-CPs. In summary, aquatic CPs exhibit much higher  $A_N$  values, RGR and mineral nutrient contents than their terrestrial counterparts and may be considered 'R-strategists'. Obviously, the very rapid growth of aquatic CPs is associated with their modular shoot structure, 'conveyor-belt' shoot growth system, rapid apical shoot growth and frequent branching (Adamec, 2011, 2018). As ecophysiological traits have not been studied in terrestrial wetland *Utricularia* (approx. 170 species), it is unclear whether their ecophysiological traits in these rootless, usually rhizomatous species resemble more typical rooted terrestrial CPs (similar ecologically) or systematically relative aquatic *Utricularia* species.

#### Mineral nutrient economy

Generally, the median foliar N and K content in terrestrial CPs is around twice lower than that in terrestrial non-CPs, while the P content is the same. It is accepted that these low N and K contents both reflect the barren, wet environments and respond to comparatively low  $A_N$  and RGR values in terrestrial CPs (Ellison and Adamec, 2011; Adamec and Pavlovič, 2018). Nutrient stoichiometry suggests co-limitation of CP growth by N + P or N + P + K. Therefore, it is beneficial for CPs to capture animal prey as the prey is an approx. 5–10× richer N and P source than CP organs (Adamec and Pavlovič, 2018). As the unspent prey carcasses still contain a great deal of nutrients, which later fertilize the soil around the plants, they can secondarily support root nutrient uptake. However, the importance of this neglected nutritional consequence of carnivory is unknown.

Mineral nutrient economy in terrestrial CPs comprises processes of foliar nutrient uptake from prey and root nutrient uptake from the soil, mineral nutrient reutilization from aged shoots, and stimulation of root nutrient uptake by foliar nutrient uptake; in CPs, all these processes differ more or less from those in non-CPs. One can assume that the uptake capacity of roots is very low, while the uptake affinity is relatively high (Adamec and Pavlovič, 2018). Gao et al. (2015), however, measured a very high  $\text{NH}_4^+$  uptake by intact *Dionaea* roots from concentrated (2 mM)  $\text{NH}_4\text{NO}_3$  solution with an 11-fold uptake preference over  $\text{NO}_3^-$ . Although the experiment showed potential capacity for root nutrient uptake, it does not reflect the nutrient uptake affinity under natural low-nutrient conditions.

Trap uptake of 16 macro- or micronutrients from prey (or mineral solutions) has been confirmed in CPs, but nutrient uptake efficiency has been estimated only for N, P, K, Ca and Mg. Generally, the N uptake efficiency is relatively low, and around a half of that for P, K and Mg, while that for Ca is usually zero; uptake efficiency for micronutrients is unknown (see Adamec and Pavlovič, 2018; cf. Capó-Bauçà et al., 2020). We note that the N uptake efficiency from prey in the field was 25 % lower than in the greenhouse (Hanslin and Karlsson, 1996), and a similar decline may also be expected for other nutrients. In which form can N be absorbed from the digestive cocktail? Presumably, traps of all CPs can absorb  $\text{NH}_4^+$ .

The  $\text{NH}_4^+$  transporters (AMTs) in the roots of non-CPs are usually constitutively expressed; however, expression of the  $\text{NH}_4^+$  transporter in *Dionaea* (DmAMT1) and its affinity for  $\text{NH}_4^+$  are enhanced by prey stimulus (Scherzer et al., 2013; Gao et al., 2015). Expression of the homologous transporter NaAMT1 is also stimulated by prey in *Nepenthes alata* (Schulze et al., 1999), indicating that CPs adapt their localization and transcriptional regulation to a carnivorous lifestyle. Similarly, prey-stimulated expression was found in *Dionaea* for the low-affinity DmKT1 channel, high-affinity DmHAK5 transporter and DmHKT1 channel which mediate  $\text{K}^+$  and  $\text{Na}^+$  uptake, respectively (Scherzer et al., 2015; Böhm et al., 2016a, b). With these channels and transporters being highly selective, new discoveries can be expected for the transport of other mineral nutrients (e.g. Mg and microelements). In contrast, the high  $\text{NH}_4^+$  concentration (0.14–0.31 mM) found in the fluid in prey-free traps of two rootless aquatic *Utricularia* species indicates low uptake affinity for the traps as compared with the shoots (Sirová et al., 2014).

Very efficient N, P and K reutilization, minimizing nutrient losses from senescent shoots in addition to slow growth, has evolved as a typical adaptive, convergent ecophysiological trait in nutrient-poor substrates, especially in terrestrial CPs of different taxa (Adamec, 1997, 2002, 2011; for a review, see Adamec and Pavlovič, 2018). Moreover, mean N and P reutilization efficiencies in terrestrial CPs are approx. 20–25 % higher than in co-occurring bog and fen non-CPs. This suggests that the selective pressure for this trait was repeatedly much higher in the phylogeny of all terrestrial lineages of CPs compared with co-occurring non-CPs. However, mean N and P reutilization efficiencies are somewhat lower in aquatic CPs in spite of their higher foliar N content (cf. Adamec, 2018). It might be due to very rapid shoot senescence and decay in aquatic CPs allowing too short a time for high efficiency. Very efficient K reutilization in terrestrial CPs contrasts with the (nearly) zero efficiency in aquatic CPs and non-CPs. The explanation is still unknown. Curiously, very effective N and P (and also K!) reutilization has evolved convergently in epiphytic or terrestrial wetland *Utricularia* species of different generic sections, again in contrast to aquatic species (Adamec, 2014), highlighting that this trait is ecologically, not systematically, related. Mg and especially Ca are not reutilized in all CPs, and this puts an emphasis on their uptake only from prey (Mg) and the soil to cover their need.

A profound stimulation of mineral nutrient uptake by roots as a result of foliar mineral uptake from prey was observed in several *Drosera* and *Pinguicula* species in growth experiments (see Adamec, 2011; Adamec and Pavlovič, 2018). Plants fed on insects or mineral nutrient solutions in long-term growth experiments grew vigorously and accumulated approx. 1.6–27× more N, P, K, Ca and Mg in total biomass (relative to unfed controls) than they could have gained directly from the prey; a higher prey dose led to a greater effect. As animal prey is a poor source of K, Ca and Mg (see below), and Ca may not be absorbed, the extent of the uptake stimulation of these cations is much higher than for N and P as root uptake has to cover all nutrient demands for increased growth. The explanation for this stimulation effect is still lacking. Which mineral nutrients absorbed from prey are responsible for this effect (only N or



P?) or in which CP genera and species does the effect occur? In three *Drosera* species, increased root biomass explained 70–85 % of the effect, while other influences analysed were marginal (Adamec, 2002). In any event, root respiration rate was unchanged and the root stimulation was uncorrelated with root or shoot mineral nutrient contents.

