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RESEARCH PAPER

# Sticky plant captures prey for symbiotic bug: is this digestive mutualism?

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## ABSTRACT

**Many plants capture and kill insects but, until relatively recently, only carnivorous plants with digestive enzymes were known to gain directly from the nutrients of those insects. Recent studies show that some carnivorous plants lack digestive enzymes and have evolved digestive mutualisms with symbiotic insects that digest their prey for them. *Rhododendron macrosepalum*, a plant with sticky leaves that captures insects, has an association with symbiotic Mirid bugs that consume the insects captured. Here, we determine what the nature of the relationship is between Mirid and plant. We find that *R. macrosepalum* has no digestive enzymes of its own but that it does not seem to have the ability to absorb hemipteran faeces through its leaf cuticle. Naturally occurring levels of $^{15}N$ and $^{14}N$ were used to determine that *R. macrosepalum* gains no nitrogen through its association with the Mirid bugs and that it obtains all of its nitrogen from the soil. The Mirids, on the other hand, seem to obtain nitrogen from insects captured by the plant, as well as from plant tissues. The relationship between plant and Mirid is not a digestive mutualism but more likely an antagonistic relationship. This study adds to our understanding of how digestive mutualisms evolve and shows that insect capture alone, or in combination with a symbiotic insect relationship does not necessarily make a plant 'carnivorous'.**

## INTRODUCTION

Facilitating the digestive process of one organism by another provides some of the most classic examples of mutualism. These digestive mutualisms are often highly specialised and obligatory for one or both of the organisms involved (*e.g.*, Quinlan & Cherrett 1979; Treseder *et al.* 1995; Nalepa *et al.* 2001; Harrington 2005). Perhaps one of the most obvious examples of digestive mutualism is the microsymbionts within the gut systems of animals that help them to digest their host's food (Breznak & Brune 1994; Stevens & Hume 1998). However, other organisms, such as leaf cutter ants, enlist the services of fungi to digest cellulose in 'gardens' outside of their digestive tracts (Currie 2001). The ants then consume some of the fungus but simultaneously prevent contamination by alien microbes that would otherwise eliminate the fungus (Currie 2001; Currie & Stuart 2001). More recently, plants have also been found to have digestive mutualisms with animals that function as 'digestive organs'. Although digestive mutualisms have long been suspected for certain insectivorous pitcher plants that lack digestive enzymes (*e.g.*, Bradshaw & Creelman 1984; Juniper *et al.*1989), the first real examples of digestive mutualism in plants were recorded in *Roridula* (Ellis & Midgley 1996), a sticky plant that captures large numbers of insects (Marloth 1903, 1910) but which has no digestive enzymes (Marloth 1925; Ellis & Midgley 1996). The lack of digestive enzymes was confirmed using a crude, but effective method of placing photographic film on the leaves of the plants. In the presence of digestive enzymes the albumin layer on the film is digested, leaving small clear patches (Heslop-Harrison & Knox 1971; Ellis & Midgley 1996).

Using flies labelled with $^{15}N$, Ellis & Midgley (1996) demonstrated that obligate-associated (Dolling & Palmer 1991; Anderson 2006) Mirid bugs facilitate the digestion of prey captured by the *Roridula* plants, and that the hemipterans and plants had co-speciated (Anderson *et al.* 2004). Using naturally occurring ratios of $^{14}N$ and $^{15}N$ isotopes, Anderson & Midgley (2002, 2003) found that *Roridula* plants have elevated $^{15}N$ levels in comparison to co-occurring plants with the same mycorrhizal systems (also see Midgley & Stock 1998). Using these isotope ratios, they calculated that *Roridula* plants obtain in excess of 70% of their nitrogen (N) from faeces of the hemipterans, which are deposited on the leaves of the plants after the hemipterans have finished consuming the prey captured by *Roridula* (Anderson & Midgley 2002, 2003). The N compounds from the faeces pass through very unusual gaps in the cuticles of *Roridula* leaves (Anderson & Midgely 2005). The presence of such gaps in carnivorous plant leaves can be reliably shown by the rapid uptake of the dye neutral red (Joel & Juniper 1982). Subsequently, similar digestive mutualisms have also been found in Bromeliaceae, which often have symbionts living within their liquid-filled traps or amongst their leaves (Romero *et al.* 2006, 2008,

2010). But perhaps the most bizarre examples include certain species of *Nepenthes* that entice rodents and shrews to feed on nectar secretions from the lids covering the traps. While feeding from these, the rodents and shrews defecate into the traps like a lavatory and the plants obtain N from these faeces (Clarke *et al.* 2009; Greenwood *et al.* 2011).

