

**Following Darwin's footsteps using 'the most wonderful plants in the world':
the ecophysiological responses of the carnivorous plant *Drosera rotundifolia* to
nitrogen availability.**

by

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A doctoral thesis submitted in partial fulfilment of the requirements for the award of

Doctor of Philosophy of Loughborough University

30 September 2014

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Abstract

Nitrogen (N) is an essential element to plants for growth, maintenance and reproduction, however most N does not exist in a form that is biologically available to plants. In order to maximise the acquisition and retention of N, plants have evolved a variety of morphological and physiological adaptations and life history strategies, as well as the ability to respond plastically to changes in resource availability in ecological time. Determining the ecophysiological responses of plants to changes in root N availability is crucial to further understanding of the mechanisms underlying competitive interactions between plants, and between plants and other organisms, that ultimately contribute to community structure and ecosystem functioning. Carnivorous plants are ideal systems for investigating ecophysiological responses to N availability as:- (i) they share a unique adaptation for obtaining supplemental N from captured prey, therefore ecological stoichiometry and energetic cost/benefit models may be explored; (ii) the trait of botanical carnivory is widely considered to have independently co-evolved as a response to N-deficient, sunny and wet environments, therefore resource allocation trade-offs between plant investment in N and carbon (C) acquisition may be observed, and (iii) they are extremely sensitive to changes in root N availability in ecological time. In this research, the carnivorous plant *Drosera rotundifolia* (round-leaved sundew) was used to address several unanswered ecophysiological and evolutionary questions relating to patterns and processes of prey capture and the N nutrition of carnivorous plants. Furthermore, the potential for reducing uncertainty in the calculation of plant reliance on carnivory using a $\delta^{15}\text{N}$ natural abundance multi-level linear mixing model was explored. A combined approach of *in-situ* and *ex-situ* studies was employed, using co-occurring non-carnivorous plants or carnivorous plant species with differing evolutionary lineages or prey capture mechanisms respectively to provide context.

Results show that the adaptations of carnivory, high reproductive investment and a relatively short life span enable *Drosera rotundifolia* to survive and thrive in an extreme, N deficient environment. Phenotypically plastic responses by the plant to light and root N availability provide evidence of resource allocation trade-offs between investment in carnivory for N acquisition and in photosynthesis for C acquisition. Plants invested less heavily in prey capture (measured as the stickiness of leaf mucilage) as N availability increased or light availability decreased. These results show that the energetic costs associated with carnivory are avoided by the plant when less costly sources of N are available for uptake and that the production of carbon-rich mucilage is only made under nutrient-limited and well-lit conditions. Results obtained from the comparison of captured insect prey with background invertebrates of potential prey indicate that *Drosera rotundifolia* is a dietary generalist, where the quantity of prey captured per plant is positively correlated with leaf stickiness and total leaf area. Plant reliance on prey-derived N decreased with increasing root N availability, providing evidence that carnivory is only of net benefit to the plant in N-deficient and well-lit environments, as the photosynthetic costs of investment in the trait are not exceeded by the energetic gain from prey N uptake in shady or dry habitats. A more accurate and precise method for calculating plant reliance on botanical carnivory is presented which incorporates the insect diet of the plant. This method has wider significance for reducing uncertainty in the calculation of relative source contributions to a mixture for most natural abundance applications using a multi-level linear mixing model. To conclude, results from this research further understanding of the ecophysiological mechanisms underlying plant responses to changes in resource availability and the selective pressures driving the evolution of plant adaptations. These results therefore assist with predicting how plants and plant communities may respond to sustained N deposition inputs and future environmental scenarios.

Key words: carnivorous plants, N deposition, prey capture, plant-insect interactions, stable isotope analysis, linear mixing model, *Drosera rotundifolia*.

Acknowledgements

Firstly, I would like to thank my primary supervisor, Dr Jonathan Millett, for the continued support and advice during these studies. I am extremely grateful for the opportunity to undertake a PhD that focusses on such an interesting research area. Thank you for always being available to answer my questions, and for offering the opportunity to undertake related teaching and research activities. Thanks also to my Research Director, Dr David Graham, for your guidance and support. I would like to extend my gratitude to Loughborough University for the provision of funding for my studies, to NERC for the provision of funding to undertake $\delta^{15}\text{N}/\delta^{13}\text{C}$ natural abundance stable isotope analyses of plant and invertebrate samples, and to the British Ecological Society for the provision of funding to undertake related botanical ID courses.

I would also like to thank the support staff in the Department of Geography, in particular Barry, Fengjuan, Richard, Stuart and Mark, in particular for advice on laboratory analyses, the provision of endless consumables and help with setting the greenhouse up. Thanks also to my fantastic colleagues in the Department who have made my time at Loughborough so enjoyable, in particular to Erika, Kate, Sally, Lewis and Andy.

I am grateful for invaluable support from Dr Joan Daniels, Senior Reserves Manager (Natural England) and Mr Mike Bailey, Senior Reserves Manager (Countryside Council for Wales) during my six months of fieldwork at Whixall Moss, Shropshire, and Cors Fochno, Ceredigion, respectively. Many thanks to Dr Jason Newton and Dr Rona McGill of the NERC Life Sciences Mass Spectrometry Facility, Glasgow, for help and advice with $\delta^{15}\text{N}/\delta^{13}\text{C}$ natural abundance stable isotope analyses. I am extremely lucky to have received the support of Dr Richard Payne, Dr James Rowson, Dr Simon Caporn and Professor Nancy Dise of the PEATBOG research team, Manchester Metropolitan University, in particular for allowing me to use sundews from their experimental plots at Cors Fochno.

I would also like to thank my family and friends outside of Loughborough University who have provided support during my studies. In particular, thanks to Al, Mum, Jen, Ro, Sue, Phil and Linda.

Finally, I would like to thank my Grandma for providing love, encouragement and inspiration, and for instilling my interest in botany as a child.

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application (1-4 days). Presented are degrees of freedom (*df*), *F* and *P* values for the percentage uptake efficiency of prey N by *D. rotundifolia* from mass-standardised flies (absorbed N_{prey}), $\delta^{15}\text{N}$ and the C : N ratio. Significant effects at *P* < 0.05 are highlighted in bold.

Table 35 Physiological characteristics of *Drosera rotundifolia* plants at two ombrotrophic bogs in the UK that vary by N deposition input. Presented are the mean \pm 1 S.E. for: total dry mass per plant (mg), mass; number of inflorescences per plant (n). Significant effects at *P* < 0.05 are highlighted in bold. 205

Table 36 Physiological characteristics of *Drosophila melanogaster* prey fed to *Drosera rotundifolia* plants growing in experimental plots at Cors Fochno that differ by ambient warming treatment. Presented are the following Tables:- (a) mean \pm 1 S.E. for:- dry mass per fly (mg), mass; C : N ratio per milled *D. melanogaster* sample (pooled where necessary) for the independent samples *t*-test output for testing the influence of fly experimental status on the dry mass and C : N ratio of flies; (b) results of 2-way (vegetation warming status of survey quadrats, time) univariate ANOVA for the dry mass of *D. melanogaster* flies. Vegetation warming statuses of survey quadrats: unwarmed (control), warmed; time = prey retention time on *D. rotundifolia* plant: 1, 2, 3 or 4 days; (c) mean \pm 1 S.E. for the C : N ratio per milled *D. melanogaster* sample (pooled where necessary) for the independent samples *t*-test output for testing the influence of vegetation warming status on the fly C : N ratio. Significant effects at *P* < 0.05 are highlighted in bold. 206

Chapter 1: Introduction

1.1 The evolution of botanical carnivory

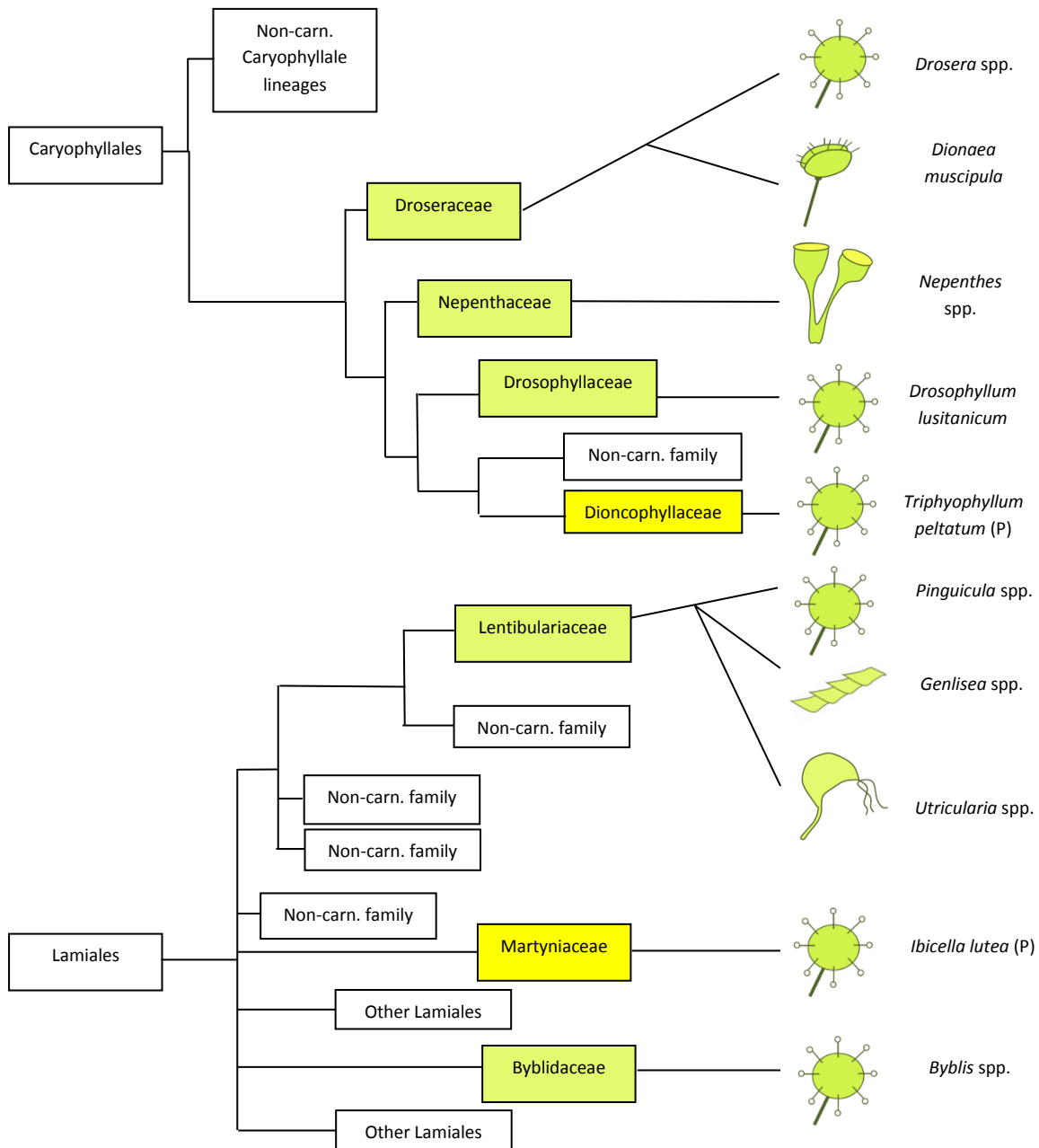
Carnivorous plants have fascinated scientists and the general public alike for centuries; they were referred to as '*miracula naturae*' by naturalists of the 18th century (Lloyd, 1942) and as the 'most wonderful plants in the world' by Charles Darwin (1898). Carnivorous plants occupy a wide geographical distribution; they are found on every continent except Antarctica, and are species-rich, with approximately 630 extant species recorded (Porembski and Barthlott, 2006). The trait of botanical carnivory, broadly defined as the ability of a plant to capture prey, absorb metabolites from prey and utilise these metabolites in growth and development (Lloyd, 1942), has independently evolved at least six times in five angiosperm orders including monocotyledons and dicotyledons (Ellison and Gotelli, 2009). A wide range of prey capture mechanisms exist, including adhesive 'flypaper' traps, snap traps, pitfall traps and bladder-like traps, with a high degree of convergence in morphological and physiological traits observed across carnivorous plant taxa. Two of these orders, the Caryophyllales and the Lamiales, contain over 95% of carnivorous plant species, and provide a good example of the evolutionary convergence of adhesive traps (Fig. 1).

Carnivorous plants are favoured as model organisms for addressing a wide range of evolutionary and ecological questions due to the multiple and independent evolution of botanical carnivory, the morphological convergence of prey capture mechanisms, their suitability for testing energetic cost/benefit models and their high sensitivity in response to small changes in root N availability in ecological time. Early studies focussed primarily on taxonomy and the physiological mechanisms underlying prey capture and digestion. More recent research has focussed on addressing why the carnivorous trait has independently evolved multiple times. The observation that carnivorous plants are predominantly restricted to similar abiotic environments underpins the theory of the energetic cost/benefit model for the evolution of botanical carnivory (Givnish *et al.*, 1984). The model proposes that carnivory is only of net benefit to plants exposed to nutrient-limited, bright and waterlogged habitats, as the photosynthetic costs of investment in the trait are not exceeded by the energetic gain from prey N uptake in shady or dry habitats.

Further to addressing the question of why the adaptive trait of carnivory has evolved, recent research has focussed on identifying and understanding the selective pressures driving the evolution of trap specialisation. Evidence from molecular phylogenetic data of the Lentibulariaceae (order Lamiales) (Müller *et al.*, 2004) shows that trap specialisation evolved rapidly in this family, from a simple, adhesive trap ancestor, to the simple adhesive traps of *Pinguicula* spp., to the semi-specialised 'corkscrew' traps of *Genlisea* spp., to the highly specialised bladder-like traps of *Utricularia* spp. (Fig.

1). These results, along with ecological evidence of differences in prey capture patterns between species, formed the basis for a proposed explanation for the selective pressures driving the evolution of trap specialisation, named the predictable prey capture hypothesis (Müller *et al.*, 2004). This theory predicts that species with relatively simple trap structures (e.g. *Pinguicula* and *Drosera* spp.) have more predictable and frequent prey capture as a result of dietary generalism than species with relatively complex trap structures (e.g. *Utricularia* spp.).

Ecological studies testing the proposals of the predictable prey capture hypothesis are scant, and results show mixed support. For species with adhesive traps which are predicted to be dietary generalists, results are mixed; one study indicates generalism by *Drosera capillaris* (Jennings *et al.*, 2010), and another indicates dietary specialism by *Pinguicula longifolia* (Antor and García, 1994). Evidence from remaining studies utilising species with adhesive traps is largely limited by the experimental approaches employed. Limitations include a lack of consideration for:- parameters of the background invertebrate population of potential prey (e.g. van Achterberg, 1973; Thum, 1986), the prey retention ability of leaves (e.g. Karlsson *et al.*, 1987; Zamora, 1995; Alcalá and Domínguez, 2003) or the size distribution of captured prey in comparison with the size distribution of potential prey (Volkova *et al.*, 2010). Therefore, it is evident that further research is required to test the proposals of the predictable prey capture hypothesis in order to offer an insight into the selective pressures driving the evolution of trap specialisation in carnivorous plants.



Key:-

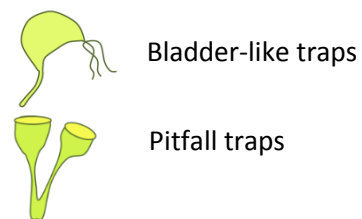
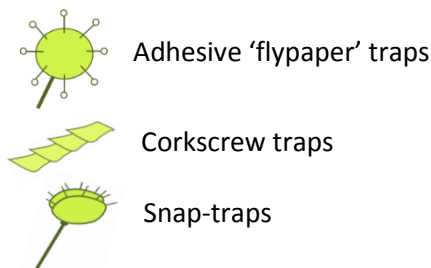


Figure 1 Phylogeny of the Caryophyllales and Lamiales; the angiosperm orders containing over 95% of carnivorous plant species. Illustrations show the prey capture mechanism of the carnivorous or protocarnivorous plant(s) in each family. Families that are exclusively carnivorous are highlighted in green; families that are mostly non-carnivorous are highlighted in yellow. Species that are protocarnivorous, or only carnivorous for part of the life cycle, are followed by (P). Adapted from Ellison and Gotelli (2009).

1.2 Carnivorous plant ecology

1.2.1 Energetics of botanical carnivory

Further to the observation that carnivorous plants are limited to nutrient-deficient, bright and waterlogged environments, Givnish *et al.* (1984) predicted three net benefits to the plant that are associated with carnivory:- “(i) an increase in a plant’s total rate of photosynthesis as a result of increased mineral absorption, through (a) an increased rate of photosynthesis per unit leaf mass, or (b) an increase in the total leaf mass that can be supported; (ii) excess nutrients obtained from trapped prey could be invested in reproduction, and (iii) carnivory may serve to replace autotrophy partly with heterotrophy as a source of chemical energy – i.e. carnivorous plants may be able to obtain carbon, in the form of sugars, from trapped prey, therefore bypassing the need for photosynthesis”.

Ecological studies testing Givnish’s proposed benefits of carnivory have concluded mixed support for the model. Investigations of the first energetic prediction presented mixed results; no significant increase in photosynthetic rate following prey assimilation has been observed in the genera of *Pinguicula* (butterworts), *Drosera* (sundews) and *Sarracenia* (North American pitcher plants) (Méndez and Karlsson, 1999; Wakefield *et al.*, 2005), a significant increase was observed in *Aldrovanda* (waterwheel plant) and a significant decrease in *Utricularia* (bladderworts) (Adamec, 2008). One possible explanation for the marginal increase or no change in photosynthetic rate following prey assimilation may be as some species, such as *Sarracenia purpurea*, store prey-derived nutrients in overwintering above-ground tissues for use during trap production at the beginning of the following growth season (Small, 1972; Butler and Ellison, 2007). However, if Givnish’s primary measure of photosynthesis is bypassed, evidence for increased plant growth following prey assimilation is observed in six genera (Darwin, 1875; Thorén *et al.*, 1996; Moran and Moran, 1998; Adamec, 2002, 2008; Farnsworth and Ellison, 2008). There is some support for Givnish’s second prediction - increased investment in vegetative or sexual reproduction has been observed in *Pinguicula* (Eckstein and Karlsson, 2001), *Sarracenia* (Ne’eman *et al.*, 2006) and *Drosera* (Thum, 1989; Stewart and Nilsen, 1992), and scant evidence to support the third prediction – heterotrophic uptake of carbon has only been observed in *Aldrovanda* and *Drosera* (Fabian-Galan and Salageanu, 1968; Dixon *et al.*, 1980).

Therefore it is evident that there is a need for further research testing the predictions of the energetic cost/benefit model in order to address why botanical carnivory has independently evolved multiple times.

1.2.2 Leaf economics

A major objective for plant ecologists is to understand how internal plant processes using carbon and nutrients vary between species, plant functional types and vegetation types, as this knowledge will enable the responses of vegetation to climate change and differing land uses to be accurately predicted (Wright *et al.*, 2004). A breakthrough towards meeting this objective was made by Wright *et al.* (2004); results show that a universal spectrum of leaf economics exists, ranging from a quick return in investment of nutrients and dry mass of leaves to a slow return in investment in nutrients and leaf dry mass. One of the scaling relationships identified is the positive correlation between photosynthetic capacity (A_{mass}) and tissue nutrient content. For carnivorous plants, recent research indicates that A_{mass} and photosynthetic nutrient use efficiencies for N and P (PNU E_N and PNU E_P) are significantly lower and therefore more conservative than those of non-carnivorous plants (Ellison, 2006; Farnsworth and Ellison, 2008), and A_{mass} does not increase following prey addition (Méndez and Karlsson, 1999; Wakefield *et al.*, 2005). Therefore the time taken by the plant to 'pay back' the carbon invested in carnivory via photosynthesis is relatively long even though traps are less costly to construct than non-carnivorous phyllodia (Karagatzides and Ellison, 2009). Therefore, this evidence indicates that carnivorous plants are positioned as outliers at the 'slow and tough' end of the leaf economics spectrum.

Subsequent research by Shipley *et al.* (2006) shows that an underlying set of leaf functional trait trade-offs are responsible for the universal spectrum of strongly correlated leaf traits between species and environments, one of these being a trade-off between long-term allocation to structural tissue versus short-term allocation to liquid phase processes, e.g. photosynthesis. Carnivorous plants are a good example of this trade-off:- as the limiting factor to growth is usually nutrients, proportionally large resource allocations are made to the structural tissues of traps at the expense of short-term energetic gain via photosynthesis, as traps are less photosynthetically efficient than non-carnivorous phyllodia (Ellison and Gotelli, 2002). Therefore, the dual functions of traps for N nutrition and photosynthesis makes carnivorous plants ideal organisms for investigating ecological stoichiometry and resource allocation trade-offs between functional traits.

1.2.3 Patterns and processes of prey capture by carnivorous plants

1.2.3.1 Investment in prey attraction

Carnivorous plants utilise various mechanisms to attract invertebrate prey to their traps, such as nectar by *Sarracenia purpurea* (Bennett and Ellison, 2009), scent (Jürgens *et al.*, 2009; Di Giusto *et al.*, 2010) and possibly UV patterning (Joel *et al.*, 1985). Leaf redness, an indicator of leaf anthocyanin content, has been proposed to serve a prey attractant role for some carnivorous plant species (Lloyd, 1942; Ichiishi *et al.*, 1999; Schaefer and Ruxton, 2008). The results of a recent *in-situ* study using *Drosera rotundifolia* (Foot *et al.*, 2014) tentatively indicate that leaf redness is not used for prey attraction by this species; plants with redder leaves had a higher probability of prey capture success than plants with greener leaves, but results also show that artificial red traps captured less prey than green traps, therefore potentially confounding factors (e.g. scent or UV patterning) may have been associated with the red colouration of leaves. Leaf anthocyanin production is linked with other functional roles and processes in non-carnivorous vascular plants, notably as a photoprotective role against UV damage of the chloroplasts by the absorption of blue-green light (Neill and Gould, 2003; Merzlyak *et al.*, 2008) and as a passive by-product of flavonoid synthesis (Karageorgou & Manetas, 2006; Archetti *et al.*, 2009; Gould *et al.*, 2010). However there is a deficit of research exploring the influence of light and root N availability on leaf anthocyanin content (and therefore leaf redness) using carnivorous plants. Therefore further research is needed to determine the functional role of leaf anthocyanin in carnivorous plants.

1.2.3.2 Investment in prey capture

A wide variety of mechanisms for trapping insect prey are observed across species of carnivorous plant, such as pitchers, adhesive traps, snap-traps and bladder-like traps. The evolution of trap complexity is linked with the degree of plant reliance on the trait of carnivory ($\%N_{dfp}$) which varies substantially between species (Schulze *et al.*, 1991). For example, *Darlingtonia californica*, which possesses relatively complex, ca. 1 m long pitchers is more reliant on carnivory ($\%N_{dfp} = 76.4\%$) than the relatively simple, adhesive traps of *Drosera erythrorhiza* ($\%N_{dfp} = 19.6\%$) (Schulze *et al.*, 1991, 1997). As discussed earlier, the evolution of trap complexity may also be linked with dietary strategy, as proposed by the predictable prey capture hypothesis (Müller *et al.*, 2004).

Carnivorous plants may also vary investment in prey capture in ecological time as a phenotypically plastic response to changes in resource availability, for example by varying trap number, trap size, trap dimensions, and physiological mechanisms for prey capture. For *Drosera rotundifolia*, an adhesive trap

species that utilises sticky mucilage to capture prey, investment in mucilage production, and therefore leaf stickiness, decreases as root N availability increases (Thorén *et al.*, 2003). For *Sarracenia purpurea*, a plant possessing pitchers for prey capture and more photosynthetically efficient phyllodia, the proportional allocation of resources to pitchers compared with phyllodia decreases as root N availability increases (Ellison and Gotelli, 2002). This phenotypically plastic ecophysiological response enables the plant to avoid the costs associated with carnivory (i.e. the long pay back times) when nutrients are available at a lower cost to the plant, therefore providing support for Givnish's energetic cost/benefit model.

This observed plasticity in resource allocation to carnivory also provides support for the carbon-nutrient balance (CNB) hypothesis (Bryant *et al.*, 1983), which proposes that the carbon-nutrient balance of plants is controlled by resource availability, and that plants prioritise investment in the production of secondary carbon compounds under nutrient-limited and well-lit conditions. The hypothesis is based on three key assumptions:- (i) plants prioritise growth over the production of secondary carbon compounds (Tuomi *et al.*, 1991); (ii) nutrients constrain growth more than photosynthesis (Bryant *et al.*, 1983), and (iii) light limitations constrain photosynthesis more than growth (Bryant *et al.*, 1988). The hypothesis was originally supported by experimental evidence of plant-herbivore interactions, whereby plant investment in defensive secondary compounds for anti-palatability against herbivory was larger under nutrient-deficient and bright environments than for plants exposed to nutrient-rich and shaded environments, regardless of the degree of grazing pressure to the plant. Application of the hypothesis to carnivorous plants therefore proposes that investment in carbon-rich mucilage production decreases as root N availability or degree of shading increases. The CNB hypothesis and the energetic cost/benefit model also supported by observed ecophysiological responses of *D. rotundifolia* to changes in light availability:- leaves of shaded plants were less sticky than the leaves of plants exposed to full sun (Zamora *et al.*, 1998; Thorén *et al.*, 2003). The plasticity of investment in prey capture by carnivorous plants therefore illustrates resource allocation trade-offs by the plant in response to changes in the environment .

1.2.3.3 Diet and dietary strategy

Carnivorous plants predominantly capture a wide variety of flying and flightless insect prey, with the two most frequently captured taxa reported to be ants (Formicidae spp.) and flies (Diptera spp.) (Ellison and Gotelli, 2009). The quantity (i.e. total prey mass) and quality (i.e. species type or size class) of prey may vary between species, within species and throughout the life span of a plant (Moran *et al.*, 1999; Ellison and Gotelli, 2009). Factors influencing between-species differences include variation in the mechanism, shape, size and prey capture efficiency of traps, growth form and in the composition

of background invertebrate spectra (Kato *et al.*, 1993; Verbeek and Boasson, 1993). Factors influencing prey spectra between plants and during a plant's life span include plasticity in trap age and morphology (e.g. trap number, size, shape and orientation), abiotic environment (e.g. degree of shading), biotic factors (e.g. inter-specific competition for prey), the degree of investment in prey capture by the plant and temporal and spatial changes in the composition of background invertebrate spectra (Gibson, 1991; Antor and García, 1994; Alcalá and Domínguez, 2003; Foot *et al.*, 2014).

However, a lack of consensus continues regarding whether variation in dietary strategy exists between carnivorous plant species. Most carnivorous plant species trap a wide diversity of invertebrate orders; only a few species capture predominantly one taxon, e.g. *Nepenthes alata* for which Formicidae constitute 98% of the plants' diet (Kato *et al.*, 1993; Ellison and Gotelli, 2009). However the experimental designs of many studies to date limit the strength of recommendations made regarding dietary strategy. Limitations include a lack of consideration for:- parameters of the background invertebrate population of potential prey (e.g. van Achterberg, 1973; Thum, 1986), the prey retention ability of leaves (e.g. Karlsson *et al.*, 1987; Zamora, 1995; Alcalá and Domínguez, 2003) or the size distribution of captured prey in comparison with the size distribution of potential prey (Volkova *et al.*, 2010). Therefore determining the dietary strategies of carnivorous plant species utilising different prey capture mechanisms is essential for further understanding selective pressures driving the evolution of patterns and processes of prey capture in carnivorous plants.

1.3 N nutrition of carnivorous plants

1.3.1 Resource allocation trade-offs and ecophysiological responses to N availability

As the pay back times associated with the costs of carnivory are relatively long, carnivorous plants preferentially allocate resources away from investment in botanical carnivory when N is available to the roots; the costs associated with root N uptake are substantially lower than those associated with N uptake from prey. Phenotypic plasticity in the reliance of carnivorous plants on carnivory is also influenced by root N availability; results of an *in-situ* study of *Drosera rotundifolia* by Millett *et al.* (2012) show that plant reliance on carnivory (measured as the proportion of prey-derived N of the total plant N budget) decreases as N deposition increases. This result shows that at high N deposition levels and therefore high root N availabilities, the marginal benefits of carnivory were lower but the costs associated with carnivory remained the same, thus providing further support for Givnish's model that carnivory is only of net benefit to the plant in nutrient-limited environments.

1.3.2 Benefits of prey capture

In addition to increased growth and reproductive investment as mentioned earlier, the benefit of prey capture to carnivorous plants is evident from the positive correlation between plant reliance on carnivory and the tissue C:N ratio of *Drosera rotundifolia* (Millett *et al.*, 2012). Earlier studies show that prey capture or addition can benefit the plant by stimulating root N uptake; results of a study by Aldenius *et al.* (1983) show that the manipulated feeding of prey to *Pinguicula vulgaris* led to *ca.* 1.6 x higher uptake of N than could have been obtained from prey N alone. This result provides further evidence of the plasticity of resource allocation in response to resource availability.

1.3.3 Competitive interactions

The growth, survival and reproduction of carnivorous plants *in-situ* are influenced by a wide variety of biotic interactions, including herbivory, parasitic/fungal attack, robbery of prey and competition with other plants and animals for resources (Adamec, 1997; Jennings *et al.*, 2010). Carnivorous plants are often assumed to be relatively poor competitors (e.g. Brewer, 2003; Wolf *et al.*, 2006) due to characteristics such as short, weakly developed root systems (Ellison and Adamec, 2011) and low maximum photosynthetic rates and relative growth rates (RGRs) in comparison with non-carnivorous vascular plants (Karagatzides and Ellison, 2009; Ellison and Adamec, 2011). Indeed, if the Competitive Index utilised by the ruderal-competitor-stress (RCS) model (Grime 1973), which ranks plant species according to their investment in the competitive features of maximum plant height, morphology, maximum potential growth rate and maximum litter production, is applied to carnivorous plant species, they would mostly generate relatively low competitive scores.

Some evidence exists to support that sympatric species of carnivorous plant that potentially compete for the same prey resources have evolved specialised different trap morphologies and/or mechanisms for prey attraction in order to avoid competition. For example, of the co-occurring Bornean pitcher plants *Nepenthes rafflesiana* and *N. gracilis*, *N. rafflesiana* possesses large, scented, and colour-contrasted pitchers and therefore attracts pollinators, whereas the smaller, unscented pitchers of *N. gracilis* attract flightless invertebrates (Moran *et al.*, 1999). However, competition avoidance by trap specialisation of three sympatric *Drosera* species in European Russia was not evident (Volkova *et al.*, 2010); species captured the same proportions of identical prey orders and therefore presumably experienced dietary overlap, however specialisation may have been evident at lower prey taxon resolutions than were measured during the study. Thus, further research is required to explore the evolution of trap and prey specialisation for sympatric carnivorous plant species.

A lack of consensus also continues regarding whether carnivorous plants interact competitively with other plants or whether they respond ecophysiologicaly to avoid competition in ecological time. Some evidence of competition for resources exists; results of an *in-situ* study by Gibson (1991) found that density-dependent intra-specific competition for insects reduced plant size in populations of *Drosera filiformis*. Evidence also exists for competition between carnivorous plants and predatory animals; *Drosera rotundifolia* plants that co-exist with predatory wolf spiders (Lycosidae) displayed reduced fitness from smaller %N_{dfp} values as a result of dietary overlap (Jennings *et al.*, 2010). Conversely, some carnivorous plants may possess mechanisms to avoid competition with other plants; results of a study exploring potential interactions between prey-denied *Sarracenia purpurea* plants and surrounding non-carnivorous plants show no evidence of below-ground competition for nutrients, despite the increased likelihood of nutrient niche overlap from prey exclusion (Karlsson *et al.*, 1991; Brewer, 2003). In response to shading by co-occurring plants, proportional investment in pitchers compared with phyllodia was reduced, thereby reducing their demand for nutrients. Therefore, these results suggest that *S. purpurea* possesses a ruderal strategy (Grime, 1979), whereby survival is dependent on frequent episodes of disturbance (as occur frequently by fire) in order to reduce the detrimental effects of continued shading by co-occurring plant species. However further research is required to assess the CSR strategy of carnivorous plant species in ecological time.

1.4 Using stable isotopes in carnivorous plant ecology

1.4.1 Introduction to the single isotope, linear mixing model

Stable isotope natural abundance analysis, the measurement of the ratio of naturally occurring isotopic forms of bioactive elements, such as $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$ (expressed as $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$), is a powerful and cost-effective technique that has provided new insight into a wide variety of ecological research areas. Examples of application of the technique include N and C cycling in ecosystem-scale research (Högberg, 1997), the trophic ecology of organisms in food webs and the feeding ecology of consumers (Kelly, 2000). In plant ecophysiological research, examples include determination of organic N forms to the N budget of plants with mycorrhizal symbioses (Hobbie and Högberg, 2012), of host plant-derived N to the N budget of epiphytes (Wania *et al.*, 2002) and of prey-derived N to the total N budget (%N_{dfp}) of carnivorous plants (Brearley, 2011). Most plant ecophysiological studies determine the proportional contribution of two sources to a mixture by use of a simple, two end-point, single isotope linear mixing model, as presented by Shearer and Kohl (1988) for estimating the contribution of atmospherically-fixed N to N₂-fixing plants. This model uses the $\delta^{15}\text{N}$ of two sources and of the mixture to determine the proportional contribution of each source to the mixture. For application of

the model to the N nutrition of carnivorous plants, the mixture is the $\delta^{15}\text{N}$ of the carnivorous plant and the sources are the $\delta^{15}\text{N}$ of the plant that has obtained 100% of N from prey (prey N end-point) and the $\delta^{15}\text{N}$ of the plant that has obtained 100% of N via the roots (root N end-point) (Schulze *et al.*, 1991).

1.4.2 Assumptions and limitations of the linear mixing model

In order to calculate $\%N_{\text{dfp}}$ of most carnivorous plant species, it is necessary to use the $\delta^{15}\text{N}$ of invertebrates and the $\delta^{15}\text{N}$ of reference non-carnivorous plants as proxies for the prey and root N end-points respectively (Fig. 2), as naturally occurring genetic variants of carnivorous plants that can only obtain N from prey or via the roots are extremely rare. Previous studies have used the mean $\delta^{15}\text{N}$ of pooled background invertebrate samples considered to be potential prey as a proxy for the prey N end-point, but no consideration was given to the actual diet of the plant. The diet of carnivorous plants varies between species, e.g. due to differences in dietary strategy and prey capture mechanism (Ellison and Gotelli, 2009), and within species, e.g. due to spatial and temporal variation in the taxa distribution and abundance of potential prey (Alcalá and Domínguez, 2003) or as a result of phenotypic plasticity in plant investment in prey capture (Antor and García, 1994). Furthermore, invertebrate $\delta^{15}\text{N}$ varies between and within taxa due to factors such as trophic level, diet and life cycle stage (Vanderklift and Ponsard, 2003). Therefore there is a need for the following research using a carnivorous plant:- (i) presentation of a method for calculating an accurate value for $\delta^{15}\text{N}_{\text{prey}}$ through incorporation of the diet of the plant; (ii) incorporation of the variability within and between orders of captured prey into the calculation of variability of $\delta^{15}\text{N}_{\text{prey}}$.

Most applications of the linear mixing model require the use of isotopic signature proxies to represent the isotopic signatures of one or both of the sources of the mixture; in the presentation of the model, Shearer and Kohl (1988) used the $\delta^{15}\text{N}$ of co-occurring non- N_2 -fixing reference plants as a proxy for the N_2 fixing plant (mixture) that has obtained 100% of N from the soil N pool. As it is difficult to quantify how representative the $\delta^{15}\text{N}$ of proxies are of the true $\delta^{15}\text{N}$ values for source end-points, values of source contributions to a mixture are considered semi-quantitative (Shearer and Kohl, 1988). It is important therefore to use proxies that are as closely representative of the end-points as possible in order to minimise potential error in the calculation of source proportions of a mixture; this was achieved by Shearer and Kohl (1988) through the use of the mean leaf $\delta^{15}\text{N}$ of a large number of co-occurring non- N_2 -fixing plant species in order to minimise the influence of varying rooting depth on $\delta^{15}\text{N}$. Previous studies estimating $\%N_{\text{dfp}}$ of *Drosera rotundifolia* (round-leaved sundew) have used co-occurring *Sphagnum* spp. (Millett *et al.*, 2003, 2012), or a selection of co-occurring non-carnivorous vascular plant species (mainly Gramineae) (Schulze *et al.*, 1991) as proxies for the root N end-point,

and samples of background invertebrates considered to be potential prey (Schulze *et al.*, 1991; Millett *et al.*, 2003, 2012) as proxies for the prey N end-point. It may be suggested that plant proxies for the root N end-point would be more representative if factors influencing $\delta^{15}\text{N}$ are considered, such as maximum rooting depth (Kohzu *et al.*, 2003) and the degree of root discrimination between ^{15}N and ^{14}N (Shearer and Kohl, 1988), and that invertebrate proxies for the prey N end-point would be more representative if factors such as the proportional abundance of prey orders to the diet of the carnivorous plant, and invertebrate tissue %N are considered. Therefore, there is need for comparison of the potential suitability of a range of proxies for the prey N and root N end-points of *Drosera rotundifolia*.

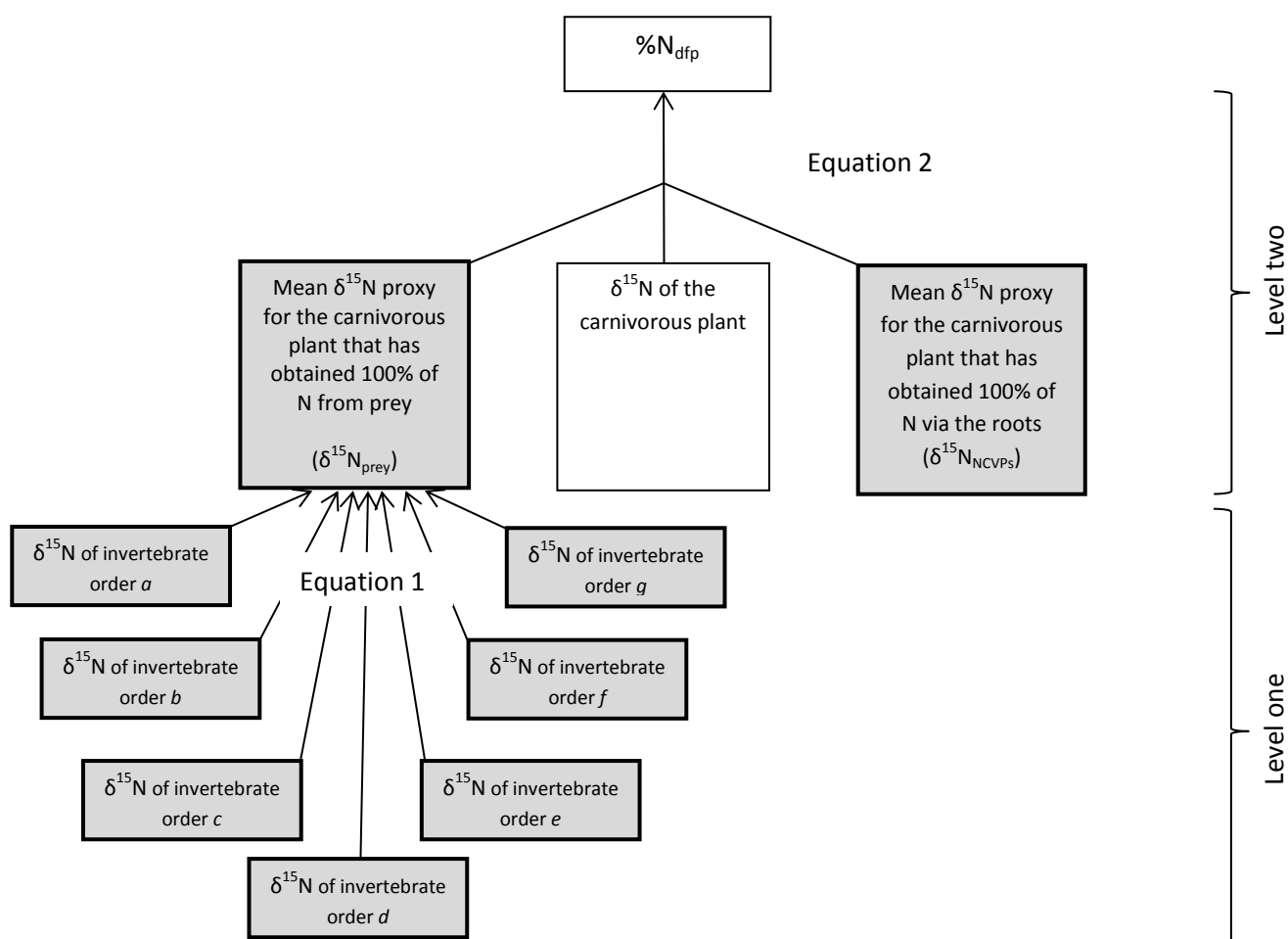


Figure 2 Schematic diagram of the multi-level, hierarchical structure of end-members contributing to the precision of the estimation of the percentage contribution of prey-derived N ($\%N_{dfp}$) to the N budget of a carnivorous plant. End-member variables are indicated by grey-filled, bold-outlined boxes. Level one contributors are the taxonomic orders of invertebrate prey captured by the carnivorous plant which contribute towards the variability in $\delta^{15}\text{N}_{prev}$.

1.4.3 Uncertainty in the calculation of source proportions to a mixture using a multi-level linear mixing model

There are several sources of uncertainty associated with use of the linear mixing model that contribute towards variability in $\%N_{dfp}$, such as uncertainty incurred due to variability in $\delta^{15}N$ of the sources used in the model. This uncertainty source has recently been remedied for single-level models by the presentation of methods for calculating the contribution of source variances to variability of a mixture, e.g. Isoerror (Phillips and Gregg, 2001), Moore Penrose (Hall-Aspland *et al.*, 2005) and Bayesian methods (Moore and Semmens, 2008; Parnell *et al.*, 2010). Following sensitivity analysis using Isoerror exploring the influence of source parameters of the difference in isotopic signatures between sources, population SD and source proportions on variability in source proportion estimates to a mixture, Phillips and Gregg (2001) showed that source difference exerts the most powerful influence on the SE of the source proportion estimate; specifically, doubling the isotopic difference between sources reduces the variability of source proportion estimates by half. Furthermore, reducing the sample size of all source parameters resulted in at least halving the variability in source proportion estimates.

However, no studies to date have explored the influence of similar parameters of level one contributors on variability in source proportion estimates using multi-level models, such as in the case of calculating $\%N_{dfp}$ of carnivorous plants that are dietary generalists, where many orders of prey (level one contributors) comprise the prey N end-point (Fig. 2). Studies to date exploring the N nutrition of carnivorous plants *in-situ* have not considered the influence of variability in invertebrate prey taxa $\delta^{15}N$, the diet of the carnivorous plant (i.e. proportional contribution of each taxa to $\delta^{15}N_{prey}$) or $SD(\delta^{15}N)$ within and between taxa on variability in $SE(\%N_{dfp})$ (Schulze *et al.*, 1991, 1997; Moran *et al.*, 2001; Millett *et al.*, 2003, 2012), therefore $SE(\%N_{dfp})$ is likely to be underestimated (Phillips and Gregg, 2001). Therefore there is a need to determine the influence of level one contributors to variability in source proportion estimates in multi-level mixing models in order to address this source of uncertainty in source proportion estimates.