In light of new findings in *Dionaea* and accepting the concept of increased  $A_N$  as a benefit from carnivory (Pavlovič and Saganová, 2015; Fusbender et al., 2017; Kruse et al., 2017), the following scenario of processes leading to root stimulation in terrestrial CPs may be hypothesized. (1) During the first 2 weeks after prey capture, N and P are absorbed from prey and allocated mainly to traps and petioles (in *Pinguicula* and *Drosera*, to whole leaves); organic C (and also P) from prey is used mainly in traps to increase their respiration and ATP production to cover increased trap functions, while N entering the petioles supports the photosynthetic apparatus. (2) After the prey is digested and spent (approx. 2 weeks), increased foliar photosynthesis provides more photosynthates for faster growth of leaves/traps and also roots. (3) Longer roots with higher biomass and higher uptake affinity and capacity are able to absorb much larger amounts of mineral nutrients from a greater soil volume, thus supporting the increased growth of leaves/traps. (4) If the plant can capture additional prey continuously, the initially limited stimulation of total photosynthesis and shoot growth is amplified in time and, by a positive feedback mechanism, root activity is also increasingly stimulated. In summary, prey-derived nutrients do not directly enter roots but mainly support photosynthesis, which then stimulates roots. We can ask which phytohormones regulate this process. Evidently, this leaf–root nutrient interaction as an adaptive trait in CPs represents both the crucial mechanism by which mineral and organic nutrients from prey support the increased plant growth, and the principal ecological advantage of CPs over non-CPs in barren wet soils. It is possible that this mechanism acts in all terrestrial CP genera, but has not been proven so far, or its extent correlates with growth rate ('faster' *Drosera* vs. 'slower' *Nepenthes*). Analogically, mineral nutrient uptake by shoots in two aquatic CPs from the ambient water appears to be stimulated by prey capture (Adamec et al., 2010), but the photosynthetic stimulation is ambiguous (Adamec, 2018).

Two important questions should be answered: what is the role of foliar or root N or P content in this scenario and what is the final change of root/shoot biomass ratio in CPs? Probably, the regulatory role of N or P content alone in leaves and roots is only marginal or zero as these values in prey-fed CPs can even significantly decline (Adamec, 2002, 2011), and the same applies for aquatics (Adamec, 2018). It may thus be crucial for initiation of the process that a small amount of N and P enters the leaves and supports photosynthesis and growth, as a result of which the tissue N and P contents decline. Alternatively, prey capture (or nutrient solution application) induces the profound processes of prey digestion and nutrient absorption (Matušiková et al., 2018; see below), which 'switch on' the cascade of gene-expressed processes leading ultimately to stimulation of root nutrient uptake and increased plant growth. The comparison of the root/shoot ratio in three *Drosera* species fed on mineral solution (mild decrease; Adamec, 2002) and in insect-fed

*Dionaea* (great increase; Gao et al., 2015) indicates different regulation of root growth in these genera.

Regardless of the physiological mechanism of utilization of prey-derived nutrients, the final ecophysiological consequence and benefit of carnivory in all CP species is significantly accelerated growth and development, leading finally to prolific flowering and seed set (Adamec, 1997, 2011; Ellison and Adamec, 2011, 2018b; Adamec and Pavlovič, 2018; Givnish et al., 2018). In various field or greenhouse growth experiments, in which different terrestrial CPs were fed prey or mineral nutrient solution, final biomass usually increased by 2–5× compared with untreated controls, and a similar growth increase could be caused by soil fertilization alone (Adamec, 1997, 2011; Ellison, 2006). However, species-specific differences in the relationship between the trap and root nutrient uptake allows three main ecophysiological groups to be distinguished: 'nutrient-requiring' species (e.g. *Drosera capillaris*, *Sarracenia flava* and *Pinguicula vulgaris*), 'root–leaf nutrient competitors' (e.g. *Drosera binata*, *D. whittakeri* and *Pinguicula villosa*) and 'nutrient-modest' species (e.g. *Drosera closterostigma*; Adamec, 1997, 2011). This suggests a great physiological diversity of interorgan regulation of mineral nutrition and growth, even in related CP species. Field growth experiments have repeatedly revealed that prey capture is the crucial factor for rapid growth of CPs in natural habitats as much more prey than is normally captured can support CP growth. Furthermore, prey capture is more important for seedlings or young plants than for adults, as successful juveniles grow faster, attain maturity sooner and have more prolific flowering and seed set (minimum size effect; see Adamec and Pavlovič, 2018). One of the possibilities to assess the ecological importance of carnivory is to estimate seasonal nutrient gain covered from carnivory. Generally, due to rather imprecise inputs, the estimated values vary intraspecifically, interspecifically, regionally and seasonally. In summary, only 1–16 % K (or less Mg), but 7–100 % (usually 30–60 %) N and P is covered from prey for dozens of species from nine genera (for a review, see Adamec and Pavlovič, 2018). Evidently, very high values depend not only on intensive prey capture, but also on low root uptake and probably also on high N and P reutilization efficiencies.

Moreover, traps of many, if not all, CP species effectively absorb soluble organic nitrogenous substances (urea and amino acids) from prey (Juniper et al., 1989; Adamec, 1997; Karagatzides et al., 2009; Fusbender et al., 2017; Kruse et al., 2017; Yilamujiang et al., 2017), and the direct gain of a mixture of organic substances also follows from the endocytosis found in the traps of six CP genera (Adlassnig et al., 2012; Koller-Peroutka et al., 2019). Trap uptake of nucleic acid bases or (oligo)nucleotides has never been studied, but is highly probable due to their high pool in digested prey. A couple of studies on double labelling have recently elucidated the fate of prey-derived C and N in *Dionaea muscipula*. In *Dionaea* fed on [ $^{13}\text{C}$ ]/[ $^{15}\text{N}$ ]glutamine instead of prey,  $^{15}\text{N}$  of applied glutamine was already separated from its  $^{13}\text{C}$  skeleton in the digestive fluid in the traps after 46 h (Fusbender et al., 2017). Most of the glutamine-based C and N absorbed was found in fed and unfed traps and less in petioles, but there was a weak representation in roots. Moreover, much of



the glutamine-based  $^{13}\text{C}$  was respired in the traps, and their respiratory metabolism was enhanced. Thus, amino acids absorbed from prey are not the only N source for the plant, but are used directly as a substrate for respiratory energy generation in traps, at least in *Dionaea* (Fasbender *et al.*, 2017). Similar results were obtained after *Dionaea*'s feeding on  $^{13}\text{C}/^{15}\text{N}$ -labelled milled insect as prey (Kruse *et al.*, 2017). During prey digestion, amino acids were absorbed from the prey and were used as respiratory substrates and for the synthesis of N-rich transport forms, asparagine and glutamine. Later, when the prey was completely digested, the amino-N helped increase petiole photosynthesis. The uptake and fate of other elements and molecules in CPs remain poorly explored.

The ecological importance of direct uptake of organic substances as a partial substitute for photosynthesis in terrestrial CPs is not generally accepted. Expression of structural and functional traits of carnivory (mainly trap development) is downregulated by the higher availability of N in traps, indicating a negative feedback loop (for reviews, see Adamec, 2018; Adamec and Pavlovič, 2018). Generally, absorbed mineral N forms in traps act much more strongly than amino acids or insect prey. Shoot N content was found in some CP species – terrestrial and aquatic – as an endogenous factor to regulate the development of traps.

#### Cost–benefit relationships in CPs

The cost–benefit model proposed by Givnish *et al.* (1984) and its modifications (Laakkonen *et al.*, 2006; Adamec, 2011; Brewer *et al.*, 2011; Pavlovič and Saganová, 2015; Givnish *et al.*, 2018) have become a conceptual framework for many ecophysiological studies on CPs. This model explains why terrestrial CPs are restricted to sunny, moist and nutrient-poor habitats. Only in this environment does the benefit from increased uptake of nutrients from animal prey exceed the cost of investment in carnivorous adaptations. The costs and benefits are in terms of photosynthesis and respiration (photosynthetic model; Givnish *et al.*, 1984; Laakkonen *et al.*, 2006) or mineral nutrients gained (nutritional model; Adamec, 2011) and the models are not mutually exclusive (Adamec and Pavlovič, 2018).