The evolution of digestive mutualism may be an important intermediate step that facilitates the evolution of carnivory in certain plant lineages. Alternatively, it may represent a mechanism by which already carnivorous plants are able to save resources by reducing the production of digestive enzymes, making the study of different stages of digestive mutualism important in understanding how such mutualisms evolve. In addition, the plethora of arthropods associated with carnivorous plants spans a wide continuum of relationship types, from mutualism to parasitism, making carnivorous plants good systems for studying the evolution and breakdown of mutualisms and exploitative relationships (e.g., Anderson 2006).

Here, we examine *Rhododendron macrosepalum* Maxim, a plant whose sticky leaves and sepals capture abundant insects (Sugiura & Yamazaki 2006). Similar to *Roridula*, this plant is also associated with Mirid bugs (*Orthotylus gotoi* Yasunaga) that occur nowhere else, but which consume the insects captured by the plant (Fig. 1; also see Sugiura & Yamazaki 2006). However, it is not known whether these Mirids are digestive mutualists similar to those associated with *Roridula*, or whether the Mirids are commensalistic or even antagonistic. The plants are most sticky during flowering time, and the stickiness is concentrated on buds, young leaves and sepals (Sugiura & Yamazaki 2006), suggesting that that the sticky leaves of *R. macrosepalum* function primarily as an anti-herbivore strategy, and that the actual capture of insects may not benefit the plants. Here, we determine the nature of the relationship by: (i) testing to see whether *R. macrosepalum* has digestive enzymes, (ii) determining whether *R. macrosepalum* is able to absorb N compounds through its leaves, and (iii) testing whether *R. macrosepalum* has elevated $^{15}N$ levels associated with an alternative source of N (*i.e.*, insects or faeces).

## METHODS

### Study site and species

The study site is situated in a temperate secondary forest at Kitayama, Kyoto, Japan (35°03′25″ N, 135°46′07″ E). The dominant tree species of this forest are *Quercus serrata* and *Pinus densiflora*. *Rhododendron macrosepalum* primarily occurs along forest edges and is one of the most common shrubs at the study site. This plant is semi-deciduous and most leaf flushing occurs in April to June, continuing until August. Flowering occurs in April to late May. *Orthotylus gotoi* bugs are nymphs during most of the spring; adults begin to appear from early June and can be found until around August. The study was conducted during May and June 2011.

**Fig. 1.** *Orthotylus gotoi* nymphs probe a fly captured by the glandular hairs of *Rhododendron macrosepalum*.

### Does *R. macrosepalum* have digestive enzymes?

The methods of Heslop-Harrison & Knox (1971) were used to determine whether *R. macrosepalum* has digestive enzymes. Processed but unexposed slide film (Velvia, 50 ASA; Fujifilm, Tokyo, Japan) was placed overnight on the sticky leaves of five *R. macrosepalum* plants. As a positive control, we performed the same procedure using five *Drosera adelae* plants because it is well known that *Drosera* does produce digestive enzymes (Joel & Juniper 1982). On the next day, the film strips were removed, allowed to dry and then examined for clear patches where the albumen layer of the film had been digested. The production of digestive enzymes is enhanced by smearing a paste made from instant yeast and water onto the leaves of carnivorous plants (Heslop-Harrison & Knox 1971). We repeated the exposure experiment after applying a thin layer of 1% yeast solution to the leaves of five *D. adelae* and five *R. macrosepalum* plants.