1.5 Ombrotrophic bogs and their plant communities

1.5.1 Introduction to ombrotrophic bogs

Peat bogs may be defined as 'ombrotrophic peatlands with the surface above the surrounding terrain or otherwise isolated from the laterally moving mineral-rich soil waters' (Rydin and Jeglum, 2006). Bogs are completely reliant on wet and dry atmospheric deposition for nutrient supply ('ombrotrophic' = rain-fed) due to their physical isolation from groundwater, and are therefore extremely nutrient-deficient environments. Bogs occupy the most acidic and nutrient-deficient end of the peatland spectrum due to the acidifying nature of *Sphagnum* moss and the poor buffering capacity of rainwater; surface water pH is typically 4 or lower (Rydin and Jeglum, 2006).

Bogs are usually limited to cold, temperate climates, predominantly occupying a boreal geographic distribution (Aselmann and Crutzen, 1989). Blanket and raised bogs are prevalent throughout the UK, notably Scotland, Wales, Ireland, and north-eastern counties of England (JNCC, 1999; McLeod *et al.*, 2005_a). Bogs are characterised by topographic heterogeneity at several geographical scales (Ivanov, 1981). At the macroscale level, landforms are categorised as raised bogs (e.g. domed and plateau bogs), non-raised bogs (e.g. flat, basin and riparian bogs), and blanket bogs (Rydin and Jeglum, 2006). The landform type and extent of bog habitat is governed by regional climate and topography of the land, with the development of ombrotrophic bog from minerotrophic peatland considered to be mostly initiated by climatic shifts (Barber, 1981). At the microtopographic level, vegetation typically forms a repeat pattern of hummocks and hollows interspersed with lawns, bare peat, and open water (Tansley, 1939; Sjörs, 1948). The microtopography of a bog surface is distinct from those of other habitat types as it is an inherent product of inhabitant vegetation (Tansley, 1939; Pearsall, 1956) and therefore is influenced by differences in the life history, morphology and environmental requirements of the inhabitant bryophytes, vascular plants and lichens (Nordbakken, 2000; Rietkerk *et al.*, 2004).

Bogs, as well as other peatland types, have provided an important source of food, water and fuel for many millennia, and are more recently acknowledged for their important hydrological roles (e.g. flood alleviation) which has led to their referral as 'the kidneys of the landscape' (Mitsch and Gosselink, 1993; Acreman *et al.*, 2003). Their significance is recognised on a global scale due to their ability to store atmospheric carbon (C); storage is estimated as the equivalent of one third of the total soil C pool and approximately two thirds of all C in the atmosphere (Post *et al.*, 1982; Houghton *et al.*, 1990; Turunen *et al.*, 2002). Peatlands can act as a sinks of atmospheric carbon dioxide (CO₂) through the accumulation of decomposed organic plant matter, and also emit the greenhouse gases CO₂ and methane (CH₄) as by-products of decomposition processes (Clymo *et al.*, 1988; Bartlett and Harriss, 1993). During the Holocene to date, the net balance of C by global peatlands has been of accumulation

and therefore a 'cooling' radiative forcing effect has been emitted of *ca.* -0.2 to -0.5 W M⁻² (Frolking and Roulet, 2007). Dise (2009) emphasises the significance of peatlands in this respect:- 'metre by metre, peatlands store more C than any other terrestrial ecosystem, despite only covering about 3% of the Earth's land area'. Therefore, peatlands are directly and indirectly valuable ecosystems.

Bogs support a notable diversity of highly adapted species, in particular bryophytes, lichens and liverworts, however the extreme environment maintains decreased species richness in comparison to poor and rich fens (Sjörs, 1948). *Sphagnum* is by far the most characteristic genus of organisms inhabiting bog ecosystems (Van Breemen, 1995). Vascular plants are present in relatively low densities; characteristic species in the UK include ericoid sub-shrubs, notably common heather *Calluna vulgaris* and cross-leaved heath *Erica tetralix*, and a limited range of graminoid monocotyledons, notably hare's-tail cottongrass *Eriophorum vaginatum*, deergrass *Scirpus cespitosus* and purple moor grass *Molinia caerulea* (Rodwell, 1991). Many rare and localised invertebrates inhabit bogs; in the UK, lowland raised bogs support BAP Priority Species of large heath butterfly *Coenonympha tullia*, bog hoverfly *Eristalis cryptarum* and mire pill beetle *Curimopsis nigrita* and the IUCN RDB species rosy marsh moth *Eugraphe subrosea* (Fowles *et al.*, 2004; JNCC, 2014). Many animal species at higher trophic levels tend to be opportunistic or seasonal visitors that also utilise more hospitable and food-rich habitat types, e.g. the European otter *Lutra lutra* (McLeod *et al.*, 2005_b) and the hen harrier *Circus cyaneus* (JNCC, 2014).

1.5.2 Plant adaptations

1.5.2.1 *Sphagnum* genus; the 'ecosystem engineers'

The bryophyte genus of *Sphagnum* (Plate 1) is responsible for peat formation, forms the bulk of living and dead biomass in bogs and is extremely diverse; at least 34 species are found in peatland throughout the UK alone (Lamers *et al.*, 2000; Charman, 2002; Atherton *et al.*, 2010). The waterlogged environment of the acrotelm is created by *Sphagnum* in two ways:- capillary spaces and apical elongation of the capitula raise the water table to the acrotelm (Hayward & Clymo, 1982) and atmospheric water is directly absorbed by *Sphagnum* via the hyaline cells (van Breemen, 1995). The degree of saturation of the surface microtopography depends largely on the morphological variability between *Sphagnum* species; hummock-forming species maintain a higher water table than hollow-forming species (Hayward and Clymo, 1982; Rydin, 1985). *Sphagnum* also acidifies the surrounding environment through the high cation exchange capacity of one of its constituents, galacturonic acid (Clymo & Hayward, 1982). These morphological and physiological characteristics therefore maintain

the acidic, wet, and anoxic environmental conditions of the acrotelm (Clymo & Hayward, 1982; Malmer *et al.*, 1994; van Breemen, 1995).



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Plate 1 Hummock-forming *Sphagnum* species at Cors Fochno estuarine raised bog, Ceredigion, UK.

In addition to the afore-mentioned characteristics, the competitive advantage of *Sphagnum* in comparison to vascular plants is further increased by their ability to act as small-scale autogenic 'ecosystem engineers' (Jones *et al.*, 1994; Svensson, 1995). *Sphagnum* capitula are located above the rhizosphere of co-existing vascular plants, and along with their ability to directly absorb and store atmosphere-derived nutrients, these characteristics enable the quantity of nutrients made available to the roots of plants to be controlled (Svensson, 1995). As pristine bog vegetation is primarily limited by nutrients (usually nitrogen), the ability of *Sphagnum* to control nutrient availability to cohabitant vascular plants enables avoidance of the inevitable shading that would result from the higher growth rates of plants should the primary limiting factor be lifted (Limpens *et al.*, 2003). The ability of several *Sphagnum* species to acquire additional N from N₂-fixation with symbiotic bacteria provides another example of an adaptation for N acquisition by these mosses (Chapman and Hemond, 1982).

1.5.2.2 Vascular plants

Vascular plant species possess a variety of morphological and physiological adaptations to the nutrient-deficient, anoxic, waterlogged environment (Table 1). Adaptations for nutrient acquisition include mycorrhizal associations and carnivory; strategies that compensate for relatively low root N availability by providing supplementary N obtained via mycorrhiza or from captured insect prey respectively. Adaptations of high internal nutrient recycling efficiencies and evergreen leaves permit high levels of nutrient retention, thus lowering plant reliance on root N uptake.

Table 1 Contrasting physiological and morphological adaptations to the ombrotrophic bog environment by four co-occurring vascular plant species that typically inhabit bogs in the UK. Adapted from Rydin and Jeglum (2006).

Adaptation	Vascular plant species			
	<i>Calluna vulgaris</i> ^a	<i>Drosera rotundifolia</i> ^a	<i>Erica tetralix</i> ^{b,c,d}	<i>Eriophorum vaginatum</i> ^{a,d}
Aerenchyma	-	-	-	x
Below-ground storage	x	-	-	x
Deep-rooted	-	-	-	x
Efficient nutrient translocation	-	-	-	x
Evergreen	x	-	x	(x)
Insect capture	-	x	-	-
Long-lived	x	-	x	x
Mycorrhiza	x	-	x	-
Regular seedling establishment	-	x	-	(x)
Tussock-forming	-	-	-	x
Woody	x	-	x	-

^a Rydin and Jeglum, 2006.

^b Aerts, 1990.

^c Grime *et al.*, 2007.

^d Miller *et al.*, 1983.

Adaptations to the waterlogged environment include aerenchyma, hollow intercellular spaces which facilitate the transport of oxygen to lower parts of the plant, therefore enabling roots to thrive in deep, anoxic zones and aiding the plant to float, e.g. *Eriophorum* spp. (Smirnoff and Crawford, 1983). The development of tussocks by many sedge and rush species (e.g. *Carex cespitosa*) enables survival in highly variable water depths, due to the raising of leaves above the water table (Rydin and Jeglum, 2006). The woody structure of *Calluna vulgaris* enables the plant to live above the water table in *Sphagnum* hummocks and provide sufficient oxygen for the symbiotic mycorrhizae, therefore compensating for the lack of aerenchyma (Rydin and Jeglum, 2006).

1.5.2.3 Study species: the carnivorous plant *Drosera rotundifolia*

The round-leaved sundew, *Drosera rotundifolia* (Plate 2(a)) is a rosette-forming, carnivorous perennial herb that occupies a circumboreal distribution. It is typically found growing in *Sphagnum* hummocks (Crowder *et al.*, 1990). The adaptations of carnivory and over-winter storage of nutrients in the resting bud for re-utilisation in the spring assist with the plants' survival and reproduction in nutrient-deficient environments (Crowder *et al.*, 1990). The species uses an adhesive trap mechanism to capture invertebrate prey; leaves are covered in trichomes that each secrete a droplet of sticky mucilage (Darwin, 1875; Plate 2(b)). An alighting insect is adhered to the leaf by the mucilage and the leaf curls around the prey. Insect movement triggers the production of digestive enzymes which are secreted by the trichomes and the insect is digested. About 7 days after capture, the leaf uncurls and the undigested chitinous exoskeleton of the prey is released. The adaptations of a shallow root system with adventitious roots enable the plant to keep pace with the upward growth of surrounding *Sphagnum* species (Crowder *et al.*, 1990; Wolf *et al.*, 2006). The species is relatively short-lived compared with most co-occurring vascular plant species; typically only living for about 5 years (Crowder *et al.*, 1990).

(a)



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(b)



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Plate 2 *Drosera rotundifolia* (round-leaved sundew). Shown are: the rosette spiral arrangement of the leaves (Fig. 2(a)); the leaf structure with protruding trichomes (Fig. 2(b)). Each trichome produces a droplet of sticky mucilage (red dots) used to adhere insects.

1.5.3 Pressures and threats to bog ecosystems

1.5.3.1 Habitat destruction

The net carbon balance and intrinsic biodiversity of bog ecosystems are influenced by a wide range of direct and indirect anthropogenic activities and processes, notably climate change, pollution and habitat destruction. The largest current threat to bogs, and indeed all peatland types, is habitat destruction; predominant uses include drainage and conversion to agricultural land or forestry, or peat cutting for fuel or horticultural use (Dise, 2009). At a global scale, destruction has resulted in the loss of about 25% of peatlands (Parish *et al.*, 2008); in the UK, only 1% of England's peatland is classified as undamaged and therefore actively sequestering carbon (Natural England, 2010).

1.5.3.2 Climate change

Aside from direct habitat destruction, longer term threats to bog functioning include climate change and pollution. Bogs are particularly vulnerable to the effects of climate change due to their predominantly circumboreal geographical distribution; increased temperatures are predicted, and have already been observed, to be greatest in high latitude regions (ACIA, 2004; Chapin *et al.*, 2005). Future climate change projections predict that upon the doubling of current atmospheric CO₂ levels, the global equilibrium mean surface area temperature is likely to rise within the range of 2.0 – 4.5 °C, most likely by 3 °C (Meehl *et al.*, 2007). A lack of consensus continues regarding the likely effects of increased global temperature projections on the net C balance of peatlands (Davidson & Janssens, 2006; Dise, 2009). Some research suggests that increased temperatures may accelerate decomposition rates of below-ground C, thereby contributing to global warming via a net output of atmospheric C in the forms of CO₂ and CH₄ (Jenkinson *et al.*, 1991; Oechel *et al.*, 2000). Other research suggests that increased temperatures may accelerate the rate of C sequestration in peatlands via increases in the rate of net primary productivity (NPP) of vegetation, causing a net reduction effect to global warming (Robinson & Moore, 2000; Turetsky *et al.*, 2000; Camill *et al.*, 2001). The lack of consensus is partly explained by the complexities associated with predicting peatland responses to warming due to multiple interacting biological processes which are influenced by many factors in addition to temperature (Davidson and Janssens, 2006; Dise, 2009). Therefore it is evident that further research is required to formulate accurate predictions of the response of the net C balance of peatlands to warming.

1.5.3.3 Pollution

Bogs are also threatened by pollution, particularly in the form of reactive nitrogen (N). *Sphagnum* species are only capable of absorbing and storing atmospheric-derived N up to a proposed critical N deposition threshold of *ca.* $18 \text{ kg N ha}^{-1} \text{ yr}^{-1}$, beyond which *Sphagnum* becomes N-saturated and loses its filtering ability, leading to increases in the availability of root N to co-occurring vascular plants (Bobbink *et al.*, 1998; Lamers *et al.*, 2000; Berendse *et al.*, 2001). Increases in root N availability may alter competitive interactions between plant species, favouring the growth of highly competitive, fast-growing plant species (Aerts and Berendse, 1988). These changes in competitive interactions may ultimately lead to vegetative succession toward terrestrial habitat types, which possess a much smaller C storage ability (Dise, 2009), and result in a net reduction in biodiversity due to the local extinction of bog specialist species (Berendse *et al.*, 2001).

1.5.4 Study sites

1.5.4.1 Cors Fochno

Cors Fochno (Plate 3) is an estuarine raised bog that lies on the Afon Dyfi estuarine floodplain in Ceredigion, Mid Wales (latitude 52 30 09 N, longitude 04 00 57 W) (JNCC, 2010_a). It receives relatively low background N deposition inputs of *ca.* 7.98 kg N ha⁻¹ yr⁻¹ (APIS, 2014_a). It is one of the largest remnants (*ca.* 653 ha) of actively growing, primary raised bog in the UK and is characterised by classic oceanic hummock-hollow microtopography (CCW, 2008_a; Plate 3). Typical raised bog *Sphagnum* species are present as well as the rare *S. pulchrum* and *S. austinii*. Vascular plants present include all three British sundews (*Drosera* spp.), bog myrtle *Myrica gale*, bog rosemary *Andromeda polifolia* and cottongrass *Eriophorum* spp. (CCW, 2008_a). In addition to rare flora, the bog supports a wide diversity of nationally scarce species assemblages, including invertebrates (e.g. bog bush-cricket *Metrioptera bracyptera*), reptiles (e.g. grass snake *Natrix natrix*), mammals (e.g. otter *Lutra lutra*) and birds (e.g. merlin *Falco columbarius*) and therefore holds numerous conservation designations (Dyfi NNR, Cors Fochno SAC, Dyfi SSSI, RAMSAR site, Dyfi Biosphere Area) (CCW_a, 2008).



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Plate 3 Classic oceanic hummock-hollow microtopography of Cors Fochno estuarine raised bog, Ceredigion, UK. The ericoid shrub bog myrtle *Myrica gale* (dark brown) is prominent.

1.5.4.2 Whixall Moss

Whixall Moss (Plate 4) is a lowland raised bog constituting part of the Fen's, Whixall and Bettisfield Mosses NNR (ca. 949 ha) and is situated on the Shropshire/Wrexham border, UK (latitude 52 55 21 N, longitude 02 45 44 W). It receives relatively high background N deposition inputs of ca. 22.54 kg N ha⁻¹ yr⁻¹ (APIS, 2014_b; JNCC 2010_b). A large proportion of the site has been damaged by peat extraction, however areas of uncut bog remain and substantial restoration efforts are being made to regenerate cut areas. Notable *Sphagnum* species include *S. papillosum*, *S. magellanicum* and *S. pulchrum*. Vascular plants supported include all three British sundews (*Drosera* spp.), bog asphodel *Narthecium ossifragum*, white beak-sedge *Rhynchospora alba*, bog rosemary *Andromeda polifolia* and cranberry *Vaccinium oxycoccos* (CCW, 2008_b). The bog supports over 1700 invertebrate species including 29 Red Data Book species (eg. Linyphiid spider *Carorita limnaea*) and many nationally scarce birds (e.g. meadow pipit *Anthus pratensis*) and mammals (e.g. water vole *Arvicola amphibius*) (Boardman, 2005; JNCC, 2010_b). The site therefore holds numerous conservation designations (Fenn's, Whixall, Bettisfield, Wem and Cadney Mosses SSSI/SAC/NNR, Midland Mires and Mosses Ramsar phase 2 site) (CCW, 2008_b).



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Plate 4 Whixall Moss lowland raised bog, Shropshire, UK. Common heather *Calluna vulgaris* (purple flowers) and cottongrass *Eriophorum* spp. (white seed heads) are present.

1.6 Thesis overview and structure

In this research, the carnivorous plant *Drosera rotundifolia* (round-leaved sundew) is used to address several unanswered ecophysiological and evolutionary questions relating to the influence of nitrogen (N) deposition on prey capture, diet and nitrogen (N) nutrition of carnivorous plants. Nitrogen deposition is one of the largest drivers of global environmental change (Sala *et al.*, 2000). Peatland ecosystems, such as ombrotrophic bogs, are typically N-limited and are therefore extremely vulnerable to sustained, elevated N deposition inputs (Limpens *et al.*, 2003). Peatlands are valuable ecosystems as they support high biodiversity, filter water, and can alleviate the negative effects of climate change by absorbing flood water and storing vast quantities of carbon (Dise, 2009). The ecophysiological responses of plant and *Sphagnum* species, and changes in the strength and nature of biotic interactions between plants and between plants and other organisms, to changes in root N availability underpin detrimental changes to the overall species diversity, functioning and carbon sequestration ability of peatland ecosystems in response to N deposition (Aerts *et al.*, 1999; Tomassen *et al.*, 2003; Bragazza *et al.*, 2005). With global deposition inputs of reactive nitrogen projected to increase from 100 Tg N yr⁻¹ in 1995 to 200 Tg N yr⁻¹ by 2050 (Galloway *et al.*, 2008), there is an urgent need to understand how highly specialised species inhabiting peatland ecosystems, such as carnivorous plants, have evolved to thrive in extreme environments and how the physiological responses of plants, and interactions between plants and other organisms, respond to changes in N availability. This research will therefore contribute towards this research base upon which (i) accurate predictions can be made of how peatland ecosystems will respond to sustained and elevated N deposition inputs and (ii) evidence-based land management policies can be developed and implemented to minimise losses to the biodiversity, functioning and carbon sequestration ability of peatland ecosystems.

Carnivorous plants are predominantly restricted to nutrient-deficient environments such as peatlands, where the trait of carnivory provides a useful supplemental source of N to the scarce root-derived N and aids survival in these extreme, adverse environments. Indeed, the trait of carnivory is widely considered have independently co-evolved as a response to nutrient-deficient, sunny but moist environments (Givnish *et al.*, 1984). These plants may therefore be considered model systems for further understanding of the influence of N deposition on plant ecophysiological responses and of the evolution of functional traits and nutrient-acquisition strategies that enable plants to thrive in extreme environments. Furthermore, the interactions between carnivorous plants and co-occurring invertebrate communities through the capture of invertebrate prey, and the plasticity of plant investment in prey capture in response to N availability, lends carnivorous plants as model systems for exploring the influence of N deposition on the strength and nature of multitrophic interactions between organisms that inhabit extreme, nutrient-deficient environments.

For a carnivorous plant to obtain nutrients from prey, it must attract (not necessarily required), capture and digest prey, and uptake prey-derived nutrients. These stages are interlinked with each stage influenced by the availability of specific resources. For example, relative investment in prey capture by *D. rotundifolia*, measured as leaf stickiness, is influenced by light and nitrogen availability and prey capture by invertebrate abundance. Therefore, in order to build a complete picture of the influence of N deposition on patterns and processes of prey capture and N nutrition, it is essential to examine each stage in detail, explore the sequential effects of the earlier stages of prey capture and diet on N nutrition, and consider the influence of abiotic and biotic factors other than N availability on plant ecophysiology. The research presented in this thesis therefore utilises a combination of *in-situ* and *ex-situ* experiments to address key ecological and evolutionary questions relating to a single or multiple stage(s) of nutrient acquisition from prey by the carnivorous plant *Drosera rotundifolia*.

Chapter 2.

In this Chapter, the influence of N deposition on patterns and processes of prey capture by *D. rotundifolia*, and subsequent effects on N nutrition, are explored. This Chapter also aims to clarify the dietary strategy utilised by *D. rotundifolia*, which will further understanding of the physiological processes and environmental pressures driving the evolution of trap specialisation and complexity in carnivorous plants. Understanding why complex functional structures such as the traps of carnivorous plants have evolved is important for enabling the prediction of how vascular plant species inhabiting extreme environments may adapt over successive generations in response to sustained, elevated N deposition inputs.

Chapter 3.

In this Chapter, $\delta^{15}\text{N}$ natural abundance stable isotope analysis is utilised to explore changes in N use in response to atmospheric N deposition for *D. rotundifolia* and co-occurring ombrotrophic bog plant species with contrasting life history strategies. Plant communities of ombrotrophic bogs are typically comprised of *Sphagnum* and a relatively low diversity of vascular plant species with highly specialised adaptations for nutrient acquisition and retention. Understanding how N uptake and use may differ between co-occurring plant species will assist with predicting how interactions between species, and therefore ultimately plant community structure, may change in response to sustained, elevated N deposition inputs.

Chapter 4.

In terms of N nutrition, carnivorous plants are unique in that N can be obtained via root uptake or from captured invertebrate prey. In order to explore the effects of resource availability on the N nutrition of carnivorous plants, it is essential to calculate the relative contribution of prey N (%N_{dfp}) to the N budget of the plant as this variable provides a measure of plant reliance on the trait of carnivory (Millett *et al.*, 2012). However, considerable uncertainty and potential error are associated with %N_{dfp} values reported in the literature. This Chapter explores the potential for reducing uncertainty in the calculation of plant reliance on carnivory using a $\delta^{15}\text{N}$ natural abundance multi-level linear mixing model. Specifically, this research aims to determine the influence of diet on accuracy and precision of the calculation of %N_{dfp} of *D. rotundifolia* and the likely influence of plant proxies for the root N endpoint of the mixing model. The presentation of a method for increasing precision in the calculation of %N_{dfp} offers wider application for ecological studies calculating the relative contribution of two sources to a sink using the natural abundance linear mixing model.

Chapter 5.

Several species of carnivorous plant vary in leaf colour from red to green as a phenotypically plastic response to changes in resource availability, however the functional role(s) of anthocyanins (pigments responsible for leaf redness) in carnivorous plants has not been clarified. The two leading hypotheses for the functional role of leaf redness in carnivorous plants are as a response to N deficiency and/or as a photoprotective response. Furthermore, leaf redness variation is only observed in some evolutionary lineages of carnivorous plants. In this Chapter, the contrasting hypotheses for the functional role(s) of anthocyanins are tested using a manipulative 2x2 factorial (+/- N, +/- shade) greenhouse experiment with three species of carnivorous plant from differing evolutionary lineages. Exploring the functional role of anthocyanins in carnivorous plants will offer an insight into the environmental pressures driving the evolution of leaf pigmentation in vascular plants.

Chapter 6.

To further understanding of nutrient acquisition by carnivorous plants, it is essential to clarify the effects of, and interactions between, abiotic and biotic factors on N uptake. Changes in ambient temperature influence a wide range of physiological processes in plants, such as photosynthetic rate, growth and tissue C: N ratios (Hughes, 2000; Day *et al.*, 2008). However, there is a deficiency of research exploring the influence of ambient temperature on root N uptake in carnivorous plants. Furthermore, root N uptake may be influenced by prey N uptake; results of a study by Adamec (2002)

showed that root N uptake was increased by an average of 63% following prey N uptake compared with control plants growing in a greenhouse environment. The research presented in this Chapter utilises the enriched ¹⁵N technique to explore the influence of ambient temperature and prey availability on root N uptake by *Drosera rotundifolia* growing *in-situ* at a pristine ombrotrophic bog in the UK. This research aims to offer an insight into how vascular plants with highly specialised adaptations for nutrient acquisition may respond ecophysiologicaly to projected rises in ambient temperature as a consequence of increasing greenhouse gas concentrations.

Chapter 2: Root N availability to the carnivorous plant *Drosera rotundifolia* influences the quantity, but not quality, of captured invertebrate prey.

2.1 Abstract

In response to increasing N availability, carnivorous plants decrease investment in prey capture and, as a result, capture smaller total masses of prey. However, considerable debate continues in the literature regarding the dietary strategy of carnivorous plants species and whether carnivorous plants can control the quality of prey captured in response to N availability. In this study, the influence of resource availability on patterns and processes of prey capture by the carnivorous plant *Drosera rotundifolia* is explored utilising plants growing *in-situ* at two ombrotrophic bogs in the UK that vary primarily by N deposition input. Abundance, order composition and size distribution data for spectra of captured prey and potential prey were collected throughout the plants' active growth season in order to determine the dietary strategy of *D. rotundifolia*. Leaf stickiness was measured in order to test for relationships between measures of captured prey and plant investment in prey capture. Abiotic environmental variables, leaf traits and life history traits of plants were measured in order to explore the influence of between-site differences in resource availability on investment in prey capture, qualitative and quantitative measures of actual prey captured, and allocational and functional trade-offs.

Results show that as dissolved inorganic N (DIN) decreases, leaf stickiness increases and as a result, the total mass of captured prey increases. Prey size ranged from 0.4 to 6.5 mm in length. Plants were most likely to capture invertebrates of the 0 to 0.9 mm size class than larger size classes, with the likelihood of prey capture success for this size class approximately threefold greater for plants exposed to low DIN than for plants exposed to high DIN. The order composition of captured prey was evenly distributed, with the exception that plants exposed to high DIN were more likely to capture Acarina than plants exposed to low DIN. The average size of prey differed significantly between orders; Acarina and Diptera captured by plants exposed to high DIN were smaller than Acarina and Diptera captured by plants exposed to low DIN. Plants exposed to high DIN were larger, possessed higher leaf area (LA) and specific leaf area (SLA), and invested more heavily in root biomass and reproduction in proportion to size than plants exposed to low DIN. Leaf colour did not differ between sites, and no relationship was found between leaf colour and the total mass of prey captured by plants.

Results show that as DIN, and presumably root N availability, decreases, plant investment in and reliance on prey capture increase. Between-site differences in the size distribution of prey captured suggest that the prey retention capacity of leaves decreases as invertebrate size increases. Results

indicate that the size distribution of prey captured by the plant represent a random sample from the population of potential prey, where the likelihood of prey retention is controlled by the stickiness of the leaves and invertebrate size. The higher probability of capturing Acarina at the 'high' DIN site is most likely to be explained by the differences in average size of this order between sites, rather than an indication of dietary specialism by the plant. No evidence of phenotypic plasticity in dietary strategy as a response to N availability was found, suggesting that prey attraction is not phenotypically plastic in response to N availability. Results indicate that leaf colour is not used as a primary mechanism for prey attraction. These results therefore support the classification of *Drosera rotundifolia* as a dietary generalist. Differences in plant reproductive effort and SLA between sites indicate exposure to low root N availability restricts plant resource allocation away from essential vegetative functions such as growth and maintenance and directs resource allocation towards leaf conservation.

2.2 Introduction

Carnivorous plants have evolved a wide variety of traps that differ in morphological and physiological mechanisms for the attraction and capture of invertebrates. One of the most hotly debated topics relating to the feeding ecology of carnivorous plants is whether species have evolved to be dietary generalists, where prey spectra represent random samples from populations of potential prey, or to be prey specialists, where the selective attraction and capture of particular taxa and/or size classes from the population of potential prey occurs. In Darwin's seminal work on carnivorous plants (Darwin, 1875), it was predicted that it may be more energetically beneficial for the plant to wait a longer period of time to capture a one large insect than to capture several small insects in a smaller period of time, however this prediction was not tested. Evidence from recent phylogenetic data had led to the presentation of the predictable prey capture hypothesis, which proposes that species with complex trapping mechanisms are more likely to have more frequent and predictable captures of prey than species with simpler trapping mechanisms, and by extension, more frequent and predictable captures may be achieved by prey specialism (Müller *et al.*, 2004). Therefore, it may be predicted that species that utilise relatively simple adhesive traps for prey capture (e.g. *Drosera* and *Pinguicula* spp.) are dietary generalists where prey spectra represent random sampling from background populations of potential prey. Evidence testing this prediction is scant with mixed results; one study indicates dietary generalism by *Drosera capillaris* (Jennings *et al.*, 2010), and another indicates dietary specialism by *Pinguicula longifolia* (Antor and García, 1994). Evidence from remaining prey capture studies utilising species with adhesive traps is largely limited by the experimental approaches taken. Limitations include a lack of consideration for:- parameters of the background invertebrate population of potential prey (e.g. van Achterberg, 1973; Thum, 1986), the prey retention ability of leaves (e.g. Karlsson *et al.*,

1987; Zamora, 1995; Alcalá and Domínguez, 2003) or the size distribution of captured prey in comparison with the size distribution of potential prey (Volkova *et al.*, 2010). Therefore, it is evident that further research is required to clarify the dietary strategy/strategies of carnivorous plant species utilising adhesive traps to capture prey.

In addition to potential differences in prey capture strategies between carnivorous plant species, evidence indicates that plant investment in prey capture by carnivorous plants is phenotypically plastic in response to changes in resource availability in ecological time. For *Sarracenia purpurea*, a northern pitcher plant with morphologically distinct pitcher structures for prey capture (phyllodia) and photosynthesis (keels), plants exposed to 'high' N and N:P ratio solutions exhibited reduced investment in prey capture through significantly higher keel to phyllodium ratios compared with plants exposed to 'low' N and N:P ratio solutions (Ellison and Gotelli, 2002). For *Drosera rotundifolia*, plants exposed to NPK solutions containing 5.0 mM N exhibited reduced investment in prey capture through significantly less sticky leaves compared with plants exposed to solutions containing *ca.* 0.05 mM N (Thorén *et al.*, 2003).

Whilst evidence exists to support the prediction that plant uptake of prey-derived N increases with decreasing root N availability (Millett *et al.*, 2012), evidence linking the negative relationship between root N availability and investment in prey capture with the proposed positive relationship between investment in carnivory with actual prey capture is scant and ambiguous. Results of a study (Alcalá and Domínguez, 2003) using five *in-situ* populations of *Pinguicula moranensis* situated along an environmental gradient of temperature, soil fertility, humidity and photosynthetically active radiation (PAR) (temperature, nitrogen, humidity, light ('TNHL')) found the mass of prey captured per unit leaf area to share a negative linear relationship with the TNHL gradient (ANOVA, $F_{(1,234)} = 12.10$, $P = 0.04$), however the strength of the relationship between prey capture and root N availability was confounded by significant relationships between captured prey and humidity, PAR and potential prey availability, and the experimental approach limited by the lack of measurement of leaf stickiness. Results of this study also provide preliminary evidence that the quality of prey captured by carnivorous plants may vary in response to root N availability; the relative abundance of prey taxa captured by *P. moranensis* plants differed significantly along the TNHL environmental gradient. Specifically, whilst Diptera were most frequently captured by all plants, plants exposed to high root N availability captured significantly higher relative proportions of Diptera and Acarina than plants exposed to low root N availability, which captured proportionally more Coleoptera. These patterns were suggested, but not proven, to be as a result of the negative relationship between prey retention capacity of the leaves and between-taxa differences in the mean size of invertebrates, rather than as a result of dietary specialism. It is evident therefore that further research is required to test the influence of root N availability on (i) plant

investment in prey capture; (ii) quantitative and qualitative measures of actual prey captured by carnivorous plants.

In this study, the influence of root N availability on prey capture and diet by a carnivorous plant species with adhesive traps is explored utilising *Drosera rotundifolia* plants growing *in-situ* at two ombrotrophic bogs in the UK that vary by N deposition input.

Specifically, the following aims are addressed:-

1. To determine the dietary strategy utilised by *D. rotundifolia*;
2. To determine whether phenotypic plasticity in the dietary strategy utilised by *D. rotundifolia* occurs in response to N deposition;
3. To investigate the influence of functional leaf traits, specifically leaf redness and leaf stickiness, on prey capture by *D. rotundifolia*;
4. To investigate the influence of N deposition on life history traits of *D. rotundifolia*.

Aim 1 is achieved by comparing abundance, order composition and size distribution data for spectra of captured prey and potential prey from the background invertebrate populations throughout the plants' active growth season. Thus, the probability of 'prey' capture (PPCS), a measure of the likelihood of a particular invertebrate order or size class being captured from the background invertebrate population, and relative abundances can be calculated and quantitative assessments of whether prey captured by *D. rotundifolia* represent random samples from potential prey (dietary generalism) or not (dietary specialism) can be made. As *D. rotundifolia* captures flying and flightless invertebrates, background invertebrates are surveyed by sweep net and pitfall traps respectively.

Aim 2 is achieved by comparing the relative abundances and PPCS values for orders and size classes of invertebrates captured by sample populations of *D. rotundifolia* plants from two sites that receive contrasting N deposition inputs. Dissolved inorganic N (DIN) is measured throughout the plants' active growth season in order to relate site N deposition inputs with DIN, and therefore presumably root N availability.

To achieve Aim 3, the stickiness of *D. rotundifolia* leaves is measured in order to relate quantitative and qualitative measures of prey capture with plant relative investment in prey capture. Leaf area is measured throughout the plants' active growth season to explore the relationship between total potential trapping area and prey capture. Leaf redness of survey plants is measured following plant collection at the end of the growth season in order to address whether leaf colour influences prey capture. To account for potential influence of between-site variation in the abiotic environment other than N deposition inputs on leaf traits, light availability to the plants is measured.

To achieve Aim 4, the number of survey plants that had senesced and the rosette area of each live plant were measured at monthly intervals throughout the growth season in order to explore the influence of site N deposition inputs on plant survival and growth. Following plant collection at the end of the growth season, the total dry mass per plant and the dry mass of individual plant parts are measured in order to explore the influence of N deposition on plant size, specific leaf area, root mass ratio and net reproductive effort. To account for potential between-site variation in the abiotic environment other than N deposition inputs, pore water pH and EC and light availability to the plants are measured.

Clarification of the dietary strategy used by *D. rotundifolia*, and of whether the strategy is phenotypically plastic in response to resource availability, will further understanding of patterns and processes of prey capture by carnivorous plants and offer an insight into selective pressures driving the evolution of trap specialisation. Exploring the relationship between leaf stickiness and prey capture for plants at sites receiving contrasting N deposition inputs will determine the influence of N availability on plant investment in prey capture and resultant effects on prey capture and diet, thus providing an insight into how plants with specialised adaptations to an extreme environment respond ecophysiologicaly to changes in resource availability. Exploring the relationship between leaf redness and prey capture will clarify whether leaf colour plays a primary role as a prey attractant, thus providing further insight into the functional role(s) of leaf redness in carnivorous plants. Exploring potential differences in life history traits between plant sample populations at each site will enable a bigger picture to be formed of the influence of N deposition on plant vigour and fitness, and how this is interconnected with prey capture and diet.

2.3 Methods

2.3.1 Study sites

Fieldwork was undertaken from May to September 2011 at two ombrotrophic raised bogs in the United Kingdom which differ by N deposition load (Table 2). Whixall Moss is a lowland raised peat bog in Shropshire, England; Cors Fochno is an estuarine lowland raised peat bog in Ceredigion, Wales.

Table 2 Locations, climatic and environmental characteristics of Cors Fochno and Whixall Moss ombrotrophic raised bogs. Table (a) summarises site characteristics for 2006 to 2011 (where possible), this time period representing the maximum potential life span of survey *Drosera rotundifolia* plants; Table (b) summarises site characteristics for 2011 during which fieldwork was undertaken.

(a)

Site	Location	Mean annual precipitation (mm yr ⁻¹) ^a	Mean temperature January / July (°C) ^a	Mean growing season length (d) ^b	Growing season average temperature (°C) ^b	N deposition (kg N ha ⁻¹ yr ⁻¹) ^c
Cors Fochno	52°30'09N, 04°00'57W	1381	3.5/14.7	320	11.6	8.0
Whixall Moss	52°92'16N, 02°76'45W	719	4.0/13.7	296	11.0	30.8

^a Based on observed meteorological data from KNMI Climate Explorer (<http://climexp.knmi.nl>) (accessed 08.09.2014). Data are mean values for 2006 - 2011 inclusive.

^b Growing season is the number of days with mean temperature $\geq 5^{\circ}\text{C}$. Data are mean values for 2011 – 2012 inclusive (earlier years unavailable). Based on observed meteorological data from automatic weather stations at each site; data accessed from Countryside Council for Wales (Cors Fochno) and Natural England (Whixall Moss).

^c Modelled N deposition data from APIS (<http://www.apis.ac.uk/>) (accessed 21.04.2014). Data are mean values for 2010-2012 inclusive (earlier years unavailable).

(b)

Site	Annual precipitation (mm yr ⁻¹) ^a	Temperature January / July (°C) ^a	Growing season length (d) ^{a,b}	Growing season average temperature (°C) ^{a,b}
Cors Fochno	1008	3.7/15.0	324	12.1
Whixall Moss	565	2.5/14.2	301	11.5

^a Data values for 2011 only. Based on observed meteorological data from automatic weather stations at each site; data accessed from Countryside Council for Wales (Cors Fochno) and Natural England (Whixall Moss).

^b Growing season is the number of days with mean temperature $\geq 5^{\circ}\text{C}$.

2.3.2 Sampling protocol

Ten survey plots were allocated at each site by selecting areas which contained *Drosera rotundifolia* plants growing in *Sphagnum* and were nearest to randomly generated GPS points. Fifteen survey *D. rotundifolia* plants were allocated and individually labelled at each plot by locating randomly selected map coordinates using a handheld GPS device, and selecting the nearest plant to each point.

2.3.2.1 Plants

At four-weekly intervals, the following morphological measurements of survey *D. rotundifolia* plants were taken:- mean rosette diameter (two cross-sectional measurements), the number of leaves and the length and width of each leaf (Aims 3 and 4). The light intensity next to each plant and the ambient light intensity above the vegetation were measured using a handheld light meter (SKP 200 PAR Quantum Sensor, Skye Instruments Ltd., Wales, UK) (Aims 3 & 4). As the ability of the leaf to capture and prevent invertebrates from escaping depends on the sticky substances on the leaf surface (Zamora *et al.*, 1998), investment in prey capture in terms of the stickiness of the leaves was measured (Aim 3). Measurements of leaf stickiness were not obtained from survey *D. rotundifolia* plants during the 2011 active growth season due to inclement weather conditions. However, *in-situ* measurements of leaf stickiness were taken during July 2012 from 50 separate, randomly selected *D. rotundifolia* plants situated in the 2011 survey plots at each site. An individual, randomly selected leaf from each *D. rotundifolia* plant was lightly pressed onto a 5 cm x 1 cm strip of filter paper attached to a handheld universal digital force gauge (Sauter FH2 model, Kern & Sohn, Balingen, Germany), ensuring all trichomes were adhered to the filter paper. Leaf stickiness was measured as the force (N) required to separate each leaf from the filter paper.

At the end of the survey season, survey *D. rotundifolia* plants were removed and rinsed with deionised water. Previous years' growth was removed, and the remaining living material pooled per survey plot.

In order to quantify leaf redness (Aim 3), the spectral reflectance of one randomly selected, mature leaf per plant was measured in a dark room using a UV-Vis fibre optic spectrometer (USB 2000+ model, Ocean Optics Ltd., Florida, USA) and accompanying spectrometric software (SpectraSuite, Ocean Optics Ltd., Florida, USA). Leaf spectrometry data was calibrated using SpectraSuite by dark and light reference files generated from the measurement of the reflected light spectrum of black and white polytetrafluoroethylene (PTFE) reference standards for every tray of 15 plants. In order to quantify leaf redness, the Commission Internationale de l'Eclairage (CIE) $L^*a^*b^*$ (CIELAB) colour space parameter of CIELAB a^* (CIE, 1978), a measurement of colour variation on a red to green scale where positive values indicate red coloration and negative values represent green coloration, was used (equation in Chen *et al.*, 2011).

Leaf area (Aim 3) was calculated by creating a regression model of actual leaf area and predicted leaf area using a sample of leaves as follows. Ten randomly selected leaves were taken from non-survey plants at regular time intervals throughout the growth season. Each leaf was scanned using a flatbed colour scanner, and the digital image adjacent to a transparent ruler uploaded into image processing software (Rasband, undated), where actual leaf area was calculated (O'Neal *et al.*, 2002). Predicted leaf area was calculated by applying the equation of an ellipsoid to the cross-sectional leaf radii measurements. Linear regression was used to determine the relationship between predicted and actual leaf area. This equation was then used to calculate leaf area for all *D. rotundifolia* plants.

Prior to oven drying, plant material was kept cold and stored in labelled paper sample bags to permit air drying, thus enhancing preservation through the minimisation of enzymatic reactions (Campbell & Plank, 1992).

2.3.2.2 Invertebrates

Invertebrate prey captured by *D. rotundifolia* and the background invertebrate populations were surveyed at four-weekly intervals throughout the active growth season of *D. rotundifolia* (Aims 1 & 2). Captured invertebrate prey were sampled by randomly selecting ten *D. rotundifolia* leaves containing freshly captured prey from non-survey plants within or adjacent to each plot. The length and width of each these leaves were also measured (Aim 3). To prevent damage to the captured invertebrates from their removal from the leaves with forceps in the field, each set of ten leaves containing the invertebrates were removed and stored in sterile plastic sample tubes pre-filled with saturated NaCl solution.

To represent potential prey, background terrestrial and airborne invertebrates were sampled by pitfall trap and sweep net respectively. Pitfall traps were constructed using plastic drinking cups filled half-way with saturated NaCl solution, with a suspended plastic roof attached to the rim to prevent rainwater seepage into the trap. Three individually labelled pitfall traps (bait-less) were submerged flush with *Sphagnum* capitula at random locations within a 40 cm radius of survey *D. rotundifolia* plants. Three sweep net surveys of the vegetation surrounding each plot were conducted at three times of day (10am, 12pm, and 2pm) for three minutes using a D-shaped sweep net appropriate for surveying short vegetation. After each four-week period, the invertebrates caught at each survey time by each sampling method per plot were pooled and counted, and placed into a sterile plastic sample tube pre-filled with saturated NaCl solution.

2.3.2.3 Abiotic variables

At four-weekly intervals peat water pH and electrical conductivity were recorded at each survey plot using standardised pH and EC testers (Hanna Instruments Ltd, UK) (Aims 3 and 4). 500 ml peat water samples were collected by squeezing *Sphagnum* and peat at each survey plot for NO_3^- -N, NH_4^+ -N and NO_2^- -N determination (Aims 2, 3 and 4). Water samples were stored in plastic sample bottles that had been soaked for at least one day with 10% HCl and rinsed with ultrapure water prior to each survey session. Water samples were filtered as soon as possible following collection using Whatman 0.7 μm GF/F glass fibre micro filters (Nollet, 2007) and a sterilised Sterifil aseptic system (Merck Millipore Ltd, UK), and stored in sterile plastic sample bottles in black bin liners at 3 – 5 °C to inhibit algal and bacterial growth.