How can the cost–benefit model be investigated in action? The easiest way is a visual observation of phenotypic plasticity. This allows a single genotype to produce a set of phenotypes; in many CPs, it is a variation in leaf forms and shapes termed heterophylly (Fig. 3). Under low-light conditions (photoperiod as well as light intensity), high nutrient or low water availability, many CPs lose the ability to produce carnivorous traps and form only assimilation leaves, because, under such conditions, the cost of producing the traps and their maintenance prevails over the potential benefit from prey [photosynthesis is limited by factor(s) other than low-nutrient availability]. Under high-light conditions, low nutrient and high water availability, CPs invest a lot of biomass in trap production, because surplus nutrients from animal prey support photosynthesis, which is not limited by any other factor. Although temperature was not involved in the original cost–benefit model, recent

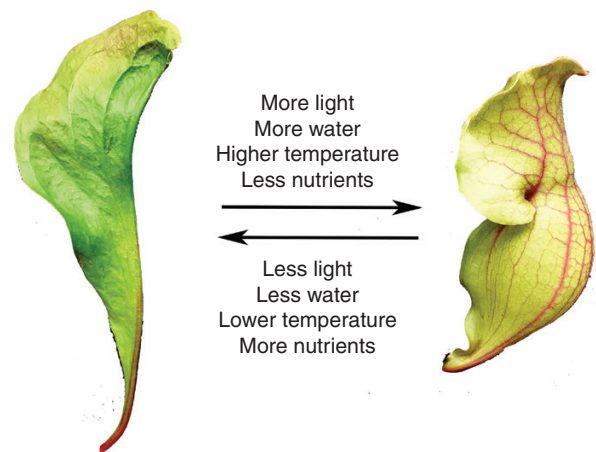


FIG. 3. Phenotypic plasticity shown as an example on *Sarracenia purpurea* ssp. *venosa*. The production of non-carnivorous leaves is favoured under low light, low water availability, low temperature and high nutrient content. The production of carnivorous pitchers is favoured under high light, high water availability, high temperature and low nutrient content.

studies indicate its importance (Fukushima *et al.*, 2017, 2021). *Cephalotus follicularis* produces trap-free leaves instead of traps under both short-day and low-temperature conditions, and *vice versa*. Lower temperatures and short days are naturally tightly coupled, and the recent discovery that the well-known photoreceptor phytochrome B is also a temperature sensor in plants may provide a molecular explanation of this phenomenon (Jung *et al.*, 2016; Legris *et al.*, 2016; Fukushima *et al.*, 2021). Temperature is the factor which affects the rate of biochemical Calvin cycle reactions and may thus represent the same constraint for photosynthesis as shortage of light and water. This factor was probably overlooked earlier and, indeed, the highest biodiversity of CPs is found in the tropics and sub-tropics. All these environmental factors may act on the phenotype separately or in combination, and unusual combinations may generate malformed phenotypes (e.g. elevated global temperature on pitcher formation of *Cephalotus*; Fukushima *et al.*, 2021). Another often overlooked factor is a high concentration of  $\text{CO}_2$ , indispensable for many aquatic CPs (Givnish *et al.*, 2018; see above). Although the effect of elevated atmospheric  $\text{CO}_2$  concentration has been well studied for decades in non-CPs, no information is available for CPs. It is tempting to assume that the elevated atmospheric  $\text{CO}_2$  concentration will support carnivory by higher availability of photosynthates for trap production, thus increasing the trap/leaf ratio *sensu stricto* the cost–benefit model. Moreover, there are no indications that an elevated atmospheric  $\text{CO}_2$  concentration alone could negatively affect CP populations directly. However, as CPs strictly require wet or aquatic habitats, the progressing global climate change (higher temperatures and drought as a result of increasing  $\text{CO}_2$  concentration) can endanger >65 % of all CP species (both terrestrial and aquatic) to local population extinction, which follows from species distribution models (Fitzpatrick and Ellison, 2018).

The phenotype may change continuously, i.e. proportionally in response to changing environmental parameters (e.g. *Sarracenia purpurea*; Fig. 3; Ellison and Gotelli, 2002), or discontinuously when the phenotype switches from one to another

after a threshold in environmental parameters is reached (e.g. *Cephalotus*; Fukushima *et al.*, 2021). Recent progress in genomics and genetic transformation of CPs enables us to partially imagine how the changes in environmental conditions are translated into trap/leaf phenotypes. The easy switch in gene expression in the formation of *Utricularia* bladders is achieved by spatial expression changes of polarity genes of the adaxially expressed *PHV* and *PHB* genes and abaxially expressed *FIL* and *KAN* genes (Whitewoods *et al.*, 2020). In *S. purpurea*, the mechanism is slightly different, but an equally easy switch in the orientation of cell divisions in the adaxial domain causes pitcher formation (Fukushima *et al.*, 2015). In *Nepenthes*, modification of the leaf into a pitcher may be associated with the altered expression of leaf polarity genes *ASYMMETRIC LEAVES1 (AS1)* and *REVOLUTA (REV)* or *HOMEODOMAIN-LEUCINE ZIPPER (HD-ZIPIII)* (Dkhar and Pareek, 2019; Dkhar *et al.*, 2020). The endogenous signal (e.g. phytohormone) transforming an environmental cue into changes of cell division leading to trap formation is not known and remains a hot topic in the developmental biology of CPs.

The original cost–benefit model (Givnish *et al.*, 1984) is based on assumptions that traps in CPs represent significant costs for the plants by producing metabolites and structures associated with prey attraction, capture and digestion. Traps are also photosynthetically partly inefficient and sometimes have an increased rate of dark respiration ( $R_D$ ; for a review, see Pavlovič and Saganová, 2015). The photosynthetic inefficiency of traps is caused by low N and chlorophyll content as well as the replacement of photosynthesizing cells by cells involved in prey capture and processing. However, after prey capture and digestion, traps absorb nutrients and the plant enhances the  $A_N$ , growth rate and reproduction as benefits. The surplus nutrients can significantly enhance  $A_N$  and exceed the cost of carnivory only if light and water are not limiting for photosynthesis (Pavlovič *et al.*, 2009). Although increased  $A_N$  after experimental feeding has been confirmed for many species (Pavlovič and Saganová, 2015), the mechanism behind increased photosynthesis has remained unknown until recently. It has been documented that *Nepenthes × ventrata* fed on different types of insect prey contained a higher content of important photosynthetic proteins (chlorophyll-binding proteins of photosystem I and II, the oxygen-evolving complex and Rubisco) and chlorophylls (Capó-Bauçà *et al.*, 2020), supporting the original cost–benefit model assumption that prey-derived N is prevalently incorporated into the photosynthetic machinery (Givnish *et al.*, 1984). Although CPs are photoautotrophic organisms, a detailed study on the composition and structure of their photosynthetic apparatus is lacking. Based on low RGR,  $A_N$  and a few  $^{13}\text{C}$  stable isotope analyses (Moran *et al.*, 2001; Pavlovič *et al.*, 2010; Capó-Bauçà *et al.*, 2020), CPs are considered to be  $C_3$  plants. Not surprisingly, their habitat preference is in strong contrast to the dry and hot habitats occupied by  $C_4$  plants. However, Crassulacean acid metabolism (CAM) cannot be completely ruled out: many Mexican *Pinguicula* species produce highly succulent, non-carnivorous leaves in the dry winter season. These leaves resemble those of succulent plants from the genus *Echeveria*, but no convincing biochemical evidence for facultative CAM in CPs exists. The peculiar lifestyle of CPs may indicate that some modifications of the photosynthetic apparatus