### Can *R. macrosepalum* absorb compounds through its leaf cuticle?

The leaves of all vascular plants are covered with a hydrophobic cuticle (Schönherr 1982; Viougeas *et al.* 1995) that forms a transport barrier to hydrophilic substances (Price 1982). But carnivorous plants and plants with digestive mutualisms normally have very thin cuticles or cuticular gaps that allow hydrophilic compounds to be absorbed into the leaf (Williams & Pickard 1969, 1974; Anderson & Midgely 2005). Joel & Juniper (1982) found that the presence of cuticular gaps could reliably be determined by the rapid uptake of the water-soluble dye, neutral red. Using the methods of Joel & Juniper (1982), (also see Anderson & Midgely 2005), we dipped five mature *R. macrosepalum* leaves into a 1% solution of neutral red. After 2 min of submersion, leaves were rinsed in water and examined for dye uptake under a dissecting microscope. As a positive control, we repeated this procedure using five *D. adelae* leaves, a known insectivorous plant with absorptive leaves.

### Determining sources of nitrogen

The approximate relative N contribution of two potential food sources (see equation 1) can be calculated using the methods of Peterson & Fry (1987); (also see Schulze *et al.* 1991; Anderson & Midgley 2002), where $\delta^{15}N_{ORG}$ is the $\delta^{15}N$ value for the organism in question (*e.g.*, the carnivorous plant or insect associated with the plant). The $\delta^{15}N_{REF1}$ is the $\delta^{15}N$ value for the reference organism: an organism that obtains all of its N from one of the potential food sources.

The $\delta^{15}N_{REF2}$ is the $\delta^{15}N$ value of an organism that obtains all of its N from the alternative food source (see equation 1).

$$\%N_{REF2} = (\delta^{15}N_{ORG} - \delta N_{REF1})/(\delta^{15}N_{REF2} - \delta^{15}N_{REF1}) \quad (1)$$

In previous studies (*e.g.*, Anderson & Midgley 2002), the actual alternative food source (*i.e.*, faeces) was used as a surrogate for $\delta^{15}N_{REF2}$; however this is likely to inflate the contribution of the alternative food source because N will fractionate after an organism consumes a particular food source. This will discriminate against the lighter $^{14}N$ isotope, so that a plant that gains all of its N from faeces should have a higher $\delta^{15}N$ value than that of the faeces.

### Reference plants and animals

If *R. macrosepalum* has digestive enzymes, it could potentially obtain N from three sources: insect prey, hemipteran faeces or the soil. In this event, the equation above cannot be used since this only discriminates the contributions from two potential N sources. If *R. macrosepalum* has no digestive enzymes, it can only obtain N from hemipteran faeces or the soil. A non-carnivorous plant can be used as a suitable reference for the soil N contribution. However it is known that microhabitat, rooting depth and mycorrhizal system (Gebauer & Schultze 1991; Hogberg 1997; Schmidt & Stewart 1997; Hobbie *et al.* 2000) can have strong effects on the $\delta^{15}N$ value of a plant. As a result, it is important to choose reference plants that grow sympatrically with the organism in question, as well as those that are closely related, as they should have similar mycorrhizal associations. As the reference plant for soil N, we chose sympatric *Rhododendron* (Ericaceae) species, *R. reticulatum*, which grows interspersed with *R. macrosepalum*. In addition to this reference plant, we also calculated $\delta^{15}N$ for four other species of sympatric Ericaceae (*Vaccinium oldhamii*, *V. hirtum*, *Pieris japonicum and Lyonia ovalifolia*) to make sure that the chosen reference plant was not unusual in any way. Since there are no sympatric species that acquire N solely from insect prey or faeces, we used actual hemipteran faeces as the alternate reference, knowing that if used, the contributions from this source would be over-inflated.

To determine the diet of the Mirids associated with *R. macrosepalum*, we used assassin bugs, which are completely insectivorous, as a reference for the insect contribution to their diet. These were immature hemipterans captured on the leaves of *R. macrosepalum* where they were feeding on insects captured by the plants. For the plant component of their diet, we used sap-sucking lace bugs (Tingidae, *Stephanitis* sp., which had been captured on the leaves of *R. macrosepalum*). In addition to these various references, we also determined the $\delta^{15}N$ values of the *R. macrosepalum* plants, the hemipterans themselves as well as the prey that they were consuming.

### Sampling methods

Eight *R. macrosepalum* plants were chosen at the field site. Approximately five young, terminal leaves were taken from each plant and placed in a paper envelope. Very young leaves were favoured since they would have very few hemipteran faeces on their surfaces. We also ensured that the leaves had no insect carcasses on them. *Rhododendron macrosepalum* plants were not randomly chosen, but were chosen only if they had a paired reference of similar size growing within 3 m. The young leaves of eight reference plant pairs were sampled as above. Young leaves of the four other ericaceous plants were sampled from the same site; however these plants were not specifically paired with any of the *R. macrosepalum* plants. Leaf samples were taken from five *V. oldhamii and L. ovalifolia* plants and from six *V. hirtum and P. japonica* plants.