2.3.3 Sample preparation and analysis

Water samples were analysed for ammonium (NH_4^+ -N), nitrate (NO_3^- -N) and nitrite (NO_2^- -N) by ion-exchange chromatography as soon as possible following sample filtration. Total dissolved inorganic N content (DIN) ($\mu\text{g l}^{-1}$) was calculated as the sum of NH_4^+ -N, NO_3^- -N and NO_2^- -N for each peat water sample. In the laboratory, invertebrates were counted and identified to order level using a Zeiss Stemi 2000 stereo microscope (Carl Zeiss Microscopy Ltd, Germany) and the length of each invertebrate measured to 0.1 mm precision using a 100 x 0.1 mm stage micrometer (Pyser-SGI Ltd, UK). Thus, species abundance, type, and size data were collated for all surveyed invertebrates (Aims 1, 2 and 3). Background invertebrates outside the length range of invertebrates captured by *D. rotundifolia* plants at both sites (0.4 - 6.5 mm in length) were excluded from the dataset. Invertebrates were rinsed thoroughly in deionised water prior to drying. Plant and invertebrate material was dried to a constant weight by placing in a forced-air oven at 70 °C for 72 hours (Campbell & Plank, 1992), and weighed to obtain dry mass measurements.

2.3.4 Data analyses

Life history traits of *D. rotundifolia* plants were calculated as follows:- root mass ratio (RMR) = the proportion of root dry mass of the total whole plant dry mass (Gregory *et al.*, 1997); survival rate = number of live plants at end of growth season divided by the total number of survey plants; net reproductive effort (RE) = the proportion of total plant biomass allocated to seeds and other reproductive structures (Reekie and Bazzaz, 1987) (Aim 4). Leaf traits were calculated as follows:- leaf area (LA) = total corrected leaf area per plant; specific leaf area (SLA) = total corrected leaf area per plant divided by leaf dry mass per plant (Evans and Poorter, 2001) (Aim 3).

To assess whether *D. rotundifolia* plants selectively capture invertebrates of a particular order or size class from the background population (Aims 1, 2 and 3), the probability of ‘prey’ capture success (cf. Foot *et al.*, 2014) was calculated for each invertebrate order / size class per site as:- the number of captured invertebrates of order/size class *x* at site *y* divided by the total number of background invertebrates of order / size class *x* (sampled by pitfall trap and sweep net) at site *y*.

The data were evaluated using ANOVA, independent-samples *t*-tests, linear regression and Pearson’s correlation. Post-hoc comparisons were conducted using Least Significant Difference (LSD) ($P < 0.05$). Data were \log_{10} -transformed where appropriate to achieve homoscedascity prior to analysis. Residual plots were used to assess for homoscedascity and normal probability plots used to test that data were normally distributed.

All statistical analyses were conducted using IBM SPSS Statistics version 21 (IBM, Chicago, USA).

2.4 Results

2.4.1 Habitat characteristics

Table 3 Abiotic characteristics of survey plots containing *in-situ Drosera rotundifolia* plants at two ombrotrophic bogs in the UK that vary by N deposition input. Presented are the mean \pm 1 S.E. for:- peat water dissolved inorganic nitrogen, DIN; proportion of ambient light available to *D. rotundifolia* plants, light; peat water pH, pH, and electrical conductivity, EC. Significant effects at $P < 0.05$ are highlighted in bold.

Site		DIN ($\mu\text{g N l}^{-1}$)	Light (%)	pH (pH units)	EC ($\mu\text{s cm}^{-1}$)
Cors Fochno	Mean	735.8	61.17	3.44	166.98
	SE	20.8	3.56	0.05	6.81
Whixall Moss	Mean	1505.4	55.28	3.81	115.81
	SE	234.1	1.67	0.15	5.09
Independent samples <i>t</i> -test results ¹		<i>P</i>			
		0.009	0.158	0.040	<0.001

¹ Comparing differences between sites.

Peat water of survey plots at Cors Fochno was significantly lower in DIN, higher in EC and more acidic than peat water of survey plots at Whixall Moss (Figs 3(a), 3(c), 3(d); Table 3). The proportion of ambient light available to *Drosera rotundifolia* plants did not differ significantly between sites (Fig. 3(b); Table 3).

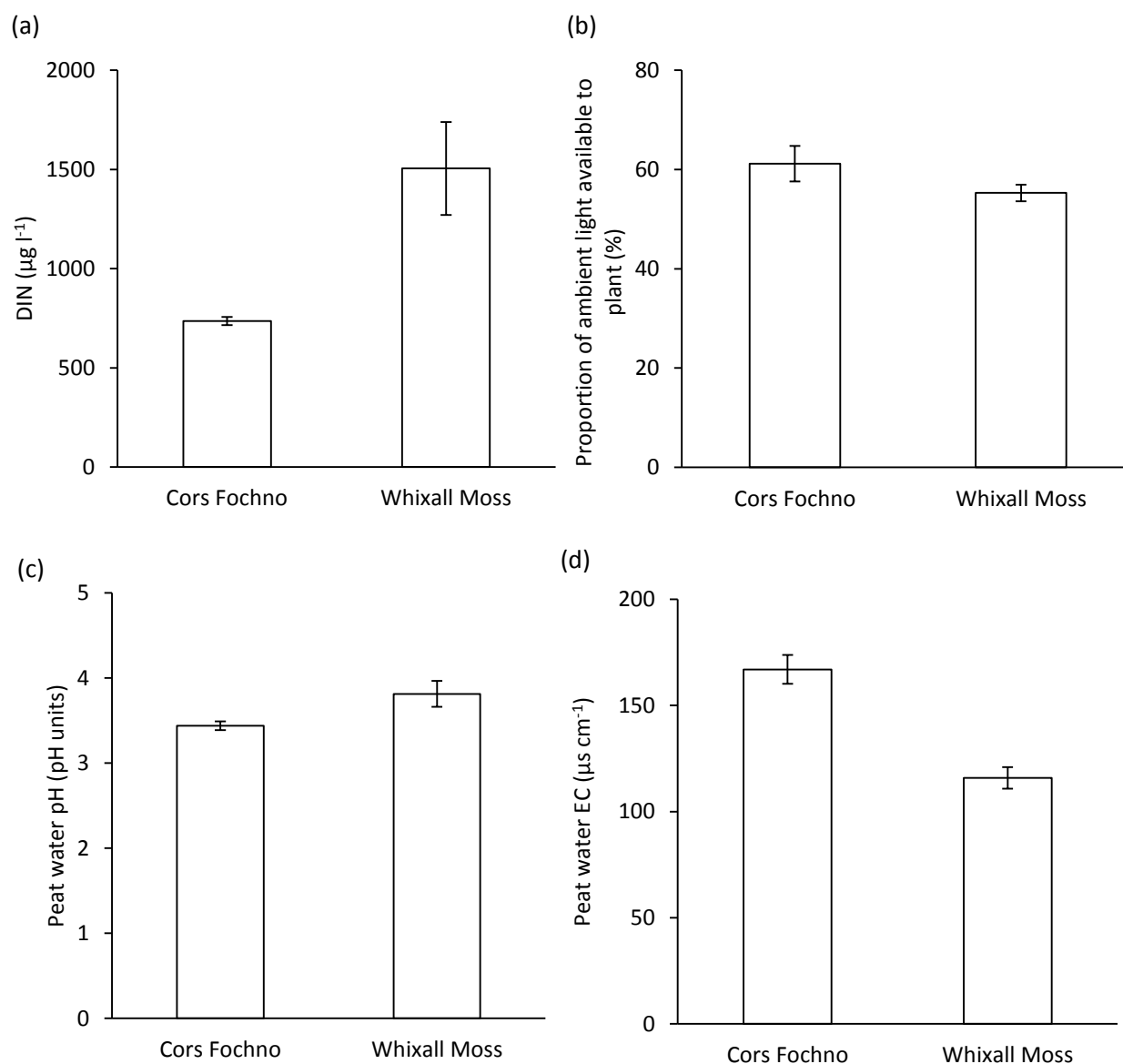


Figure 3 Abiotic characteristics of survey plots containing *Drosera rotundifolia* plants at two ombrotrophic bogs in the UK. Presented are the mean \pm 1 S.E. for:- (a) peat water dissolved inorganic nitrogen, DIN; (b) proportion of ambient light available to *D. rotundifolia* plants; (c) peat water pH; (d) peat water electrical conductivity, EC.

2.4.2 Prey capture and diet of *Drosera rotundifolia*

2.4.2.1 Prey capture

Table 4 Invertebrate prey capture by *Drosera rotundifolia* plants growing at two ombrotrophic bogs in the UK that vary by N deposition input. Presented are the mean \pm 1 S.E. per plant for:- dry mass of captured prey per unit leaf area, prey capture. Significant effects at $P < 0.05$ are highlighted in bold.

Site		Prey capture (mg cm ⁻²)
Cors Fochno	Mean	4.65
	SE	0.19
Whixall Moss	Mean	2.00
	SE	0.15
Independent samples <i>t</i> -test results ¹		$P < 0.001$

¹ Comparing differences between sites.

Drosera rotundifolia plants at Cors Fochno captured significantly greater dry mass of invertebrates per unit leaf area than plants at Whixall Moss (Fig. 4; Table 4).

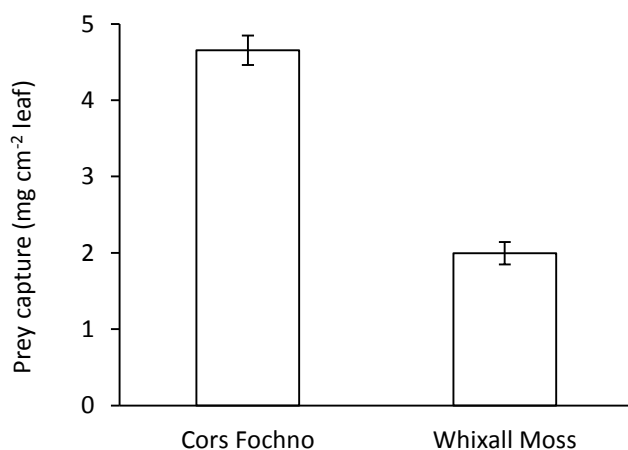


Figure 4 Prey capture by *Drosera rotundifolia* plants growing in two ombrotrophic bogs in the UK that vary by N deposition input. Presented is the mean \pm 1 S.E. for dry mass of invertebrates captured by *D. rotundifolia* per unit area of leaves that captured prey per site.

2.4.2.2 Diet

The sample population of background invertebrates surveyed by sweep net was dominated by species capable of flight, with Diptera the most abundant taxonomic order (49.19%) across both sites, followed by Coleoptera (21.26%) and Hemiptera (13.15%) (Figs. 5(a), 5(b)). Between sites, the relative abundance of Diptera and Araneae were greater at Cors Fochno (58.6% and 15.2% respectively) (Fig. 5(b)) than at Whixall Moss (44.2% and 8.5% respectively) (Fig. 5(a)), whereas the relative abundance of Hemiptera was greater at Whixall Moss (14.6%) than at Cors Fochno (10.5%). Background invertebrates surveyed by pitfall trap constituted predominantly flightless, ground-dwelling species, with Araneae the most abundant order (40.46%) of the sample populations across both sites, followed by Formicidae (37.73%) and Coleoptera (7.86%) (Figs. 5(c), 5(d)). Between sites, the relative abundances of Araneae and Formicidae were greater at Cors Fochno (43.3% and 39.8% respectively) (Fig. 5(d)) than at Whixall Moss (38.0% and 35.9% respectively) (Fig. 5(c)), whereas the relative abundance of Coleoptera was greater at Whixall Moss (10.9%) than at Cors Fochno (4.4%).

The order most frequently captured by *D. rotundifolia* at both sites was Diptera (percentage abundance of sample population:- 37.5%, Whixall Moss; 61.5%, Cors Fochno), followed by Formicidae (14.7%) and Coleoptera, Collembola and Hemiptera (8.1%) at Whixall Moss, and Formicidae (11.4%) and Hemiptera (6.6%) at Cors Fochno (Figs. 5(e), 5(f)). The observed frequencies of the taxonomic distribution of the captured prey sample population were distributed heterogeneously across invertebrate orders at the 0.01 significance level at Whixall Moss (χ^2 , (11, 320) = 431.95, $P < 0.01$) and Cors Fochno (χ^2 , (10, 361) = 1242.47, $P < 0.01$).

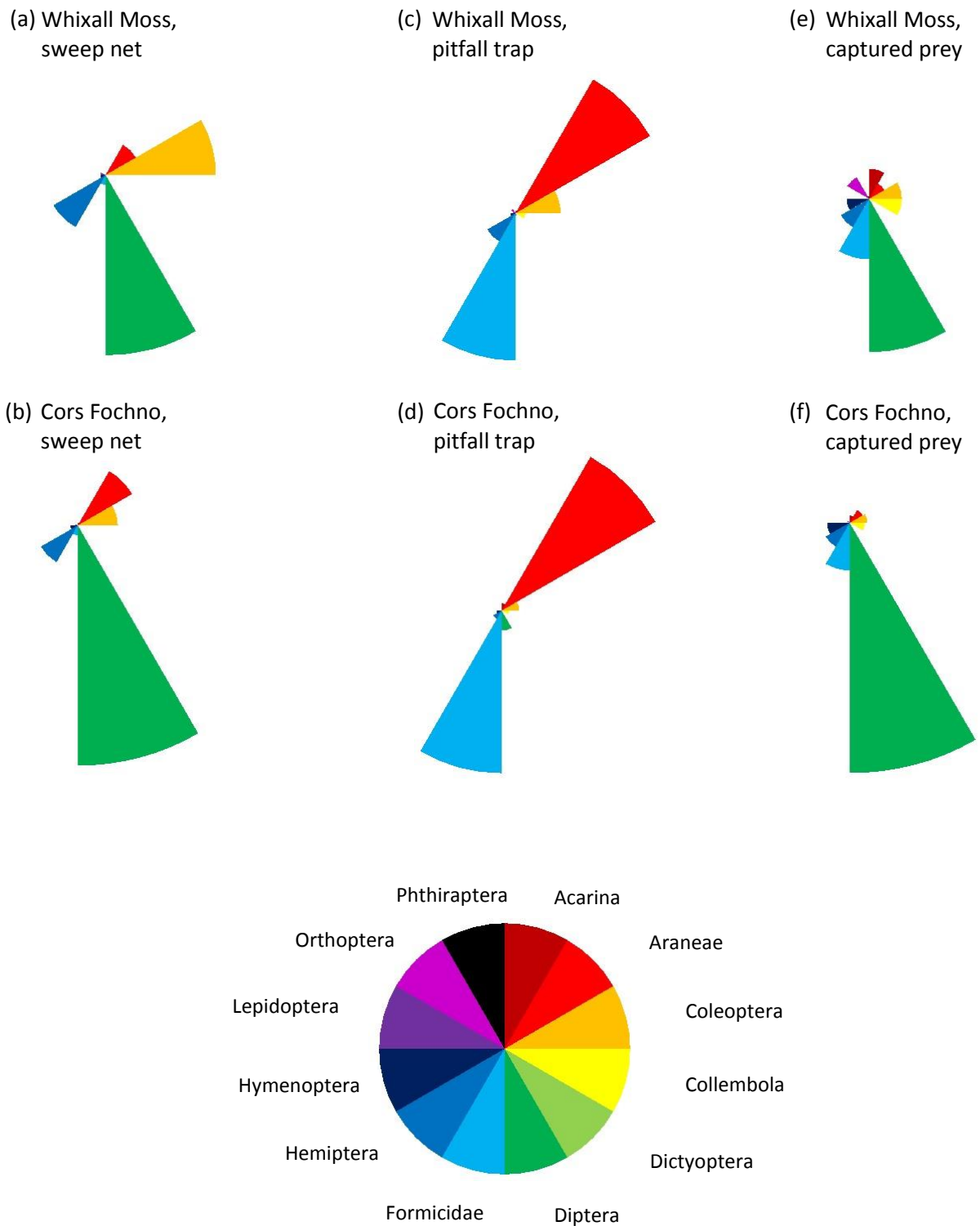


Figure 5 Spectra of background invertebrates (representing potential prey) and prey captured by *Drosera rotundifolia* plants at two ombrotrophic bogs that vary by N deposition input in the UK. Presented are proportional abundance values of each taxonomic order (family in the case of the Formicidae) of sample populations of background invertebrates (sampled by sweep net and pitfall trap) and invertebrates captured by *D. rotundifolia* plants.

The mean size class of the background invertebrate sample population surveyed by sweep net at Whixall Moss (mean \pm 1 S.E. = 2.8 ± 0.1 mm) was larger than the mean size class of the sample population at Cors Fochno (2.5 ± 0.1 mm), as illustrated by the stronger positive skew of the percentage abundance data at Whixall Moss (Figs. 6(a), 6(b)). The sweep net sample population at Cors Fochno followed a weak bimodal size distribution, with peaks at the 3.0 – 3.4 mm size class (20.9% of the sample population) and the 1.0 – 1.4 mm size class (17.3% of the sample population). A stronger tapering of the distribution of the sweep net invertebrate sample population at Cors Fochno across the larger size classes was evident compared to those of Whixall Moss, with 8.4% of the sample population at Cors Fochno present within the 4.0 – 6.9 mm size classes compared to 22.3% at Whixall Moss.

The mean size of the background invertebrate sample population surveyed by pitfall trap was larger than those surveyed by sweep net at Whixall Moss (mean \pm 1 S.E. = 3.5 ± 0.0 mm, pitfall trap; 2.8 ± 0.1 mm, sweep net) and at Cors Fochno (4.0 ± 0.0 mm, pitfall trap; 2.5 ± 0.1 mm, sweep net) (Figs. 6(c), 6(d)). The pitfall trap sample population at Whixall Moss was positively skewed with 45% of the sample population occurring in the 2.5 – 3.9 mm size classes, whereas the pitfall trap sample population at Cors Fochno was negatively skewed with 44.3% of the sample population occurring in the 4.0 – 5.4 mm size classes, illustrating the smaller mean size of pitfall trap invertebrates at Whixall Moss compared to Cors Fochno. A weak bimodal size distribution was evident at both sites, with a secondary abundance peak at the 1.5 – 1.9 mm size class (12.3% of the sample population) at Whixall Moss and a broader secondary abundance peak across the 1.0 – 1.9 mm size classes (12.5% of the sample population) at Cors Fochno.

Drosera rotundifolia plants at Cors Fochno captured, on average, smaller prey (mean \pm 1 S.E. = 1.8 ± 0.1 mm) than plants at Whixall Moss (2.1 ± 0.1 mm) (Figs. 6(e), 6(f)). They also captured fewer invertebrates within the size range of 4.0 – 6.9 mm (1.4% of the sample population) than plants at Whixall Moss (4.7% of the sample population).

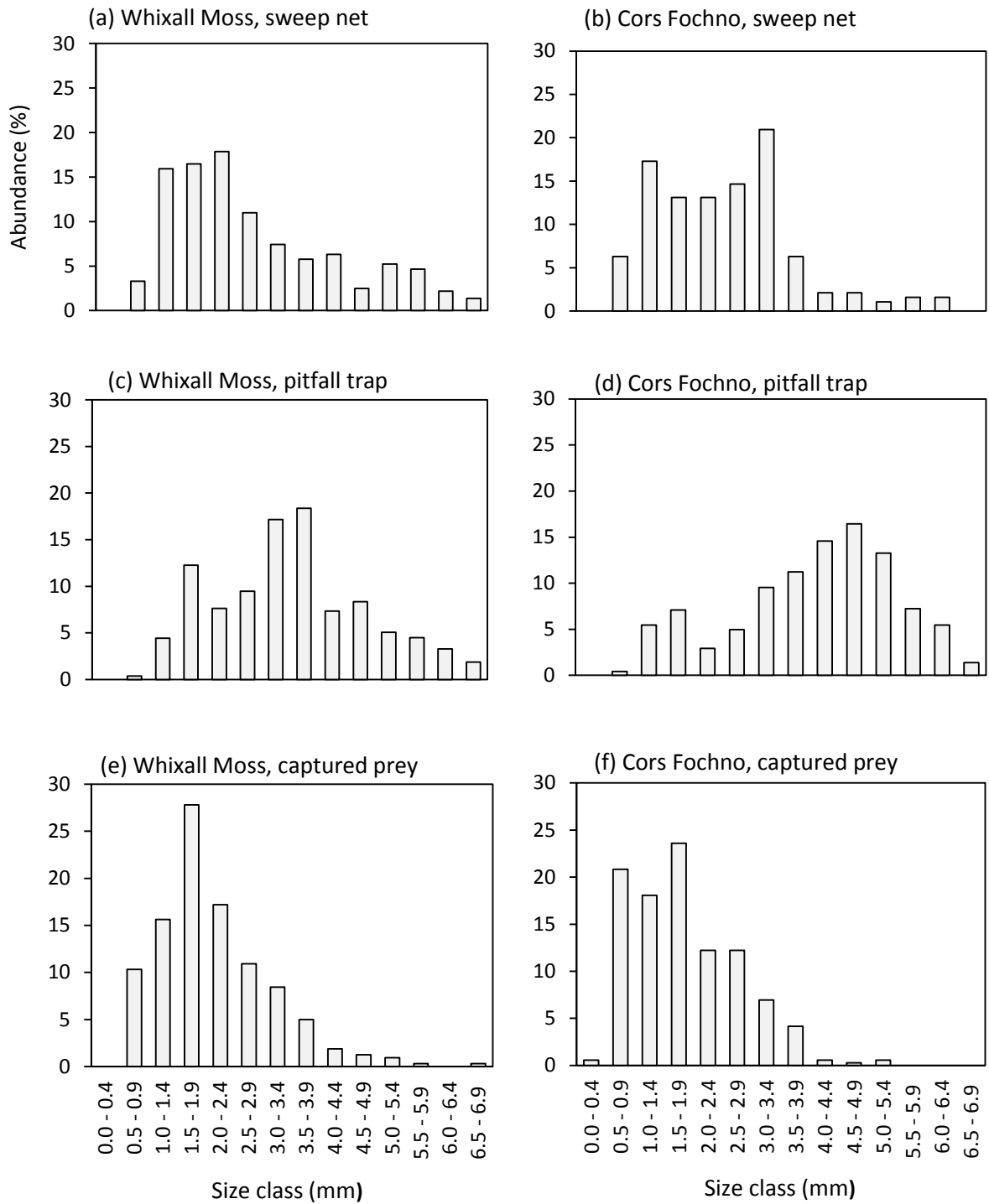


Figure 6 Size distributions of background invertebrates (representing potential prey) and captured prey of *Drosera rotundifolia* plants growing at two ombrotrophic bogs that vary by N deposition input in the UK. Presented are abundance values (%) of each size class (mm) of background invertebrates (sampled by sweep net and pitfall trap) and invertebrates captured by *D. rotundifolia* plants.

Table 5 Results of 2-way (site, invertebrate order) univariate ANOVAs for the mean length per invertebrate order for prey captured by *Drosera rotundifolia* plants growing at two ombrotrophic bogs that vary by N deposition input in the UK. Presented are degrees of freedom (*df*), *F* and *P* values for the mean length per invertebrate order of prey captured by *D. rotundifolia* at each site over the active growth season of the plants. Invertebrate orders: Acarina, Araneae, Coleoptera, Collembola, Dictyoptera, Diptera, Formicidae (family level), Hemiptera, Hymenoptera, Lepidoptera, Orthoptera, Phthiraptera. Significant effects at $P < 0.05$ are highlighted in bold.

	Mean invertebrate length per order of captured prey (mm)		
	<i>df</i>	<i>F</i>	<i>P</i>
Order	11, 680	15.826	<0.001
Site	1, 680	2.673	0.103
Site x order	8, 680	3.342	0.001

¹ Data were \log_{10} -transformed before analysis.

The mean length per order of captured prey was marginally larger at Cors Fochno for all orders except Coleoptera, however for each order except Diptera, significant between-site differences in size were not found (Fig. 7). Diptera captured by plants at Cors Fochno were significantly larger than Diptera captured by plants at Whixall Moss. The captured prey orders of Acarina at Cors Fochno and Whixall Moss, Hymenoptera at Whixall Moss and Dictyoptera at Cors Fochno were significantly smaller than the remaining orders per site, and did not significantly differ in size from each other.

At Cors Fochno, Diptera were significantly smaller than Hemiptera, Coleoptera, Formicidae and Lepidoptera, and did not significantly differ in size from the remaining orders. At Whixall Moss, Diptera were significantly smaller than Orthoptera, Hemiptera, Coleoptera and Formicidae, significantly larger than Acarina and Hymenoptera, and did not significantly differ in size from the remaining orders.

Statistically significant differences in the mean length per order for prey captured by *Drosera rotundifolia* between orders were found (Table 5); Acarina, Dictyoptera and Phthiraptera were significantly smaller than remaining orders. Diptera did not differ significantly in size from Hymenoptera, Araneae, Collembola, Orthoptera, Phthiraptera and Lepidoptera.

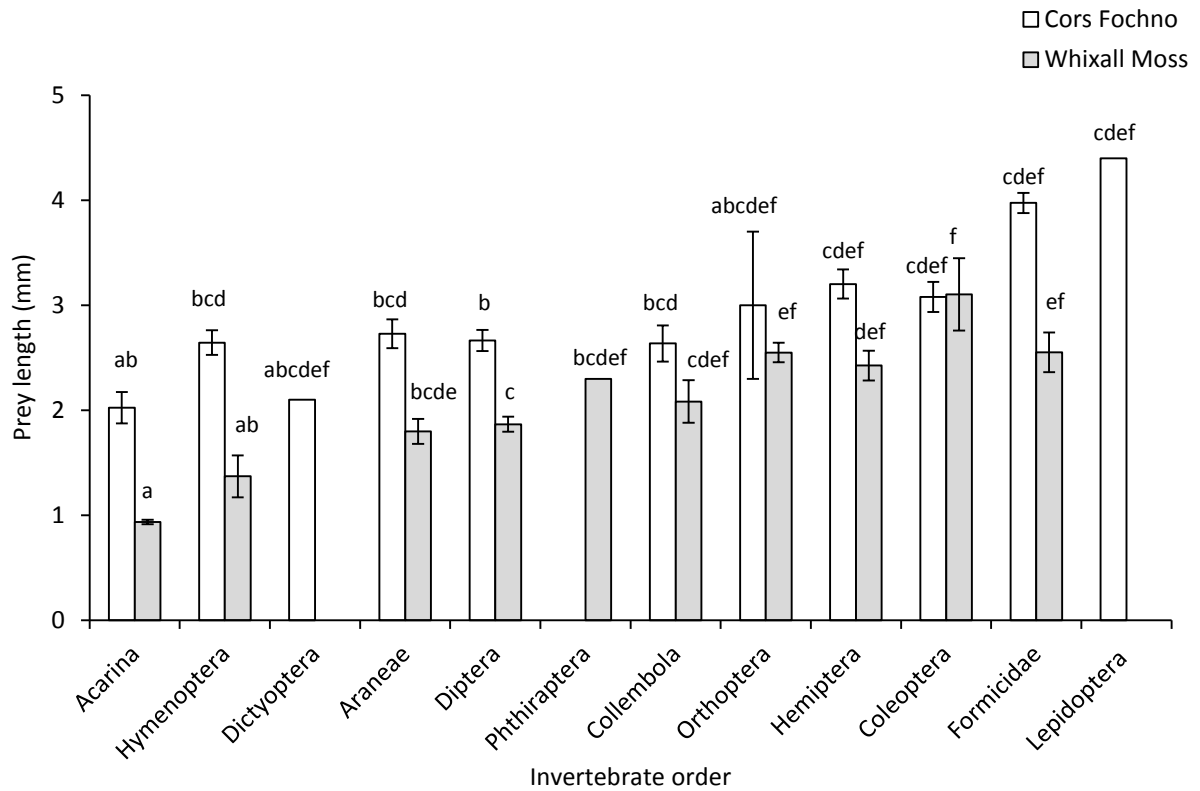


Figure 7 Order and size characteristics of invertebrates captured by *Drosera rotundifolia* plants growing at two ombrotrophic bogs in the UK that vary by N deposition input. Presented are the mean \pm 1 S.E. of invertebrate length per order of prey captured by *D. rotundifolia* plants at each site across the plants' active growth season. Sites: Cors Fochno, Whixall Moss; invertebrate orders captured by *D. rotundifolia*: Acarina, Araneae, Coleoptera, Collembola, Dictyoptera, Diptera, Formicidae (family level), Hemiptera, Hymenoptera, Lepidoptera, Orthoptera, Phthiraptera. Bars with different letters are significantly different from each other (Fisher's least significant difference, $P < 0.05$). Note: plants at Whixall Moss did not capture Dictyoptera or Lepidoptera; plants at Cors Fochno did not capture Phthiraptera.

Table 6 Results of 2-way (site, invertebrate order or size class) univariate ANOVAs for the probability of ‘prey’ capture success (PPCS) by *Drosera rotundifolia* plants. Presented are degrees of freedom (*df*), *F* and *P* values for the PPCS by (a) order of captured invertebrates; (b) size class of captured invertebrates. Sites: Cors Fochno, Whixall Moss; invertebrate orders: Acarina, Araneae, Coleoptera, Collembola, Diptera, Formicidae (family level), Hemiptera, Hymenoptera, Lepidoptera, Orthoptera; invertebrate size classes (mm): 0.0 – 0.9, 1.0 – 1.9, 2.0 – 2.9, 3.0 – 3.9, 4.0 – 4.9, 5.0 – 5.9, 6.0 – 6.9. Significant effects at *P* < 0.05 are highlighted in bold.

(a)

	Probability of ‘prey’ capture success ¹		
	<i>df</i>	<i>F</i>	<i>P</i>
Order	9, 159	11.716	<0.001
Site	1, 159	1.472	0.227
Site x order	9, 159	5.945	<0.001

(b)

	Probability of ‘prey’ capture success ¹		
	<i>df</i>	<i>F</i>	<i>P</i>
Size class	6, 135	51.199	<0.001
Site	1, 135	14.146	<0.001
Site x size class	6, 135	4.024	0.001

¹ Data were \log_{10} -transformed before analysis.

Significant differences in the probability of ‘prey’ capture success (PPCS) were found between invertebrate orders (Fig. 8(a); Table 6); *D. rotundifolia* plants were most likely to capture Acarina, followed by Diptera and Collembola. The interaction effect of site x invertebrate order was statistically significant; *D. rotundifolia* plants at Cors Fochno were most likely to capture Collembola, Diptera, Hymenoptera, Hemiptera and Lepidoptera, with no significant differences in PPCS between these orders found. *Drosera rotundifolia* plants at Whixall Moss were significantly more likely to capture Acarina than the remaining invertebrate orders.

Significant differences in PPCS were found between size classes (Fig. 8(b); Table 6); invertebrates of 0.0 – 0.9 mm in length were most likely to be captured by *D. rotundifolia* plants, followed by invertebrates in the size class of 1.0 – 1.9 mm. Invertebrates in the size class of 6.0 – 6.9 mm were least likely to be captured. The main effect of site on PPCS reached statistical significance; invertebrates captured by *D. rotundifolia* plants at Cors Fochno were significantly larger in length (mean \pm 1 S.E. = 1.57 ± 1.04 mm) than invertebrates captured by *D. rotundifolia* at Whixall Moss (1.30 ± 1.04 mm). The interaction effect of site x invertebrate size class reached statistical significance; *D. rotundifolia* plants at Cors Fochno were over three times more likely to capture invertebrates of 0.0 – 0.9 mm in length and

approximately twice as likely to capture invertebrates of 1.0 – 1.9 mm in length than *D. rotundifolia* plants at Whixall Moss.

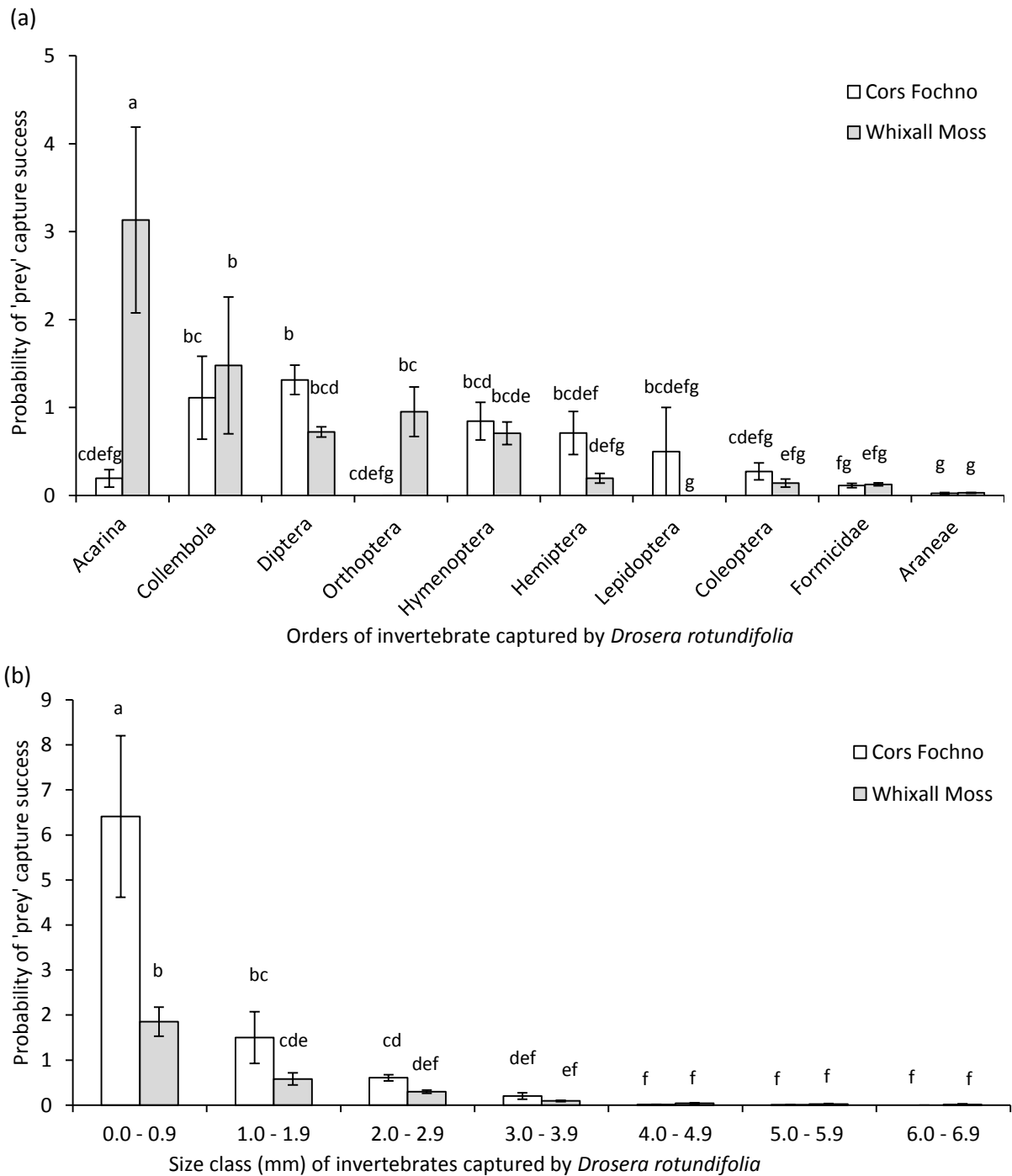


Figure 8 Influence of invertebrate characteristics on the probability of ‘prey’ capture success by *Drosera rotundifolia* plants growing at two ombrotrophic bogs in the UK that vary by N deposition input. Presented are the mean \pm 1 S.E. of the probability of ‘prey’ capture success by *D. rotundifolia* plants for:- (a) orders of captured invertebrates; (b) size classes of captured invertebrates. Bars with different letters are significantly different from each other (Fisher’s least significant difference, $P < 0.05$).

Table 7 Results of 3-way (site, prey measure, invertebrate order/size class) univariate ANOVAs for the proportion of each invertebrate order/size class per captured prey population per survey plot as calculated using two measures of prey specialisation for *Drosera rotundifolia* plants growing at two ombrotrophic bogs in the UK that vary by N deposition input. Presented are the degrees of freedom (*df*), *F* and *P* values for the proportion of each invertebrate order/size class per captured prey population per survey plot (*n* = 10 per site) by (a) invertebrate order; (b) invertebrate size class. Sites: Cors Fochno, Whixall Moss; prey specialisation measures (PSMs): relative abundance (%), probability of ‘prey’ capture success (PPCS) of each order as a proportion of total PPCS per survey plot (%); invertebrate orders: Acarina, Araneae, Coleoptera, Collembola, Diptera, Formicidae (family level), Hemiptera, Hymenoptera, Lepidoptera, Orthoptera; invertebrate size classes (mm): 0.0 – 0.9, 1.0 – 1.9, 2.0 – 2.9, 3.0 – 3.9, 4.0 – 4.9, 5.0 – 5.9, 6.0 – 6.9. Significant effects at *P* < 0.05 are highlighted in bold. See Appendix 1 for site level data.

(a)

	<i>df</i>	Proportion (%) ¹	
		<i>F</i>	<i>P</i>
Site (S)	1, 360	6.715	0.010
Prey specialisation measure (PSM)	1, 360	0.186	0.666
Invertebrate order (O)	9, 360	32.104	<0.001
S x PSM	1, 360	2.190	0.140
S x O	9, 360	7.207	<0.001
PSM x O	9, 360	9.247	<0.001
S x PSM x O	9, 360	1.767	0.074

¹ Data were log₁₀ transformed (log₁₀(*x* + 1)) before analysis.

(b)

	<i>df</i>	Proportion (%) ²	
		<i>F</i>	<i>P</i>
Site (S)	1, 276	4.543	0.034
Prey specialisation measure (PSM)	1, 276	4.811	0.029
Invertebrate size class (SC)	6, 276	169.560	<0.001
S x PSM	1, 276	0.109	0.741
S x SC	6, 276	2.347	0.032
PSM x SC	6, 276	9.789	<0.001
S x PSM x SC	6, 276	0.059	0.999

² Data were square root transformed (sqrt(*x* + 1)) before analysis.

Significant differences between the RA and PPCS of individual invertebrate orders were found; the interaction effect of prey specialisation measure (PSM) x order was statistically significant (Table 7(a); Fig. 9(a)). Specifically, RA was significantly larger than PPCS for Diptera, Formicidae and Araneae. RA was significantly smaller than PPCS for Hymenoptera, Collembola and Acarina.

Size class proportion values calculated using RA were significantly higher than proportion values calculated using PPCS (Table 7(b)). Significant differences between the RA and PPCS of individual size classes were found; the interaction effect of PSM was statistically significant. Specifically, RA values were significantly larger than PPCS values for the invertebrate size classes of 1.0 – 1.9 mm, 2.0 – 2.9 mm and 3.0 – 3.9 mm (Fig. 9(b)). RA values were significantly smaller than the PPCS values for the 0.0 – 0.9 mm size class. No significant differences between RA and PPCS values were found for size classes of 4 mm and above.

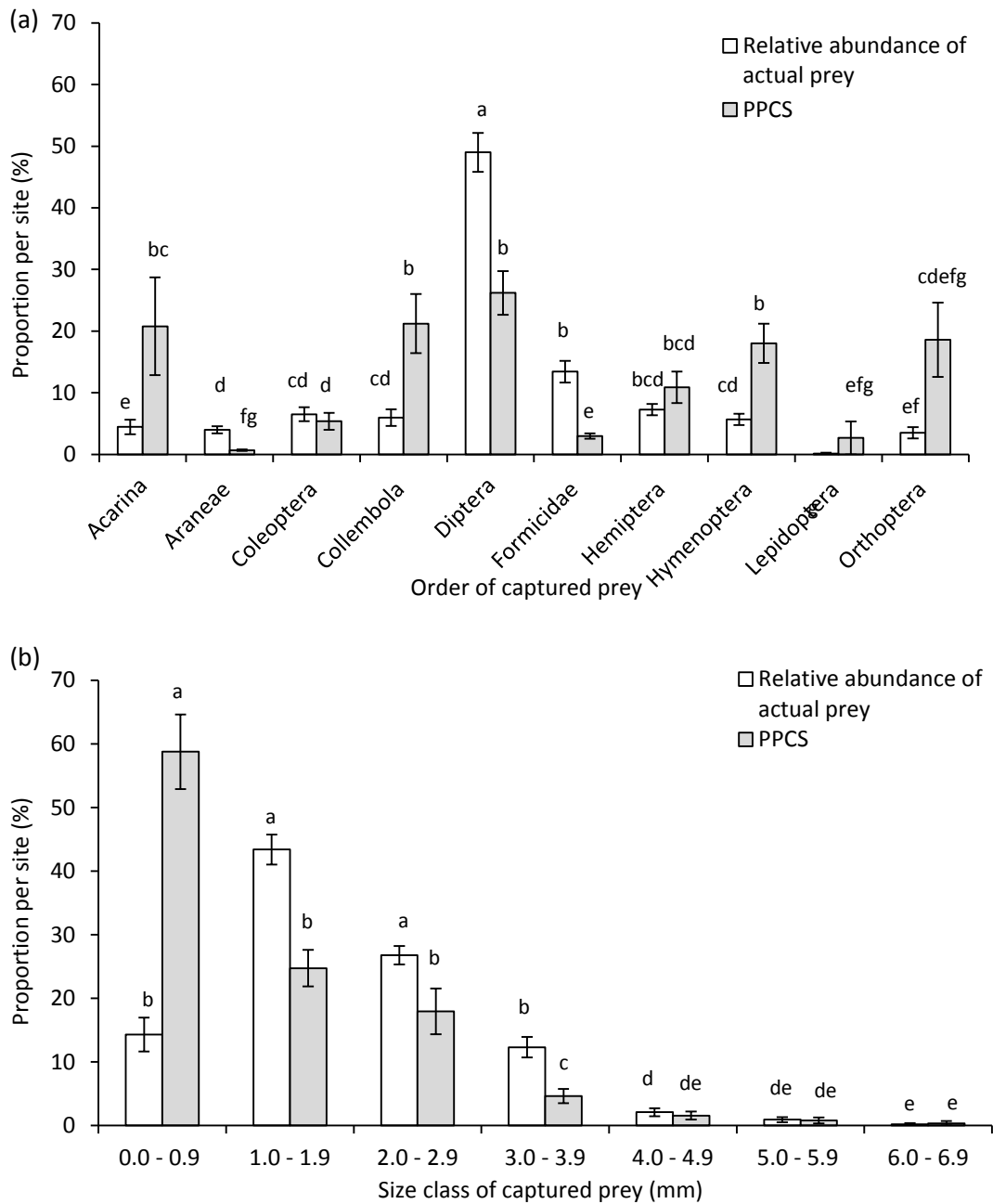


Figure 9 Influence of prey specialisation measure (PSM) and invertebrate parameters on the proportion of each invertebrate order/size class of prey captured by *Drosera rotundifolia* plants growing at two ombrotrophic bogs in the UK that vary by N deposition input. Presented are the mean \pm 1 S.E. proportion per captured prey population per survey plot calculated using two PSMs for: (a) invertebrate order; (b) invertebrate size class. Sites: Cors Fochno, Whixall Moss; PSMs: relative abundance (%), probability of ‘prey’ capture success (PPCS) of each order as a proportion of total PPCS per survey plot (%); invertebrate orders: Acarina, Araneae, Coleoptera, Collembola, Diptera, Formicidae (family level), Hemiptera, Hymenoptera, Lepidoptera, Orthoptera; invertebrate size classes (mm): 0.0 – 0.9, 1.0 – 1.9, 2.0 – 2.9, 3.0 – 3.9, 4.0 – 4.9, 5.0 – 5.9, 6.0 – 6.9. Bars with different letters are significantly different from each other (Fisher’s least significant difference, $P < 0.05$).