may be expected. For example, recent studies have shown that the chloroplast genome of many CPs lacks some or all NADPH dehydrogenase (NDH) genes (Wicke *et al.*, 2014; Silva *et al.*, 2016; Cao *et al.*, 2019; Nevill *et al.*, 2019). The NDH complex is not essential for photosynthesis under normal conditions; its importance becomes more evident under stress conditions by preventing over-reduction of the chloroplast stroma (Shikanai *et al.*, 2007). The lost *ndh* genes are reminiscent of other plants that no longer entirely rely on photosynthesis for energy and nutrients, such as hemiparasitic plants or partial mycoheterotrophs (Graham *et al.*, 2017; Wicke and Naumann, 2018; see above). Besides reduced photosynthesis in traps, intensive respiration has been documented in CPs with active traps. The Venus flytrap temporarily increased  $R_D$  after the generation of action potentials (Pavlovič *et al.*, 2011), but  $R_D$  of *Utricularia* traps remains permanently high (Adamec, 2006). Positively selected cysteine motifs in COX in *Utricularia* may account for this (Jobson *et al.*, 2004). Modifications of the respiratory electron transport chain for the peculiar lifestyle of CPs deserve further investigation and may bring many surprising discoveries.

## BIOCHEMISTRY OF PREY DIGESTION AND NUTRIENT ABSORPTION

### *Prey digestion traits*

Specialized digestive glands for prey digestion and nutrient uptake are usually sessile or stalked multicellular structures derived from the epidermis (Fig. 4; Owen and Lennon, 1999; Thornhill *et al.*, 2008). Their ultrastructure revealed a labyrinthine-like cell wall organization, lack of chloroplasts, numerous mitochondria and the extensive development of a secretory system. A consistent feature of digestive glands is an endodermoid cell layer which resembles the well-known Casparian strip in the roots and serves the same function, i.e. restriction of apoplastic flow of nutrients (Juniper *et al.*, 1989; Owen *et al.*, 1999). All digestive glands have also evolved cuticular discontinuities (or cuticular gaps) to facilitate transport. The current state of knowledge on enzyme composition of the digestive fluid secreted by these glands is still confined to certain CP genera and individual enzyme types, and has been recently reviewed by Ravee *et al.* (2018) and Matušková *et al.* (2018). Early proteomic analyses of the digestive fluid led to identification of many enzymes, but soon the method was hampered by an unusual amino acid composition of the proteins and by the limited representation of carnivorous plants in the genomic/proteomic databases. Recent combined transcriptome and proteome profilings of traps and their digestive fluid have significantly accelerated new enzyme discoveries (Lee *et al.*, 2016; Wan Zakaria *et al.*, 2019) and have emphasized that there are likely to be no specific proteins (genes) attributable to prey digestion. Instead, some of those involved in plant biotic and abiotic interactions have been co-opted (Mithöfer, 2011; Schulze *et al.*, 2012; Bemm *et al.*, 2016; Lee *et al.*, 2016; Dkhar *et al.*, 2020). Enzymes represent the typical component of prey decomposition machinery in CPs, but growing evidence points to the contribution of accompanying secondary metabolites, and this has not been thoroughly studied. Scents and



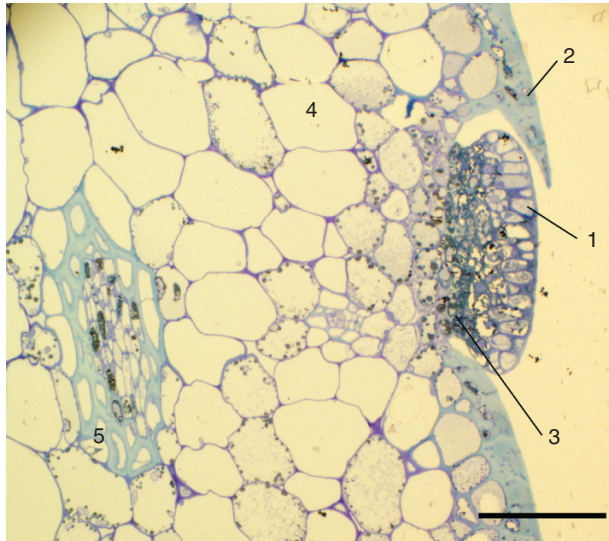


FIG. 4. Anatomy of the *Nepenthes* × *Mixta* trap. Semi-thin section of the digestive zone of the trap stained with toluidine blue and basic fuchsin through the digestive zone with digestive glands; digestive gland (1), epidermal ridge protecting the digestive gland (2), endodermoid layer (3), trap mesophyll (4), vascular bundles (5); scale bar = 100  $\mu$ m.

other compounds described in non-CPs may administer anaesthetic or even toxic effects on prey in *Nepenthes khasiana* and *Sarracenia* (reviewed in Hatcher et al., 2020). The enormously variable chemical properties of these metabolites have possibly conditioned their functional divergence not only to facilitate prey digestion but also to enhance prey capture efficiency or retention in traps. Recent analysis of the *Nepenthes* × *ventrata* metabolome has concluded that a few exclusively polar, N- and P-containing glycosylated metabolites accumulate differently in *Drosophila melanogaster*-fed pitchers (Dávila-Lara et al., 2020); unfortunately, the nature of many of them remains unknown due to the absence of reference molecules in biologically relevant databases. Available data on metabolite contents for CPs are still rather fragmented, but many compounds involved in prey digestion appear rather consistent across different trap types, underlining their necessity for carnivorous functions (Hatcher et al., 2020).

For some taxa (e.g. *Sarracenia*) that had been considered poor enzyme producers if at all, enzymes and their secretion have recently been identified (Fukushima et al., 2017; Koller-Peroutka et al., 2019), complementing the data on plant-governed enzymatic decay from *Nepenthes*, *Dionaea*, *Drosera*, *Cephalotus* and *Pinguicula*. The synthesis of digestive hydrolases and their release to traps is either constitutive or induced by mechanical or chemical stimulation from captured prey (Fig. 5). The cascade of subsequent events in traps takes place in the specific environment of trap fluid (except for *Dionaea* where the fluid is released only in response to prey stimuli), the properties and composition of which differ among genera (Gaume et al., 2019). The fluid undergoes energy-dependent acidification soon after prey capture, and this not only enhances prey confinement and increases the prey killing rate by suffocation, but also allows for chemical decomposition of the prey (Bazile et al., 2015; Gilbert et al., 2020). The acidification is important not only for optimal enzyme activity, but also for the