The $\delta^{15}N$ values of ten randomly chosen adult *O. gotoi* hemipterans as well as three assassin bugs were determined. The lace bugs were too small to be individually sampled and as a result, we ground five of them together, which was sufficient to make up one sample. The insect prey captured by *R. macrosepalum* is quite diverse and consists of various taxa of various sizes from different trophic levels, and probably with very different $\delta^{15}N$ values (Sugiura & Yamazaki 2006; Table S1). To incorporate the contributions of all these different taxa, we collected all of the live prey items that we could find on *R. macrosepalum* leaves (35 individuals; see Table S1). All prey items were ground together and eight replicates were taken from this single mixed sample. Lastly, we collected the faeces from *O.gotoi*. Approximately 300 hemipterans were placed in glass Petri dishes for 3 days. Hempterans were given cotton wool soaked in water to drink. After 3 days, the hemipteran faecal deposits were scraped from the glass Petri dishes. Sufficient faeces were collected for four isotope samples.

### Isotope methods

All samples were placed in a drying oven (60 °C) for 3 days. Samples were removed and ground into a fine powder using a mortar and pestle before placing each sample into a separate Eppendorf tube. Samples were folded into tin capsules and N isotope ratios were measured using an isotope ratio mass spectrometer (Delta V plus; Thermo Fisher Scientific, USA) coupled with an elemental analyser (Flash 2000; Thermo Fisher Scientific, Waltham, MA, USA) *via* a Conflo-IV interface (Thermo Fisher Scientific) at the Center for Ecological Research, Kyoto University. The measured values were corrected using reference material CERKU-01, 02 (Tayasu *et al.* 2011) for the internationally accepted standard, atmospheric N. The values were expressed as a $\delta^{15}N‰$, which are differences between the isotopic ratios in the sample from those in the standard and measured as parts per thousand. Analytical error (SD) of the on-line procedure was 0.055‰.

## RESULTS AND DISCUSSION

Plants may benefit from insect capture if they are able to produce digestive enzymes to digest such prey. However, the albumen layer of film placed on the leaves of *R. macrosepalum* was never digested, irrespective of whether yeast solution was added or not. This suggests that *R. macrosepalum* produces no digestive enzymes in its leaves. As a positive control, we found that the albumen layer of film placed on five *Drosera adelae* plants was consistently digested, leaving clear spots on the film (Fig. S1). This was true for both the

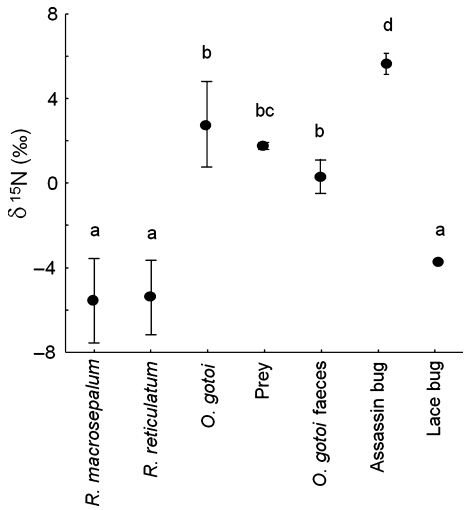


**Fig. 2.** Mean ± SD $\delta^{15}$N values for *Rhododendron macrosepalum*, its soil reference, *Rhododendron reticulatum*, the symbiotic hemipteran *Orthotylus gotoi*, the prey that it feeds on, its faeces, as well as insectivorous reference hemipterans (assassin bugs, Reduvidae) and herbivorous reference hemipterans (lace bugs, Tingidae).