2.4.3 Leaf traits of *Drosera rotundifolia*

Table 8 Leaf traits of *Drosera rotundifolia* plants growing at two ombrotrophic bogs in the UK that vary by N deposition input. Presented are the mean \pm 1 S.E. per plant for: leaf area, LA; specific leaf area, SLA; CIELAB a* score of leaf redness, colour; stickiness per unit leaf area, stickiness. Significant effects at $P < 0.05$ are highlighted in bold.

Site		LA (cm ²)	SLA (m ² kg ⁻¹)	Colour (CIELAB a*) ²	Stickiness (Newtons cm ⁻² leaf)
Cors Fochno	Mean	0.460	29.48	8.09	0.516
	SE	0.023	3.26	0.64	0.025
Whixall Moss	Mean	0.683	72.19	7.80	0.228
	SE	0.042	10.07	0.72	0.015
Independent samples	t-test results ¹				
	<i>P</i>	<0.001	0.001	0.766	<0.001

¹ Comparing differences between sites.

² Positive values represent a red coloration and negative values a green coloration.

Drosera rotundifolia plants at Whixall Moss possessed significantly larger leaf area (LA) and specific leaf area (SLA) than plants at Cors Fochno (Fig.s 10(a), 10(b); Table 8). The leaves of plants at Cors Fochno were marginally redder than the leaves of plants at Whixall Moss, however a statistically significant difference in leaf redness between sites was not found (Fig. 10(c); Table 8).

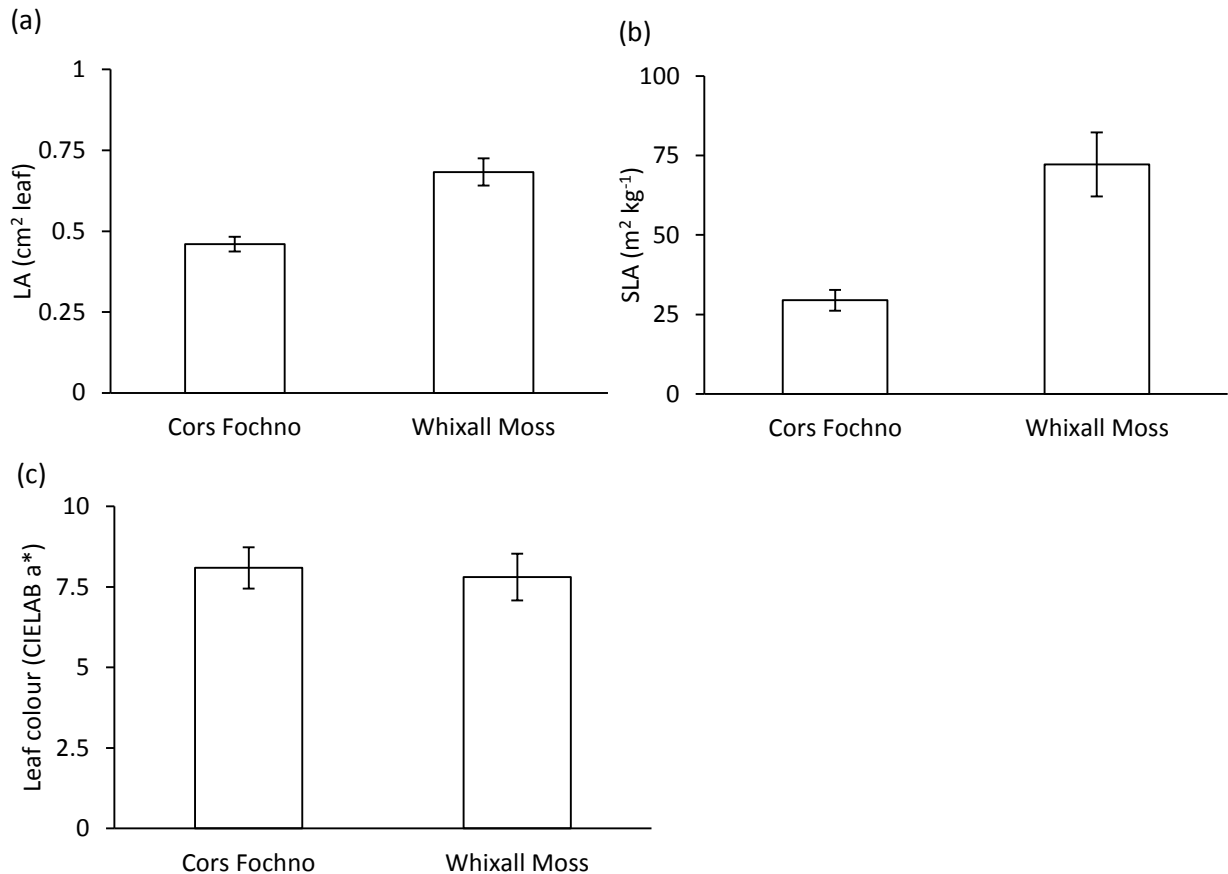


Figure 10 Leaf traits of *Drosera rotundifolia* plants growing in two ombrotrophic bogs in the UK that vary by N deposition input. Presented are the mean \pm 1 S.E. for: (a) leaf area, LA; (b) specific leaf area, SLA; (c) CIELAB a* score of leaf colour from green to red.

The leaves of *D. rotundifolia* plants at Cors Fochno were significantly stickier than the leaves of plants at Whixall Moss (Fig. 11(a); Table 8); the leaves of plants at Cors Fochno were over twice as sticky as the leaves of plants at Whixall Moss. For plants at Cors Fochno, a significant negative correlation was found between leaf stickiness and total dry mass per plant (Pearson's correlation coefficient, r , = -0.709, P < 0.022, n = 10) (Fig. 11(b)) however no relationship between these variables was found for plants at Whixall Moss (r = 0.335, P = 0.345, n = 10) (Fig. 11(b)).

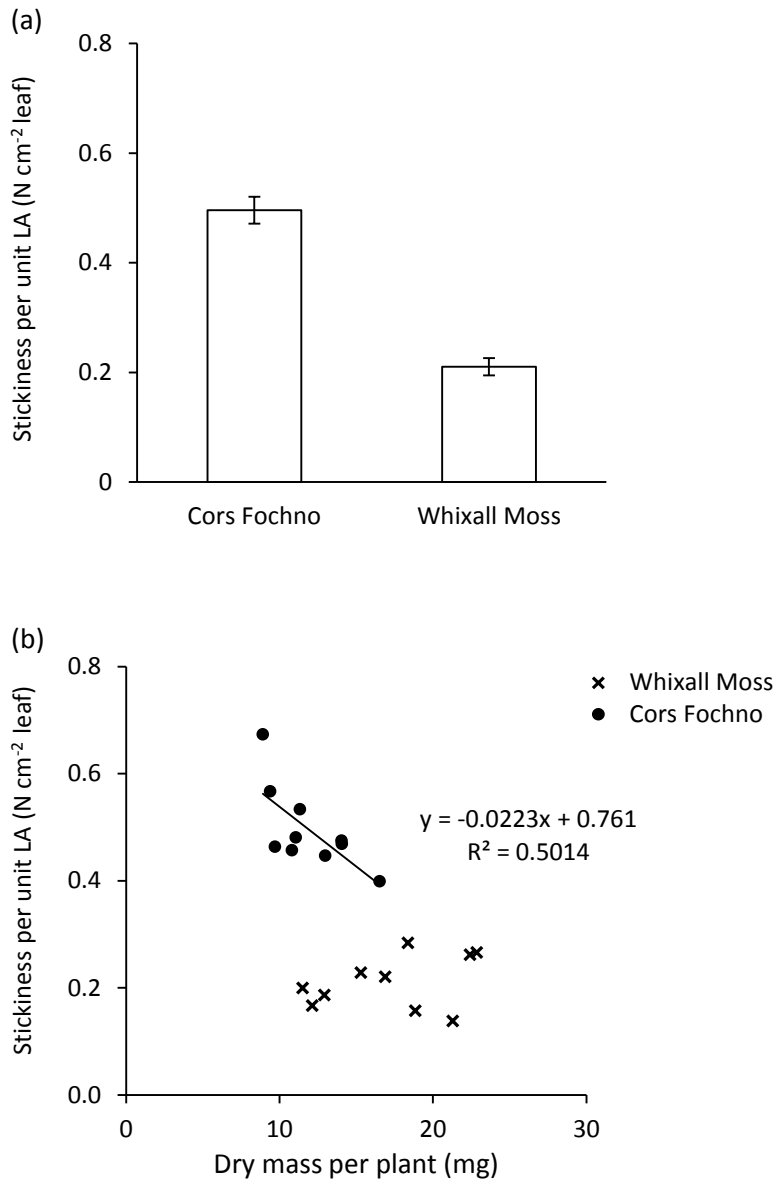


Figure 11 Parameters influencing leaf stickiness of *Drosera rotundifolia* plants growing in two ombrotrophic bogs in the UK that vary by N deposition input. Presented are: (a) mean \pm 1 S.E. for stickiness per unit leaf area, and (b) scatterplot displaying the relationship between stickiness per unit leaf area and plant dry mass. Each data point represents the mean values from 15 *D. rotundifolia* plants per plot (n = 10 per site).

Table 9 Results of Pearson’s correlation between leaf traits and parameters of prey capture for *Drosera rotundifolia* plants growing at two ombrotrophic bogs in the UK that vary by N deposition input. The following variables are presented: leaf area, LA; leaf colour (CIELAB a* score of leaf redness), colour; the probability of ‘prey’ capture success (PPCS) by *D. rotundifolia* for all invertebrate orders, PPCS_{total} and for the three most frequently captured invertebrate orders of Diptera, PPCS_{Diptera}, Formicidae, PPCS_{Formicidae}, and Hemiptera, PPCS_{Hemiptera}. Significant effects at $P < 0.05$ are highlighted in bold.

		Colour	PPCS _{total}	PPCS _{Diptera}	PPCS _{Formicidae}	PPCS _{Hemiptera}
LA	Pearson’s correlation	0.268	-0.225	-0.558	0.412	-0.300
	<i>P</i>	0.253	0.340	0.011	0.071	0.212
Colour	Pearson’s correlation		0.129	-0.197	0.249	0.112
	<i>P</i>		0.588	0.406	0.290	0.647

No significant correlations were found between leaf colour and PPCS variables, or between leaf colour and leaf area (Table 9).

2.4.4 Life history and physiological traits of *Drosera rotundifolia*

Drosera rotundifolia plants growing at Whixall Moss were significantly larger than plants growing at Cors Fochno (Fig. 12(a); Table 10). Plants at Whixall Moss possessed significantly higher RMR and significantly lower RE than plants at Cors Fochno (Fig. 12(b), 12(d); Table 4). Plant survival rate did not differ significantly between sites (Fig. 12(c); Table 10).

Table 10 Life history and physiological traits of *Drosera rotundifolia* plants growing at two ombrotrophic bogs in the UK. Presented are the mean \pm 1 S.E. for: total dry mass per live plant, mass; root to mass ratio per live plant, RMR; survival rate and net reproductive effort (proportion of dry mass of reproductive structures of the total plant dry mass), RE. Significant effects at $P < 0.05$ are highlighted in bold.

Site		Mass ¹ (mg)	RMR	Survival %	Net RE
Cors Fochno	Mean	11.91	0.121	98	0.184
	SE	0.59	0.008	1.1	0.202
Whixall Moss	Mean	17.56	0.098	95	0.368
	SE	0.76	0.004	1.8	0.226
Independent samples results ²	<i>t</i> -test <i>P</i>	<0.001	0.015	0.126	<0.001

¹ Data were log₁₀-transformed before analysis.

² Comparing differences between sites.

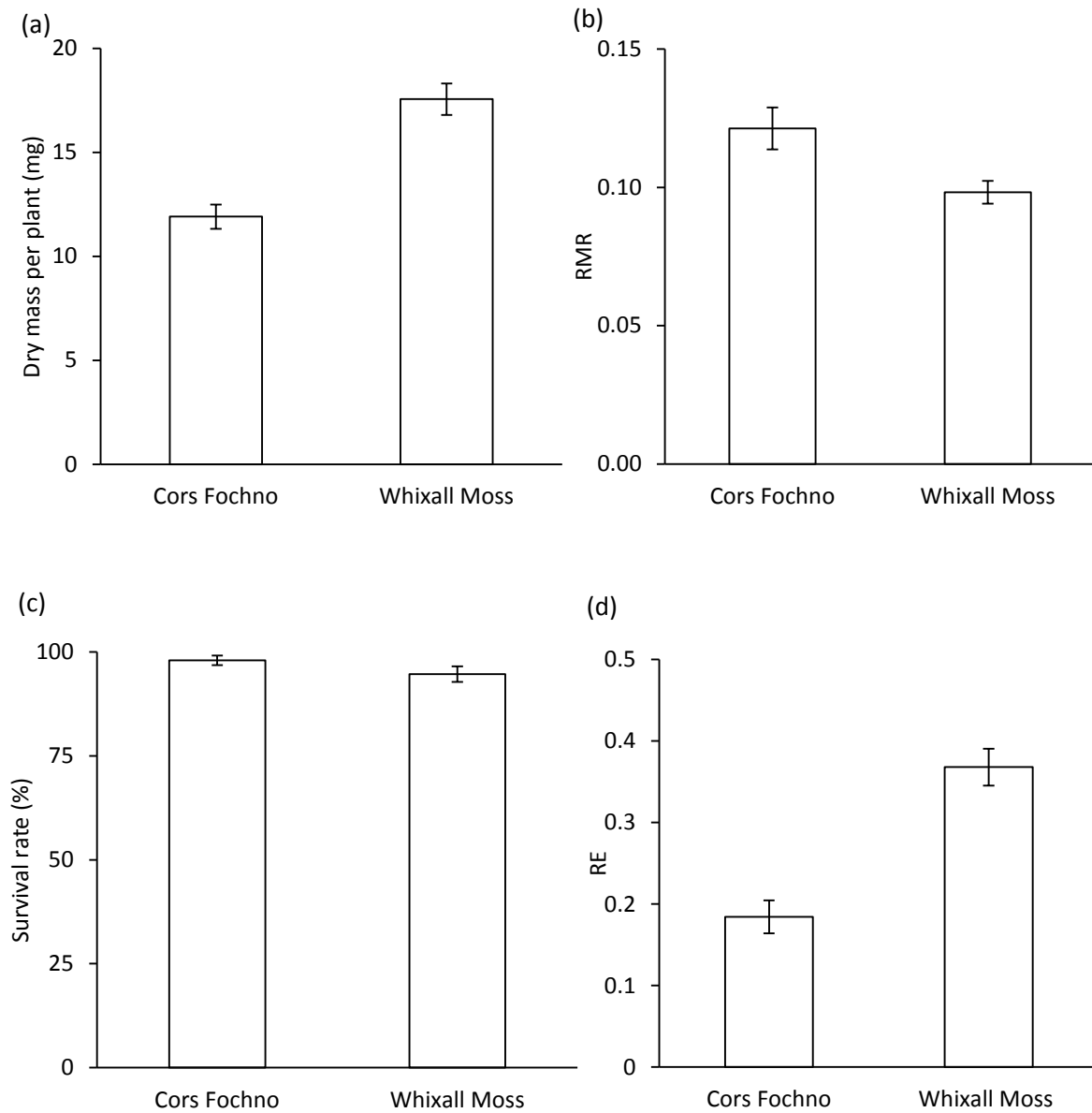


Figure 12 Life history traits of *Drosera rotundifolia* plants growing in two ombrotrophic bogs in the UK that vary by N deposition input. Presented are the mean ± 1 S.E. for: (a) dry mass per live plant; (b) root mass ratio per live plant, RMR; (c) survival rate (percentage of plant sample population alive at the end of the experimental period); (d) net reproductive effort per live plant, RE.

2.5 Discussion

Arguably the most important question to be addressed by this study is whether evidence from the data exists to support the classification of *Drosera rotundifolia* as a prey generalist or specialist; to be eligible as the latter, the plant species must possess the ability to selectively attract and capture particular size class(es) and/or order(s) from the background invertebrate population. Results for the probability of 'prey' capture (PPCS) and actual prey (relative abundance, (RA)) data show that the 'diet' of *D. rotundifolia* plants was comprised of twelve invertebrate orders; a result corresponding with the wide taxonomic range of captured prey reported by earlier studies (Darwin, 1875; Schmid, 1912; van Achterberg, 1973, Thum, 1986; Crowder et al., 1990; Volkova et al., 2010; Foot et al., 2014) and providing preliminary support for the classification of the species as a dietary generalist. Results showing that plants at Cors Fochno were significantly more likely to capture invertebrates of the smallest size class of 0.0 – 0.9 mm and marginally more likely to capture invertebrates of the 1.0 – 3.9 mm size classes than plants at Whixall Moss, are likely to reflect between-site differences in plant investment in prey capture; leaves of plants at Cors Fochno were stickier and therefore presumably possessed higher prey retention capacities than the leaves of plants at Whixall Moss. This result therefore further supports the classification of *D. rotundifolia* as a generalist, where the prey spectrum represents a random sample of potential prey, with prey size class distribution representing the prey retention capacity of the leaves.

The incremental decrease of PPCS with increasing invertebrate size class indicates that larger invertebrates were more likely to escape once adhered to the leaf than smaller invertebrates. This indication corresponds with the results of a study of *Pinguicula vallisneriifolia* (Zamora, 1995), which found the prey retention capacity of the plants to be positively correlated with leaf stickiness, and that prey retention capacity decreases as prey size increases, and of a study exploring invertebrate prey escape using spider-less orb webs by Nentwig (1982), which showed web escape time of prey to decrease with increasing prey size. Results of this study further show that the majority of invertebrate orders shared similar PPCS values, indicating that positive selection of particular orders did not occur and therefore supporting the classification of *D. rotundifolia* as a generalist. The exception to the pattern, of significantly higher PPCS for Acarina by plants at Whixall Moss, is more likely to reflect the average invertebrate size per order rather than the positive selective attraction and capture of the order; Acarina captured at Whixall Moss were significantly smaller than remaining orders per site, therefore they were presumably less likely to escape once adhered to leaf mucilage than orders with larger average sizes.

Results comparing the influence of prey specialisation measures of the RA of actual prey and PPCS show substantial influence of the measure used on the evenness and distribution of captured prey by order and size class. RA results showed a large peak of *ca.* 50% for Diptera, indicating a higher degree of prey specialisation by *D. rotundifolia* than PPCS results, which showed a more even distribution across orders. In terms of invertebrate size distribution, RA values were significantly larger than PPCS values; RA peaked at 1.0 – 1.9 mm size class, whereas PPCS values peaked at 0.0 – 0.9 mm size class. Therefore, RA data suggest that *D. rotundifolia* displays a higher degree of prey specialism through the capture of Diptera that are mostly within the 1.0 – 1.9 mm size class, whereas PPCS data suggest that *D. rotundifolia* is best described as a generalist that captures any order of invertebrate that lie mostly within the 0.0 – 0.9 mm size class. Therefore the prey specialisation calculation measure used directly influences the recommendation that may be made regarding the dietary strategy of *D. rotundifolia*, and therefore raises the question of which measure is most representative of the degree of dietary specialisation of *D. rotundifolia*. Both methods have advantages and disadvantages:- RA as a measure of actual prey capture is precise, but only when the abundance of background potential prey is equally distributed across orders / size classes that are captured by *D. rotundifolia* – likely to be an almost non-existent occurrence for *in-situ* invertebrate communities. PPCS offers the advantage of incorporating background availability of potential prey, however the sampling method(s) used to survey background invertebrates may not collect sample populations that are fully representative of abundance and distribution of orders and size classes of potential prey to *D. rotundifolia*. In this study, PPCS is considered to be most representative measure of the degree of dietary specialisation by *D. rotundifolia* as the abundance of orders and size classes of the background invertebrate sample populations were heterogeneous within and between sites, thus negating the use of RA. Therefore, these results indicate *D. rotundifolia* to be a dietary generalist that is most likely to capture prey within the 0.0 – 0.9 mm size class.

Comparisons of the results of this study, and of results in the literature, illustrate the substantial influence of sampling method on the order and size class distribution and abundance of background invertebrates captured, and therefore on the PPCS per order / size class. Most studies that have surveyed background invertebrate populations of potential prey to carnivorous plant species utilising ‘adhesive’ prey capture mechanisms have used artificial sticky traps placed near to the plants *in-situ* (Karlsson *et al.*, 1987; Zamora, 1990, 1995; Antor and Garcia, 1994; Alcalá and Domínguez, 2003; Jennings *et al.*, 2010; Foot *et al.*, 2014), with the exception of a prey specialisation study of three sympatric *Drosera spp.* by Volkova *et al.* (2010), which utilised window flight-traps and pitfall traps to sample flying and flightless invertebrates respectively. In this study, background potential prey data from the use of artificial sticky traps were not obtained as the glue lost its adhesiveness following heavy rainfall, therefore background potential prey data was represented by the aggregated sample

population of invertebrates captured by pitfall trap (representing flightless species) and sweep net (representing flying species).

Results of this study show large differences in the abundance of background invertebrates captured by each sampling method influenced the precision of the PPCS data. The abundance of invertebrates captured by pitfall trapping was nearly fivefold larger than the abundance captured by sweep netting; a difference that may more strongly reflect variation in potential trapping time between the sampling methods (pitfall trapping was conducted continuously throughout the course of the experiment whereas sweep netting was conducted at short, discrete intervals) rather than the differences in the absolute numbers of orders captured predominantly by each method. This difference in absolute numbers of background invertebrates collected by each sampling method directly affected PPCS data per order and per size class. The PPCS of orders comprised of mostly flying species that were captured predominantly by sweep net (e.g. Diptera, Coleoptera) were likely to be over-represented, whereas the PPCS of orders comprised of flightless species (e.g. Araneae, Formicidae) that were captured predominantly by pitfall trap were likely to be under-represented; this is indicated by the PPCS results (Fig. 12(a)). Thus, actual PPCS by *D. rotundifolia* may have been more evenly distributed across invertebrate orders than is suggested by the results.

In terms of size distribution of background invertebrate sample populations, invertebrates captured by sweep net were smaller on average than invertebrates captured by pitfall (possibly as larger flying invertebrates are typically less likely to be captured by sweep net than by pitfall trap), and the magnitude of difference in size was larger at Cors Fochno than at Whixall Moss. Thus, the PPCS values for smaller size classes, particularly 0.0-0.9 mm, may have been over-represented at both sites and over-represented by a larger magnitude at Cors Fochno, implying that the actual prey retention capacity of plants at both sites for larger prey was greater, and that the actual prey retention capacity of plants at Cors Fochno for larger prey was greater than plants at Whixall Moss. Therefore, whilst the precision of the PPCS results was influenced by the limitations of background sampling methods, results still support the classification of *D. rotundifolia* as a dietary generalist.

For studies utilising artificial sticky traps to survey background invertebrates of potential prey, results show that orders of flying species were most frequently captured e.g. Diptera, whereas orders comprised of predominantly flightless species (e.g. Formicidae) were rarely captured (Zamora, 1990, 1995; Antor and Garcia, 1994; Alcalá and Domínguez, 2003; Jennings *et al.*, 2010; Foot *et al.*, 2014). Thus, for *Drosera rotundifolia*, for which the capture of flying and flightless species is well documented (e.g. Crowder *et al.*, 1990; Jennings *et al.*, 2010; Foot *et al.*, 2014), the background sample population collected using this sampling method may not be fully representative of potential prey to the plant. This inference is apparent upon comparison of the results of relative abundances of potential prey captured using sticky artificial traps and actual prey captured by *D. rotundifolia* presented by Jennings

et al. (2010); artificial traps captured a larger proportion of Diptera (*ca.* 40 %) and a smaller proportion of Formicidae (*ca.* 12 %) than plant leaves (Dipt. = *ca.* 33 %; Form. = *ca.* 24 %), therefore indicating that PPCS by the plant is under-represented for Diptera and over-represented for Formicidae. This may have implications for the recommendation made regarding the dietary strategy of the plant; the under-representation of the PPCS for Diptera may imply that true prey capture indicates that *D. rotundifolia* is a dietary specialist preferring Diptera, as opposed to indication of a dietary generalist that was made. Therefore, selection of the appropriate sampling methods for surveying background invertebrate population of potential prey requires consideration of the typical diet and micro-habitat of the plant. In the case of *Drosera rotundifolia*, as a low-lying plant that captures flying and flightless orders of prey, combined with the results from this study showing the contrasting advantages and disadvantages of each sampling method of background invertebrates, indicate that future prey capture studies would benefit from employing a multi-sampling approach to surveying background potential prey, where artificial sticky traps, pitfall trapping and sweep netting are used.

The result showing *Drosera rotundifolia* plants at Cors Fochno to capture significant greater mass of invertebrates than plants at Whixall Moss may be explained by the significantly greater investment in prey capture by plants at Cors Fochno of increased leaf stickiness. However, inferences that may be made with certainty using captured prey mass data alone are limited; prey mass differences may be confounded by between-site variation in prey availability:- specifically, differences in the abundance or average mass of invertebrates constituting the background population of 'potential' prey. Therefore, the probability of prey capture success (PPCS_{total}), arguably represents a more realistic parameter of the degree of prey specialisation or generalisation by the plant. Results of studies in the literature show that as root N availability to carnivorous plants increases, plant investment in prey capture and reliance on carnivory decreases (Thorén *et al.*, 2003; Millett *et al.*, 2012). It is assumed, therefore, that PPCS (and therefore reliance on prey capture) increases as plant reliance on carnivory increases. The results of this study show that *D. rotundifolia* plants at Cors Fochno were smaller and displayed higher PPCS_{total} than plants at Whixall Moss, and that pore water at Cors Fochno was significantly lower in DIN compared with pore water at Whixall Moss, indicating, for the first time, that plant reliance on prey capture decreases with increasing DIN and therefore [assumed] increasing root N availability. This result corresponds with the results of studies indicating the phenotypic plasticity of *D. rotundifolia* to changes in N availability in terms of investment and reliance on the trait of carnivory, and provides support for the energetic cost/benefit model for the evolution of carnivory as proposed by Givnish *et al.* (1984). However, it should be acknowledged that captured prey were collected from a fixed number of randomly sampled leaves on which captured prey were present at each site, and therefore this measure is not fully representative of the prey capture reliance of the plant population at each site. Future studies would benefit from taking a random sample of a fixed number of leaves at each

site, recording the number and area of leaves containing prey and the number and area of leaves with prey absent, so that the probability of prey capture per leaf / leaf area can be calculated.

The results showing *Drosera rotundifolia* plants growing at Whixall Moss to be larger in size and display higher fitness than plants growing at Cors Fochno are likely, but not proven, to be as a result of the significantly higher DIN (and therefore assumed higher root N availability) at Whixall Moss compared with Cors Fochno; positive relationships between root N availability and plant biomass, and between root N availability and net reproductive effort, for angiosperms of N-limited ecosystems are well documented (Bloom *et al.*, 1985; Güsewell and Koerselman, 2002). The result of significantly larger biomass of plants at Whixall Moss is in agreement with the results of the majority of studies investigating the influence of root N availability on the biomass of *Drosera* species (Karlsson *et al.*, 1991; Adamec *et al.*, 1992) and other carnivorous plant genera (Harder and Zemlin, 1967; Christensen, 1976; Aldenius *et al.*, 1983), and therefore supports the classification of *D. rotundifolia* as a “nutrient-requiring” species of carnivorous plant (Adamec, 1997), where new biomass is produced upon uptake of N; in this case predominantly via the roots. The significantly lower net reproductive effort of *D. rotundifolia* plants growing at Cors Fochno suggests that low root N availability restricted the allocation of resources away from essential vegetative functions such as growth and maintenance (Bazzaz *et al.*, 1987). This result appears to contrast with those of a manipulative study by Thorén *et al.* (2003), which showed that differences in substrate N concentrations of 0.05 mM and 5.0 mM exerted no statistically significant effect on the number of inflorescences produced by *D. rotundifolia*, however this difference may be explained by the production of larger inflorescences by plants exposed to high N availability; inflorescence dry mass was not measured.

The implied greater degree of N-limitation acting on *D. rotundifolia* plants growing at Cors Fochno is supported by results for allocational traits such as investment in prey capture and the shoot to root ratio. The significantly stickier leaves of plants at Cors Fochno demonstrate a phenotypically plastic resource allocation shift from investment in N uptake via the roots to investment in N uptake via the leaves from increased prey capture; N acquisition via the roots is less energetically costly than N acquisition from the capture and digestion of invertebrate prey (Karagatzides and Ellison, 2009). This result is supported by the literature; reliance on the trait of carnivory by *Drosera rotundifolia* decreased along an N deposition gradient from 1.9 kg N ha⁻¹ yr⁻¹ to 11.3 kg N ha⁻¹ yr⁻¹ (Millett *et al.*, 2012), and leaves of *D. rotundifolia* plants exposed to a substrate N concentration of ca. 5 mM were significantly less sticky than the leaves of plants exposed to a substrate N concentration of 0.05 mM (Thorén *et al.*, 2003). Plants growing at Cors Fochno showed higher proportional resource allocation to root biomass, as indicated by significantly higher RMRs, suggesting partial functional compensation for limited root N availability and providing support for the optimal partitioning model (*cf.* Gedroc *et al.*, 1996; Sultan, 2000). The result of no significant difference in plant survival rate between sites suggests

DIN (and assumed therefore root N availability) did not influence plant survival; however it is acknowledged that it would be more appropriate to measure this variable over the entire lifespans of the survey plants (maximum lifespan of *ca.* 5 years reported for *D. rotundifolia* (Crowder *et al.*, 1990)).

The results relating to functional leaf traits of *Drosera rotundifolia* provide evidence for the influence of resource availability (in this case, root N availability) on plant trade-offs between allocation towards resource acquisition and the conservation of existing resources (Lienin and Kleyer, 2011). The greater LA and SLA of plants growing at Whixall Moss compared with plants growing at Cors Fochno indicates that plants at Whixall Moss were exposed to higher root N availability through the well documented positive correlations of SLA with soil fertility and leaf N concentration (Garnier *et al.*, 1997; De Frenne *et al.*, 2011) and displayed higher potential growth rates (e.g. Garnier, 1992; Lambers and Poorter, 1992) than plants at Cors Fochno, the latter indication being supported by the significantly greater biomass of plants at Whixall Moss. The smaller SLA of plants growing at Cors Fochno indicates plastic responses to low root N availability by the larger proportional allocation of resources to leaf conservation and therefore preservation of plant N (Reich *et al.*, 1999).

Benefits of increased plant investment in prey capture were evident; leaf stickiness showed strong, positive correlations with the dry mass of invertebrates captured per unit leaf area, $PPCS_{total}$, $PPCS_{Diptera}$ and $PPCS_{Hemiptera}$, presumably as the prey retention capacity per unit leaf area increased with increasing leaf stickiness. Results showing a significant negative relationship between LA and leaf stickiness, and that the probability of prey capture ($PPCS_{total}$) was uninfluenced by LA but positively correlated with leaf stickiness, indicate a leaf area-stickiness resource trade-off. Additionally, the result showing leaf stickiness to decrease as plant size increases indicates that the trade-off is size-dependent, with proportionally higher investment made towards increasing leaf area as opposed to stickiness as plants grow larger. This trade-off may also therefore be influenced by root N availability, due to the significant influence of root N availability on plant size. Similar leaf trait resource trade-offs are reported in the literature; a leaf size-area trade-off for *D. rotundifolia* plants is suggested to be influenced by resource availability by Foot *et al.* (2014), illustrating the complexity of the influence of resource availability on leaf trait relationships and of the allocational trade-offs between multiple traits. No evidence of selective prey attraction by *D. rotundifolia* was found by the results of this study, thus supporting the classification of the species as a generalist; no relationships were found between leaf colour and the probability of prey capture success of the three most frequently captured invertebrate orders, $PPCS_{Diptera}$, $PPCS_{Formicidae}$ and $PPCS_{Hemiptera}$. However, it should be noted that other potentially confounding mechanisms for prey attraction by *D. rotundifolia* of UV patterning and/or scent (Joel *et al.*, 1985; Jürgens *et al.*, 2009) were not addressed by this study; hypotheses that warrant further investigation.

Exploration of within-site patterns between relative investment in prey capture and plant dry mass for *D. rotundifolia* plants showed leaf stickiness increases as plant size decreases for plants at Cors Fochno, but no relationship between these variables was found for plants at Whixall Moss. This seems a likely result; small-sized plants at Cors Fochno were exposed to low root N availability and would have had greater demand for prey-derived N, and therefore invested more heavily in leaf stickiness, than small-sized plants at Whixall Moss, which would be able to acquire a greater amount of N via the roots. This suggestion is in keeping with the results of Millett *et al.* (2012), which show that plant reliance on prey-derived N by *D. rotundifolia* decreases as root N availability increases. However, the validity of this result is limited by the measurement of leaf stickiness and plant mass in different years; the potential influence of interannual, within-site variation in resource availability (e.g. root N or light availability) on either physiological variable cannot be discounted. Therefore, further data collection using identical plants during the same growth season at both sites is required to clarify the nature of the relationship between these variables.

Results indicate that differences in invertebrate form and behaviour between orders may influence the PPCS by *D. rotundifolia*; significant positive correlations were found between leaf stickiness and $PPCS_{Diptera}$ and $PPCS_{Hemiptera}$, but no significant relationship was found between leaf stickiness and $PPCS_{Formicidae}$. Indeed, results of Nentwig (1982) found Diptera and Hemiptera to exhibit longer web escape times than Formicidae and that this is likely to be explained by between-order differences in morphology (e.g. wing presence/absence or area), physiology (e.g. kinetic energy) or behaviour (e.g. web thread biting by Formicidae). The classification of *Drosera rotundifolia* as a passive prey capture strategist is further supported by the results of $PPCS_{total}$ to be uninfluenced by leaf colour, and of leaf colour to not differ significantly between sites; if colour was utilised by the plant as a primary mechanism of prey attraction and therefore be classified a parameter of investment in prey capture, then it would be expected to be negatively correlated with DIN (and therefore root N availability). This result contrasts with results reported by Foot *et al.* (2014), which showed redder leaves of *D. rotundifolia* to have a higher PPCS than greener leaves; a difference that is likely to be explained by the lack of measurement of prey capture variables, e.g. leaf stickiness, in the latter study, as acknowledged by the authors. Thus, results of this study indicate *D. rotundifolia* to be a dietary generalist, a classification that is supported by several previous studies of *D. rotundifolia* and other *Drosera* species (Jennings *et al.*, 2010; Foot *et al.*, 2014). However, it is acknowledged that PPCS incorporates the probability of invertebrate capture and the probability of invertebrate escape once trapped, of which only the former is a measure of prey attraction by the plant. In order therefore to provide definitive support for the classification of *D. rotundifolia* as a passive prey capture strategist, further manipulative studies that independently investigate each of these two prey capture parameters are required.

2.6 Conclusions

Findings of this study provide support for the classification of *Drosera rotundifolia* as a dietary generalist, where the order composition and size distribution of the captured prey spectrum represents random sampling from the potential prey population within the limitations of the prey retention capacity of the leaf mucilage. Plants exposed to 'low' root N availability possessed stickier leaves and captured greater dry mass of invertebrates per unit leaf area than plants exposed to 'high' root N availability, suggesting a phenotypically plastic response to resource availability and providing support for the cost/benefit model for the evolution of botanical carnivory (Givnish et al., 1984). No evidence of prey specialisation in terms of size and/or order in response to between-site differences in resource availability was found, suggesting that *D. rotundifolia* does not forage optimally in response to root N availability. Further evidence of phenotypic plasticity by *D. rotundifolia* in response to resource availability was found; plants at Whixall Moss were larger, invested more heavily in reproduction but made smaller proportional mass allocation to roots, indicating that, for plants exposed to low root N availability at Cors Fochno, allocation was directed away from essential vegetative functions such as growth and reproduction and invested into N uptake. Results showing that the total mass or type/size class of prey captured by the plant were uninfluenced by leaf colour, and the lack of a significant relationship between leaf colour and DIN, further indicate that leaf colour does not fulfil a primary prey attraction role.

The results of this study indicate that PPCS offers a more precise measure of the degree of dietary specialisation by carnivorous plants than the RA of actual prey, however consideration should be taken to select the most appropriate sampling method(s) in order to obtain a representative sample of potential prey to the carnivorous plant. Results from this study, and comparison with those of earlier studies, indicate that a multi-sampling method approach is the most appropriate. Future work using the data collected for this study would benefit from testing the influence of site, invertebrate order and invertebrate size on the PPCS for order and size class data combined, e.g. calculation of the PPCS for Diptera within 0.0-0.9 mm size class, etc. The following follow-up studies would address the gaps remaining in the research base of strategies for prey attraction and capture by carnivorous plants utilising adhesive prey capture mechanisms:-

As PPCS incorporates the probability of an invertebrate being captured by the plant, and the prey retention capacity of the plant, further manipulative research should explore whether (a) invertebrate size; (ii) invertebrate type influences each of these parameters independently.

Is the prey retention capacity of *D. rotundifolia* leaves controlled by mucilage adhesiveness alone, or does trichome density or droplet size also play a role? Does root N availability and/or prey availability influence leaf trichome density / droplet size?

Investigation of the suitability of artificial sticky traps for surveying potential prey to *D. rotundifolia* by (a) utilising identically sized artificial traps covered in non-drying glue of varying levels of adhesiveness; (b) quantifying the relative adhesiveness of the glue used in comparison to the adhesiveness of leaf mucilage, and (c) comparing the influence of the level of adhesiveness on the species composition and size distribution of invertebrates captured by traps and plants.

Is scent and/or UV patternation used for prey attraction by *D. rotundifolia*? If so, does plant investment in prey attraction increase as root N availability decreases or prey availability increases?

2.7 References

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2.8 Appendices

Appendix 1 Abundance and probability of ‘prey’ capture success (PPCS) data for invertebrates captured by *Drosera rotundifolia* plants growing at two ombrotrophic bogs in the UK. Presented are values for the relative abundance and PPCS (as a percentage of each order/size class to the total PPCS per site) for captured prey at each site by (a) order; (b) size class. Data of each column are ranked from highest to lowest percentage values.

(a)

Cors Fochno		Whixall Moss	
Relative abundance (%)	PPCS (%) ¹	Relative abundance (%)	PPCS (%) ²
1. Diptera (61.5)	1. Diptera (25.8)	1. Diptera (37.5)	1. Acarina (41.8)
2. Formicidae (11.4)	2. Collembola (21.8)	2. Formicidae (14.7)	2. Collembola (19.7)
3. Hemiptera (6.6)	3. Hymenoptera (16.6)	3. Coleoptera (8.1)	3. Orthoptera (12.7)
4. Hymenoptera (5.8)	4. Hemiptera (14.0)	4. Collembola (8.1)	4. Diptera (9.7)
5. Coleoptera (4.4)	5. Lepidoptera (9.8)	5. Hemiptera (8.1)	5. Hymenoptera (9.5)
6. Araneae (3.6)	6. Coleoptera (5.4)	6. Acarina (7.2)	6. Hemiptera (2.6)
7. Collembola (3.6)	7. Acarina (3.8)	7. Orthoptera (6.3)	7. Coleoptera (1.9)
8. Acarina (1.7)	8. Formicidae (2.2)	8. Hymenoptera (5.3)	8. Formicidae (1.7)
9. Orthoptera (0.8)	9. Araneae (0.5)	9. Araneae (4.4)	9. Araneae (0.4)
10. Dictyoptera (0.3)	10. Orthoptera (0.0)	10. Phthiraptera (0.3)	
11. Lepidoptera (0.3)			

¹ PPCS for Dictyoptera at Cors Fochno was not calculable as background Dictyoptera were not captured.

² PPCS for Phthiraptera at Whixall Moss was not calculable as background Phthiraptera were not captured.

(b)

Cors Fochno		Whixall Moss	
Relative abundance (%)	PPCS (%)	Relative abundance (%)	PPCS (%)
1. 1.0 to 1.9 mm (41.7)	1. 0.0 to 0.9 mm (73.3)	1. 1.0 to 1.9 mm (43.4)	1. 0.0 to 0.9 mm (63.7)
2. 2.0 to 2.9 mm (24.4)	2. 1.0 to 1.9 mm (17.2)	2. 2.0 to 2.9 mm (28.1)	2. 1.0 to 1.9 mm (20.0)
3. 0.0 to 0.9 mm (21.4)	3. 2.0 to 2.9 mm (7.0)	3. 3.0 to 3.9 mm (13.4)	3. 2.0 to 2.9 mm (10.3)
4. 3.0 to 3.9 mm (11.1)	4. 3.0 to 3.9 mm (2.3)	4. 0.0 to 0.9 mm (10.3)	4. 3.0 to 3.9 mm (3.2)
5. 4.0 to 4.9 mm (0.8)	5. 4.0 to 4.9 mm (0.1)	5. 4.0 to 4.9 mm (3.1)	5. 4.0 to 4.9 mm (1.4)
6. 5.0 to 5.9 mm (0.6)	6. 5.0 to 5.9 mm (0.1)	6. 5.0 to 5.9 mm (1.3)	6. 5.0 to 5.9 mm (0.9)
7. 6.0 to 6.9 mm (0.0)	7. 6.0 to 6.9 mm (0.0)	7. 6.0 to 6.9 mm (0.3)	7. 6.0 to 6.9 mm (0.5)

Chapter 3: Changing plant N use in response to atmospheric N deposition: differences among plant species with contrasting life history strategies.

3.1 Abstract

In this study, the effects of nitrogen (N) deposition on N uptake and use by co-occurring plant and bryophyte species were explored at two ombrotrophic raised bogs in the UK subject to 8.0 and 30.8 kg N ha⁻¹ yr⁻¹. Tissue N concentrations and natural abundance $\delta^{15}\text{N}$ signatures of *Calluna vulgaris*, *Drosera rotundifolia*, *Erica tetralix*, *Eriophorum vaginatum* and *Sphagnum fuscum* are reported. The influence of N deposition on the reliance of the carnivorous plant *Drosera rotundifolia* on the trait of carnivory was determined using the $\delta^{15}\text{N}$ natural abundance mixing model (Moran *et al.*, 2001).

Tissue N concentrations varied between species and between sites. Tissue N concentration increased with increased N deposition, indicating higher root N uptake at the high N deposition site. The difference in tissue N increase between sites varied between species, indicating the influence of species differences in functional type and N acquisition strategy on N uptake and retention.

The proportion of prey-derived N of the plant N budget of *Drosera rotundifolia* decreased from 49.41 % to 25.47 % as N deposition increased, providing evidence that plant reliance on the trait of botanical carnivory decreases as N deposition increases.

Tissue $\delta^{15}\text{N}$ signatures of the study species were variable, ranging from -3.8 to -11.9 ‰. Tissue $\delta^{15}\text{N}$ varied between sites, with some species showing larger differences than others. Implications of these results in light of species differences in functional type, N source preference, mycorrhizal status, and strategies for N acquisition and retention are discussed.