autoactivation of some proteases from proenzymes (Athauda et al., 2004; Risør et al., 2016). Enzymatic decomposition is achieved by a cocktail of hydrolases that are very stable, possess broad substrate specificity, resist proteases and act in acidic conditions. Prey lysis is probably also a partially non-enzymatic process, mainly by the activity of certain secondary metabolites and reactive oxygen species (Chia et al., 2004; Eilenberg et al. 2010), but our knowledge of these events remains limited. Using 3-D electron tomography, Gergely et al. (2018) described in detail the structural organization of the secretory organelles in the stimulated digestive gland cells of *Dionaea*: the secretory apparatus of the resting glands appears ‘overbuilt’ to quickly upregulate hydrolytic enzyme secretion in response to prey capture without assembling new membrane systems. The increase in synthesis of secretory proteases requires only the recruitment of more polysomes to endoplasmic reticulum (ER) membranes, while the existing ER to Golgi COPII vesicle transport system exhibits excess transport capacity to maintain the increased amounts of lytic enzymes produced in the ER. Vesicle transport is mediated by actin that allows for better control and faster nutrition trafficking. Temporal differences have been demonstrated for secretion of different proteases upon feeding on bovine serum albumin, providing further evidence of aspartic and cysteine proteases as major enzymes responsible for peptide hydrolase activity in *Dionaea* (Schulze et al., 2012; Libiaková et al., 2014). The dynamic sequential changes of the digestive gland ultrastructure have also been recently described for the related *Aldrovanda* (Atsuzawa et al., 2020).

The occurrence of enzymes such as proteases, RNases, chitinases and phosphatases in traps appears well substantiated in the context of carnivory, given the extensive N and P gain from the animal diet. In contrast,  $\alpha$ -amylases and  $\beta$ -1,3-glucanases (apart from executing pathogen-related functions) probably digest carbohydrates occasionally from pollen, spores or leaf detritus, as suggested for the *Pinguicula* and some *Drosera* and *Nepenthes* species growing beneath the forest canopy (Pavlovič and Saganová, 2015; Michalko et al., 2017; Kocáb et al., 2020). Surprisingly, nucleases – especially those cleaving DNA – have received little attention so far, though their substrate, rich in both N and P, is present in any type of living prey. The fate of insect DNA in traps is unknown, but pathogen resistance mediated by extracellular DNA in root cap slime (Wen et al., 2009), which is closely comparable with digestive mucilage, allows one to speculate that this analogy might also be relevant for plant carnivory. The roles and functional characteristics of other enzyme types in CPs are only suggested, based on sequence conservation among the diverse members of particular enzyme groups, or on functional blocks that probably mediate enzyme activity. Further progress to date has been hampered by the unknown structural relationships among these regions, unknown effects on substrate specificity and missing experimental proof.

Besides prey digestion, hydrolases constitutively present in the traps of some CPs are assumed to be plant investment into protection against ubiquitous microbes (e.g. certain chitinases in some *Droseraceae* and *Nepenthes*), or as an adaptation to nutrient-poor habitats to opportunistically gain P (e.g. RNases in *Cephalotus*, *Dionaea* and *D. adela* or phosphatases in *Genlisea* and aquatic *Utricularia*). The glands undergoing the digestion (Fig. 4) simultaneously

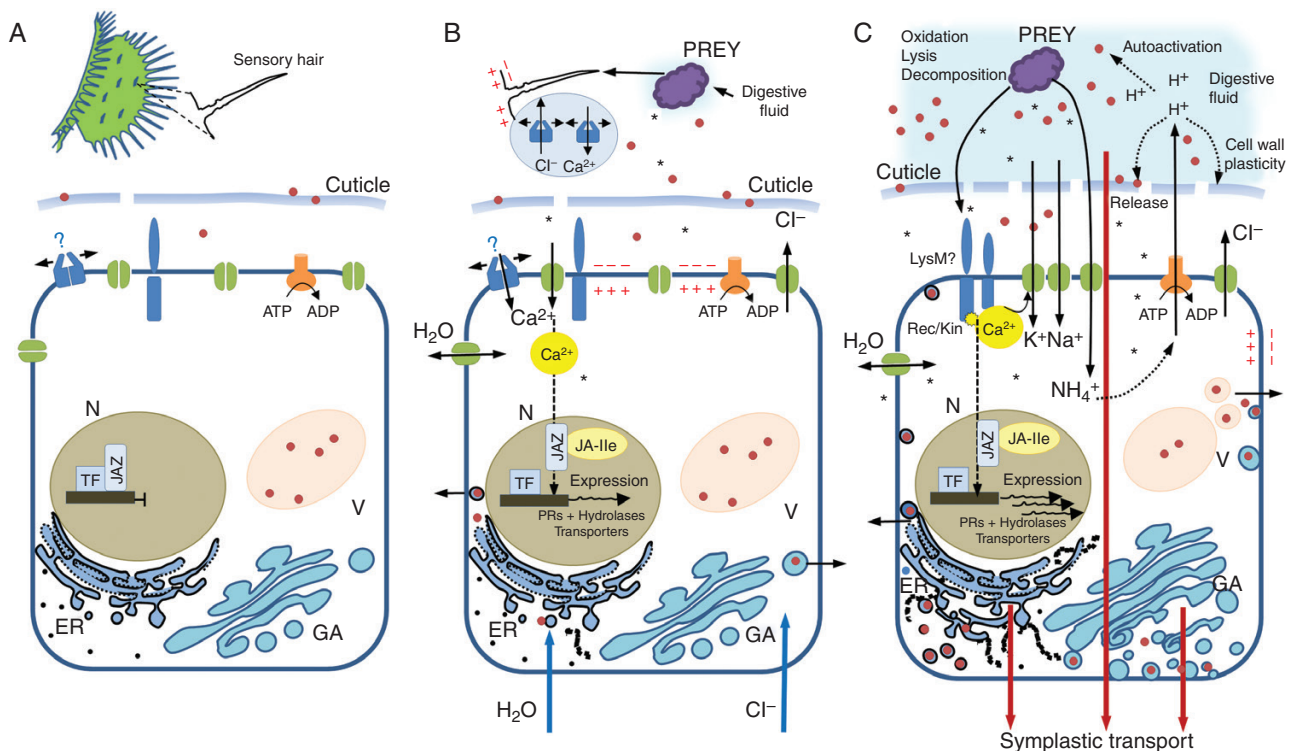


FIG. 5. Biochemistry of prey decomposition in the digestive glands in the *Dionaea* trap. (A) In the resting gland, no enzyme production occurs and free ribosomes are only visible (black dots). (B) In the early phase, prey stimulates mechanosensitive, chloride-permeable channels (FLYC) by touching the sensory trigger hairs that, with other, possibly Ca<sup>2+</sup>-permeable channels, mediate membrane depolarization. The generated action potentials and Ca<sup>2+</sup> wave are propagated through plasmodesmata along the outer cell layers and lead to trap closure and activation of the JA signalling pathway in the digestive glands. Similar to non-CPs, jasmonic acid (JA) conjugates with isoleucine (JA-Ile), mediates degradation of JASMONATE ZIM-DOMAIN (JAZ) repressors and soon activates gene expression in the nucleus (N) to produce hydrolases (red dots). (C) In the later phase, substances (stimulants) derived from captured prey (\*) penetrate the cuticular gaps. Ammonium ions released by deamination in traps induce H<sup>+</sup> secretion into the fluid, and extremely low amounts of low molecular weight nitrogenous elicitors (amino acids and amines) enter the gland cell. Specific prey-derived molecules (e.g. chitin) activate receptors with a LysM domain and different kinases (Rec/Kin, blue membrane proteins, respectively), which mediate Ca<sup>2+</sup>-dependent and JA-mediated activation of further downstream responses. Zymogens are also gradually released from the vacuole (V). Enzymes (red dots) released from the cutinized wall directly elicit gap formation in the cuticle and secretion of digestive enzymes. Enzymes stored in the wall of the secretory cells may release active residues from prey proteins that further stimulate the gland. With progressing digestion, hydrolases and various transporters (green membrane channels) are intensively produced. The prey is decomposed in the fluid, which becomes strongly acidic and contains autoactivated proteases and oxidases (red dots). The digestive gland readily absorbs the released nutrients either by transporters or by endocytosis, and polysomes utilize them to synthesize novel proteins on membranes of the endoplasmic reticulum (ER). By the end of prey decomposition, the digestive gland cells are rich in exporting vesicles from the ER or Golgi apparatus (GA), and absorbed nutrients are also transported symplastically. The time course and overlap (in any) of the individual steps of mechanically (B) and chemically activated processes (C) are not fully imaged, and only the most typical components are indicated in both panels.