**Table 1.** Mean ± SD $\delta^{15}$ N values of all organisms used in the study, including the four alternative reference plants.

| species | classification | average $d^{15}$N values | SD |
|---|---|---|---|
| *Rhododendron macrosepalum* | Target plant | 5.53 | 1.87 |
| *Rhododendron reticulatum* | Soil reference | 5.38 | 1.69 |
| *Vaccinium oldhamii* | Alternative soil reference | −3.11 | 1.26 |
| *Vaccinium hirtum* | Alternative soil reference | −6.32 | 0.25 |
| *Pieris japonica* | Alternative soil reference | −2.54 | 1.51 |
| *Lyonia ovalifolia* | Alternative soil reference | −2.55 | 1.46 |
| *Orthotylus gotoi* | Target hemipteran | 2.69 | 2.01 |
| prey | Alternative plant N source | 1.76 | 0.2 |
| faeces | Alternative plant N source | 0.28 | 0.79 |
| reduvidae | Carnivorous hemipteran reference | 6.64 | 0.4 |
| tingidae | Herbivorous hemipteran reference | −3.61 | |

five *D. adelae* that had yeast solution added as an enzyme catalyst and for plants without the addition of yeast.

An alternative route for insect N to enter the plant would be if hemipteran faecal N were absorbed directly through the leaf cuticle. However, we found that *R. macrosepalum* was unable to absorb the dye neutral red through its cuticle, making it unlikely that faecal N could be absorbed by this method. In contrast, neutral red dye was quickly absorbed into specialised cells on the leaves of *D. adelae*, and the absorptive cells were clearly observed as red spots on the leaves of the plant (Fig. S2).

To further test the possibility that *R. macrosepalum* can gain nutritional benefits from prey capture, we used naturally occurring ratios of N isotopes to determine the potential sources of N in *R. macrosepalum*. We found that $\delta^{15}$N varied significantly when it came from organisms of different trophic levels, *e.g.,* Ericaceae plants, hemipterans and faeces ($F = 39.48$, $P < 0.000001$; Fig. 2, Tables 1 and 2). However, $\delta^{15}$N values for *R. macrosepalum* were not significantly different from the primary soil reference plant *R. reticulatum* ($P = 0.831$; Fig. 2). Of the alternative reference plants, none had significantly lower $\delta^{15}$N values (Table 2) than *R. reticulatum*. The similar or higher values of all reference plants make it unlikely that *R. macrosepalum* is obtaining N from higher trophic levels. Since *R. macrosepalum* has no digestive enzymes, is unable to absorb N through its leaf cuticle, and shows no signature of insect N in its leaves, it is unlikely that the plants gain any nutritional benefits from prey capture. Furthermore, it seems unlikely that they gain any benefits from the symbiotic association with *O. gotoi.*

On the other hand, *O. gotoi* has $\delta^{15}$N values that are significantly higher than herbivorous Tingid hemipterans ($P < 0.003$; Fig. 2) and significantly lower than the insectivorous assassin bugs ($P = 0.003$; Fig. 2), suggesting that *O. gotoi* is possibly omnivorous. Using equation 1, we calculate that *O. gotoi* obtains approximately 31% of its N from the *R. macrosepalum* plants and the rest from insect prey, suggesting that the relationship is antagonistic. One weakness of this calculation is that we are not sure of the host plant for Tingidae, and whether the values reflected for this reference are a good reflection of a herbivore living on *R. macrosepalum*. However, although *O. gotoi* has not yet been unequivocally observed probing plant tissues (Sugiura & Yamazaki 2006; Anderson B., personal observation.), the provision of host plant material increased Mirid longevity (Sugiura & Yamazaki 2006; Anderson B., personal observation). This also suggests that plant sap could be an important part of their diet, supporting the idea of an antagonistic relationship between plant and bug.

Most Mirid bugs are in fact sap-sucking herbivores and many have adapted to a species-specific life on glandular plants (Schuh 1995; Wheeler 2001). On glandular plants, Mirids have frequently turned to carnivory, or display mixed feeding strategies to exploit the insect prey captured by the plants (Schuh 1995). The ease of dietary switching in Mirids may be an important step in the evolution of carnivory for plants with glandular hairs such as *Roridula, Byblis, Drosera* and *Pinguicula*, all of which have associations with Mirid bugs (China & Carvalho 1951; China 1953; Dolling & Palmer 1991; Zamora 1995). It is most likely that the stickiness first evolves as a herbivore deterrent, which then enables the Mirids to make the dietary switch to captured insects. In support of this, the stickiest parts of *R. macrosepalum* are the buds, sepals and young leaves (Sugiura & Yamazaki 2006), which are in most need of defence. From here, it seems just a small step to the evolution of cuticular gaps that make the passage of N into plant leaves possible.