3.2 Introduction

Ombrotrophic peatland ecosystems receive inorganic N inputs solely via atmospheric deposition, and thus are predominantly N-limited (Lamers *et al.*, 2000). Mosses, particularly *Sphagnum* species, dominate ombrotrophic bogs and engineer the abiotic environment, which is characterised by waterlogging, anoxia, high acidity and nutrient-deficiency (Berendse *et al.*, 2001). Thus, compared with other ecosystem types, vascular plant density is low and only small numbers of plant species are able to withstand the harsh environment (Lamers *et al.*, 2000). Plant functional types of ombrotrophic bog communities are typically dominated by dwarf shrubs, e.g. *Calluna vulgaris* and *Erica tetralix*, graminoids, e.g. *Eriophorum vaginatum* and *Molinia caerulea*, and to a lesser extent by forbs, e.g. *Drosera rotundifolia* and *D. anglica* (Rodwell, 1991). The availability of inorganic N to vascular plants is controlled by co-occurring *Sphagnum spp.*, which intercept and absorb inorganic N from atmospheric deposition (Bragazza *et al.*, 2005). However, at N deposition inputs above the threshold of *ca.* 20 kg N ha⁻¹ yr⁻¹, the N filtering capacity of *Sphagnum spp.* ceases to function and N is released into the rhizosphere (Lamers *et al.*, 2000; Bragazza *et al.*, 2005). Therefore as N deposition increases, tissue N concentrations of bryophytes and plants increase (Limpens *et al.*, 2003; Bragazza *et al.*, 2005). These plant N nutrition changes trigger an increase in plant productivity, leading to community dominance by fast-growing nitrophilous species and to subsequent decline in species diversity and ecosystem functioning (Aerts *et al.*, 1992; Hartley and Amos, 1999; Tomassen *et al.*, 2003). Peatland ecosystems are therefore particularly vulnerable to increasing global rates of N deposition (Lamers *et al.*, 2000).

In order to acquire sufficient N for growth and maintenance under conditions of very low inorganic N availability, co-existing plant species of contrasting functional types have evolved a wide range of physiological adaptations for maximising nitrogen uptake and retention and nutrient use efficiency (NUE), and minimising plant N demand (Table 11). Many peatland species possess physiological adaptations and strategies that enable the offsetting of inter-specific competition for inorganic N, and the uptake of more energetically favourable forms of organic N, for example amino acid acquisition via mutualistic symbioses with mycorrhizal fungi (Marschner and Dell, 1994), direct absorption of free amino acids from the substrate (Chapin *et al.*, 1993), or from the absorption of prey-derived N obtained via the trait of botanical carnivory (Darwin, 1875). Ericoid shrubs maintain high rates of N retention and NUE through their evergreen habit, whereby the thick and waxy morphological traits of leaves minimise N loss via leaching, and by low tissue nutrient concentrations and long tissue life-spans (Aerts, 1995). However, a trade-off between these adaptations and productivity exists; evergreen species typically possess lower rates of growth and maximum photosynthesis than co-occurring deciduous species (Reich *et al.*, 1998; Shipley *et al.*, 2006). For graminoids, high relative growth rates compared with forbs are achievable largely due to high NUE as a result of high N

resorption rates from senescing tissues to new growth, and sequential leaf development (Fetcher and Shaver, 1983; Jonasson and Chapin, 1985).

Table 11 Characteristics of four co-habiting vascular plant species that frequently occur in plant assemblages of ombrotrophic raised bogs in the UK. Presented are: life history, life-form, functional type, established strategy, the presence and type of mycorrhizal association (ERM = ericoid mycorrhizal fungi; VA = vesicular arbuscular fungi) and maximum rooting depth.

Characteristic	Plant species			
	<i>Calluna vulgaris</i>	<i>Drosera rotundifolia</i>	<i>Erica tetralix</i>	<i>Eriophorum vaginatum</i>
Life history	Polycarpic perennial ^a	Polycarpic perennial ^a	Polycarpic perennial ^a	Polycarpic perennial ^a
Life-form	Woody chaemaephyte / phanerophyte ^a	Helophyte ^a	Woody chaemaephyte / phanerophyte ^a	Helophyte ^a
Functional type	Dwarf shrub ^a	Forb ^a	Dwarf shrub ^a	Graminoid ^a
Established strategy	Intermediate between stress-tolerant competitor and stress-tolerator ^a	Stress-tolerant ruderal ^a	Intermediate between stress-tolerant competitor and stress-tolerator ^a	Intermediate between stress-tolerant competitor and stress-tolerator ^a
Mycorrhizal association?	Yes ^a	Maybe ^{a,b,c}	Yes ^a	No ^d
Mycorrhizal type	ERM ^e	VA ^a	ERM ^f	-
Maximum rooting depth (cm)	40 ^g	<6 ^h	23 ⁱ	20 ^j

^a Grime *et al.*, 2007.

^b Quilliam and Jones, 2010.

^c Rydin and Jeglum, 2013.

^d Chapin *et al.*, 1993.

^e Johansson, 2000.

^f Yesmin *et al.*, 1996.

^g Aerts *et al.*, 1992.

^h Crowder *et al.*, 1990.

ⁱ Aerts *et al.*, 1989.

^j Chapin *et al.*, 1979.

Evidence in the literature supports the prediction that N deposition inputs to ombrotrophic bogs trigger changes in plant N uptake and use within- and between-species (Limpens *et al.*, 2003; Nordbakken *et al.*, 2003; Tomassen *et al.*, 2003). As N deposition increases, rates of mineralization and nitrification increase, leading to increased availability of NH₄-N and NO₃-N to plants, and shifts in the dominant inorganic N form (Nordbakken *et al.*, 2003; de Graaf *et al.*, 1998). N uptake by different plant functional types varies in response to N deposition due to differences in N form preference, rooting depth, competitive ability and plant N demand, the latter a product of relative growth rate, size, and

NUE (Aerts, 1990; Nordbakken *et al.*, 2003). For mycorrhizal plant species, plant reliance on mycorrhizal symbionts decreases as N deposition increases (Yesmin *et al.*, 1996; Michelsen *et al.*, 1998; Hobbie *et al.*, 2000). For carnivorous plants, changes in N deposition input have been linked to changes in N source uptake; an *in-situ* study of *D. rotundifolia* (Millett *et al.*, 2012) found plant reliance to switch from predominantly prey-derived N at an N deposition input of 1.9 kg N ha⁻¹ yr⁻¹ to predominantly root-derived N at N deposition inputs of 3.8 and 11.3 kg N ha⁻¹ yr⁻¹. This phenotypic plasticity of plant reliance on prey-derived N by carnivorous plants in response to changes in N deposition inputs, along with the difference in $\delta^{15}\text{N}$ between the relatively enriched prey N and the relatively depleted root N that comprise the $\delta^{15}\text{N}$ of the carnivorous plant, may lend the use of the $\delta^{15}\text{N}$ of a carnivorous plant species as an indicator of relative differences in N deposition inputs between sites.

However, the need for further data acquisition investigating plant N uptake patterns under field conditions remains (Näsholm *et al.*, 2009). Over the last few decades, the application of natural abundance $\delta^{15}\text{N}$ analysis as a tool for plant ecophysiology research has increased in popularity (Hobbie and Högberg, 2012), but only a handful of studies exist that use the technique for exploring plant N uptake and use in ombrotrophic bogs. The $\delta^{15}\text{N}$ signatures of plant species are highly variable, and may be influenced by plant functional type (Asada *et al.* 2005), mycorrhizal status and form (Michelsen *et al.*, 1998), ability to absorb and source different chemical forms of N (Schulze *et al.*, 1994) and maximum rooting depth (Kohzu *et al.*, 2003). Therefore, natural abundance $\delta^{15}\text{N}$ analysis represents a comprehensive method for exploring differences in N uptake and use by plant species of ombrotrophic bog communities.

In this study, the influence of N deposition on N uptake and use by wetland plant and bryophyte species with contrasting life history strategies, growth forms and mycorrhizal statuses is explored using co-occurring plants growing at two ombrotrophic bogs in the UK that receive contrasting N deposition inputs. Specifically, the following aims are addressed:-

1. To investigate the influence of N deposition on N uptake by plant species with contrasting life history strategies.
2. To investigate the influence of N deposition on N nutrition of plant species with contrasting life history strategies.
3. To test the hypothesis that plant reliance on prey-derived N by the carnivorous plant *Drosera rotundifolia* decreases with increasing N deposition.
4. To explore the potential use of tissue $\delta^{15}\text{N}$ of *Drosera rotundifolia* plants as a relative indicator of N deposition inputs acting on peatland sites.

Aims 1 and 2 are achieved by comparing N uptake and nutrition for plants varying by life history strategy (*Drosera rotundifolia* (carnivorous forb), *Calluna vulgaris* (shrub), *Erica tetralix* (ericoid shrub), *Eriophorum vaginatum* (graminoid) and *Sphagnum fuscum* (moss)) growing at two ombrotrophic bogs in the UK that receive contrasting N deposition inputs.

Aim 3 is achieved by comparing plant reliance on the trait of botanical carnivory for *Drosera rotundifolia* plants growing at two ombrotrophic bogs in the UK that receive contrasting N deposition inputs. Plant reliance on carnivory, defined as the relative contribution of prey-derived N to the total N budget of the plant (%N_{dfp}), is calculated using the $\delta^{15}\text{N}$ natural abundance linear mixing model. Tissue $\delta^{15}\text{N}$ values of *D. rotundifolia*, co-occurring non-carnivorous plants, and invertebrate prey captured by *D. rotundifolia* plants are entered into the equation of the mixing model in order to calculate %N_{dfp}.

Aim 4 is achieved by quantifying the relationship between *Drosera rotundifolia* tissue $\delta^{15}\text{N}$ and N deposition inputs at the site level using data for *D. rotundifolia* sample populations at the sites used in this study and data for *D. rotundifolia* sample populations at other sites reported in the literature.

These data are used to form recommendations as to how N uptake and use by plant species varying by functional type, mycorrhizal status and N acquisition and retention strategies respond to changes in the abiotic environment, in particular the impact of increased inorganic N availability as a result of increased N deposition.

3.3 Methods

3.3.1 Study sites

Fieldwork was undertaken from May to September 2011 at two ombrotrophic raised bogs in the United Kingdom which differ primarily by N deposition load (Table 12). Whixall Moss is a lowland raised peat bog in Shropshire, England, and Cors Fochno is an estuarine lowland raised peat bog in Ceredigion, Wales.

Table 12 Locations, climatic and environmental characteristics of Cors Fochno and Whixall Moss ombrotrophic raised bogs. Table (a) summarises site characteristics for 2006 to 2011 (where possible), this time period representing the maximum potential life span of survey *Drosera rotundifolia* plants; Table (b) summarises site characteristics for 2011 during which fieldwork was undertaken.

(a)

Site	Location	Mean annual precipitation (mm yr ⁻¹) ^a	Mean temperature January / July (°C) ^a	Mean growing season length (d) ^b	Growing season average temperature (°C) ^b	N deposition (kg N ha ⁻¹ yr ⁻¹) ^c
Cors Fochno	52°30'09N, 04°00'57W	1381	3.5/14.7	320	11.6	8.0
Whixall Moss	52°92'16N, 02°76'45W	719	4.0/13.7	296	11.0	30.8

^a Based on observed meteorological data from KNMI Climate Explorer (<http://climexp.knmi.nl>) (accessed 08.09.2014). Data are mean values for 2006 - 2011 inclusive.

^b Growing season is the number of days with mean temperature ≥ 5°C. Data are mean values for 2011 – 2012 inclusive (earlier years unavailable). Based on observed meteorological data from automatic weather stations at each site; data accessed from Countryside Council for Wales (Cors Fochno) and Natural England (Whixall Moss).

^c Modelled N deposition data from APIS (<http://www.apis.ac.uk/>) (accessed 21.04.2014). Data are mean values for 2010-2012 inclusive (earlier years unavailable).

(b)

Site	Annual precipitation (mm yr ⁻¹) ^a	Temperature January / July (°C) ^a	Growing season length (d) ^{a,b}	Growing season average temperature (°C) ^{a,b}
Cors Fochno	1008	3.7/15.0	324	12.1
Whixall Moss	565	2.5/14.2	301	11.5

^a Data values for 2011 only. Based on observed meteorological data from automatic weather stations at each site; data accessed from Countryside Council for Wales (Cors Fochno) and Natural England (Whixall Moss).

^b Growing season is the number of days with mean temperature ≥ 5°C.

3.3.2 Sampling protocol

Ten survey plots were allocated at each site by selecting areas which contained *Drosera rotundifolia* plants growing in *Sphagnum* and were nearest to randomly generated GPS points. In order to quantify the reliance of *D. rotundifolia* on the trait of carnivory (Aims 1, 2 and 3), samples of invertebrate prey and background invertebrates were collected at monthly intervals throughout the plants' active growth season. Fifteen survey *D. rotundifolia* plants were allocated and individually labelled at each plot by locating randomly selected map coordinates using a handheld GPS device, and selecting the nearest plant to each point.

3.3.2.1 Plants

In order to analyse plant material for $\delta^{15}\text{N}$, N and C content (Aims 1-4), material from each selected plant species was collected at the end of the survey season. *Drosera rotundifolia* plants were removed and rinsed with deionised water. Previous growth material was removed, and the remaining living material pooled per survey plot. A 10 cm³ sample of *S. fuscum* capitula surrounding each survey *D. rotundifolia* plant was collected, rinsed with deionised water, and pooled per survey plot. The leaves and stems of each selected type of vascular non-carnivorous plant species present on each survey plot (*Calluna vulgaris*, *Erica tetralix* and *Eriophorum vaginatum*) were removed, rinsed in deionised water, and pooled per species per plot. All plant material was stored in labelled paper sample bags to permit air drying, thus enhancing preservation through the minimisation of enzymatic reactions (Campbell & Plank, 1992).

3.3.2.2 Invertebrates

In order to calculate a precise value of the proportion and amount of prey-derived N of the total N budget of *D. rotundifolia* (Aim 1-3), order composition of the invertebrate diet of the plants was determined and incorporated into the prey $\delta^{15}\text{N}$ end-point of the $\delta^{15}\text{N}$ natural abundance mixing model (Moran *et al.*, 2001). Background sampling (sweep nets and pitfall traps) were then used to collect sufficient mass of each order of captured prey for $\delta^{15}\text{N}$ isotopic natural abundance determination. Invertebrate prey captured by *D. rotundifolia* and the background invertebrate populations were surveyed at three four-weekly intervals throughout the active growth season of *D. rotundifolia*. Invertebrates captured by survey *D. rotundifolia* plants were not removed as this would influence the proportion of prey-derived N of the plants. Captured invertebrate prey were sampled by randomly selecting ten *D. rotundifolia* leaves containing freshly captured prey from non-survey plants within or adjacent to each plot. To prevent damage to the captured invertebrates that may have been

incurred from their removal from the leaves with forceps in the field, each set of ten leaves containing the invertebrates was removed and stored in sterile plastic sample tubes pre-filled with saturated NaCl solution. The inorganic, carbon-free preservation method of saturated NaCl solution was chosen as the instant freezing of invertebrate samples was not feasible in this study; other preservation solutions such as formaldehyde and ethanol are not as effective sample storage methods prior to $\delta^{15}\text{N}$ isotopic natural abundance determination as they have a greater alteration effect on the $\delta^{15}\text{N}$: $\delta^{13}\text{C}$ ratio of invertebrate tissue (Ponsard & Amlou, 1999).

Background terrestrial and airborne invertebrates were sampled by pitfall trap and sweep net respectively. Pitfall traps were constructed using plastic drinking cups filled half-way with saturated NaCl solution, with a suspended plastic roof attached to the rim to prevent rainwater seepage into the trap. At each survey plot, three individually labelled pitfall traps (bait-less) were submerged flush with *Sphagnum capitula* at random locations within a 40 cm radius of survey *D. rotundifolia* plants. Three sweep net surveys of the vegetation surrounding each plot were conducted at three times of day (10am, 12pm, and 2pm) for three minutes using a D-shaped sweep net appropriate for surveying short vegetation. After each four-week period, the invertebrates caught at each survey time by each sampling method per plot were pooled and counted, and placed into a sterile plastic sample tube pre-filled with saturated NaCl solution.

3.3.2.3 Abiotic variables

At four survey times at four-weekly intervals, the pH and electrical conductivity of pore water were recorded at each survey plot using standardised pH and EC testers (Hanna Instruments Ltd, UK). 500 ml pore water samples, for inorganic N determination, were collected by squeezing the upper 10 cm layer of peat at each survey plot. Water samples were stored in plastic sample bottles that had been soaked for at least one day with 10% HCl and rinsed with ultrapure water prior to each survey session. Water samples were filtered as soon as possible following collection using Whatman 0.7 μm GF/F glass fibre micro filters (Nollet, 2007) and a sterilised Sterifil aseptic system (Merck Millipore Ltd, UK), and stored in sterile plastic sample bottles in black bin liners at 3 – 5 °C to inhibit algal and bacterial growth.

3.3.3 Sample preparation and analysis

3.3.3.1 Abiotic variables

To assess root N availability to the plants, pore water samples were analysed for ammonium ($\text{NH}_4^+\text{-N}$), nitrate ($\text{NO}_3^-\text{-N}$) and nitrite ($\text{NO}_2^-\text{-N}$) by ion-exchange chromatography as soon as possible following sample filtration. Total dissolved inorganic N content (DIN) ($\mu\text{g l}^{-1}$) was calculated as the sum of $\text{NH}_4^+\text{-N}$, $\text{NO}_3^-\text{-N}$ and $\text{NO}_2^-\text{-N}$ for each pore water sample.

3.3.3.2 $\delta^{15}\text{N}$ isotopic natural abundance determination

In the laboratory, invertebrates were counted and identified to order level using a Zeiss Stemi 2000 stereo microscope (Carl Zeiss Microscopy Ltd, Germany) and the length of each invertebrate measured to 0.1 mm precision using a 100 x 0.1 mm stage micrometer (Pyser-SGI Ltd, UK). Thus, species abundance, type, and size data were collated for all surveyed invertebrates. Background invertebrates outside the length range of invertebrates captured by *D. rotundifolia* plants at both sites (0.4 - 6.5 mm in length) were excluded from the dataset, and remaining background invertebrates pooled per order per plot for $\delta^{15}\text{N}$ isotopic natural abundance determination.

Invertebrates were rinsed thoroughly in deionised water prior to drying. Plant and invertebrate material was dried to a constant weight by placing in a forced-air oven at 70 °C for 72 hours (Campbell & Plank, 1992), and weighed to obtain dry mass measurements. *Sphagnum fuscum* and *E. vaginatum* were pre-selected as the reference non-carnivorous genera required for estimation of the root $\delta^{15}\text{N}$ end-point of the $\delta^{15}\text{N}$ natural abundance mixing model for the calculation of the proportion of prey-derived N of the N budget of *D. rotundifolia* (Moran *et al.*, 2001). Of the species used in this study, *S. fuscum* and *E. vaginatum* possess maximum rooting depths closest to that of *D. rotundifolia*, and therefore, as the $\delta^{15}\text{N}$ signature of plant species increases with increasing rooting depth (Hobbie and Högberg, 2012), these species are most closely representative of the root $\delta^{15}\text{N}$ uptake of *D. rotundifolia* plants deriving 100% of the plant N budget via the roots (Schulze *et al.*, 1991; Moran *et al.*, 2001). *D. rotundifolia* and invertebrate samples were ground to a fine powder using a pestle and mortar, and *Sphagnum* and non-carnivorous vascular plant material using a ball mill (Retsch MM200, Retsch, Haan, Germany) to ensure sample homogeneity (Baker & Thompson, 1992). Plant and invertebrate material were pre-weighed into tin capsules and analysed for $\delta^{15}\text{N}$ (Aims 1-4) at the NERC Life Science Mass Spectrometry Facility, East Kilbride, Scotland. Carbon and nitrogen isotopes were analysed by continuous flow using a Thermo Scientific DELTA V Plus isotope ratio mass spectrometer (Thermo Scientific, Bremen, Germany) interfaced with a Costech ECS 4010 elemental analyser (Costech

Instruments, Milan, Italy). Three in-house standards (gelatine, glycine, alanine) were run every ten samples for quality assurance. All data are reported with respect to the international standard of atmospheric N₂ for δ¹⁵N. Results are reported in δ notation as the deviation from standards in parts per thousand (‰), where $\delta X = [(R_{\text{sample}} - R_{\text{reference}}) - 1] \times 1000$, and $R = {}^{15}\text{N} : {}^{14}\text{N}$, and $X = {}^{15}\text{N}$. Precision was 0.2 ‰ for δ¹⁵N. Total N and C contents of plant and invertebrate material were also obtained during the δ¹⁵N analysis (Aims 1 and 2).

3.3.4 Data analyses

3.3.4.1 Calculation of the relative contribution of N derived from invertebrate prey (%N_{dfp}) to the total N budget of *Drosera rotundifolia*

The two end-member δ¹⁵N linear mixing model as presented by Shearer and Kohl (1988) for estimating the contribution of atmospherically-fixed N to plants, and later adapted for use in carnivorous plant research by Schulze *et al.* (1991), was used to calculate the relative contributions of invertebrate prey N and root N to the total N budget of *D. rotundifolia* (Equation 1):

Equation 1

$$\%N_{dfp} = \frac{\delta^{15}N_A - \delta^{15}N_B}{\delta^{15}N_C - \delta^{15}N_B}$$

Where %N_{dfp} is the relative contribution of invertebrate prey N to the total N budget of *D. rotundifolia* (%), δ¹⁵N_A is the δ¹⁵N of *D. rotundifolia*, δ¹⁵N_B is the mean δ¹⁵N of *S. fuscum* and *E. vaginatum* from the corresponding survey plot, and δ¹⁵N_C is the δ¹⁵N of the invertebrate prey, which was calculated using the mean δ¹⁵N of captured invertebrate prey orders weighted to take into account the relative capture rate of these different orders by *D. rotundifolia* and the N content of prey (Equation 2):

Equation 2

$$\delta^{15}N_C = \sum_i^n \left[aA_i \times \left(\frac{aB_i}{100} \right) \times \left(\frac{aC_i}{aD} \right) \right]$$

where δ¹⁵N_C is the weighted mean δ¹⁵N of invertebrate prey captured by *D. rotundifolia*, n is the total number of captured prey invertebrate orders at site a, aA_i is the δ¹⁵N value (‰) of the *i*th invertebrate

order per survey plot at site a , aB_i is the percentage N by weight of the i th invertebrate order per survey plot at site a , aC_i is the dry mass (mg) of the i th invertebrate order per survey plot at site a , and aD is the total dry mass (mg) of captured invertebrate prey per survey plot at site a .

3.3.4.2 Statistical analyses

The data were evaluated using independent-samples t -tests, analyses of variance (ANOVAs) and Pearson's correlation. Two-way ANOVAs were used to test for differences in tissue N concentration and $\delta^{15}\text{N}$ between sites and between plant/bryophyte species, and to test for differences in the adjusted amount of N per *D. rotundifolia* plant between sites and between N sources (Aims 1 and 2). Independent-samples t -tests were used to test for differences in environmental characteristics and $\%N_{\text{dfp}}$ and tissue C:N ratios of *Drosera rotundifolia* plants between sites (Aim 3). Pearson's product-moment correlation (two-tailed) was used to test for a linear relationship between the $\delta^{15}\text{N}$ of *D. rotundifolia* and site N deposition input, using data collated from this study and previous research in the literature (Aim 4). Post-hoc comparisons were conducted using Fisher's Least Significant Difference (LSD) (0.05 significance level). Data were \log_{10} -transformed prior to analysis where data did not conform to the assumptions of homoscedascity. Residual plots were used to assess for homoscedascity and normal probability plots used to test that data were normally distributed.

All statistical analyses were conducted using IBM SPSS Statistics version 21 (IBM, Chicago, USA).

3.4 Results

3.4.1 Abiotic characteristics of the study sites

Table 13 Results of independent samples *t*-tests for environmental characteristics of the ombrotrophic bogs in the UK used in this study. Presented are mean \pm 1 S.E. of each peat water variable throughout the 2011 field season per site:- ammonium (NH₄-N) content, nitrate (NO₃-N) content, nitrite (NO₂-N) content, total dissolved inorganic N (DIN) content, pH and electrical conductivity (EC). Significant effects at $P < 0.05$ are highlighted in bold.

		NH ₄ -N ($\mu\text{g l}^{-1}$)	NO ₃ -N ($\mu\text{g l}^{-1}$)	NO ₂ -N ($\mu\text{g l}^{-1}$)	DIN ($\mu\text{g l}^{-1}$)	pH (pH units)	EC ($\mu\text{s cm}^{-1}$)
Cors Fochno	Mean	0	137	710	735.8	3.439	166.983
	SE	0	22	5	20.5	0.052	6.808
Whixall Moss	Mean	468	284	782	1505.4	3.814	115.812
	SE	184	68	17	234.1	0.152	5.086
Independent samples <i>t</i> -test results ¹	<i>P</i>	0.032	0.070	0.003	0.009	0.040	<0.001
	<i>df</i>	9	10	10	9	11	18

¹ Comparing differences between sites.

Peat water at the Cors Fochno survey plots contained 100% and 9% less available N in the form of NH₄-N and NO₂-N respectively throughout the active growth season than peat water at the Whixall Moss survey plots (Table 13). Between-site differences in peat water NO₃-N were not statistically significant. As a result, concentrations of total dissolved inorganic N (DIN) in the peat water at Whixall Moss were approximately twofold higher than peat water at Cors Fochno. Peat water pH at Cors Fochno was lower than that at Whixall Moss, whereas EC was higher (Table 13).

Table 14 Results of two-way univariate ANOVAs for N characteristics of five co-existing vascular plant and bryophyte species growing in two ombrotrophic bogs in the UK that vary by N deposition input. Presented are degrees of freedom (*df*), *F* and *P* values from the analyses of tissue N concentration (percentage N by weight) (%N) and $\delta^{15}\text{N}$ (‰) of plant tissue ($\delta^{15}\text{N}$). Significant effects at $P < 0.05$ are highlighted in bold.

Effect	%N			$\delta^{15}\text{N}$		
	<i>df</i>	<i>F</i>	<i>P</i>	<i>df</i>	<i>F</i>	<i>P</i>
Site	1, 99	171	<0.001	1, 99	7	0.012
Species	4, 99	87	<0.001	4, 99	61	<0.001
Site x species	4, 99	3	0.024	4, 99	20	<0.001

3.4.2 Tissue N concentrations of plant species

Tissue N concentration (%N) differed significantly between species, but these differences were site dependent (Table 14) (Aim 1). Specifically, at Cors Fochno tissue N concentration was lower for *S. fuscum* than the other species, and tissue N concentration of *E. vaginatum* was higher than the other species (Fig. 13). At Whixall Moss the tissue N concentration of all species was higher than the same species at Cors Fochno, and the ranking of concentrations stayed the same (Table 14; Fig. 13). However, the magnitude of increase in tissue N concentration per species between plants growing at Cors Fochno and plants growing at Whixall Moss differed according to species and was largest for *D. rotundifolia* (39.9%) followed by *E. vaginatum* (36.0%) and *C. vulgaris* (33.1%), and smallest for *E. tetralix* (29.9%).

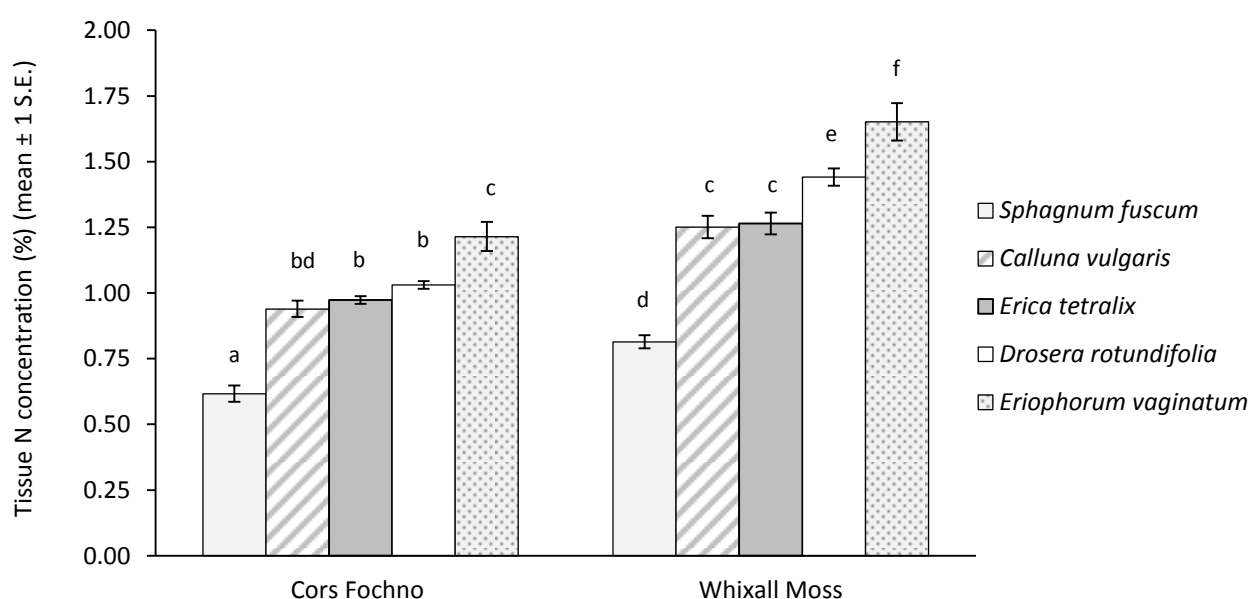


Figure 13 Tissue N concentrations (%N by weight) (mean \pm 1 S.E.) of co-existing plant and bryophyte species growing at two ombrotrophic bogs in the UK. Bars with different letters are significantly different from each other (Fisher's least significant difference, $P < 0.05$).

3.4.3 Tissue $\delta^{15}\text{N}$ of plant species

Calluna vulgaris and *E. tetralix* at Cors Fochno were significantly more $\delta^{15}\text{N}$ -depleted than remaining species per site, but did not differ from each other (Fig. 14) (Aim 2). *Drosera rotundifolia* at Cors Fochno was the most $\delta^{15}\text{N}$ -enriched of the species per site, but did not differ significantly in $\delta^{15}\text{N}$ from *E. vaginatum* at Cors Fochno and *D. rotundifolia* at Whixall Moss (Aim 2). Plant tissue $\delta^{15}\text{N}$ varied significantly between species (Table 14) (Aim 2); *D. rotundifolia* was significantly more $\delta^{15}\text{N}$ -enriched

than the remaining species. *Calluna vulgaris* and *E. tetralix* were significantly more $\delta^{15}\text{N}$ -depleted than the remaining species, but did not significantly differ from each other.

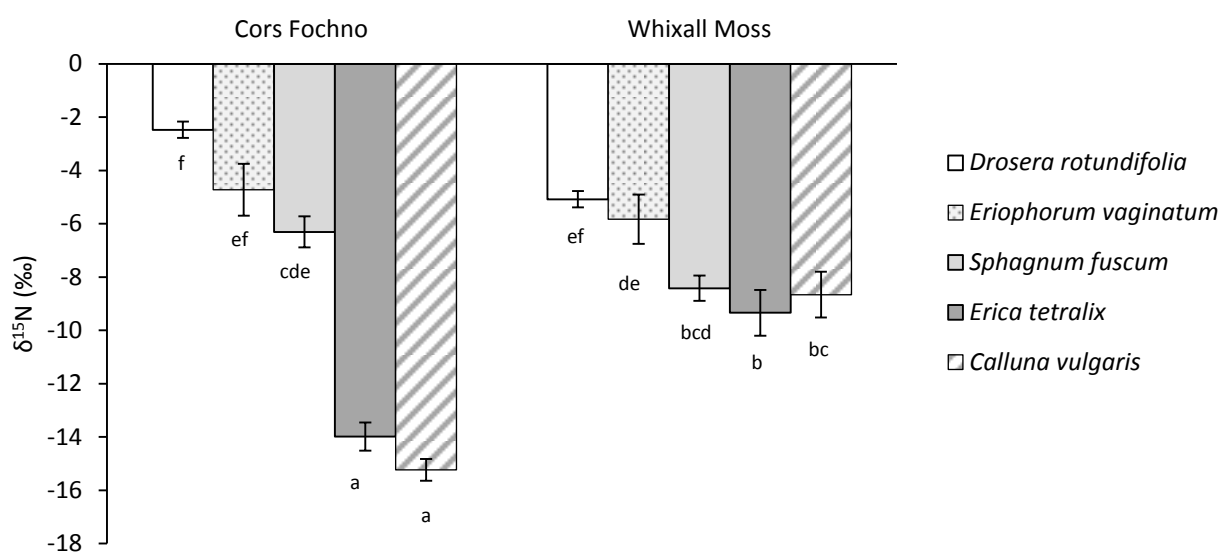


Figure 14 Tissue $\delta^{15}\text{N}$ (‰) (mean \pm 1 S.E.) of co-existing plant and bryophyte species growing at two ombrotrophic bogs in the UK. Bars with different letters are significantly different from each other (Fisher's least significant difference, $P < 0.05$).

3.4.4 N nutrition of *Drosera rotundifolia*

Table 15 N nutrition of *Drosera rotundifolia* plants at two ombrotrophic bogs in the UK that vary by N deposition input. Presented are the mean \pm 1 S.E. for the following parameters of *D. rotundifolia*: the percentage contribution of prey-derived N to the total N budget, $\%N_{\text{dfp}}$; tissue C : N ratio. Significant effects at $P < 0.05$ are highlighted in bold.

Site		$\%N_{\text{dfp}}$	C : N ratio
Cors Fochno	Mean	49.4	45.6
	SE	8.8	0.7
Whixall Moss	Mean	25.5	33.8
	SE	4.9	0.8
Independent samples t -test results ¹	P	0.029	<0.001
	df	18	18

¹ Comparing differences between sites.

Drosera rotundifolia plants at Cors Fochno obtained significantly greater %N_{dfp} than plants at Whixall Moss (Fig. 15; Table 15) (Aim 3).

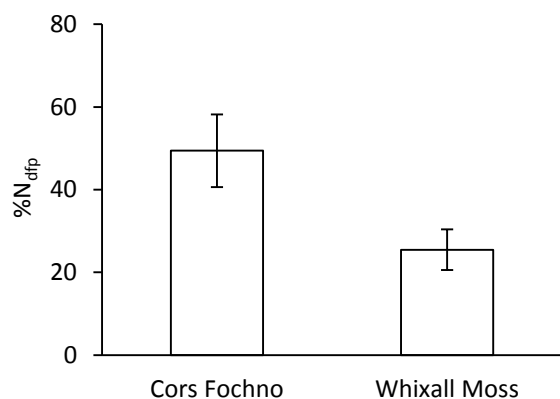


Figure 15 The percentage contribution of prey-derived N to the total N budget (%N_{dfp}) of *Drosera rotundifolia* plants growing at two ombrotrophic bogs that vary by N deposition input.

Tissue C : N ratios of *Drosera rotundifolia* plants at Cors Fochno were significantly higher than plants at Whixall Moss (Fig. 16; Table 15) (Aim 3).

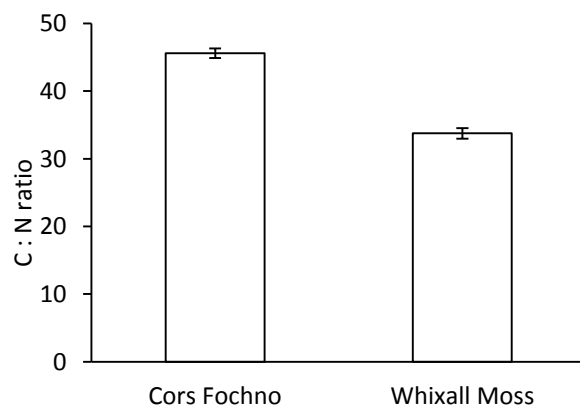


Figure 16 The C to N ratio (mean ± 1 S.E.) of *Drosera rotundifolia* plants growing at two ombrotrophic bogs receiving contrasting N deposition inputs in the UK.

Table 16 Results of 2-way (site, N source) univariate ANOVAs for the amount of prey-derived and root-derived N per plant by *Drosera rotundifolia* plants growing at two ombrotrophic bogs in the UK that vary by N deposition input. Presented are degrees of freedom (*df*), *F* and *P* values for the amount of adjusted N of *D. rotundifolia*. N sources: prey-derived, root-derived; sites: Cors Fochno, Whixall Moss. Significant effects at $P < 0.05$ are highlighted in bold.

	Adjusted N (μg per plant)		
	<i>df</i>	<i>F</i>	<i>P</i>
N source (NS)	1, 39	16.507	<0.001
Site	1, 39	25.074	<0.001
NS x site	1, 39	22.323	<0.001

When adjusted for plant mass, the amount of N_{dfp} of *D. rotundifolia* plants did not significantly differ between sites (Fig. 17), but plants at Whixall Moss contained significantly greater mass-adjusted amounts of N_{dfr} than plants at Cors Fochno (Table 16) (Aim 3).

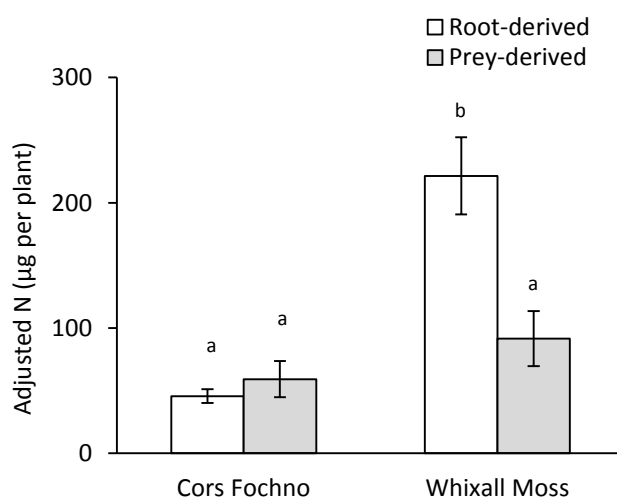


Figure 17 N nutrition of *Drosera rotundifolia* plants growing at two ombrotrophic bogs that vary by N deposition input. Presented are the mean \pm 1 S.E. for the amounts of prey-derived N (N_{dfp}) and root-derived N (N_{dfr}), both at a common plant mass of 14.6 mg and corrected for the covariate relationship with mass. Bars with different letters are significantly different from each other (Fisher's least significant difference, $P < 0.05$).

Upon comparison of this study's data with the results of earlier studies reporting $\delta^{15}\text{N}$ values of *in-situ* *D. rotundifolia* plants at sites that vary by N deposition input (Table 17), no relationship was found between $\delta^{15}\text{N}$ and N deposition input (Pearson's correlation coefficient, r (two-tailed): $r_{(9)} = -0.129$, $p = 0.741$) (Aim 4).

Table 17 Summary of data reported in the literature for tissue $\delta^{15}\text{N}$ of *Drosera rotundifolia* plants growing *in-situ* at peatland sites that vary by N deposition input.

Site name	Location	N deposition (kg N ha ⁻¹ yr ⁻¹)	$\delta^{15}\text{N}$ (‰) (mean \pm 1 S.E.)	Plant part(s) used for $\delta^{15}\text{N}$ analysis	Source
Whixall Moss	UK (Wales)	30.8 ^a	-5.1 \pm 0.3	Current year's above- and below-ground material	This study
Cors Fochno	UK (West England)	8.0 ^a	-2.5 \pm 0.3	Current year's above- and below-ground material	This study
Åkerlänna Römossa	Sweden (Central)	3.8 ^b	-2.1 \pm 0.3	Current year's above- and below-ground material	Millett <i>et al.</i> , 2012
Saxnäs Mosse	Sweden (South)	11.3 ^b	-1.6 \pm 0.2	Current year's above- and below-ground material	Millett <i>et al.</i> , 2012
Lappmyran	Sweden (North)	1.9 ^b	-0.8 \pm 0.2	Current year's above- and below-ground material	Millett <i>et al.</i> , 2012
Lake District	UK (North England)	28.4 ^c	1.4 \pm 0.2	Whole plant	Millett <i>et al.</i> , 2003
Rannoch Moor	UK (Scotland)	17.9 ^c	2.3 \pm 0.2	Whole plant	Millett <i>et al.</i> , 2003
New Forest (valley mire)	UK (Southern England)	17.5 ^c	2.6 \pm 0.2	Whole plant	Millett <i>et al.</i> , 2003
Southern Kisselbergmossen	Norway (South)	7.9 ^d	6.5 \pm 1.6	Above-ground material	Nordbakken <i>et al.</i> , 2003

^a Modelled N deposition data from APIS (<http://www.apis.ac.uk/> (accessed 21.04.2014)). Data are mean values for 2010-2012 inclusive.

^b Modelled N deposition data from EMEP model (<http://www.emep.int> (accessed 30.09.2011)). Data are mean values for 2004-2009 inclusive.

^c Modelled N deposition data from DEFRA (<http://pollutantdeposition.defra.gov.uk/data>) using northing and easting Cartesian coordinates of Ordnance Survey grid references provided by Millett *et al.*, 2003 (accessed 22.04.2014)). Data are 2004 values (pre-2004 data unavailable).

^d Estimated total N deposition, see Nordbakken *et al.*, 2003, for further details. Erratum of the unit of measurement of N deposition stated in the paper is acknowledged by the author (Nordbakken, 2014, pers. comm.).

3.5 Discussion

The results of this study show that as the N deposition input to ombrotrophic bogs increases, the inorganic N content of pore water situated in the upper 10 cm of the *Sphagnum* layer increases. This result is in agreement with the results of several studies exploring the influence of N deposition on bog plant communities, which show the inorganic N content of pore water to increase/decrease in accordance with changes in N deposition (Limpens *et al.*, 2003; Bragazza *et al.*, 2005). The very large difference in DIN between the two sites indicates that the additional N deposited onto Whixall Moss is not being taken up by *Sphagnum* species, and is therefore available to vascular plants. This result corresponds with those of Lamers *et al.* (2000) which show that the 'N-filtering' capacity of *Sphagnum* is saturated at N deposition inputs above the threshold of 18 kg N ha⁻¹ yr⁻¹ and is therefore released by *Sphagnum* into the rhizosphere.

Plant species with contrasting life history strategies differed in their response to increases in root N availability. The tissue N concentration of all species increased in response to increased N deposition, indicating that N becomes available to all species. However there were small differences between species in the degree of tissue N concentration increase; *D. rotundifolia* underwent the largest degree of tissue N concentration increase than the other species and *E. tetralix* underwent the lowest degree of tissue N concentration increase than the other species. This indicates that these species may be (a) more/less able to utilise this additional N; (b) have more/less need for this additional N, or (c) be able to alter resource allocation in order to take advantage of the changing availability of this less energetically costly N source. These results also suggest changes in specific adaptations to this low nutrient environment; there is evidence of decreased reliance on carnivory by *D. rotundifolia* and on mycorrhizal-derived N by *C. vulgaris* and *E. tetralix* as root N availability increases.

Drosera rotundifolia possessed the second highest tissue %N of the study species, indicating that the additional source of N obtained from captured prey provides this species with an energetic advantage compared with non-carnivorous co-occurring species (Givnish *et al.*, 1984). The capitula of *Sphagnum fuscum* possessed significantly lower tissue %N than the plant species, reflecting the difference in functional type, and N acquisition and retention strategies of *Sphagnum spp.* compared with vascular plants. *Sphagnum spp.* possess very high N use efficiency due to their ability to directly absorb dissolved inorganic N across their entire surface area (Gerdol *et al.*, 2006), however capitula N content is maintained at low levels due to the translocation of up to 90% of plant N to the stems where it is stored as amino acids (Aldous, 2002).