assimilate nutrients released from prey (Owen *et al.*, 1999), while the cutinized trap epidermis alone can be impermeable to nutrients. A few members of the gland ‘transportome’ have been identified in certain genera, some of which have been mentioned in previous parts of this review. The massive uptake of nutrients by individual plasma membrane transporters in digestive glands is regulated at the transcriptional level (unlike those constitutively active in roots in non-CPs, Scherzer *et al.*, 2013), and complements uptake by endocytosis and via a symplastic route (Adlassnig *et al.*, 2012). Whether this process is co-ordinated, and how, remains unexplored. A comprehensive scheme of the processes occurring in a *Dionaea* digestive gland is depicted in Fig. 5.

#### Regulation of enzyme activity

Carnivorous plants regulate digestive enzyme activity to save available resources. Some enzymes in certain CP genera showed

tissue-specific, constitutive or development-dependent expression (Nishimura *et al.*, 2013; Wan Zakaria *et al.*, 2019; Dkhar *et al.*, 2020; Arai *et al.*, 2021). Recent protein depletion experiments suggest that the level of many enzymes is perceived and autoregulated in the digestive fluid of *Nepenthes*, indicating the existence of feedback mechanisms for enzyme replenishment in the fluid (Wan Zakaria *et al.*, 2019; Goh *et al.*, 2020). However, many enzymes are clearly upregulated or completely newly synthesized by prey stimuli. As far as is known, CPs have not built up a completely new system of regulation but co-opted the existing one from plant defence mechanisms (Pavlovič and Saganová, 2015; Bemm *et al.*, 2016; Matušiková *et al.*, 2018). The concept that botanical carnivory and plant defence responses are inter-related is old (Fig. 1, Juniper *et al.*, 1989), but the discovery that they use the same signalling pathway is relatively new. If non-CPs are attacked by biotrophic pathogens, the salicylic acid (SA)-mediated defence signalling pathway is activated. When herbivores or necrotrophic pathogens attack non-CPs, the jasmonic acid (JA)-mediated signalling pathway



is activated (Li *et al.*, 2019). To date, there is no direct evidence that SA is involved in activation of the CP digestive system (studies on the genera *Drosera* and *Nepenthes*; Matušiková *et al.*, 2005; Buch *et al.*, 2015; Krausko *et al.*, 2017; Jakšová *et al.*, 2021), but this cannot be completely ruled out as not all carnivorous genera have been investigated in this respect. On the other hand, the involvement of JA in the induction of digestive enzyme expression has been confirmed at least in the order Caryophyllales. In non-CPs, the true bioactive ligand in JA signalling is jasmonic acid–isoleucine conjugate (JA-Ile) which binds to the CORONATINE INSENSITIVE1 (COI1) receptor and mediates the ubiquitin-dependent degradation of JASMONATE ZIM-DOMAIN (JAZ) repressors, resulting in the activation of jasmonate-dependent gene expression (Thines *et al.*, 2007; Fonseca *et al.*, 2009; Sheard *et al.*, 2010). An increased level of JA-Ile after experimental feeding was found in carnivorous plants of genera *Aldrovanda*, *Drosera*, *Dionaea* and *Nepenthes* (Nakamura *et al.*, 2013; Libiaková *et al.*, 2014; Mithöfer *et al.*, 2014; Yilamujiang *et al.*, 2016; Krausko *et al.*, 2017; Pavlovič *et al.*, 2017; Jakšová *et al.*, 2020, 2021), and external application of jasmonates triggered expression and secretion of digestive enzymes (Libiaková *et al.*, 2014; Paszota *et al.*, 2014; Buch *et al.*, 2015; Bemm *et al.*, 2016; Böhm *et al.*, 2016a; Krausko *et al.*, 2017; Pavlovič *et al.*, 2017; Jakšová *et al.*, 2021). In the Venus flytrap, mechanical stimuli from struggling prey translated into action potentials (APs) and the Ca<sup>2+</sup> wave (Suda *et al.*, 2020) can induce a high pool of jasmonates sufficient to trigger enzyme secretion and activity (Pavlovič *et al.*, 2017; Fig. 5B). This sequence of events is remarkably similar to the activation of plant defence (Pavlovič and Saganová, 2015). Later, the process of digestion is maintained by chemical stimuli released from insect prey which keep the jasmonate level and enzyme production high (Fig. 5C; Libiaková *et al.*, 2014; Bemm *et al.*, 2016; Jakšová *et al.*, 2020). This two-step digestive process activation is advantageous, helping to optimize digestive enzyme production in response to stimuli from prey. Even within each step, the digestive process is fine-tuned by the number of mechanical stimuli (and, thus, the number of APs generated), or by the type of chemical compounds (e.g. protein or chitin) detected (Libiaková *et al.*, 2014; Bemm *et al.*, 2016; Böhm *et al.*, 2016a; Jakšová *et al.*, 2020). The experiments on the passive pitcher traps of *Nepenthes* indicate that chemical stimulation alone is sufficient to induce jasmonate accumulation (Yilamujiang *et al.*, 2016), although this ability has probably evolved independently of that in Droseraceae (Palfalvi *et al.*, 2020). How these chemical stimuli induce jasmonate synthesis is not exactly known, but it is tempting to assume that it is activated by a LysM receptor analogue for chitin sensing in non-CPs (Miya *et al.*, 2007), protein deamination and subsequent membrane depolarization (Scherzer *et al.*, 2013; Bemm *et al.*, 2016; Fig. 5). Jasmonates activate the transcription of genes encoding digestive enzymes in a similar way to that in non-CPs in a COI1- and JAZ-dependent manner, as shown by experiments with coronatine-*O*-methyloxime which prevents COI1–JAZ interaction (Bemm *et al.*, 2016; Böhm *et al.*, 2016a). However, it seems that not all carnivorous taxa co-opted this ancient signalling pathway which is present with some modifications already in Bryophyta (Monte *et al.*, 2020).