The results of this paper also serve as a 'control' for other digestive mutualism experiments, demonstrating that isotopes are a useful method for confirming whether plants are gaining N from sources other than the soil (either through direct carnivory or through digestive mutualisms). These

**Table 2.** P values for post hoc LSD test.

| | *Rhododendron macrosepalum* | *Rhododendron reticulatum* | *Vaccinium oldhamii* | *Vaccinium hirtum* | *Pieris japonica* | *Lyonia ovalifolia* | *Orthotylus gotoi* | prey | faeces | reduvid | tingidae |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Rhododendron macrosepalum* | | 0.831 | 0.005 | 0.325 | 0.000 | 0.001 | 0.000 | 0.000 | 0.000 | 0.000 | 0.220 |
| *Rhododendron reticulatum* | 0.831 | | 0.009 | 0.239 | 0.001 | 0.001 | 0.000 | 0.000 | 0.000 | 0.000 | 0.259 |
| *Vaccinium oldhamii* | 0.005 | 0.009 | | 0.001 | 0.522 | 0.547 | 0.000 | 0.000 | 0.001 | 0.000 | 0.753 |
| *Vaccinium hirtum* | 0.325 | 0.239 | 0.001 | | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.092 |
| *Pieris Japonica* | 0.000 | 0.001 | 0.522 | 0.000 | | 0.991 | 0.000 | 0.000 | 0.004 | 0.000 | 0.498 |
| *Lyonia ovalifolia* | 0.001 | 0.001 | 0.547 | 0.000 | 0.991 | | 0.000 | 0.000 | 0.006 | 0.000 | 0.508 |
| *Orthotylus gotoi* | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | | 0.184 | 0.007 | 0.003 | 0.000 |
| prey | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.184 | | 0.104 | 0.000 | 0.001 |
| faeces | 0.000 | 0.000 | 0.001 | 0.000 | 0.004 | 0.006 | 0.007 | 0.104 | | 0.000 | 0.021 |
| reduvid | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.003 | 0.000 | 0.000 | | 0.000 |
| tingidae | 0.220 | 0.259 | 0.753 | 0.092 | 0.498 | 0.508 | 0.000 | 0.001 | 0.021 | 0.000 | |

methods could very easily be applied to the long list (see Juniper *et al.* 1989; Chase *et al.* 2009) of potential but unconfirmed carnivorous plants to determine whether they are able to obtain N from the prey that they capture. Moreover, it demonstrates that in addition to evolving trapping mechanisms and an association with a carnivorous insect, plants also require further adaptations to facilitate the absorption of N compounds through their cuticles. Some controversy has surrounded the question of whether plants that digest their prey solely through digestive mutualisms are truly carnivorous (see Anderson & Midgley 2003; Chase *et al.* 2009). However, the acquisition of trapping mechanisms as well as absorptive cells on the leaves may be sufficient to classify some of these plants as carnivores (see Givnish *et al.* 1984). Alternatively, the growing number of plants with digestive mutualisms suggests that this is a syndrome that could encompass a broader range of plants than just those that trap insects and digest them with the aid of a mutualist. This syndrome could be given a different name, *e.g.,* coprophagous plants.

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## AUTHOR CONTRIBUTIONS

BA and AK formulated the idea and did the sampling. BA wrote the manuscript and IT oversaw the isotopic analysis.

## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Film strips placed overnight on *Rhododendron macrosepalum* and *Drosera adelae* leaves. The digestion of the albumen layer can clearly be seen on the strips placed on Drosera but not on the strips placed on Rhododendron.

**Figure S2.** The uptake of neutral red by specialized absorptive cells on the leaf of *Drosera adelae*. Dark red cells on the right hand side indicate the part of the leaf that was submerged in the dye. The lighter red cells on the left were not submerged in the dye.

**Table S1.** A list of live prey captured on *R. macrosepaulm*. Prey are identified to order level and where possible to family. The average length of each species was recorded using calipers and where possible the trophic level is given. For many of these insects the trophic level was not known or the adults and nymphs feed on different trophic levels.

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