The statistically significant between-species variation in %N reported in this study reflects the variation in functional type, growth form, and N acquisition and retention strategies of the species investigated. *Eriophorum vaginatum* contained the highest tissue %N of the study species. This result is indicative of

the growth form and N acquisition and retention strategies of the species. *Eriophorum vaginatum* is a tussock-forming clonal sedge that exhibits high productivity and long tussock lifespan (Fetcher and Shaver, 1983; Gebauer *et al.*, 1995), and possesses one of the highest rates of nutrient resorption efficiency of vascular plants (Jonasson and Shaver, 1999). This high efficiency is achieved through the characteristic graminoid trait of sequential leaf development and the ability of the plant to resorb over 80% of leaf N content of senescing leaves into new growth (Jonasson and Chapin, 1985). In addition, *E. vaginatum* is able to directly absorb organic N in the form of free amino acids in the peat layer, which are estimated to constitute for *ca.* 60% of the plants' N budget (Chapin *et al.*, 1993), thus largely bypassing inter-specific competition for inorganic forms of soil N.

Significant between-species variation was found in the magnitude at which tissue %N was greater for plants growing at Whixall Moss compared with plants growing at Cors Fochno. *Drosera rotundifolia* showed the largest magnitude of tissue %N increase between plants growing at the two sites, indicating that upon exposure to high root N availability, plants were highly efficient at root N uptake, and/or that prey-derived N provides a useful additional source of N. *Erica tetralix* demonstrated the smallest magnitude of increase in tissue %N between sites, perhaps indicating minor differences in N uptake via mycorrhiza between sites, or reflecting the relatively high mean residence time of N in the plant of this evergreen species (Aerts, 1990).

Results of this study found significant between-species differences in tissue $\delta^{15}\text{N}$, ranging from -3.8 to -11.9 ‰. This result is likely to be indicative of the variation in growth form, N source preference, rooting depth and/or mycorrhizal status between the study species. *Drosera rotundifolia* was significantly more $\delta^{15}\text{N}$ -enriched, and *E. tetralix* and *C. vulgaris* were significantly more $\delta^{15}\text{N}$ -depleted, than the remaining species. This pattern supports previous field evidence from the literature that plant $\delta^{15}\text{N}$ is clustered by growth form in the order of shrubs < bryophytes < forbs, perhaps reflecting differences in N-acquiring strategies and isotopic fractionation during N assimilation and translocation processes (Pate *et al.*, 1993; Asada *et al.*, 2005).

The observation that plant species become more $\delta^{15}\text{N}$ -depleted as maximum rooting depth increases (Kohzu *et al.*, 2003) is supported by the results of this study:- species $\delta^{15}\text{N}$ decreased from -3.8 to -11.9 ‰ as rooting depth increased from < 6 to 40 cm. The $\delta^{15}\text{N}$ signatures of the ericoid mycorrhizal (ERM) species of *E. tetralix* and *C. vulgaris* were on average 6.5 ‰ more $\delta^{15}\text{N}$ -depleted than the non-mycorrhizal species of *E. vaginatum*. This result is in agreement with similar wetland plant studies which report ERM species to be $\delta^{15}\text{N}$ -depleted compared with co-occurring non-mycorrhizal species (Nordbakken *et al.*, 2003; Asada *et al.*, 2005), and concurs with the outcome of a meta-analysis of plant $\delta^{15}\text{N}$ data by Crane *et al.* (2009) which found ERM species of a wide variety of habitat types to be $\delta^{15}\text{N}$ -depleted by 5.9 ‰ on average than non-mycorrhizal species. The significantly enriched tissue $\delta^{15}\text{N}$ of *D. rotundifolia* in comparison to the other study species is likely to reflect the carnivorous trait

of the species (Schulze *et al.*, 1991); prey-derived N is relatively $\delta^{15}\text{N}$ -enriched compared with root-derived N due to the retention of $\delta^{15}\text{N}$ compared with ^{14}N at each trophic level of food webs (Post, 2002), and may also reflect larger differentiation of root N uptake in the form of relatively $\delta^{15}\text{N}$ -enriched $\text{NH}_4\text{-N}$, as *Drosera* species display limited capacity for root $\text{NO}_3\text{-N}$ uptake (Chandler and Anderson, 1976).

The results of this study show that, overall, species growing at Whixall Moss were significantly more $\delta^{15}\text{N}$ -enriched than species growing at Cors Fochno, however this result mostly reflects the large degree of $\delta^{15}\text{N}$ enrichment of *E. tetralix* and *C. vulgaris* plants growing at Whixall Moss (-14.0 ‰ and -15.2 ‰ respectively) compared to those growing at Cors Fochno (-9.3 ‰ and -8.7 ‰ respectively); between-site differences in $\delta^{15}\text{N}$ for each of the remaining species failed to reach statistical significance. Ericoid mycorrhizal-derived N of plants is more $\delta^{15}\text{N}$ -depleted than non-mycorrhizal root-derived N, widely considered to be either due to the transfer of proportionally more ^{14}N than ^{15}N from the fungus to the host plant either as a result of isotopic fractionation processes by the fungus, or due to non-mycorrhizal plants obtaining N from relatively $\delta^{15}\text{N}$ -enriched sources compared with mycorrhizal plants (Emmertson *et al.*, 2001; Hobbie and Hogberg, 2012). As no significant between-site differences in the $\delta^{15}\text{N}$ of non-ERM plant species were found, the results of this study provide tentative support for those of earlier studies indicating that the reliance of mycorrhizal plants on mycorrhizal-derived N increases as root N availability decreases (Yesmin *et al.*, 1996; Hobbie *et al.*, 2000). An alternative explanation for the significantly more depleted $\delta^{15}\text{N}$ of *C. vulgaris* plants growing at Whixall Moss than plants growing at Cors Fochno may be due to N source uptake preference; *C. vulgaris* showed reduced growth in response to high soil $\text{NH}_4\text{-N}$ content, but no growth difference in response to high soil $\text{NO}_3\text{-N}$ content, suggesting plant N uptake preference for the relatively $\delta^{15}\text{N}$ -depleted N form of $\text{NO}_3\text{-N}$ (Yesmin *et al.*, 1996; Van den Berg *et al.*, 2008).

Results of this study show that *Drosera rotundifolia* plants growing at Whixall Moss contained significantly smaller proportion of prey-derived N ($\%N_{\text{dfp}}$) of the total plant N budget (mean \pm 1 S.E. = 25.47 ± 4.90 %) than *D. rotundifolia* plants growing at Cors Fochno (49.41 ± 8.81 %). This result supports the results of a recent study (Millett *et al.*, 2012) investigating N nutrition of *D. rotundifolia* plants growing at three sites situated along a latitudinal N deposition gradient that showed plant reliance on the trait of botanical carnivory to decrease as N deposition increases, and provides evidence in an ecological context to support the evolutionary cost-benefit model as proposed by Givnish *et al.*, 1984. The $\%N_{\text{dfp}}$ results from this study lie within the range of 22.2 to 56.7 % as reported by Millett *et al.* (2012) for *D. rotundifolia* plants growing at sites receiving N deposition input range of 1.9 to 11.3 $\text{kg N ha}^{-1} \text{yr}^{-1}$. However, plant $\%N_{\text{dfp}}$ for the five sites from both studies, and from the three sites used by Millett *et al.* (2012), does not increase sequentially with increasing site atmospheric N deposition, possibly reflecting differences in the nature and precision of the mixing model end-points

used to estimate %N_{dfp} between studies (Moran *et al.*, 2001), and/or variation in root N availability as a result of geographical variation in ammonification and nitrification flux rates (Houlton *et al.*, 2007).

Upon consideration of the influence of N deposition on the $\delta^{15}\text{N}$ signature of *Drosera rotundifolia*, it may be predicted that plant tissue would become significantly $\delta^{15}\text{N}$ -depleted as N deposition increases, due to plant reliance on prey-derived N decreasing as N deposition increases (results of this study, and Millett *et al.*, 2012), and due to the relative $\delta^{15}\text{N}$ -enrichment of prey-derived N compared with root-derived N (Schulze *et al.*, 1991). However, the results of this study, and of comparison of the results of this study with the results of earlier $\delta^{15}\text{N}$ natural abundance studies of *D. rotundifolia in-situ* (Table 17), do not provide support for this prediction. In this study, *D. rotundifolia* plants growing at Whixall Moss were marginally $\delta^{15}\text{N}$ -depleted (mean \pm 1 S.E. = -5.08 ± 0.67 ‰) compared with plants growing at Cors Fochno (-2.47 ± 0.67 ‰), however this difference failed to reach significance at the 95% confidence level. Upon comparison of the results of this study with those of earlier studies (Table 17), no significant relationship between N deposition and $\delta^{15}\text{N}$ was found.

Several explanations may be suggested for these surprising results. Firstly, variation exists in the parts of the plant used for $\delta^{15}\text{N}$ natural abundance analysis between some of the studies listed in Table 17:- the relatively enriched $\delta^{15}\text{N}$ value of 6.5 for *D. rotundifolia* reported by Nordbakken *et al.* (2003) may reflect the samples' composition of above-ground plant material only; flowers and leaves are reported to be $\delta^{15}\text{N}$ -enriched compared with roots (Millett *et al.*, 2003). Secondly, between-site differences in plant uptake of root N sources which vary by degree of $\delta^{15}\text{N}$ -enrichment may have occurred, as a result of changes in plant preference (Michelsen *et al.*, 1998), variation in mycorrhizae species composition and/or degree of mycorrhizal association (Michelsen *et al.*, 1998), and/or geographical variation in soil ammonification and nitrification flux rates (Houlton *et al.*, 2007). Lastly, as large variation in $\delta^{15}\text{N}$ occurs within and between invertebrate orders (Schulze *et al.*, 2001; Feldhaar *et al.*, 2009), potential between-site differences in the prey dietary composition of *D. rotundifolia* may have influenced tissue $\delta^{15}\text{N}$, either as a result of between-site differences in the $\delta^{15}\text{N}$ of potential invertebrate prey population, or plant variation in prey attraction strategy or capture success.

3.6 Conclusions

The results of this study offer an insight into how bog communities may respond to long-term inputs of relatively high N deposition. Evidence shows that the 'N filtering' ability of *Sphagnum* is lost at N deposition inputs of *ca.* 30.8 kg N ha⁻¹ yr⁻¹, therefore leading to the release of N into the rhizosphere of vascular plants. All plants benefit from this newly available N, but the magnitude of tissue N uptake varies between species, reflecting differences in the plants' life history strategy. These differences in N uptake and nutrition suggest that the nature and strength of biotic interactions between plant species will vary according to the level of N deposition input, and offer an insight at the physiological level as to why high N-demanding species such as shrubs and graminoids are more abundant at high N deposition inputs than less N-demanding species, such as forbs. Future research would benefit from exploring the influence of plant functional traits at the physiological level on the nature and strength of plant to plant interactions along an N deposition gradient in order to enable the production of evidence-based peatland management plans which minimise biodiversity loss and maximise the carbon sequestration potential of ombrotrophic bogs under future scenarios of projected, sustained high N deposition inputs.

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Chapter 4: Reducing uncertainty of source partitioning in multi-level stable isotope mixing models:- a case study using the N nutrition of a carnivorous plant.

4.1 Abstract

The two end-point, single isotope linear mixing model is the most widely used method for determining source proportional contributions to a mixture using natural abundance isotopic data. There is however considerable uncertainty and potential error in source proportion estimates due to inherent model assumptions and limitations. For multi-level model application, uncertainty in source proportion estimates due to variability within and between end-points contributing to one of the sources has not yet been addressed. Upon application of the multi-level model to the calculation of the proportion of prey-derived N of the total N budget (%N_{dfp}) of carnivorous plants, further potential error can be incurred from the use of proxies that are not most representative of the prey N and root N end-points. Furthermore, research to date has not incorporated the diet of the carnivorous plant in calculations of %N_{dfp}, therefore potentially causing inaccuracy and uncertainty in the variability of the prey N end-point of the model and therefore in %N_{dfp}.

This study utilises sample populations of *in-situ Drosera rotundifolia*, captured prey, background invertebrates, *Sphagnum* and non-carnivorous plants at two ombrotrophic bogs in the UK that vary by N deposition input to present an accurate method for determining %N_{dfp} of carnivorous plants. The influence of the inclusion of plant diet on the value of and variability in the prey N end-point was explored. Sensitivity analyses were performed to determine the influence of parameters of isotopic contributors to variability in source proportion estimates using the example of the N nutrition of *D. rotundifolia*. The potential suitability of *Sphagnum fuscum*, *Eriophorum vaginatum*, *Erica tetralix* and *Calluna vulgaris* as proxies for the root N end-point of the model was inferred by comparison with each other.

The $\delta^{15}\text{N}$, tissue percentage N content and proportional abundance of invertebrate orders to total captured prey differed significantly between sites and between orders. The inclusion of these order parameters did not significantly influence the value of $\delta^{15}\text{N}_{\text{prey}}$ but the variability of $\delta^{15}\text{N}_{\text{prey}}$ was fivefold to eightfold less than the variability of unweighted $\delta^{15}\text{N}_{\text{prey}}$. Comparison of plant proxy $\delta^{15}\text{N}$ values showed that the $\delta^{15}\text{N}$ of *S. fuscum* and *E. vaginatum* were closer to the $\delta^{15}\text{N}$ of *D. rotundifolia* at both sites than the $\delta^{15}\text{N}$ of remaining proxies, and more ^{15}N -enriched than remaining proxies. Sensitivity analyses showed that the SD of prey orders exerted the most powerful influence on $\text{SE}(\%N_{\text{dfp}})$; as $\text{SD}(\delta^{15}\text{N})$ of each prey order doubled, $\text{SE}(\%N_{\text{dfp}})$ was doubled. For all prey order parameters, the influence of sample size was substantial; increasing the sample size from 2 to 10 reduced $\text{SE}(\%N_{\text{dfp}})$ by over a half.

In conclusion, variability in %N_{dfp} of generalist carnivorous plant species may be minimised by:- (i) incorporating plant diet into the calculation of the variability in $\delta^{15}\text{N}_{\text{prey}}$; (ii) maximising the number of replicates (preferably at least 10) of each taxon of captured prey per survey plot; (iii) identifying prey and background invertebrates to as fine a taxonomic level as feasible. In this study, results indicate that the mean $\delta^{15}\text{N}$ of *S. fuscum* and *E. vaginatum* is the most representative proxy for the prey N end-point, however future research is required to verify the suitability of plant and invertebrate $\delta^{15}\text{N}$ proxies with the true value for the $\delta^{15}\text{N}$ of *D. rotundifolia* that has obtained 100% of N via the roots or from prey.

4.2 Introduction

Natural abundance stable isotope analysis is used for a wide variety of ecological research, including carnivorous plant ecology (Högberg, 1997). The linear mixing model (Shearer and Kohl, 1988) is a favoured method for calculating the proportion of prey-derived N of the total N budget (%N_{dfp}) of a carnivorous plant, i.e. plant reliance on the trait of carnivory. It is necessary to use the $\delta^{15}\text{N}$ of invertebrates and the $\delta^{15}\text{N}$ of reference non-carnivorous plants as proxies for the prey and root N end-points respectively of the linear mixing model (Shearer and Kohl, 1988), as naturally occurring genetic variants of carnivorous plants that can only obtain N from prey or via the roots are extremely rare. Previous studies have used the mean $\delta^{15}\text{N}$ of pooled background invertebrate samples considered to be potential prey as a proxy for the prey N end-point, but no consideration was given to the actual diet of the plant. The diet of carnivorous plants varies between species, e.g. due to differences in dietary strategy and prey capture mechanism (Ellison and Gotelli, 2009), and within species, e.g. due to spatial and temporal variation in the taxa distribution and abundance of potential prey (Alcalá and Domínguez, 2003) or as a result of phenotypic plasticity in plant investment in prey capture (Antor and García, 1994). Furthermore, invertebrate $\delta^{15}\text{N}$ varies between and within taxa due to factors such as trophic level, diet and life cycle stage (Vanderklift and Ponsard, 2003). Therefore there is a need for the following research using a carnivorous plant:- (i) presentation of a method for calculating an accurate value for $\delta^{15}\text{N}_{\text{prey}}$ through incorporation of the diet of the plant; (ii) incorporation of the variability within and between orders of captured prey into the calculation of variability of $\delta^{15}\text{N}_{\text{prey}}$.

Most applications of the linear mixing model require the use of isotopic signature proxies to represent the isotopic signatures of one or both of the sources of the mixture. It is important therefore to use proxies that are as closely representative of the end-points as possible in order to minimise potential error in the calculation of source proportions of a mixture; this was achieved by Shearer and Kohl (1988) through the use of the mean leaf $\delta^{15}\text{N}$ of a large number of co-occurring non-N₂-fixing plant species in order to minimise the influence of varying rooting depth on $\delta^{15}\text{N}$. Previous studies

estimating %N_{dfp} of *Drosera rotundifolia* (round-leaved sundew) have used co-occurring *Sphagnum* spp. (Millett *et al.*, 2003, 2012), or a selection of co-occurring non-carnivorous vascular plant species (mainly Gramineae) (Schulze *et al.*, 1991) as proxies for the root N end-point, and samples of background invertebrates considered to be potential prey (Schulze *et al.*, 1991; Millett *et al.*, 2003, 2012) as proxies for the prey N end-point. It may be suggested that plant proxies for the root N end-point would be more representative if factors that influence $\delta^{15}\text{N}$ are considered, such as maximum rooting depth (Kohzu *et al.*, 2003) and the degree of root discrimination between ^{15}N and ^{14}N (Shearer and Kohl, 1988), and that invertebrate proxies for the prey N end-point would be more representative if factors such as the proportional abundance of prey orders to the diet of the carnivorous plant, and invertebrate tissue %N are considered. Therefore, there is need for comparison of the potential suitability of a range of proxies for the prey N and root N end-points of *Drosera rotundifolia*.

There are several sources of uncertainty associated with use of the linear mixing model that contribute towards variability in %N_{dfp}, such as uncertainty incurred due to variability in $\delta^{15}\text{N}$ of the sources used in the model. Following sensitivity analysis using Isoerror exploring the influence of source parameters of the difference in isotopic signatures between sources, population SD and source proportions on variability in source proportion estimates to a mixture, Phillips and Gregg (2001) showed that source difference exerts the most powerful influence on the SE of the source proportion estimate. Specifically, doubling the isotopic difference between sources reduces the variability of source proportion estimates by half. Furthermore, reducing the sample size of all source parameters resulted in at least halving the variability in source proportion estimates. However, no studies to date have explored the influence of similar parameters of level one contributors on variability in source proportion estimates using multi-level models, such as in the case of calculating %N_{dfp} of carnivorous plants that are dietary generalists, where many orders of prey (level one contributors) comprise the prey N end-point. Studies to date exploring the N nutrition of carnivorous plants *in-situ* have not considered the influence of variability in invertebrate prey taxa $\delta^{15}\text{N}$, the diet of the carnivorous plant (i.e. proportional contribution of each taxa to $\delta^{15}\text{N}_{\text{prey}}$) or SD($\delta^{15}\text{N}$) within and between taxa on variability in SE(%N_{dfp}) (Schulze *et al.*, 1991, 1997; Moran *et al.*, 2001; Millett *et al.*, 2003, 2012), therefore SE(%N_{dfp}) is likely to be underestimated (Phillips and Gregg, 2001). Therefore there is a need to determine the influence of level one contributors to variability in source proportion estimates in multi-level mixing models in order to address this source of uncertainty in source proportion estimates.

This $\delta^{15}\text{N}$ natural abundance study utilises sample populations of *in-situ* *Drosera rotundifolia*, captured prey, background invertebrates, *Sphagnum* and non-carnivorous plants at two ombrotrophic bogs with contrasting N deposition inputs to present an accurate method for the estimation of the proportional

contribution of prey-derived N to the N budget ($\%N_{dfp}$) in carnivorous plants and to address uncertainty in the calculation of $\%N_{dfp}$. Specifically, the following aims are addressed:

1. To present and evaluate an accurate method for determining the proportional contribution of prey-derived N to the N budget ($\%N_{dfp}$) of a carnivorous plant.
2. To compare the potential suitability of co-occurring non-carnivorous plant species as $\delta^{15}N$ proxies for the $\delta^{15}N$ of a carnivorous plant that has obtained 100% of N via the roots.
3. To determine the sensitivity of SE of source proportions to a mixture to level one and level two contributors using the example of the calculation of $SE(\%N_{dfp})$ of a carnivorous plant.
4. To explore the influence of N deposition on the N nutrition of *Drosera rotundifolia*.

Aim 1 is achieved by incorporating $\delta^{15}N$, tissue %N and proportional mass contribution of each invertebrate order to the total mass of prey captured by *Drosera rotundifolia* into a weighted prey $\delta^{15}N$ value. The $\delta^{15}N$ of weighted prey, $\delta^{15}N$ of co-occurring, non-carnivorous reference plant species and $\delta^{15}N$ of *D. rotundifolia* are then used to calculate $\%N_{dfp}$ using the two end-member $\delta^{15}N$ natural abundance linear mixing model (Shearer and Kohl, 1988). The potential influence of using weighted prey $\delta^{15}N$ as the prey end-member of the linear mixing model is evaluated by: (i) testing for differences in $\delta^{15}N$, tissue %N and proportional mass contribution to the total prey mass between orders of captured prey; (ii) testing whether prey $\delta^{15}N$ weighting status (unweighted, weighted) influences the value of and variability in $\delta^{15}N_{prey}$, and (iii) exploring the influence of prey $\delta^{15}N$ weighting status on the value of and variability in $\%N_{dfp}$ of *D. rotundifolia*.

Aim 2 is achieved by using *Drosera rotundifolia* and four co-occurring non-carnivorous plant species (*Calluna vulgaris* (common heather), *Eriophorum vaginatum* (hare's-tail cottongrass), *Erica tetralix* (cross-leaved heath) and *Sphagnum fuscum* (rusty bog-moss)) to test whether: (i) differences in $\delta^{15}N$ values are found between plant species and between sites; (ii) non-carnivorous plant proxy type used for the root N end-member of the linear mixing model influences the value of and variability in $\%N_{dfp}$ of *D. rotundifolia*; (iii) the mean $\delta^{15}N$ of the non-carnivorous plant species selected as proxies for the root N end-member of the linear mixing model for this study differs between sites.

Aim 3 is achieved by conducting sensitivity analyses to determine the influence of the following on $SE(\%N_{dfp})$ of *D. rotundifolia*: (i) parameters of level one contributors (mean difference in $\delta^{15}N$ between prey orders, number of invertebrate orders, sample size per order and $SD(\delta^{15}N)$ of each order), and (ii) parameters of level two end-member sources (mean difference in $\delta^{15}N$ between end-members, proportional contribution of either source to the mixture, sample size per source and $SD(\delta^{15}N)$ of each order).

Aim 4 is achieved by testing for between-site differences in the following nutritional parameters of *Drosera rotundifolia*: (i) %N_{dfp}; (ii) tissue C:N ratio, and (iii) amounts of prey-derived and root-derived N per plant. In order to test for between-site differences in the root N availability to the survey plants, dissolved inorganic N (DIN) of pore water was used as a proxy for root N availability and measured at each site throughout the course of the experiment.

Results of this study will therefore provide a method for calculating accurate %N_{dfp} for carnivorous plants and offer evidence-based recommendations of how variability in source proportion estimates using multi-level mixing models can be minimised.

4.3 Methods

Fieldwork was undertaken from May to September 2011 at two ombrotrophic raised bogs in the United Kingdom which differ primarily by N deposition input (Table 18). Whixall Moss is a lowland raised peat bog in Shropshire, England; Cors Fochno is an estuarine lowland raised peat bog in Ceredigion, Wales.

Table 18 Locations, climatic and environmental characteristics of Cors Fochno and Whixall Moss ombrotrophic raised bogs. Table (a) summarises site characteristics for 2006 to 2011 (where possible), this time period representing the maximum potential life span of survey *Drosera rotundifolia* plants; Table (b) summarises site characteristics for 2011 during which fieldwork was undertaken.

(a)

Site	Location	Mean annual precipitation (mm yr ⁻¹) ^a	Mean temperature January / July (°C) ^a	Mean growing season length (d) ^b	Growing season average temperature (°C) ^b	N deposition (kg N ha ⁻¹ yr ⁻¹) ^c
Cors Fochno	52°30'09N, 04°00'57W	1381	3.5/14.7	320	11.6	8.0
Whixall Moss	52°92'16N, 02°76'45W	719	4.0/13.7	296	11.0	30.8

^a Based on observed meteorological data from KNMI Climate Explorer (<http://climexp.knmi.nl>) (accessed 08.09.2014). Data are mean values for 2006 - 2011 inclusive.

^b Growing season is the number of days with mean temperature ≥ 5°C. Data are mean values for 2011 – 2012 inclusive (earlier years unavailable). Based on observed meteorological data from automatic weather stations at each site; data accessed from Countryside Council for Wales (Cors Fochno) and Natural England (Whixall Moss).

^c Modelled N deposition data from APIS (<http://www.apis.ac.uk/>) (accessed 21.04.2014). Data are mean values for 2010-2012 inclusive (earlier years unavailable).

(b)

Site	Annual precipitation (mm yr ⁻¹) ^a	Temp. Jan / July (°C) ^a	Growing season length (d) ^b	Growing season average temp. (°C) ^{a,b}	DIN (µg N l ⁻¹) ^c
Cors Fochno	1008	3.7/15.0	324	12.1	735.8
Whixall Moss	565	2.5/14.2	301	11.5	1505.4

^a Data values for 2011 only. Based on observed meteorological data from automatic weather stations at each site; data accessed from Countryside Council for Wales (Cors Fochno) and Natural England (Whixall Moss).

^b Growing season is the number of days with mean temperature ≥ 5°C.

^c DIN values were obtained from the analysis of pore water samples taken from each survey plot at four weekly intervals during the course of the experiment in 2011. Significant differences were found between sites (independent samples *t*-test:- $t_{(9)} = 3.275$, $P = 0.009$).

Ten survey plots were allocated at each site by selecting areas which contained *Drosera rotundifolia* plants growing in *Sphagnum* and were nearest to randomly generated GPS points. Fifteen survey *D.*

rotundifolia plants were allocated and individually labelled at each plot by locating randomly selected map coordinates using a handheld GPS device, and selecting the nearest plant to each point. At four-weekly intervals, 500 ml pore water samples were collected by squeezing the upper 10 cm layer of peat at each survey plot for ammonium ($\text{NH}_4^+\text{-N}$), nitrate ($\text{NO}_3^-\text{-N}$) and nitrite ($\text{NO}_2^-\text{-N}$) determination (Aim 4). Water samples were stored in plastic sample bottles that had been soaked for at least one day with 10% HCl and rinsed with ultrapure water prior to each survey session. Water samples were filtered as soon as possible following collection using Whatman 0.7 μm GF/F glass fibre micro filters (Nollet, 2007) and a sterilised Sterifil aseptic system (Merck Millipore Ltd, UK), and stored in sterile plastic sample bottles in black bin liners at 3 – 5 °C to inhibit algal and bacterial growth. Water samples were analysed for ammonium ($\text{NH}_4^+\text{-N}$), nitrate ($\text{NO}_3^-\text{-N}$) and nitrite ($\text{NO}_2^-\text{-N}$) by ion-exchange chromatography as soon as possible following sample filtration. Total dissolved inorganic N content (DIN) ($\mu\text{g l}^{-1}$) was calculated as the sum of $\text{NH}_4^+\text{-N}$, $\text{NO}_3^-\text{-N}$ and $\text{NO}_2^-\text{-N}$ for each pore water sample.

4.3.1 Incorporating diet into the estimation of the proportion of prey-derived N of the total N budget (%N_{dfp}) of *Drosera rotundifolia*

Invertebrate sampling constituted two distinct phases. The first was to determine the diet of *D. rotundifolia* (Aim 1); the second was to determine the $\delta^{15}\text{N}$ of each prey order of the diet (Aims 1 and 3). The diet of *D. rotundifolia* was determined by collecting invertebrate prey from ten randomly selected leaves containing freshly captured prey from non-survey plants within or adjacent to each plot. Invertebrates captured by survey *D. rotundifolia* plants were not removed as this would influence %N_{dfp} of the plants. To prevent damage to the captured invertebrates that may have been incurred from their removal from the leaves with forceps in the field, each set of ten leaves containing the invertebrates were removed and stored in sample tubes pre-filled with saturated NaCl solution. In a laboratory, invertebrates were counted and identified to order level using a Zeiss Stemi 2000 stereo microscope (Carl Zeiss Microscopy Ltd, Germany) and the length of each invertebrate measured to 0.1 mm precision using a 100 x 0.1 mm stage micrometer (Pyser-SGI Ltd, UK). Thus, species abundance, type, and size data of invertebrates were collated.

In order to determine the $\delta^{15}\text{N}$ of each invertebrate order of prey captured by *D. rotundifolia*, samples of background invertebrates were collected at four weekly intervals throughout the plants' active growth season in order to obtain sufficient dry mass for $\delta^{15}\text{N}$ stable isotope analysis. As the diet of *D. rotundifolia* is constituted of flying and flightless invertebrates, background invertebrates were sampled by sweep netting and pitfall trapping respectively. At each survey plot, three bait-less pitfall traps, constructed from plastic cups with suspended roofs, were submerged flush with *Sphagnum capitula* at random locations within a 40 cm radius of survey *D. rotundifolia* plants. Three sweep net surveys of the vegetation surrounding each plot were conducted at set times (10am, 12pm, and 2pm) for three minutes. After each four-week period, the invertebrates caught at each survey time by each sampling method per plot were pooled, counted and stored in saturated NaCl solution. The inorganic, carbon-free preservation method of saturated NaCl solution was chosen as the instant freezing of invertebrate samples was not feasible in this study; other preservation solutions such as formaldehyde and ethanol are not as effective sample storage methods prior to $\delta^{15}\text{N}$ isotopic natural abundance determination as they have a greater alteration effect on the $\delta^{15}\text{N}$: $\delta^{13}\text{C}$ ratio of invertebrate tissue (Ponsard & Amlou, 1999). Invertebrates were identified to order level and the length of each specimen measured (as above). Background invertebrates outside the length range of invertebrates captured by *D. rotundifolia* plants at both sites (0.4 - 6.5 mm in length) were excluded from the dataset, and remaining background invertebrates pooled per order per plot for $\delta^{15}\text{N}$ isotopic natural abundance determination.

Invertebrates were rinsed thoroughly in deionised water and dried to a constant weight by placing in a forced-air oven at 70 °C for 72 hours (Campbell & Plank, 1992), and weighed to obtain dry mass measurements (Aim 1). Samples were ground to a fine powder using a pestle and mortar to ensure sample homogeneity (Baker & Thompson, 1992). Invertebrate material was pre-weighed into tin capsules and analysed for $\delta^{15}\text{N}$ at the NERC Life Science Mass Spectrometry Facility, East Kilbride, Scotland (Aims 1, 3 & 4). Nitrogen isotopes were analysed by continuous flow using a Thermo Scientific DELTA V Plus isotope ratio mass spectrometer (Thermo Scientific, Bremen, Germany) interfaced with a Costech ECS 4010 elemental analyser (Costech Instruments, Milan, Italy). Three in-house standards (gelatine, glycine, alanine) were run every ten samples for quality assurance. All data are reported with respect to the international standard of atmospheric N_2 for $\delta^{15}\text{N}$. Results were reported in δ notation as the deviation from standards in parts per thousand (‰), where $\delta X = [(R_{\text{sample}} / R_{\text{reference}}) - 1] \times 1000$, and $R = {}^{15}\text{N} : {}^{14}\text{N}$, and $X = {}^{15}\text{N}$. Precision was 0.2 ‰ for $\delta^{15}\text{N}$. Total N and C contents of invertebrate material were also obtained during the $\delta^{15}\text{N}$ analysis (Aim 4).

The weighted mean $\delta^{15}\text{N}$ of invertebrate prey captured by *D. rotundifolia* plants (Aim 1) was calculated by incorporating the $\delta^{15}\text{N}$ and percentage N of dry mass of each invertebrate order per survey plot and the proportional dry mass of each order of captured prey to the total dry mass of captured prey per survey plot (Equation 1):

$$\delta^{15}\text{N}_C = \sum_i^n \left[aA_i \times \left(\frac{aB_i}{100} \right) \times \left(\frac{aC_i}{aD} \right) \right] \quad (1)$$

where $\delta^{15}\text{N}_C$ represents the weighted mean $\delta^{15}\text{N}$ of invertebrate prey captured by *D. rotundifolia*, n is the total number of orders of captured invertebrate prey at site a , aA_i is the $\delta^{15}\text{N}$ value (‰) of the i th invertebrate order per survey plot at site a , aB_i is the percentage N by weight of the i th invertebrate order per survey plot at site a , aC_i is the dry mass (mg) of the i th invertebrate order per survey plot at site a , and aD is the total dry mass (mg) of captured invertebrate prey per survey plot at site a .

The two end-member $\delta^{15}\text{N}$ linear mixing model as presented by Shearer and Kohl (1988) for estimating the contribution of atmospherically-fixed N to plants, and later adapted for use in carnivorous plant research by Schulze *et al.* (1991), was used to calculate the relative contributions of invertebrate prey N and root N to the total N budget in *D. rotundifolia* (Equation 2):

$$\%N_{\text{dfp}} = \frac{\delta^{15}\text{N}_A - \delta^{15}\text{N}_B}{\delta^{15}\text{N}_C - \delta^{15}\text{N}_B} \quad (2)$$

where $\%N_{\text{dfp}}$ represents the relative contribution of invertebrate prey N to the total N budget of *D. rotundifolia* (%), $\delta^{15}\text{N}_A$ represents the $\delta^{15}\text{N}$ of *D. rotundifolia*, $\delta^{15}\text{N}_B$ represents the mean $\delta^{15}\text{N}$ of

appropriate non-carnivorous plant and bryophyte species, *S. fuscum* and *E. vaginatum*, from the corresponding survey plot, and $\delta^{15}\text{N}_c$ represents the mean weighted $\delta^{15}\text{N}$ of invertebrate prey captured by *D. rotundifolia*.

Univariate analyses of variance (ANOVAs) or independent samples *t*-tests were conducted to explore the influence of invertebrate order / prey capture parameters incorporated into the calculation of weighted prey $\delta^{15}\text{N}$ (Eqn. 1) on weighted prey $\delta^{15}\text{N}$ and differences in weighted prey $\delta^{15}\text{N}$ between-sites (Aim 1). To compare the influence of prey $\delta^{15}\text{N}$ weighting status on $\%N_{dfp}$, $\%N_{dfp}$ was also calculated using unweighted mean prey $\delta^{15}\text{N}$ as the prey N end-point of the mixing model (Aim 1). The influences of site and prey $\delta^{15}\text{N}$ weighting status on $\%N_{dfp}$ were tested using two-way univariate analysis of variance (ANOVA) (Aim 1).

4.3.2 The use of appropriate plant proxies for the root N end-point of the mixing model

At the end of the field season, survey *D. rotundifolia* plants were removed and leaves and stems from co-occurring bryophyte (*Sphagnum fuscum*) and non-carnivorous vascular plant species (*Calluna vulgaris*, *Erica tetralix* and *Eriophorum vaginatum*) were collected for $\delta^{15}\text{N}$ stable isotope analysis to explore the influence of plant proxy type on $\%N_{\text{dfp}}$ of *D. rotundifolia* (Aim 2), to provide a $\delta^{15}\text{N}$ proxy for the root N end-member of the linear mixing model (Aim 4) and to provide context for the $\delta^{15}\text{N}$ of *D. rotundifolia* (Millett *et al.*, 2003). *D. rotundifolia* plants were rinsed with deionised water, previous growth material removed and the remaining living material pooled per survey plot. A 10 cm³ sample of *Sphagnum* capitula surrounding each survey *D. rotundifolia* plant and the above-ground material of 2-3 plants of each type of non-carnivorous plant species was collected, rinsed with deionised water, and pooled per survey plot. Prior to oven drying, plant material was kept cold and stored in labelled paper sample bags to permit air drying, thus enhancing preservation through the minimisation of enzymatic reactions (Campbell & Plank, 1992).

Plant material was dried and weighed to obtain dry mass measurements (see invertebrate sample drying procedure). *D. rotundifolia* samples were ground to a fine powder using a pestle and mortar, and *Sphagnum* and non-carnivorous vascular plant material using a ball mill (Retsch MM200, Retsch, Haan, Germany). Plant material was pre-weighed into tin capsules and analysed for $\delta^{15}\text{N}$, and total N and C content (see procedure for $\delta^{15}\text{N}$ analysis of invertebrate material) (Aims 2 and 4).

In order to explore the influence of plant proxy type for the root N end-point of the mixing model on $\%N_{\text{dfp}}$ of *D. rotundifolia* (Aim 2), $\%N_{\text{dfp}}$ values were calculated using the $\delta^{15}\text{N}$ of each co-occurring plant/bryophyte species and the mean $\delta^{15}\text{N}$ of all species as the root N end-points and weighted prey $\delta^{15}\text{N}$ as the prey N end-point of the mixing model. The influence of site and plant proxy type on $\%N_{\text{dfp}}$ was tested using two-way univariate ANOVA (Aim 2).

4.3.3 Data analyses

4.3.1.1 The use of a multi-level mixing model: an example using the N nutrition of a carnivorous plant

Sensitivity analyses were conducted to determine the sensitivity of SE of source proportions of a mixture to parameters of level one and level two contributors (Aim 3). The calculation of the reliance of a carnivorous plant on botanical carnivory was used as an example. In this case, level one contributors are the $\delta^{15}\text{N}$ of the orders of invertebrate prey constituting the diet of the carnivorous plant, the two sources (level two contributors) are the prey $\delta^{15}\text{N}$ and root $\delta^{15}\text{N}$ end-points of the linear mixing model, and the mixture is the $\delta^{15}\text{N}$ of the carnivorous plant.

The sensitivity of $\text{SE}(\%N_{\text{dfp}})$ to invertebrate order parameters (number of orders, within-order sample size and SD of each order) and source parameters (isotopic difference in $\delta^{15}\text{N}$ between sources, within-source sample size, SD of each source, source proportion) was determined using the approach of Phillips and Gregg (2001). $\%N_{\text{dfp}}$ and $\text{SE}(\%N_{\text{dfp}})$ were calculated using the Isoerror spreadsheet (Phillips and Gregg, 2001). Each invertebrate order / source parameter was varied in turn within the specified value range with remaining factors set to default values (Appendices 2 and 3). The population SDs and sample sizes were kept equal to each other, and varied together. All sensitivity analyses were conducted using Microsoft Excel (2010).

4.3.1.2 Calculating the N nutrition of *Drosera rotundifolia* and differences between sites

In order to calculate a more accurate value of $\%N_{\text{dfp}}$ for *D. rotundifolia* than values calculated using methodologies of previous studies (Millett *et al.*, 2003, 2012) (Aims 1 and 4), weighted prey $\delta^{15}\text{N}$ (Eqn. 1) was used as the prey $\delta^{15}\text{N}$ end-point of the mixing model. The mean $\delta^{15}\text{N}$ of *S. fuscum* and *E. vaginatum* was incorporated as the root $\delta^{15}\text{N}$ end-point of the mixing model as these species possess maximum rooting depths closest to that of *D. rotundifolia*, and therefore, as the $\delta^{15}\text{N}$ of plant species increases with increasing rooting depth (Kohzu *et al.*, 2003), these species were considered most closely representative of the root $\delta^{15}\text{N}$ uptake of *D. rotundifolia* plants deriving 100% of the plant N budget via the roots (Schulze *et al.*, 1991; Moran *et al.*, 2001). $\%N_{\text{dfp}}$ was calculated using Equation 2. $\%N_{\text{dfp}}$ and $\%N_{\text{dfr}}$ data used in statistical analyses were calculated by taking the mean and SE of $\%N_{\text{dfp}}$ per survey plot at each site. Percentage N, $\%N_{\text{dfp}}$ and the dry mass of *D. rotundifolia* were used to calculate total N per plant, total prey-derived N (N_{dfp}) per plant and total root-derived N (N_{dfr}) per plant (Aim 4).

Strong, significant correlations were reported between N_{dfp} per plant per survey plot and the mean dry mass of live plants per survey plot (Pearson's correlation coefficient, $r = 0.620$, $P = 0.004$, $n = 20$), and

between the amount of root-derived N (N_{dfr}) per plant per survey plot and mean dry mass of live plants per survey plot (Pearson's correlation coefficient, $r = 0.724$, $P < 0.001$, $n = 20$). Therefore, plant total dry mass was used as a covariate for between-site comparisons of amounts of N, N_{dfp} and N_{dfr} (Aim 4). Analysis outcomes are reported as unadjusted for size unless stated otherwise. The data were evaluated using ANOVA, independent-samples t -tests, Pearson's correlation, and linear regression. Post-hoc comparisons were conducted using Fisher's Least Significant Difference (LSD) (0.05 significance level). Data were \log_{10} -transformed prior to analysis where data did not conform to the assumptions of homoscedasticity. Residual plots were used to assess for homoscedasticity and normal probability plots used to test that data were normally distributed.

All statistical analyses were conducted using IBM SPSS Statistics version 21 (IBM, Chicago, USA).

4.4 Results

4.4.1 Incorporating diet into the estimation of %N_{dfp}

Table 19 Results of 2-way (site, invertebrate order or weighting status) univariate ANOVAs for the $\delta^{15}\text{N}$ of invertebrate orders captured by *Drosera rotundifolia* plants growing at two ombrotrophic bogs in the UK that vary by N deposition input. Presented are degrees of freedom (*df*), *F* and *P* values for (a) $\delta^{15}\text{N}$ of orders of captured invertebrates; (b) $\delta^{15}\text{N}$ of pooled captured prey. Invertebrate orders: Acarina, Araneae, Coleoptera, Collembola, Diptera, Formicidae (family level), Hemiptera, Hymenoptera, Lepidoptera, Orthoptera; sites: Cors Fochno, Whixall Moss; weighting statuses: unweighted (average $\delta^{15}\text{N}$ of all invertebrate orders captured by *D. rotundifolia* plants, weighted (average $\delta^{15}\text{N}$ of captured prey incorporating the percentage N by dry mass of each order and the proportional contribution of dry mass of each prey order to the total dry mass of captured prey). Significant effects at $P < 0.05$ are highlighted in bold.

(a)

	<i>df</i>	$\delta^{15}\text{N}$ <i>F</i>	<i>P</i>
Invertebrate order (O)	9, 136	53.969	<0.001
Site	1, 136	8.441	0.004
O x site	9, 136	1.703	0.096

(b)

	<i>df</i>	$\delta^{15}\text{N}_{\text{prey}}$ <i>F</i>	<i>P</i>
Weighting status (WS)	1, 39	74.650	<0.001
Site	1, 39	39.083	<0.001
WS x site	1, 39	39.860	<0.001

Invertebrate $\delta^{15}\text{N}$ differed significantly between sites (Table 19(a)); invertebrates at Cors Fochno were significantly more $\delta^{15}\text{N}$ -depleted than invertebrates at Whixall Moss (Fig. 18) (Aim 1).