In the order Lamiales, the genera *Pinguicula* and *Utricularia* do not accumulate a significant amount of jasmonates in response to prey capture, and *Pinguicula* does not secrete digestive enzymes in response to exogenous jasmonate application either (Kocáb *et al.*, 2020; Jakšová *et al.*, 2021). Jasmonates are probably dispensable in the regulation of enzyme activity in *Utricularia*, because enzyme production is rather constitutive (Sirová *et al.*, 2003; Jakšová *et al.*, 2021) and partly relies on micro-organisms (Sirová *et al.*, 2018b). However, the bioactive substance activating digestive processes in *Pinguicula* remains unknown. Although the similar genes were co-opted for digestive physiology among non-related taxa of CPs, their regulation may differ as additional study on RNases indicates (Nishimura *et al.*, 2013). Thus, coding sequences and gene promoters were probably under different selective pressures in different evolutionary lineages of CPs. How the enzyme activity is regulated outside the Caryophyllales remains an important question for further study.

The activation of digestive processes by electrical signals and jasmonates resembles the activation of plant defence reactions in response to pathogen or herbivore attack (Pavlovič and Mithöfer, 2019). Many proteins and enzymes found in the digestive fluid of CPs belong to the pathogenesis-related (PR) proteins (Mithöfer *et al.*, 2011; Schulze *et al.*, 2012; Lee *et al.*, 2016), and their expression can also be induced by wounding in CPs (Krausko *et al.*, 2017; Pavlovič *et al.*, 2017, 2020). Whereas electrical signals in non-CPs often propagate systemically to neighbouring leaves priming the leaves for defence (Mousavi *et al.*, 2013), in the Venus flytrap, sundews and probably also *Aldrovanda*, they are restricted to the local trap which actually captured the prey (Krausko *et al.*, 2017; Pavlovič *et al.*, 2017). The redirection of electrical signalling in these CPs from a systemic to a local response may save available resources as it does not activate the costly systemic enzyme production in traps which have not captured any prey, a trait otherwise beneficial in plant defence reactions. The molecular mechanism of electrical signalling probably includes mechanosensitive chloride channels (FLYCATCHER, FLYC) and osmosensitive calcium gates triggering a flux of ions in sensory hairs and tentacles, probably as the initiating step of the digestion cycle in Droseraceae (Procko *et al.*, 2021; Fig. 5). However, JA itself can be transported from local to systemic leaves in non-CPs (Li *et al.*, 2020) and, moreover, an exogenous application of coronatine (a molecular mimic of JA-Ile) can induce digestive processes in the systemic traps of the Venus flytrap (Escalante-Pérez *et al.*, 2011) and *Drosera aliciae* (A. Pavlovič, pers. obs.). Yet it seems that accumulation of endogenous levels of JA in a local trap does not affect the accumulation of JA in systemic leaves in the Venus flytrap and the sundew (Krausko *et al.*, 2017; Pavlovič *et al.*, 2017). Jasmonates are a group of hormones with a broad action spectrum as signals in biotic and abiotic stress responses and plant development (Wasternack and Hause, 2013). Could a potentially increased JA level in an assimilatory organ of CPs, induced by, for example, wounding or herbivore attack, induce expression of the same sub-set of carnivory-related genes as in the trap tissue? Some studies indicate that organ-specific expression of digestive enzymes may be under the control of promoter methylation or tissue-specific transcription factors (Nishimura *et al.*, 2013; Arai *et al.*, 2021).

Tissue-specific expression can be the factor ensuring that the ubiquitous hormone JA activates carnivory-related genes only at the site where they are necessary (trap), but our understanding of the tissue-specific expression of digestive enzymes is still very fragmentary and needs further investigations.

#### Evolution of digestive enzymes

Research on CP nutrition identified mechanisms that strongly resemble the well-described defence responses of non-CPs (Pavlovič and Saganová, 2015; Bemm et al., 2016). The hypothesis that plant carnivory recruits (root) equipment against microbial pathogens has been best demonstrated on members of PR proteins, but parallels can be found in several stages of plant immunity. Co-option has been suggested as a mechanism that facilitates evolutionary emergence of new functions (Renner and Specht, 2012). Carnivorous plants with independent origins repeatedly co-opted the same plant defence protein lineages to acquire digestive functions (Fukushima et al., 2017). For example, >100 pathogen (chitin) receptor homologues are activated in the digestive glands in *Dionaea* upon feeding (Bemm et al., 2016) that are analogous to receptor-like kinase complexes in non-CPs mediating plant immunity (Wan et al., 2008), cell death (Ye et al., 2020) or symbiosis (Zhang et al., 2015). Considering the array of various chitin degradation products liberated during prey decomposition, activation of receptors with diverse affinity for prey-derived molecules probably mediates distinct downstream responses. If so, the extent to which the signals for triggering prey digestion, defence or possibly cell death might overlap is unclear.

In CPs, evolutionary constraints have selected for highly stable enzymes with different substrate specificities and cleavage patterns from those found in animal digestive enzymes (Butts et al., 2016b). Chitinases are enzymes that are one of the best fits to the phenomenon of plant carnivory as their true substrate (chitin) in plants is unknown. For this reason, their ancestral role in non-CPs is hypothesized to lyse chitin in cell walls of invading fungal pathogens. Since their discovery in the 1980s and assignment as members of so-called PR proteins, their functions have been gradually broadened for several processes in plant physiology and development (Grover, 2012). It is thus not surprising that only one-third of the presently known chitinases in *Nepenthes* are linked to prey digestion (at least to date; Rottloff et al., 2016; Filyushin et al., 2019; Dkhar et al., 2020).

In *Arabidopsis*, the chitinase gene family members were sub-classified according to their sequence, structure and phylogenetic relationship to classes I–V (Passarinho and de Vries, 2002). Consistent differential activities of homologous vacuolar sub-class Ia and extracellular Ib chitinases were demonstrated in *Drosera* (Matušíková et al., 2005; Libantová et al., 2009) and *Nepenthes* (Eilenberg et al. 2006). Chitinases protecting against pathogens underwent a rapid evolution in non-CPs. In the active site cleft of class I-type enzymes, amino acid replacements are frequent as a flexible evolutionary weapon against a wide variety of pathogenic fungi (Bishop et al., 2000; Tiffen, 2004). In carnivorous Caryophyllales, such an adaptive change in the active site cleft is manifested as a single substitution of

Phe276 in the chitinase sub-class Ia in *Nepenthes* (Renner and Specht, 2013). The substitution probably affects the enzyme properties; nevertheless, its positional homology with Phe276 in other species and its putative allocation in the vacuole suggest a role in defence against pathogens (Renner and Specht, 2013). On the other hand, selection pressure driving the loss of a vacuole targeting signal in sub-class Ib chitinases enables secretion from the digestive cells for prey decomposition, thus Ib chitinases shifted to a role specific to carnivory (Eilenberg et al., 2006; Renner and Specht, 2012). Renner and Specht (2013) hypothesized that a duplication event preceding the evolution of the Caryophyllales could generate gene copies with complementary degenerative mutations that each persisted in the genome (Force et al., 1999; Cannon et al., 2004). These copies allowed a sub-class specialization and initiation of processes that led to functional diversification in CPs (Renner and Specht, 2013). Surprisingly, the sub-class Ib chitinase genes appeared in species that have partly (*Triphyophyllum*) or completely (*Ancistrocladus*) lost their carnivorous habit; these genes might potentially have been converted to pseudogenes after loss of functional domains, probably during the transition from a plant carnivore to a part-time carnivore or non-CP (Renner and Specht, 2013). Such a loss of chitinase function in the Caryophyllales is supported by the occurrence of *Nepenthes* sub-class Ib chitinase homologues with premature stop codons (Renner and Specht, 2013).