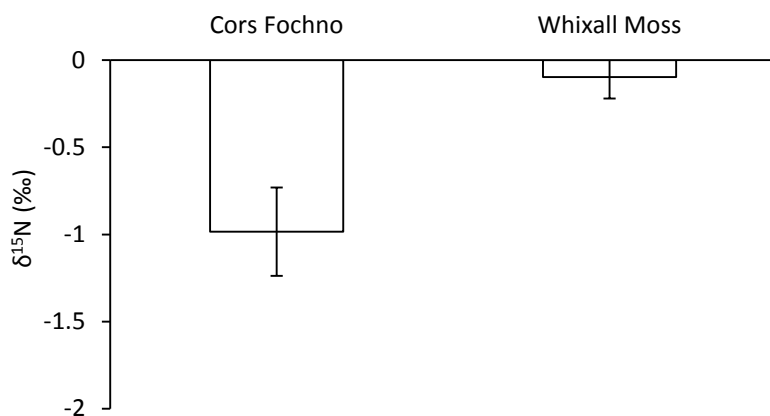


Figure 18 $\delta^{15}\text{N}$ (mean \pm 1 S.E.) of background invertebrates of all orders captured by *Drosera rotundifolia* plants at each site.

The $\delta^{15}\text{N}$ of invertebrates differed significantly between orders comprising the diet of *Drosera rotundifolia* (Table 19(a)). Specifically, Diptera and Hymenoptera were more $\delta^{15}\text{N}$ -enriched and Lepidoptera, Hemiptera and Coleoptera were more $\delta^{15}\text{N}$ -depleted than the remaining orders (Fig. 19; Appendix 4) (Aim 1).

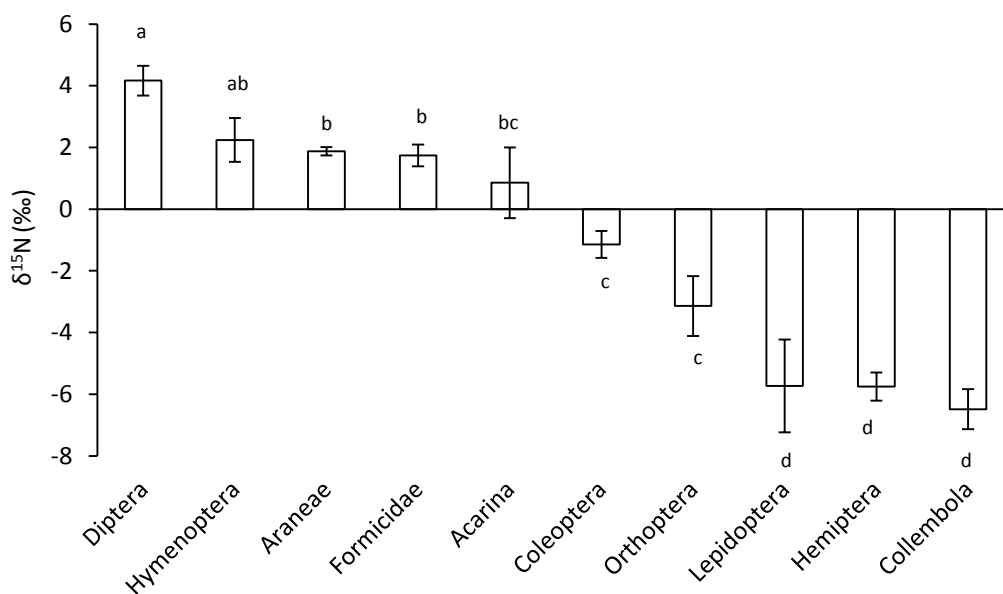


Figure 19 Background invertebrate $\delta^{15}\text{N}$ (mean \pm 1 S.E.) of the orders captured by *Drosera rotundifolia*. Bars with different letters are significantly different from each other (Fisher's least significant difference, $P < 0.05$).

Table 20 Results of 2-way (site, invertebrate order) univariate ANOVA for the percentage N content of tissue dry mass of invertebrate orders constituting the diet of *Drosera rotundifolia* plants. Presented are degrees of freedom (*df*), *F* and *P* values for tissue percentage N content of orders of captured invertebrates. Invertebrate orders: Acarina, Araneae, Coleoptera, Collembola, Diptera, Formicidae (family level), Hemiptera, Hymenoptera, Lepidoptera, Orthoptera; sites:- Cors Fochno, Whixall Moss. Significant effects at $P < 0.05$ are highlighted in bold.

	<i>df</i>	%N (wt) <i>F</i>	<i>P</i>
Invertebrate order (O)	9, 137	15.621	<0.001
Site	1, 137	22.536	<0.001
O x site	9, 137	2.650	0.008

The percentage N content (%N) of invertebrate tissue differed significantly between sites (Table 20; Fig. 20(a)) (Aim 1); %N of invertebrates at Whixall Moss was significantly greater than the %N of invertebrates at Cors Fochno. Invertebrate %N per order per site differed significantly (Table 20; Fig. 20(b)) (Aim 1). Specifically, the %N of Araneae at Cors Fochno was significantly lower than the %N of Araneae at Whixall Moss, and the %N of Collembola at Cors Fochno was significantly lower than the %N of Collembola at Whixall Moss. At Cors Fochno, the %N of Collembola did not significantly differ from the %N of Orthoptera and Lepidoptera, whereas at Whixall Moss, the %N of Collembola was significantly lower than the %N of remaining orders.

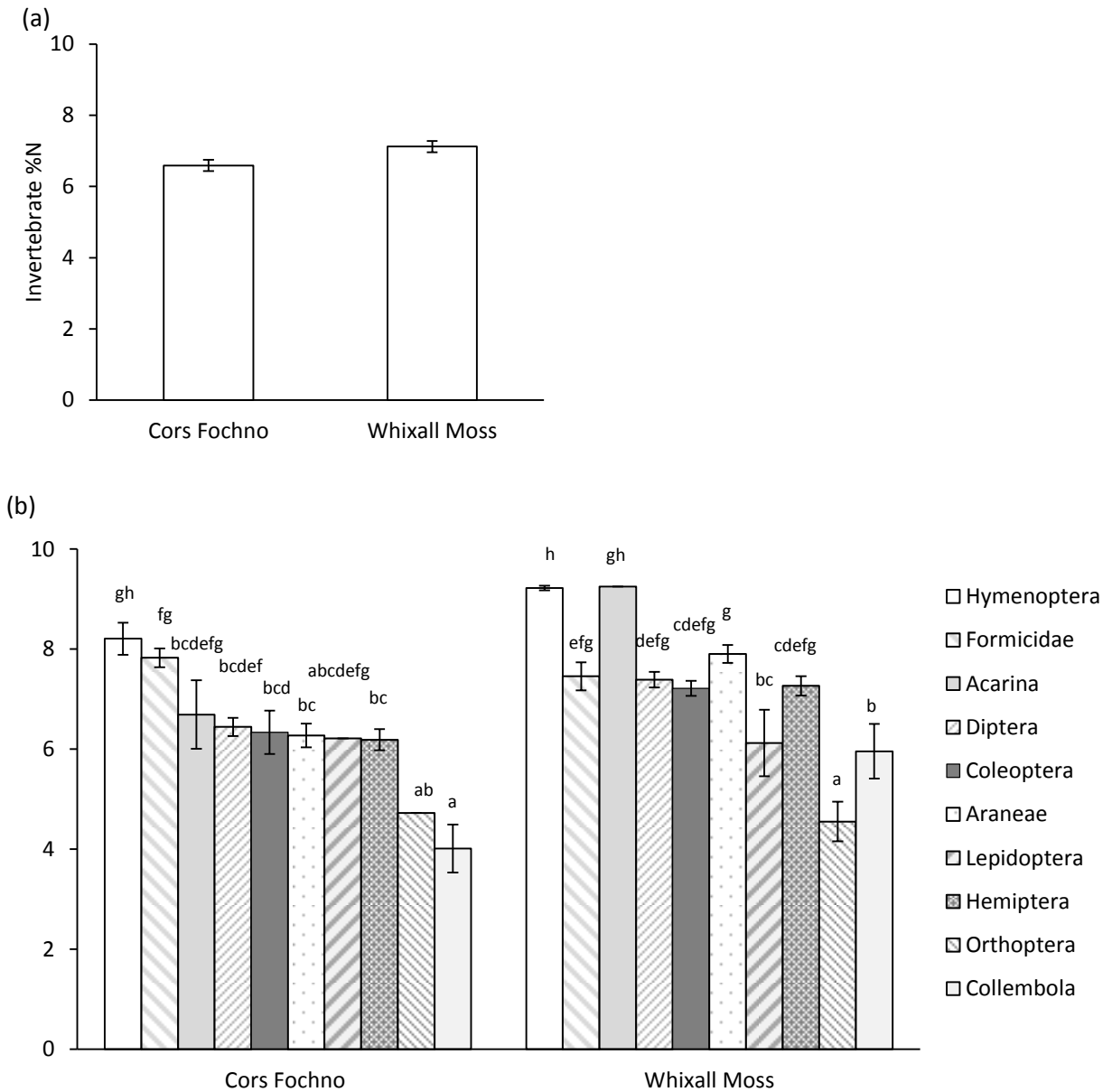


Figure 20 Tissue percentage N content (%N) of the invertebrate orders captured by *Drosera rotundifolia*. Presented are mean \pm 1 S.E. for: (a) %N per site; (b) %N per order of captured prey per site. Invertebrate orders: Acarina, Araneae, Coleoptera, Collembola, Diptera, Formicidae (family level), Hemiptera, Hymenoptera, Lepidoptera, Orthoptera; sites:- Cors Fochno, Whixall Moss. Bars with different letters in Fig. (b) differ significantly from each other (Fisher's least significant difference, $P < 0.05$).

Table 21 Results of 2-way (site, invertebrate order) univariate ANOVA for the proportional contribution of the dry mass of invertebrate prey orders to the total dry mass of prey captured by *Drosera rotundifolia* plants. Presented are degrees of freedom (*df*), *F* and *P* values for the proportional contribution of the dry mass of invertebrate prey orders to the total dry mass of prey captured by *D. rotundifolia* per order per site. Invertebrate orders: Acarina, Araneae, Coleoptera, Collembola, Diptera, Formicidae (family level), Hemiptera, Hymenoptera, Lepidoptera, Orthoptera; sites: Cors Fochno, Whixall Moss. Significant effects at $P < 0.05$ are highlighted in bold.

	<i>df</i>	<i>F</i>	<i>P</i>
Invertebrate order (O)	9, 199	59.489	<0.001
Site	1, 199	0.001	0.981
O x site	9, 199	8.525	<0.001

Drosera rotundifolia plants at Cors Fochno captured significantly greater mass proportion of Diptera and smaller mass proportion of Coleoptera than plants at Whixall Moss (Table 21; Fig. 21) (Aim 1). At Cors Fochno, the mass proportion of Diptera was significantly larger than remaining orders, whereas at Whixall Moss the mass proportion of Diptera was not significantly different to the mass proportion of Formicidae captured.

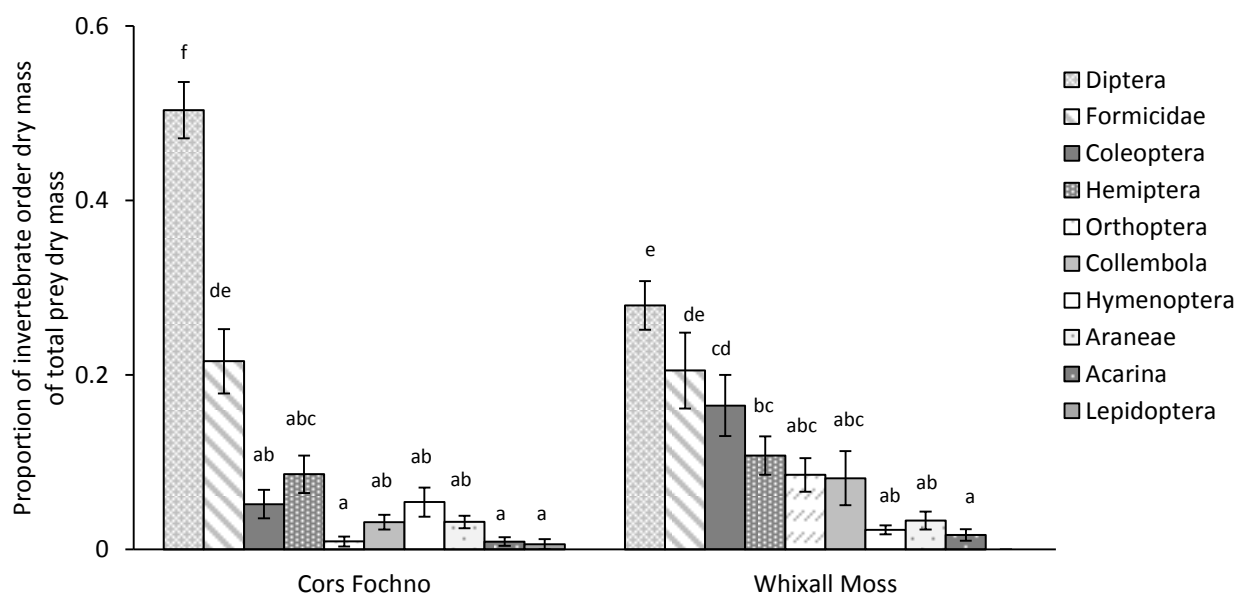


Figure 21 Proportional contribution of the dry mass of invertebrate prey orders to the total dry mass of prey captured by *Drosera rotundifolia*. Presented are mean \pm 1 S.E. for the proportion of invertebrate order dry mass of the total prey dry mass per order per site. Invertebrate orders: Acarina, Araneae, Coleoptera, Collembola, Diptera, Formicidae (family level), Hemiptera, Hymenoptera, Lepidoptera, Orthoptera; sites: Cors Fochno, Whixall Moss. Bars with different letters are significantly different from each other (Fisher's least significant difference, $P < 0.05$).

The total dry mass of invertebrate prey captured by *D. rotundifolia* plants at Cors Fochno was significantly greater than the total prey mass captured by plants at Whixall Moss (independent samples *t*-test, $t_{(18)} = -9.990$, $P < 0.001$; Fig. 22) (Aim 1).

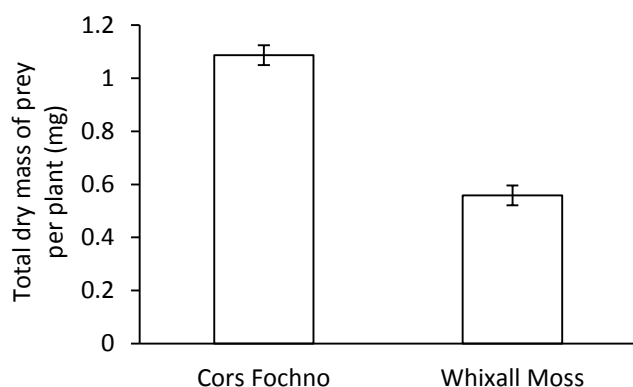


Figure 22 Total dry mass (mean \pm 1 S.E.) of invertebrate prey captured by *Drosera rotundifolia* plants at two ombrotrophic bogs in the UK that vary by N deposition input.

Unweighted $\delta^{15}\text{N}_{\text{prey}}$ for *D. rotundifolia* plants at Cors Fochno was significantly more $\delta^{15}\text{N}$ -depleted than the unweighted $\delta^{15}\text{N}_{\text{prey}}$ for plants at Whixall Moss (Fig. 23; Table 18(b)) (Aim 1). The $\delta^{15}\text{N}_{\text{prey}}$ of weighted samples from each site were not significantly different from each other (Aim 1). For invertebrates at Cors Fochno, the SE of unweighted $\delta^{15}\text{N}_{\text{prey}}$ (0.15 ‰) was fivefold larger than the SE of weighted $\delta^{15}\text{N}_{\text{prey}}$ at Cors Fochno (0.03 ‰) (Aim 1). For invertebrates at Whixall Moss, the SE of unweighted $\delta^{15}\text{N}_{\text{prey}}$ (0.24 ‰) was eightfold larger than the SE of weighted $\delta^{15}\text{N}_{\text{prey}}$ (0.03 ‰) (Aim 1).

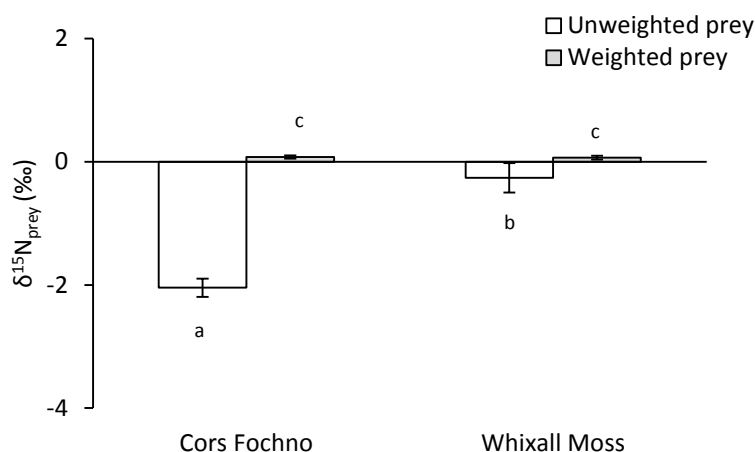


Figure 23 $\delta^{15}\text{N}$ of pooled samples of background invertebrates of the same orders that were captured by *Drosera rotundifolia* plants at two ombrotrophic bogs in the UK that vary by N deposition input. Presented are mean \pm 1 S.E. for the $\delta^{15}\text{N}$ of unweighted and weighted pooled invertebrate samples, where unweighted = mean $\delta^{15}\text{N}$ of all prey orders (Appendix 4), and weighted = mean $\delta^{15}\text{N}$ of all prey orders incorporating the $\delta^{15}\text{N}$, proportional abundance by dry mass and percentage N of dry mass for each order (Eqn. 1). Bars with different letters are significantly different from each other (Fisher's least significant difference, $P < 0.05$).

Table 22 Results of 2-way (site, prey weighting status) univariate ANOVA for the reliance on botanical carnivory by *Drosera rotundifolia* plants growing at two ombrotrophic bogs in the UK that vary by N deposition input. Presented are degrees of freedom (*df*), *F* and *P* values for the percentage contribution of prey-derived N to the total N budget (%N_{dfp}) of *D. rotundifolia* calculated using two weighting statuses of prey $\delta^{15}\text{N}$: mean $\delta^{15}\text{N}$ of all prey orders, unweighted; mean $\delta^{15}\text{N}$ of all prey orders incorporating the $\delta^{15}\text{N}$, proportional abundance by dry mass and percentage N of dry mass for each order, weighted. Significant effects at $P < 0.05$ are highlighted in bold.

	<i>df</i>	%N _{dfp} <i>F</i>	<i>P</i>
Prey weighting status (WS)	1, 39	1.163	0.288
Site	1, 39	14.162	0.001
PWS x site	1, 39	0.632	0.432

The weighting status of prey $\delta^{15}\text{N}$ exerted no statistically significant influence on the proportion of prey-derived N of the total N budget (%N_{dfp}) of *Drosera rotundifolia* plants (Fig. 24; Table 22) (Aim 1). The SE(%N_{dfp}) was reduced by ca. 24% by incorporating weighted $\delta^{15}\text{N}_{\text{prey}}$ (SE(%N_{dfp}) = 5.62 %) into the linear mixing model compared with incorporating unweighted $\delta^{15}\text{N}_{\text{prey}}$ (SE(%N_{dfp}) = 7.44 %) into the model (Aim 1).

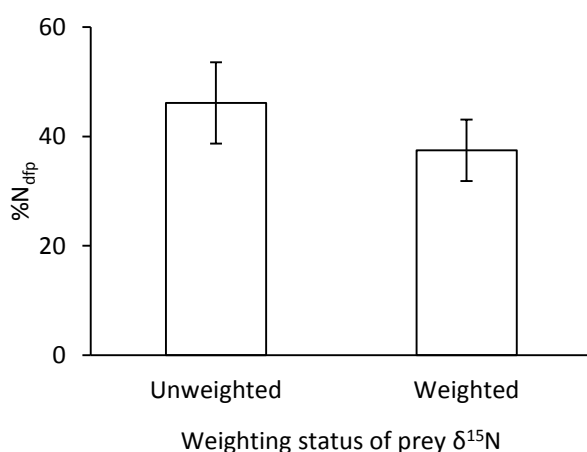


Figure 24 Reliance on botanical carnivory by *Drosera rotundifolia* plants calculated using different weighting statuses for the $\delta^{15}\text{N}$ of the prey end-member of the linear mixing model. Presented are mean \pm 1 S.E. for the percentage contribution of prey-derived N to the total N budget (%N_{dfp}) of *Drosera rotundifolia* plants using the following prey weighting statuses: mean $\delta^{15}\text{N}$ of all prey orders, unweighted; mean $\delta^{15}\text{N}$ of all prey orders incorporating the $\delta^{15}\text{N}$, proportional abundance by dry mass and percentage N of dry mass for each order, weighted.

4.4.2 The use of appropriate plant proxies for the root N end-point of the mixing model

Table 23 Results of 2-way (site, species) univariate ANOVA for the $\delta^{15}\text{N}$ of *Drosera rotundifolia* and co-occurring plant and bryophyte species growing at two ombrotrophic bogs in the UK that vary by N deposition input. Presented are degrees of freedom (*df*), *F* and *P* values for $\delta^{15}\text{N}$ of each plant species per site. Species: *Calluna vulgaris*, *Drosera rotundifolia*, *Erica tetralix*, *Eriophorum vaginatum*, *Sphagnum fuscum*; sites:- Cors Fochno, Whixall Moss. Significant effects at $P < 0.05$ are highlighted in bold.

	<i>df</i>	$\delta^{15}\text{N}$ <i>F</i>	<i>P</i>
Plant species	4, 99	61.179	<0.001
Site	1, 99	6.511	0.012
Species x site	4, 99	19.982	<0.001

Calluna vulgaris and *E. tetralix* at Cors Fochno were significantly more $\delta^{15}\text{N}$ -depleted than remaining species per site, but did not differ from each other (Fig. 25(a)) (Aim 2). *Drosera rotundifolia* at Cors Fochno was the most $\delta^{15}\text{N}$ -enriched of the species per site, but did not differ significantly in $\delta^{15}\text{N}$ from *E. vaginatum* at Cors Fochno and *D. rotundifolia* at Whixall Moss (Aim 2). Plant tissue $\delta^{15}\text{N}$ varied significantly between species (Table 23) (Aim 2); *D. rotundifolia* was significantly more $\delta^{15}\text{N}$ -enriched than the remaining species. *Calluna vulgaris* and *E. tetralix* were significantly more $\delta^{15}\text{N}$ -depleted than the remaining species, but did not significantly differ from each other. Plant/bryophyte tissue $\delta^{15}\text{N}$ differed significantly between sites (Table 23); species at Whixall Moss were significantly more $\delta^{15}\text{N}$ -enriched compared with species at Cors Fochno.

The mean proxy $\delta^{15}\text{N}$ for plants co-occurring with *D. rotundifolia* at Cors Fochno was significantly more $\delta^{15}\text{N}$ -depleted than the mean proxy $\delta^{15}\text{N}$ of co-occurring plants at Whixall Moss (independent samples *t*-test, $t_{(4)} = 4.033$, $P = 0.001$) (Fig. 25(b)) (Aim 2).

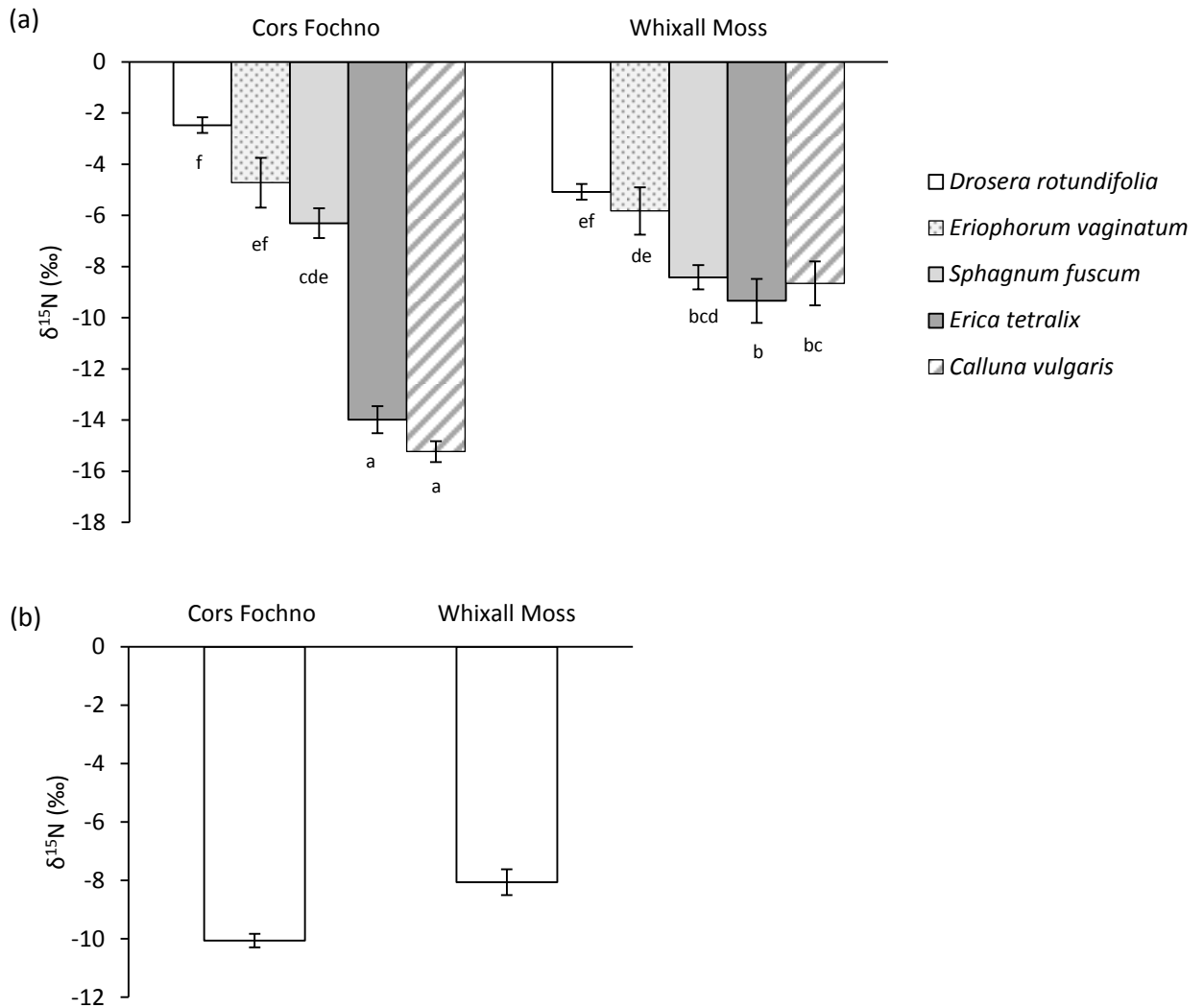


Figure 25 $\delta^{15}\text{N}$ (mean ± 1 S.E.) of *Drosera rotundifolia* and co-occurring bryophyte and non-carnivorous vascular plant species growing at two ombrotrophic bogs in the UK that vary by N deposition input. Presented are mean ± 1 S.E. for: (a) $\delta^{15}\text{N}$ of each plant species per site; (b) $\delta^{15}\text{N}$ of pooled proxy plant species (*Calluna vulgaris*, *Erica tetralix*, *Eriophorum vaginatum* and *Sphagnum fuscum*). Bars with different letters in Fig. (a) differ significantly from each other (Fisher's least significant difference, $P < 0.05$).

Table 24 Results of 2-way (site, proxy type) univariate ANOVAs for the reliance on botanical carnivory by *Drosera rotundifolia* plants growing at two ombrotrophic bogs in the UK that vary by N deposition input. Presented are degrees of freedom (*df*), *F* and *P* values for the proportion of prey-derived N of the total N budget (%N_{dfp}) of *D. rotundifolia* calculated using each plant proxy type for the *Drosera* root $\delta^{15}\text{N}$ end-member of the linear mixing model per site. Sites: Cors Fochno, Whixall Moss; root $\delta^{15}\text{N}$ proxy type: mean $\delta^{15}\text{N}$ of all co-occurring plant species (*Sphagnum fuscum*, *Calluna vulgaris*, *Erica tetralix* and *Eriophorum vaginatum*), all, $\delta^{15}\text{N}$ of *Sphagnum fuscum* only, $\delta^{15}\text{N}$ of *Calluna vulgaris* only, $\delta^{15}\text{N}$ of *Erica tetralix* only, $\delta^{15}\text{N}$ of *Eriophorum vaginatum* only. Significant effects at *P* < 0.05 are highlighted in bold.

	<i>df</i>	%N _{dfp} <i>F</i>	<i>P</i>
Proxy type (PT)	4, 99	5.295	0.001
Site	1, 99	8.291	0.005
PT x site	4, 99	0.244	0.912

Plant proxy type for the root $\delta^{15}\text{N}$ end-member of the linear mixing model exerted a significant effect on the proportion of prey-derived N of the total N budget (%N_{dfp}) of *Drosera rotundifolia* (Fig. 26; Table 24) (Aim 2). Mean %N_{dfp} values using all proxy types varied from -33.7 % to 55.1 %. The %N_{dfp} value calculated using the proxy of the $\delta^{15}\text{N}$ of *Eriophorum vaginatum* was significantly lower than the %N_{dfp} values calculated using the remaining proxies, and *E. vaginatum* was the only proxy type to produce negative %N_{dfp} values. The %N_{dfp} values calculated using the $\delta^{15}\text{N}$ of the remaining proxies did not significantly differ from each other.

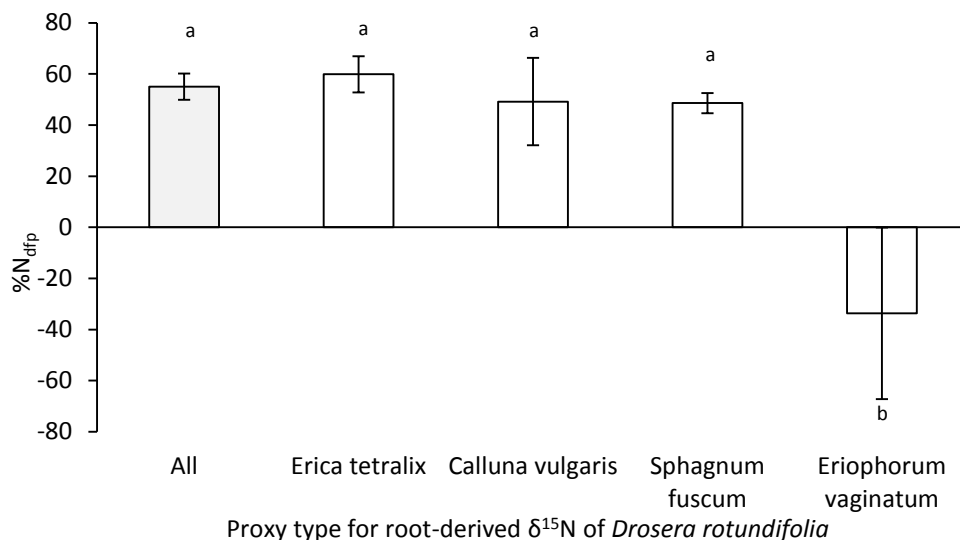


Figure 26 Reliance on botanical carnivory by *Drosera rotundifolia* plants calculated using different plant proxies for the $\delta^{15}\text{N}$ of the root end-member of the linear mixing model. Presented are mean \pm 1 S.E. for the proportion of prey-derived N of the total N budget ($\%N_{\text{dfp}}$) of *D. rotundifolia* plants using the following plant proxies: mean $\delta^{15}\text{N}$ of all plant/bryophyte species (*Erica tetralix*, *Calluna vulgaris*, *Sphagnum fuscum* and *Eriophorum vaginatum*), all, $\delta^{15}\text{N}$ of *E. tetralix*, $\delta^{15}\text{N}$ of *C. vulgaris*, $\delta^{15}\text{N}$ of *S. fuscum*, and $\delta^{15}\text{N}$ of *E. vaginatum*. Bars with different letters are significantly different from each other (Fisher's least significant difference, $P < 0.05$).

The mean $\delta^{15}\text{N}$ of non-carnivorous plant species (*S. fuscum* and *E. vaginatum*) selected as proxies for the root N end of the linear mixing model ($\delta^{15}\text{N}_{\text{NCVPs}}$) for *D. rotundifolia*, differed significantly between sites (independent samples t -test: $t_{(18)} = -2.199$, $P = 0.041$); $\delta^{15}\text{N}_{\text{NCVPs}}$ was significantly more $\delta^{15}\text{N}$ -depleted for plants at Whixall Moss than the $\delta^{15}\text{N}_{\text{NCVPs}}$ for plants at Cors Fochno (Fig. 27) (Aim 2).

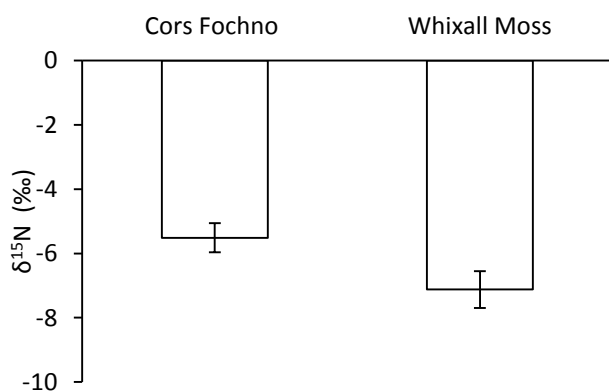


Figure 27 $\delta^{15}\text{N}$ (mean \pm 1 S.E.) of pooled non-carnivorous plant species (*Sphagnum fuscum*, *Eriophorum vaginatum*) selected as proxies for the root N end-member of the linear mixing model for the calculation of the proportional contribution of prey-derived N to the N budget of *Drosera rotundifolia*.

4.4.3 The use of a multi-level mixing model: an example using the N nutrition of a carnivorous plant.

4.4.3.1 The influence of parameters of level one contributors on variability in %N_{dfp}.

The influence of sample size of $\delta^{15}\text{N}$ per invertebrate order on $\text{SE}(\%N_{\text{dfp}})$ is considerable; for all invertebrate order parameters, increasing the sample size from 2 to 10 reduces $\text{SE}(\%N_{\text{dfp}})$ by over a half (Figs. 28(a), 28(b), 28(c)) (Aim 3). For example, for a total of 10 invertebrate orders:- if the sample size within each order = 2, $\text{SE}(\%N_{\text{dfp}}) = 1.77\%$; if the sample size within each order = 10, $\text{SE}(\%N_{\text{dfp}}) = 0.79\%$ (Fig 28(b)). The $\text{SE}(\%N_{\text{dfp}})$ is most sensitive to differences in $\text{SD}(\delta^{15}\text{N})$ within invertebrate orders (Fig. 28(c)) (Aim 3). The positive linear relationship between $\text{SE}(\%N_{\text{dfp}})$ and $\text{SD}(\delta^{15}\text{N})$ reflects that $\text{Var}(\%N_{\text{dfp}})$ constitutes the sum of $\text{SD}(\delta^{15}\text{N})$ of all invertebrate orders of captured prey; therefore as the $\text{SD}(\delta^{15}\text{N})$ within each invertebrate order is doubled, the $\text{SE}(\%N_{\text{dfp}})$ is doubled. Varying the mean difference in $\delta^{15}\text{N}$ between orders or the number of orders constituting the diet of the carnivorous plant exerts no influence on $\text{SE}(\%N_{\text{dfp}})$ (Figs. 28(a), 28(b)); variation in $\text{SE}(\%N_{\text{dfp}})$ reflects within-order differences in sample size only (Aim 3).

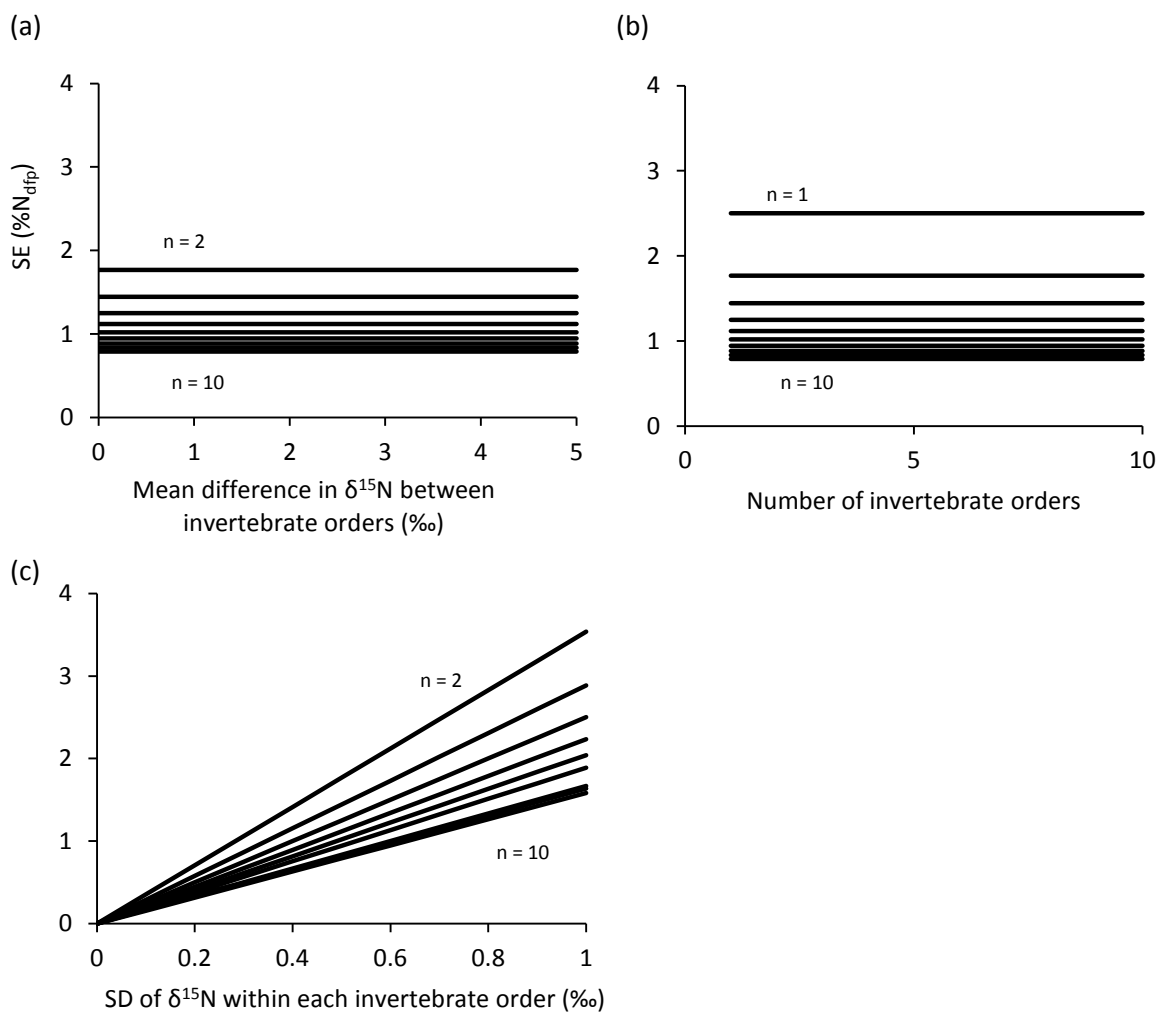


Figure 28 Influence of parameters of level one $\delta^{15}\text{N}$ contributors on variability in the percentage contribution of the associated level two source to the isotope mixture using the N nutrition of a carnivorous plant as a case study. Here, level one contributors are represented by the $\delta^{15}\text{N}$ of invertebrate orders constituting the diet of the carnivorous plant, the associated level two source is represented by the mean $\delta^{15}\text{N}$ of captured prey, and the isotope mixture is represented by the $\delta^{15}\text{N}$ of the carnivorous plant. Presented are the influences of the following invertebrate order parameters on the variability in the percentage contribution of prey-derived N to the total N budget (%N_{dfp}) of the carnivorous plant: (a) differences in $\delta^{15}\text{N}$ between invertebrate orders of 0 to 5 ‰ for each sample size (excluding n = 1); (b) differences in the number of invertebrate orders; (c) differences in SD of $\delta^{15}\text{N}$ within each invertebrate order. Lines represent sample sizes of 2 (top line) to 10 (bottom line), except for (b) where the top line represents a sample size of 1. Parameters used in the analyses are given in Appendix 2.

4.4.3.2 The influence of parameters of level two sources on variability in %N_{dfp}

The results of sensitivity analyses exploring the influence of various source factors on variation in the percentage contribution of prey-derived N of the N budget (%N_{dfp}) of a carnivorous plant show that the difference in $\delta^{15}\text{N}$ between prey N and root N end-members (sources) exerts the most powerful influence on the SE(%N_{dfp}) (Fig. 29(a)) (Aim 3). Specifically, a curved negative relationship exists between source difference in $\delta^{15}\text{N}$ and SE(%N_{dfp}), where a steep increase in SE(%N_{dfp}) occurs for sources with $\delta^{15}\text{N}$ differences of $< ca. 6 \text{ ‰}$. For example, for source sample sizes of 5:- when $\delta^{15}\text{N}$ difference = 2, SE(%N_{dfp}) = 36.08 ‰, when $\delta^{15}\text{N}$ difference = 6 ‰, SE(%N_{dfp}) = 12.03 ‰. A shallow curvilinear relationship exists between the proportion of either source to the mixture and SE(%N_{dfp}) (Fig. 29(b)); if source proportions = 0 or 1, SE(%N_{dfp}) values are about twofold larger than SE(%N_{dfp}) value when source proportion = 0.5 (Aim 3). A positive linear relationship is observed between the SD of source $\delta^{15}\text{N}$ and SE(%N_{dfp}), with the gradient of the line increasing as sample size decreases (Aim 3).

The influence of sample size is substantial; for all source factors, increasing the sample size from 2 to 10 decreases SE(%N_{dfp}) by more than half, e.g. where SD($\delta^{15}\text{N}$) of the prey or root N end-member = 2.5:- at n = 2, SE(%N_{dfp}) = 12.50; at n = 10, SE(%N_{dfp}) = 5.59 (Fig. 29(c)) (Aim 3).