A distinct evolutionary history has been proposed for some other PR members. The class III chitinase in *Nepenthes* has evolved to code for an enzyme with dual roles in both defence and carnivory (Rottloff et al., 2011). S-like RNases underwent convergent amino acid changes in phylogenetically non-related *C. follicularis* on the one hand and *Nepenthes*, *Dionaea* and *Drosera* on the other (Nishimura et al., 2014). Orthologous relationships were also observed for PR-1-like proteins, thaumatin-like proteins and phosphatases from CPs of multiple origins (Fukushima et al., 2017). Apart from these examples of stress-related compounds, evolutionary relatedness with carnivory has been shown for the class V  $\beta$ -1,3-glucanase gene from *Drosera rotundifolia* (Michalko et al., 2017). In contrast to chitinases, an ancestral function of  $\beta$ -1,3-glucanases in non-CPs plausibly relates to cell division, yet they belong to PR proteins as some of them restrict pathogen spreading (Doxey et al., 2007). Promoter analyses suggest that *DrGln1* plays a role in plant development and responds to environmental cues. Though not detected in traps, the class V  $\beta$ -1,3-glucanases could represent a unique example of non-defence-related genes that have been co-opted for carnivory (Michalko et al., 2017). If indeed so, it is tempting to hypothesize a similar mechanism for  $\alpha$ -amylase, which appears specific to the *Pinguicula* secretome (Kocáb et al., 2020).

Co-option to carnivory does not necessarily require a change in gene sequences. Pitchers in *N. hemsleyana* and some closely related *Nepenthes* species have adapted to use and absorb bat-derived urea (Yilamujiang et al., 2017). Instead of developing a specific urease, the plant utilizes an available enzyme that recycles N from the endogenous urea. Although these plants with a unique nutrient sequestration strategy have been suspected of a partial loss of carnivory in favour of coprophagy (Fleischmann et al., 2018), *N. hemsleyana* still expresses the whole set of enzymes found



in typical insectivorous *N. rafflesiana* (Kocáb *et al.*, 2021). Similarly, the ‘omnivorous’ *N. ampullaria*, which acquires significant amounts of nutrients from leaf litter, accumulated only a few unique enzymes and the data do not provide evidence for an adaptive evolution towards a breakdown of leaf litter (Zulkapli *et al.*, 2021). However, the plant actively reduces the acidity level for trapping living micro-organisms and arthropod larvae which probably also contribute to breakdown of leaf litter (Moran *et al.*, 2010).

The evolution of other enzymes has not been studied in detail. Advances in high-throughput analyses probably will allow more comprehensive structural comparisons to be conducted, revealing putative functional divergences in the context of carnivory. Such an analysis of a large group of (probably functional) proteases from *D. capensis*, for instance, has revealed sequential and structural features not comparable with reference enzymes from other (non-)CPs that could have evolved to effectively digest the diverse spectrum of proteins from prey (Butts *et al.*, 2016a, b). Functional differences among these proteases are likely to be adaptive and may imply a variety of substrate preferences, physico-chemical properties and cleavage patterns in addition to the standard spectrum of their counterparts in non-CPs.

#### CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Plant carnivory combines adaptations to both nutrient-poor and wet soils, of which wet anoxic soils appear to be supreme and are more limiting for CP survival at sites. The extant species of CPs are a result of at least ten independent evolutionary lineages. Therefore, it has also become ecologically beneficial for several ancestral plant taxa to capture animal prey by special foliar-derived traps and utilize animal-derived nutrients. Generally, three evolutionary strategies to increase the benefit of carnivory (and thus to extend the costs) may be distinguished (Ellison and Adamec, 2018a): (1) to capture more prey or larger prey; (2) to better digest captured prey and more effectively absorb nutrients from it; and (3) to stimulate photosynthesis and root nutrient uptake by prey-derived nutrients in order to accelerate plant growth and development. Typical physiological processes occurring in CPs are also common individually in non-CPs, but in CPs they are usually coupled in series, forming together a co-ordinated cluster of traits: the carnivorous syndrome. Prey capture leads to stimulation of digestive enzyme production, secretion and nutrient absorption from digested prey through specific upregulation of gene expression. Later, due to prey-derived N, photosynthesis in leaves and plant growth are increased and root nutrient uptake is stimulated. How the leaf and root nutrient uptake are co-ordinated on the level of ion channels and nutrient transporters remains to be investigated. Though CP roots grow permanently in anoxic soil conditions and their root porosity is limited, the characteristics of their anaerobic metabolism (fermentation) are almost unknown as the dominant pieces of knowledge have been obtained under aerobic conditions (Adamec, 2005). Yet CP roots, though relatively small, are metabolically very active and able to take up all necessary mineral nutrients for prey-stimulated growth.

Hatcher *et al.* (2020) hypothesized that metabolite diversity provided a mechanism for the evolution of CPs and facilitated their rapid occupation of new environments. Under low-nutrient conditions, both divergent and convergent trends resulted in the evolution of five different trap types, which share many common features and allow generalization (at least partly) of the costs and benefits of carnivory, the mechanism of evolution by WGD and gene duplication or the mechanism of prey digestion. Unfortunately, available knowledge is still mostly focused on a few carnivorous ‘model’ species and digestive enzyme types. Recent modern analyses and combined approaches have revealed many molecular details on plant carnivory. The most exciting aspect, however, is probably the orchestration of the individual underlying mechanisms. These might be co-opted from defence, signalling or pollinating and dispersal mechanisms in non-CPs, but could also have coevolved to best meet conditions for prey attraction, capture and digestion and nutrient absorption. Experimental proofs should be obtained for the as yet understudied non-enzymatic digestive components actively contributing to prey digestion. The major challenge is to characterize the dynamic metabolic changes during stimulation of traps, starting with prey capture until its consumption. Our view on defence-related enzymes as the sole source of components for carnivory will probably broaden to other compounds and processes in non-CPs, as, for example, all mutualistic interactions with insects could have been opportunistically re-cruited and (partly) exploited in plant carnivory.

Phytohormones control all aspects of plant growth and development, so it was expected for a long period they also have functions in botanical carnivory. However, in contrast to non-CPs, only a few studies have been devoted to hormonal signalling in CPs, mainly confined to jasmonates. In the last decade, the role of jasmonates in the induction of digestive processes has been elucidated in four genera of Caryophyllales, but how the remaining CP genera activate digestive process is still unknown. Moreover, what is the role of other phytohormones in botanical carnivory? Auxin (IAA) has also been suspected to participate somehow in leaf bending reaction in *Drosera* (La Porta *et al.*, 2019) and ABA for digestive fluid regulation in *Nepenthes* (Wan Zakaria *et al.*, 2019), but these findings are still very fragmentary. How do these phytohormones interact with the JA signalling pathway? Except for an antagonistic effect of ABA and JA in *Dionaea* (Escalante-Pérez *et al.*, 2011), almost nothing is known about hormone cross-talk in CPs. How do phytohormones participate in phenotypic plasticity and trap formation in response to environmental conditions? What is the cost of hormonal signalling? Jasmonate-controlled re-direction of gene expression from photosynthesis and growth to plant defence in non-CPs (Pavlovič and Saganová, 2015) may have important implications for the cost–benefit model and needs further investigation.

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