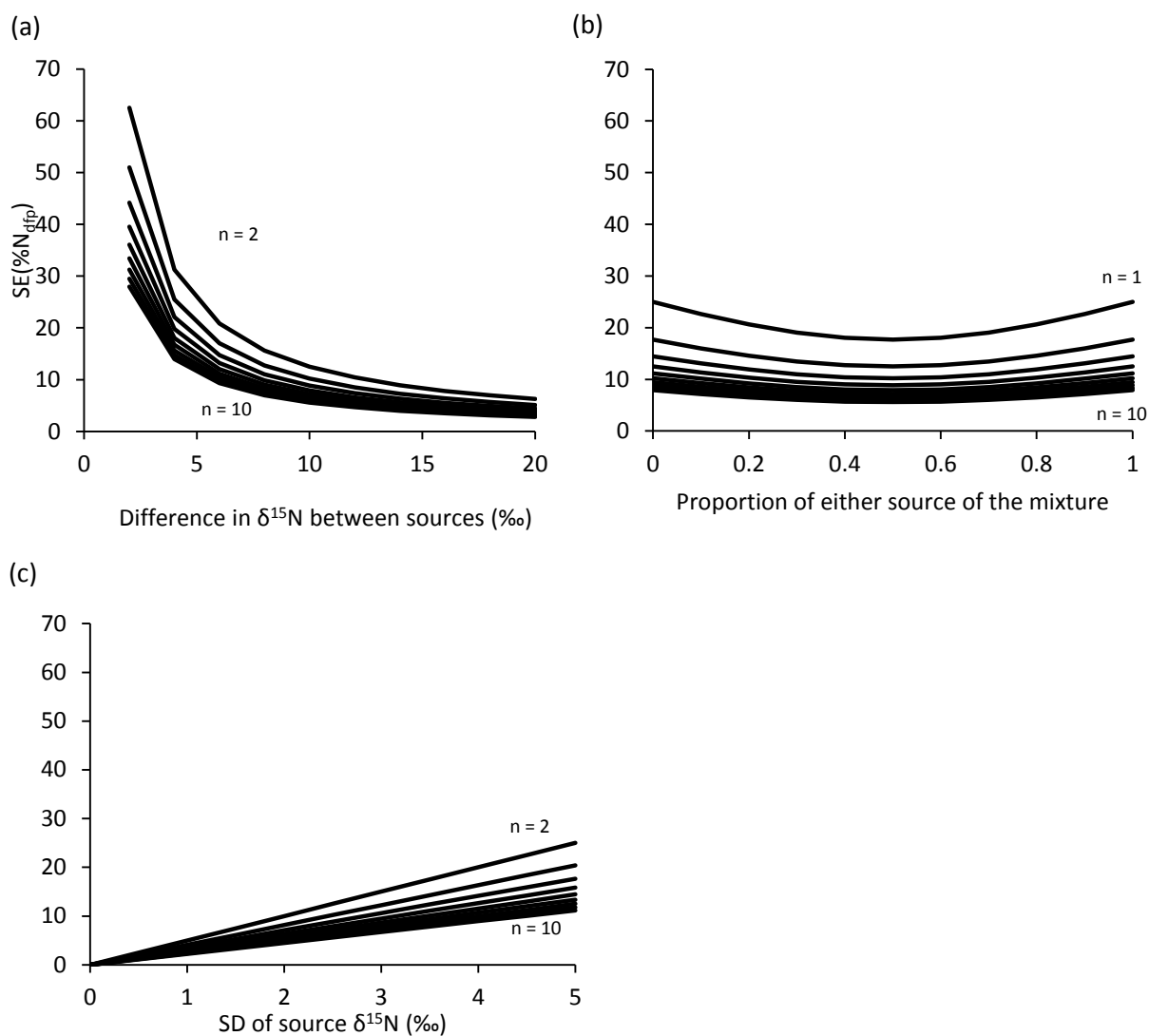


Figure 29 Influence of parameters of level two end-members on variability in source proportions of the stable isotope. Here, level two end-member sources are represented by the $\delta^{15}\text{N}$ of the prey N end-member and the $\delta^{15}\text{N}$ of the root N end-member of the N nutrition of a carnivorous plant; source proportion is represented as the percentage contribution of prey-derived N of the total N budget (%N_{dfp}) of the carnivorous plant. Presented are the influences of the following parameters of prey N / root N end-members on the SE of %N_{dfp}: (a) differences in isotopic $\delta^{15}\text{N}$ signature between sources of 0 to 20 ‰ for each sample size; (b) proportion of either source $\delta^{15}\text{N}$ to the $\delta^{15}\text{N}$ of the plant; (c) differences in SD of $\delta^{15}\text{N}$ of each source. Lines represent sample sizes of 2 (top line) to 10 (bottom line), except for (b) where the top line represents a sample size of 1. Parameters used in the analyses are given in Appendix 3.

4.4.4 The N nutrition of *Drosera rotundifolia* and differences between sites

Table 25 N nutrition of *Drosera rotundifolia* plants at two ombrotrophic bogs in the UK that vary by N deposition input. Presented are the mean \pm 1 S.E. for the following parameters of *D. rotundifolia*: the percentage contribution of prey-derived N to the total N budget, %N_{dfp}; tissue C : N ratio. Significant effects at $P < 0.05$ are highlighted in bold.

Site		%N _{dfp}	C : N ratio
Cors Fochno	Mean	49.4	45.6
	SE	8.8	0.7
Whixall Moss	Mean	25.5	33.8
	SE	4.9	0.8
Independent samples <i>t</i> -test results ¹		<i>P</i>	
		0.029	<0.001

¹ Comparing differences between sites.

Drosera rotundifolia plants at Cors Fochno obtained significantly greater %N_{dfp} than plants at Whixall Moss (Fig. 30; Table 25) (Aim 4).

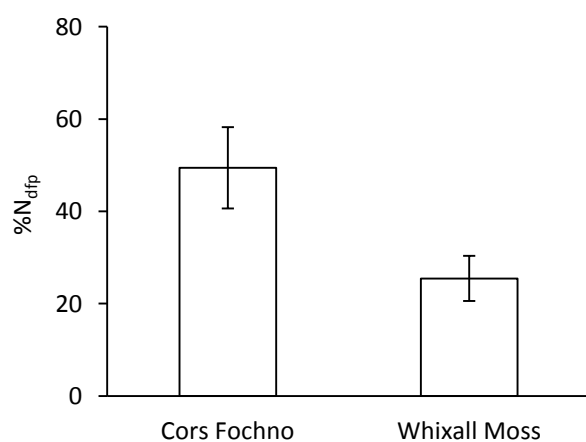


Figure 30 The percentage contribution of prey-derived N to the total N budget (%N_{dfp}) of *Drosera rotundifolia* plants growing at two ombrotrophic bogs receiving contrasting N deposition inputs in the UK.

Plants at Cors Fochno contained significantly higher tissue C : N ratios than plants at Whixall Moss (Fig. 31; Table 25) (Aim 4).

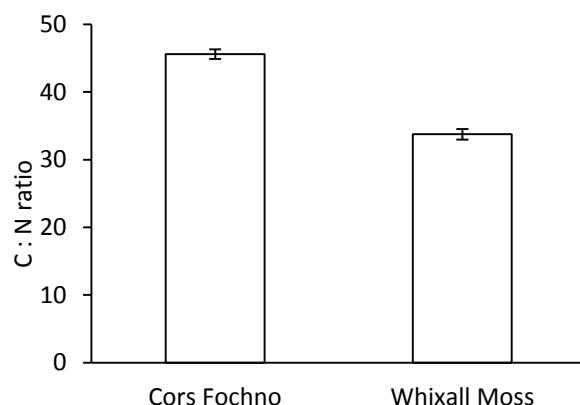


Figure 31 Tissue C to N ratios (mean \pm 1 S.E.) of *Drosera rotundifolia* plants growing at two ombrotrophic bogs that vary by N deposition input in the UK.

Table 26 Results of 2-way (site, N source) univariate ANOVA for the amount of prey-derived and root-derived N per plant by *Drosera rotundifolia* plants growing at two ombrotrophic bogs in the UK that vary by N deposition input. Presented are degrees of freedom (*df*), *F* and *P* values for the amount of adjusted N of *D. rotundifolia*. N sources: prey-derived, root-derived; sites:- Cors Fochno, Whixall Moss. Significant effects at $P < 0.05$ are highlighted in bold.

	Adjusted N (μg per plant)		
	<i>df</i>	<i>F</i>	<i>P</i>
N source (NS)	1, 39	16.507	<0.001
Site	1, 39	25.074	<0.001
NS x site	1, 39	22.323	<0.001

When adjusted for plant mass, the amount of N_{dfr} of *D. rotundifolia* plants did not significantly differ between sites (Fig. 32), but plants at Whixall Moss contained significantly greater mass-adjusted amounts of N_{dfr} than plants at Cors Fochno (Table 26) (Aim 4).

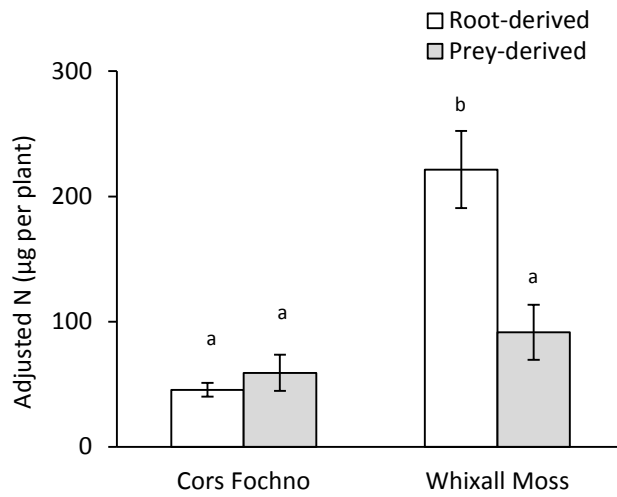


Figure 32 N nutrition of *Drosera rotundifolia* plants growing at two ombrotrophic bogs receiving contrasting N deposition inputs in the UK. Presented are the mean \pm 1 S.E. for the amounts of prey-derived N (N_{dfp}) and root-derived N (N_{dfr}), both at a common plant mass of 14.6 mg and corrected for the covariate relationship with mass. Bars with different letters are significantly different from each other (Fisher's least significant difference, $P < 0.05$).

4.5 Discussion

4.5.1 Incorporating diet into the estimation of %N_{dfp}

The results showing significant differences in invertebrate $\delta^{15}\text{N}$, tissue %N and proportional mass contribution to total prey mass between orders justify the incorporation of these prey parameters in the calculation of weighted prey $\delta^{15}\text{N}$, and the incorporation of weighted prey $\delta^{15}\text{N}$ in the calculation of %N_{dfp}. The variation in these invertebrate parameters is well documented; differences in invertebrate order $\delta^{15}\text{N}$ and %N are primarily attributed to variation in trophic position (Vander Zanden and Rasmussen, 2001) and diet (Vanderklift and Ponsard, 2003). As the $\delta^{15}\text{N}$ of invertebrate consumers is enriched by *ca.* 1.4 ‰ at each trophic level due to the preferential retention of the heavier isotope of N (Deniro and Epstein, 1981; McCutchan *et al.*, 2003), broader patterns that may be inferred from the data include:- the more $\delta^{15}\text{N}$ -enriched orders (e.g. Araneae, Formicidae) are likely to be predominantly composed of consumer types occupying higher trophic levels such as predators, parasites and omnivores, and the more $\delta^{15}\text{N}$ -depleted orders (e.g. Hemiptera and Lepidoptera) are likely to be predominantly composed of consumer types occupying lower trophic levels such as herbivores and detritivores, where $\delta^{15}\text{N}$ reflects the relatively $\delta^{15}\text{N}$ -depleted signatures of the food sources of plant and decaying organic matter respectively (Spence and Rosenheim, 2005).

Between-site differences in invertebrate $\delta^{15}\text{N}$ and %N are likely to reflect between-site differences in N deposition input; increased DIN leads to increased plant C:N ratios (Heijmans *et al.*, 2002) and therefore increased %N and $\delta^{15}\text{N}$ of herbivores and detritivores, a pattern that is continued throughout higher trophic levels of the food web (Vanderklift and Ponsard, 2003). The result showing between-site differences in the proportional abundance of prey orders is not unusual or limited to *D. rotundifolia* (Alcalá and Domínguez, 2003; Volkova *et al.*, 2010), and may be attributed to factors such as spatial variation in the relative abundance of potential prey orders or between-order differences in size and therefore the probability of prey escape from the plant. Variation in these prey parameters are therefore likely to occur *in-situ* throughout the variety of habitats in which carnivorous plants are found, therefore highlighting the importance of inclusion of weighted prey $\delta^{15}\text{N}$ in the calculation of %N_{dfp}. The importance of using weighted prey $\delta^{15}\text{N}$ is further indicated by results showing significant between-site differences in unweighted prey $\delta^{15}\text{N}$ but no between-site difference in weighted prey $\delta^{15}\text{N}$. Whilst prey $\delta^{15}\text{N}$ weighting status exerted no statistically significant influence on %N_{dfp} of *D. rotundifolia* in this study, the marginally lower %N_{dfp} values obtained from using weighted prey $\delta^{15}\text{N}$ suggests that %N_{dfp} may have been overestimated by previous studies of *D. rotundifolia* that used unweighted prey $\delta^{15}\text{N}$ as the prey N end-point of the linear mixing model (e.g. Schulze *et al.*, 1991; Millett *et al.*, 2012).

Results showing weighted $\delta^{15}\text{N}_{\text{prey}}$ to possess fivefold to eightfold less variability than the equivalent unweighted $\delta^{15}\text{N}_{\text{prey}}$, and that incorporation of weighted $\delta^{15}\text{N}_{\text{prey}}$ into the linear mixing model results in about a quarter less variability in $\%N_{\text{dfp}}$ compared with incorporation of unweighted $\delta^{15}\text{N}_{\text{prey}}$ indicate that use of weighted $\delta^{15}\text{N}_{\text{prey}}$ reduces uncertainty in the estimation of the reliance of *D. rotundifolia* on carnivory. Therefore, future studies may substantially reduce uncertainty in the estimation of the reliance of carnivorous plants on carnivory by incorporating diet, $\%N$ and the total mass of captured prey into the linear mixing model.

4.5.2 The use of appropriate plant proxies for the root N end-point of the mixing model

The results of this study highlight the strong influence of plant proxy $\delta^{15}\text{N}$ on $\%N_{\text{dfp}}$; of the three $\delta^{15}\text{N}$ constituents of the linear mixing model equation (Eqn. 2), weighted $\delta^{15}\text{N}_{\text{prey}}$ and $\delta^{15}\text{N}_{\text{Drosera}}$ did not differ between sites, therefore significant differences in $\%N_{\text{dfp}}$ found between sites predominantly reflect the significant between-site differences in $\delta^{15}\text{N}_{\text{NCVPs}}$. Therefore, the species of plant used as a proxy for the root N end-point of the linear mixing model directly influences the conclusions made regarding the reliance of *D. rotundifolia* on the trait of botanical carnivory. In this study, use of the mean $\delta^{15}\text{N}$ of *E. vaginatum* and *S. fuscum* as a proxy for the root N end-point resulted in $\%N_{\text{dfp}}$ values indicates that (i) *D. rotundifolia* plants at the low N deposition site were significantly more reliant on carnivory than plants at the high N deposition site, and (ii) N source reliance by *D. rotundifolia* switches from predominantly prey to root at N deposition threshold of *ca.* 8 kg N ha⁻¹ yr⁻¹. The latter result contrasts with the results of Millett *et al.*, 2012, which indicate N source reliance to switch from predominantly prey to root at an N deposition threshold of < 4 kg N ha⁻¹ yr⁻¹. This contrast reflects differences in plant/bryophyte species used as proxies for the root N end-point of the mixing model between studies; the *Sphagnum* only proxy used by Millett *et al.* (2012) was more $\delta^{15}\text{N}$ -enriched than $\delta^{15}\text{N}_{\text{NCVPs}}$ used in this study but $\delta^{15}\text{N}$ values for *D. rotundifolia* and prey were similar between studies, showing that $\%N_{\text{dfp}}$ increases as the difference between $\delta^{15}\text{N}_{\text{prey}}$ and $\delta^{15}\text{N}_{\text{NCVPs}}$ increases, therefore reducing the N deposition threshold at which *D. rotundifolia* switches from predominantly prey to root reliance. Conversely, if $\delta^{15}\text{N}_{\text{NCVPs}}$ was identical between sites but $\delta^{15}\text{N}_{\text{prey}}$ differed significantly, this would have an identical effect on $\%N_{\text{dfp}}$. Therefore, it is of critical importance to select proxies that are most representative of the carnivorous plant species that has obtained 100% of N via the roots or from prey.

An assessment of the suitability of $\delta^{15}\text{N}_{\text{NCVPs}}$ as a proxy for the $\delta^{15}\text{N}$ of *D. rotundifolia* that has obtained 100% of N via the roots was not possible in this study as gland-less mutants of this carnivorous plant species are not known to exist. Therefore presented values of $\%N_{\text{dfp}}$ represent semi-quantitative estimates of true $\%N_{\text{dfp}}$. However, it may be suggested the plant proxies used in this study are more

representative of *D. rotundifolia* that has obtained 100% of N via the roots than other co-occurring plant species, and more representative than proxies used by previous studies exploring the N nutrition of this species. The mean $\delta^{15}\text{N}$ of *Eriophorum vaginatum* and *Sphagnum fuscum* was selected as the proxy for the root $\delta^{15}\text{N}$ end-point of the mixing model as these species are reported to possess maximum rooting depths closest to that of *D. rotundifolia* (Nordbakken *et al.*, 2003), and therefore, as the $\delta^{15}\text{N}$ of plant species increases with increasing rooting depth (Kohzu *et al.*, 2003), these species were considered most representative of the $\delta^{15}\text{N}$ of *D. rotundifolia* that has obtained 100% of N via the roots. The results of this study support this observation; the $\delta^{15}\text{N}$ of *E. vaginatum* was not significantly different to the $\delta^{15}\text{N}$ of *D. rotundifolia* at either site, and the $\delta^{15}\text{N}$ of *E. vaginatum* and *S. fuscum* did not differ from each other, suggesting that the rooting depths of these species were closer to the rooting depth of *D. rotundifolia* than *E. tetralix* and *C. vulgaris*. Therefore, *E. vaginatum* and *S. fuscum* are considered more representative of the $\delta^{15}\text{N}$ of *D. rotundifolia* that has obtained 100% of N via the roots than the remaining co-occurring plant species.

One of the assumptions of the linear mixing model is that the degree of root discrimination between ^{15}N and ^{14}N is identical for the carnivorous plant and the reference plants used as a proxy for the root N end-point (Shearer and Kohl, 1988). This assumption is arguably met by choice of plant proxy used in this study due to the similarities in the $\delta^{15}\text{N}$ of *E. vaginatum* and *S. fuscum* with the lower $\delta^{15}\text{N}$ values of *D. rotundifolia*. Use of the selected plant proxies also provides the best balance of N uptake and assimilation strategies that are most representative of *D. rotundifolia*: root N availability to *D. rotundifolia* is typically controlled and supplied by co-occurring *Sphagnum* spp. (Lamers *et al.*, 2000), and it is assumed that no significant N fractionation by *Sphagnum* occurs during the direct absorption of atmospheric N (Bragazza *et al.*, 2005), therefore the $\delta^{15}\text{N}$ of *S. fuscum* may be considered most representative of the $\delta^{15}\text{N}$ available to *D. rotundifolia*. In addition, most vascular plant species undergo internal N fractionation processes which may alter $\delta^{15}\text{N}$ (Evans, 2001), therefore incorporation of the $\delta^{15}\text{N}$ of the co-occurring vascular plant species of *E. vaginatum*, which shares a similar rooting depth and the ability to absorb both isotopically distinct sources of N ($\text{NO}_3\text{-N}$ and $\text{NH}_4\text{-N}$) via the roots with *D. rotundifolia* (Crowder *et al.*, 1990; Koch *et al.*, 1991; Evans, 2001), considers the potential changes to $\delta^{15}\text{N}$ of *D. rotundifolia* during uptake and assimilation of root N. However, in order to quantify the amount of error introduced to the calculation of $\%N_{\text{dfp}}$ by the use of reference non-carnivorous plant species as proxies for the $\delta^{15}\text{N}$ of *D. rotundifolia* that has obtained 100% of N via the roots, there is a need to conduct a manipulative experiment under controlled conditions that compares the $\delta^{15}\text{N}$ and $\%N_{\text{dfp}}$ of *D. rotundifolia* plants raised from the point of trap maturity with no access to potential prey (representing 100% of N uptake via the roots) with genetically identical plants raised with potential prey available, and assess differences in $\delta^{15}\text{N}$ between *D. rotundifolia* plants and co-occurring non-carnivorous plant/bryophyte species.

There are potential limitations to the use of invertebrate $\delta^{15}\text{N}$ as a proxy for the $\delta^{15}\text{N}$ of a carnivorous plant that has obtained 100% of N from prey. It is currently undetermined whether *D. rotundifolia* discriminates against prey ^{15}N or ^{14}N during the digestion, assimilation and absorption of prey N:- if the assumption of the mixing model that no discrimination occurs by the plant is not met, then this would influence the precision of $\%N_{\text{dfp}}$ estimates (Shearer and Kohl, 1988; Millett *et al.*, 2012). Differential fractionation of stable isotopes of N occurs through trophic levels in animal food chains, with consumer tissue usually $\delta^{15}\text{N}$ -enriched compared to that of corresponding prey species (DeNiro and Epstein, 1981; Minagawa and Wada, 1984). Whilst differential fractionation of invertebrate $\delta^{15}\text{N}$ in the context of carnivorous plants has not yet been explored, it may be predicted to occur in plant tissues during prey absorption and assimilation, as carnivorous plants and their invertebrate prey differ in terms of the primary biochemical form of N excretion, 'diet', and tissue / organ composition (Vanderklift and Ponsard, 2003). This therefore highlights the need for further research to quantify the amount of error introduced to the calculation of $\%N_{\text{dfp}}$ by the use of prey $\delta^{15}\text{N}$ as a proxy for the $\delta^{15}\text{N}$ carnivorous plant that has obtained 100% of N from prey by determining whether discrimination by *D. rotundifolia* occurs against prey ^{15}N or ^{14}N during the digestion, assimilation and absorption of prey N, and if so, to what degree.

4.5.3 The use of a multi-level mixing model: an example using the N nutrition of a carnivorous plant.

Results of sensitivity analyses of the influence of parameters of prey orders on variability in $SE(\%N_{\text{dfp}})$ showing that $SD(\delta^{15}\text{N})$ within each prey order is the only parameter to influence $SE(\%N_{\text{dfp}})$ correspond with the results of Phillips and Gregg (2001); $SD(\delta^{15}N_{\text{prey}})$ is the sum of $SD(\delta^{15}\text{N})$ of all invertebrate orders constituting the diet of the plant. The results of the number of prey orders and the isotopic difference between orders on $SE(\%N_{\text{dfp}})$ demonstrate that the degree of prey specialisation of a carnivorous plant, or the variation in $\delta^{15}\text{N}$ between prey orders constituting the diet, exert no influence on variability in plant reliance on carnivory. The latter result contrasts with the positive relationship between the difference in source $\delta^{15}\text{N}$ and $SE(\%N_{\text{dfp}})$, and reflects that it is the difference between the mean $\delta^{15}N_{\text{prey}}$ and $\delta^{15}N_{\text{NCVPs}}$ constituting the denominator of the linear mixing model equation that influence $SE(\%N_{\text{dfp}})$. Therefore, the only feasible method of reducing prey-derived variability in $\%N_{\text{dfp}}$ is to increase the sample size of each invertebrate order constituting the diet of the plant. This result highlights the importance of obtaining sufficient replicates of all prey orders (preferably at least $n = 10$) from the background invertebrate population, including those that are either proportionally less abundant or more difficult to sample than other orders, such as Dictyoptera and Phthiraptera in this study. Additionally, $SD(\delta^{15}N_{\text{prey}})$ may be reduced by decreasing the taxonomic level to which invertebrates are classified to, e.g. identifying to family or species level, as the increased number of invertebrate categories constituting the prey N end-point would not influence $SE(\%N_{\text{dfp}})$ but

the sum of $SD(\delta^{15}N)$ is likely to be smaller than the equivalent $SE(\delta^{15}N)$ for level classification. This would be particularly beneficial for prey orders which are highly variable in $\delta^{15}N$ as a result of within-order differences in diet and/or trophic level, such as Lepidoptera in this study.

Whilst $SE(\%N_{dfp})$ is only influenced by the prey order parameter of $SD(\delta^{15}N)$, the source parameters of difference in $\delta^{15}N$ between sources, source proportion and $SD(\delta^{15}N)$ of each source each influence $SE(\%N_{dfp})$ as shown originally by Phillips and Gregg (2001) and by the sensitivity analyses of this study. In the case of the N nutrition of carnivorous plants, variability in $\%N_{dfp}$ is likely to reflect root N availability and therefore N deposition input; when DIN is low or high, the source proportion of N_{dfp} is likely to be closer to one or zero respectively, and therefore $SE(\%N_{dfp})$ will be larger compared with $SE(\%N_{dfp})$ of plants exposed to intermediate DIN. The influence of source difference in $\delta^{15}N$ on variability in $\%N_{dfp}$ is illustrated by the results of a study (Moran *et al.*, 2001) using the sympatric pitcher plants *Nepenthes albomarginata* and *Nepenthes rafflesiana* which specialise on contrasting prey types that differ in $\delta^{15}N$; the former captures mostly termites (mean $\delta^{15}N = -4$ ‰) whereas the latter captures mostly ants (mean $\delta^{15}N = 2$ ‰). The difference between $\delta^{15}N_{prey}$ and $\delta^{15}N_{NCVPs}$ was smaller for *N. albomarginata* than for *N. rafflesiana*, resulting in a relatively larger value for $SE(\%N_{dfp})$ of 7.3 % compared with 2.4 % for the latter. As stated by Phillips and Gregg (2001), the influence of difference in $\delta^{15}N$ between sources and source proportions are out of the control of the researcher. However, the influence of $SD(\delta^{15}N)$ of sources can be minimised by increased sample sizes of prey and of non-carnivorous plant species constituting proxies for the prey N and root N end-points of the mixing model, respectively.

The mixing model, as originally proposed by Shearer and Kohl (1988), is subject to several inherent limitations and potential error sources. As discussed previously, the use of representative proxies is paramount to minimise potential error in the calculation of $\%N_{dfp}$. The model is also limited by scenarios relating to differences in the $\delta^{15}N$ values used in the equation. For example, negative and therefore unrealistic $\%N_{dfp}$ values for some survey plots at both sites were obtained upon the use of the $\delta^{15}N$ of *E. vaginatum* only as a proxy for the root N end-point as a result of a negative value of $\delta^{15}N_{Droseira}$ and a smaller negative value of $\delta^{15}N_{NCVPs}$ constituting the numerator of the equation. Inclusion of these erroneous results led to the underestimation of $\%N_{dfp}$ at both sites, thus highlighting the importance of using mean $\delta^{15}N$ of reference non-carnivorous plant/bryophyte species as the proxy for the root N end-point (Shearer and Kohl, 1988).

There are a couple of specific assumptions and limitations that arise in the calculation of and error associated with $\%N_{dfp}$ that result from model application to this study. Firstly, the order composition, proportional mass contribution and %N of invertebrates captured by co-occurring non-survey *D. rotundifolia* plants at each survey plot was used to calculate the mean weighted prey $\delta^{15}N$ value. This prey $\delta^{15}N$ value was incorporated, along with the $\delta^{15}N$ value of survey *D. rotundifolia* plant tissue, into

the two end point mixing model equation to calculate the contribution of prey-derived N of total plant N budget (%) of survey *D. rotundifolia* plants. It is assumed, therefore, that the order composition, proportional mass contribution and %N of the diet of survey *D. rotundifolia* plants was identical to the diet of non-survey *D. rotundifolia* plants at each survey plot. This seems to be a reasonable assumption; within-site variability in %N_{dfp} of survey plants was small at both sites. Secondly, the calculation of %N_{dfp} assumes that *D. rotundifolia* digests and assimilates 100% of prey N, and that this is consistent across all orders and size classes of prey. There is some evidence to suggest this may not be the case, however; invertebrate exoskeletons are composed primarily of chitin which contains N, and most of the exoskeleton is undigested by the plant (Crowder *et al.*, 1990). Thus, an unquantified proportion of invertebrate N content contained within chitin is unabsorbed by *D. rotundifolia*. Furthermore, the percentage of chitin of total invertebrate dry mass may vary between-species due to anatomical differences and within-species due to life cycle stage (Kramer *et al.*, 1995). Therefore, future research is required to determine (i) the average proportion of prey N of the total invertebrate N that is absorbed by the plant; (ii) whether prey size and/or order exert a statistically significant influence on the proportion of prey N absorbed by the plant.

4.5.4 The N nutrition of *Drosera rotundifolia* and differences between sites

The results of this study showing that the proportion of prey-derived N (N_{dfp}) of the N budget of *D. rotundifolia* is significantly lower for plants at Whixall Moss compared with the proportion of N_{dfp} for plants at Cors Fochno, and that DIN is significantly higher at Whixall Moss than at Cors Fochno, correspond with the results of Millett *et al.* (2012) that show the reliance of *D. rotundifolia* on carnivory decreases with increasing N deposition input. The significantly higher N_{dfp} of plants at Cors Fochno compared with plants at Whixall Moss indicates that plant investment in prey capture is phenotypically plastic in response to changes in resource quality such as root N availability, and is consistent with the results of previous studies indicating plant investment in carnivory to decrease as root N availability increases (Ellison and Gotelli, 2002; Thorén *et al.*, 2003). Specifically, *D. rotundifolia* plants at Cors Fochno obtained 49.4% of N_{dfp} and plants at Whixall Moss obtained 25.5 %, indicating that a plant shift from a predominantly prey to root reliance occurs at a N deposition threshold of *ca.* 8.0 kg N ha⁻¹ yr⁻¹.

The uptake of prey N by plants was statistically insignificant between sites following adjustment for plant mass, suggesting that plant investment in prey capture, such as trap stickiness, was similar between sites, but that this represented a substantially larger relative energetic cost proportional to plant mass to the smaller plants at Cors Fochno. Results showing that *D. rotundifolia* plants at Cors Fochno to contain significantly higher %N_{dfp} and tissue C : N ratios than plants at Whixall Moss are

consistent with the results of earlier studies showing a positive correlation between tissue C : N ratio and %N_{dfp} or mass-adjusted N_{dfp} (Millett *et al.*, 2003, 2012), and indicate a positive benefit of prey capture to the plant. Results of this study therefore demonstrate the substantial influence of root N availability on phenotypic plasticity in terms of plant reliance on botanical carnivory, and provide support for the energetic cost-benefit model proposed by Givnish *et al.* (1984).

4.6 Conclusions

Results of this study show that variability in $\%N_{dfp}$ of *Drosera rotundifolia* is reduced by the incorporation of the prey parameters of invertebrate order, $\%N$ and proportional order mass contribution to the total mass of prey captured by the plant, thus justifying the preferential use of weighted $\delta^{15}N_{prey}$ compared with unweighted $\delta^{15}N_{prey}$ as the prey N end-point of the mixing model. The application of the $\delta^{15}N_{prey}$ weighting should be applicable to the majority of studies using multi-level single isotope data where calculation of the relative contribution of two sources to a mixture is required, e.g. prey and root N source contributions to the N budget of other generalist carnivorous plant species; atmospheric and host plant N source contributions to the N budget of epiphytes (e.g. Wania *et al.*, 2002), and mycorrhizal and root-derived source contributions to the N budget of plant species with mycorrhizal associations (e.g. Gebauer and Meyer, 2003). At the broader community level, the weighting of the isotope of level one contributors should be applicable to food web studies using lower trophic levels of food webs (e.g. Galván *et al.*, 2011), however further research is required to assess the suitability of the method for food web data using more than two trophic levels.

However, whilst the incorporation of level one parameters reduces variability in $\%N_{dfp}$, the potential error associated with $\%N_{dfp}$ due to the use of proxies for the prey N and root N end-points remains unquantified, highlighting the need for further research. Sensitivity analyses exploring the influence of invertebrate order and source parameters on variability in $\%N_{dfp}$ indicate that sample size and SD of each order or source exert the most powerful influences. These results highlight the importance of experimental design that minimises the SD of level one and level two contributors e.g. by the identification of invertebrates to as fine a taxonomic level as feasible, and to obtain sample sizes of a minimum of 10 per invertebrate taxon or reference non-carnivorous plant species.

The results of this study relating to the N nutrition of *D. rotundifolia* are consistent with findings reported in the literature that the plant reliance on botanical carnivory decreases as N deposition increases, and provide further evidence of the phenotypic plasticity of *D. rotundifolia* in response to resource availability. The increased tissue C : N ratio of *D. rotundifolia* plants at Cors Fochno provides evidence of a benefit of increased relative investment in carnivory proportional to plant size, and provides support for the cost/benefit model for the evolution of the trait of botanical carnivory. These results therefore provide further understanding of how carnivorous plants have adapted to thrive in extreme, hostile environments.

4.7 References

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4.8 Appendices

Appendix 2 Values of invertebrate prey parameters used in the sensitivity analyses for variability in the percentage contribution of prey-derived N to the total N budget ($\%N_{dfp}$) of a carnivorous plant. Each parameter was varied individually over the ranges indicated, with other values set at their default values, with the exception of sample sizes which were run for each parameter set.

Parameter	Minimum	Maximum	Default
Isotopic difference between invertebrate orders (‰)	0	5	2 ^a
Number of invertebrate orders	1	10	5
Sample size (within order)	1	10	10
SD of each invertebrate order (‰)	0.0	1	0.5

^a Based on the mean differences in $\delta^{15}N$ between invertebrate orders at Whixall Moss (1.37 ‰) and Cors Fochno (1.61 ‰).

Appendix 3 Values of source parameters used in the sensitivity analyses for variability in the percentage contribution of prey-derived N to the total N budget ($\%N_{dfp}$) of a carnivorous plant. Sources constituted Source A ($\delta^{15}N_{prey}$) and Source B ($\delta^{15}N_{NCVPS}$). Each parameter was varied individually over the ranges indicated, with other values set at their default values, with the exception of sample sizes which were run for each parameter set.

Parameter	Minimum	Maximum	Default
Isotopic difference between sources (‰)	2	20	10
Sample size (within source)	1	10	
SD of each source (‰)	0	5	2.5
Source proportion	0.0	1.0	0.5

Appendix 4 $\delta^{15}\text{N}$ values (mean \pm 1 S.E., minimum and maximum values) for orders of invertebrate prey captured by *Drosera rotundifolia* plants at two ombrotrophic bogs in the UK. Sample size (n) represents the number of pooled survey plot samples.

Order	Site	$\delta^{15}\text{N}$ (‰)			
		$\bar{x} \pm \text{S.E.}$	n	Min.	Max.
Acarina	Cors Fochno	1.2 \pm 1.9	2	-0.7	3.1
	Whixall Moss	0.2	1	-	-
Araneae	Cors Fochno	2.1 \pm 0.1	10	1.5	2.7
	Whixall Moss	0.7 \pm 0.2	10	1.0	3.2
Coleoptera	Cors Fochno	-1.4 \pm 0.7	10	-6.7	0.4
	Whixall Moss	-0.9 \pm 0.5	10	-3.2	1.8
Collembola	Cors Fochno	-7.3 \pm 0.8	3	-8.2	-5.7
	Whixall Moss	-6.1 \pm 0.9	6	-9.5	-3.1
Diptera	Cors Fochno	3.5 \pm 0.6	10	0.6	6.9
	Whixall Moss	4.8 \pm 0.7	10	1.3	8.8
Formicidae	Cors Fochno	0.7 \pm 0.2	10	0.0	1.8
	Whixall Moss	2.7 \pm 0.5	10	1.1	6.7
Hemiptera	Cors Fochno	-6.4 \pm 0.2	9	-10.8	-3.6
	Whixall Moss	-5.2 \pm 0.4	10	-7.0	-2.7
Hymenoptera	Cors Fochno	-0.6 \pm 1.1	5	-2.0	3.2
	Whixall Moss	3.6 \pm 0.5	6	2.3	5.4
Lepidoptera	Cors Fochno	-11.0	1	-	-
	Whixall Moss	-4.9 \pm 1.4	6	-9.8	1.1
Orthoptera	Cors Fochno	-2.4	1	-	-
	Whixall Moss	-3.2 \pm 1.1	7	-7.9	0.8

Appendix 5 $\delta^{15}\text{N}$ values (mean \pm 1 S.E., minimum and maximum values) for *Drosera rotundifolia*, co-occurring vascular plant and bryophyte species and invertebrates from two ombrotrophic bogs in the UK. Invertebrate values presented are:- the mean $\delta^{15}\text{N}$ for all background invertebrates captured at each site (invertebrates (unweighted)), and the weighted mean $\delta^{15}\text{N}$ of invertebrates captured by *D. rotundifolia* at each site, which incorporates $\delta^{15}\text{N}$, %N, and proportional contribution to plant 'diet' of each invertebrate order of captured prey (invertebrates (weighted)). Means comprise 10 pooled survey plot samples per site.

Species	Site	$\delta^{15}\text{N}$		
		$\bar{x} \pm \text{S.E.}$	Min.	Max.
<i>Drosera rotundifolia</i>	Cors Fochno	-2.5 \pm 0.3	-3.9	-1.0
	Whixall Moss	-5.1 \pm 0.3	-6.5	-3.7
<i>Sphagnum fuscum</i>	Cors Fochno	-6.3 \pm 0.6	-8.7	-3.1
	Whixall Moss	-8.4 \pm 0.5	-10.2	-6.2
<i>Calluna vulgaris</i>	Cors Fochno	-15.2 \pm 0.4	-17.2	-12.8
	Whixall Moss	-8.7 \pm 0.9	-10.3	-1.6
<i>Erica tetralix</i>	Cors Fochno	-14.0 \pm 0.5	-15.4	-11.2
	Whixall Moss	-9.3 \pm 0.9	-11.7	-3.8
<i>Eriophorum vaginatum</i>	Cors Fochno	-4.7 \pm 1.0	-10.2	-0.5
	Whixall Moss	-5.8 \pm 0.9	-9.1	-0.5
Invertebrates (unweighted)	Cors Fochno	-2.0 \pm 0.1	-2.6	-1.2
	Whixall Moss	-0.7 \pm 0.2	1.0	1.7
Invertebrates (weighted)	Cors Fochno	0.1 \pm 0.0	-0.1	0.2
	Whixall Moss	0.1 \pm 0.0	-0.1	0.2

5.1 Introduction

The trait of botanical carnivory evolved independently at least six times across five angiosperm orders, with notable functional and morphological convergence observed between lineages (Ellison & Gotelli, 2009). The evolution of the trait is considered by some as an example of Hobson's choice – representing the least detrimental choice from two options in a bad situation; net energy gain as a result of the construction costs of prey capturing organs is low (Ellison, 2006). As a result, carnivorous plants possess relatively slow growth rates, low photosynthetic rates and low photosynthetic nitrogen use efficiencies (PNUE) in comparison to non-carnivorous vascular plants, placing them at the 'slow and tough' end of the leaf economics spectrum (Wright *et al.*, 2004).

Whilst large variation in prey capture mechanism, life history strategy, and ecological niche exists across all species of carnivorous plant, several traits are shared by species from different evolutionary pathways and that differ by mechanism of prey capture. One of these traits is leaf colour; several families show leaf colour variation within the green to red spectrum range in ecological time, e.g. Droseraceae (Lloyd, 1942; Kruse *et al.*, 2013), Nepenthes (Moran & Moran, 1998), and Sarraceniaceae (Bennett & Ellison, 2009). Similar to vascular plants in general, there is a current lack of agreement amongst the scientific community as to the nature of the functional role(s) of leaf redness of carnivorous plants. Two competing hypotheses have recently been proposed:- (i) the photoprotection hypothesis, which proposes leaf redness to increase in response to light stress in order to reduce the degree of photoinhibition (Drumm-Herrel, 1984), and (ii) the 'prey attraction' hypothesis, where leaf redness is suggested to occur as a visual signal to attract invertebrate prey (Lloyd, 1942). A variant of the prey attraction hypothesis is explored by this study, named the 'N deficiency' hypothesis, where leaf redness is proposed to increase as a phenotypically plastic stress response to root N deficiency.

Leaf redness of vascular plants is predominantly caused by the accumulation of anthocyanins, a group of water-soluble, N-free, non-photosynthetic pigments belonging to the parent family of flavonoids, are responsible for red pigmentation all plant organs of terrestrial vascular plants except some families of the Caryophyllales (cacti, beet), where they are replaced by betalains (Chalker-Scott, 1999; Brockington *et al.*, 2011). Anthocyanins absorb the green to yellow range (approx. 500-550 nm) of the PAR spectrum, thus leaves with high anthocyanin content appear red due to the remainder of the spectrum reflecting off the leaf surfaces (Neill & Gould, 2000). However, leaf redness is often only weakly correlated to foliar anthocyanin content; foliar chlorophyll content and leaf morphology can also be contributory factors (Neill & Gould, 2000; Gould, 2004). Anthocyanins are synthesised in the cytoplasm, after which they are actively pumped into cell vacuoles (Marrs *et al.*, 1995). Leaf colour

variation to red is often observed at specific developmental stages of the life cycle, e.g. the transient reddening of juvenile leaves (Kubasek *et al.*, 1992; Karageorgou & Manetas, 2006) and of senescing leaves (Wheldale, 1916; Sanger, 1971; Lee *et al.*, 2003), and /or as a phenotypically plastic response to changes in the environment, particularly due to stress (Chalker-Scott, 1999; Close & Beadle, 2003; Gould, 2004). A heated debate continues in the scientific community as to whether anthocyanins serve an ecophysiological function, and if so, the nature of this function(s), or if they are simply a passive by-product of flavonoid synthesis (Karageorgou & Manetas, 2006; Archetti *et al.*, 2009; Gould *et al.*, 2010). Anthocyanins are widely considered to accumulate as a stress response to abiotic and biotic factors, notably PAR, but also UV light (Burger & Edwards, 1996), nutrient deficiency (Kumar & Sharma, 1999), heat (Shao *et al.*, 2007), cold (Christie *et al.*, 1994), drought (Hughes *et al.*, 2010), heavy metal contamination (Mobin & Khan, 2007), wounding (Stone *et al.*, 2001), and attack by pathogens such as fungi (Hipskind *et al.*, 1996). Thus, it is evident that anthocyanins are capable of offering a versatile response to stress factors in vascular plants.

The most widely favoured functional theory, the photoprotection hypothesis, proposes that, under light-saturated conditions, anthocyanins reduce the severity of, and aid recovery from, photoinhibition through the absorption of surplus light photons, thus protecting photosynthetic apparatus in the chloroplasts from photo-oxidative damage (Drumm-Herrel, 1984). There is a deficit in the literature of studies testing the hypothesis using carnivorous plants; results of an *in-situ* study using *Pinguicula vulgaris* found supplemental UV-B treated plants to increase leaf anthocyanin by twofold compared with control plants, however the influence of PAR on leaf anthocyanin content was not explored (Méndez *et al.*, 1999). However, strong support for the photoprotection hypothesis exists in the literature using non-carnivorous vascular plants (Krol *et al.*, 1995; Dodd *et al.*, 1998; Smillie & Hetherington, 1999; Gould *et al.*, 2002; Pietrini *et al.*, 2002; Solovchenko & Chivkunova, 2011). For example, upon exposure to high light availability, the degree of photoinhibition of *Oxalis triangularis* leaves was significantly lower for red-leaved plants than for green-leaved plants (Nielsen & Simonsen, 2011). There is strong evidence in the literature for anthocyanin synthesis to be triggered by light, e.g. synthesis by cells of mustard seedlings is activated by the red to far red PAR wavelength range (Nick *et al.*, 1993). However, the ecophysiological responses of several plant species to light fail to support the photoprotection hypothesis (Esteban *et al.*, 2008; Zeliou *et al.*, 2009). Thus, it is evident that the functional role(s) of anthocyanins may vary depending on the species' evolutionary history and current environmental restraints, and therefore requires exploration using carnivorous plants.

The alternative theory to explain the functional role of anthocyanins in carnivorous plants, the 'prey attraction' hypothesis was first suggested by Lloyd (1942), who upon observing the red-coloured mucilage glands of *Drosera* spp., proposed the role of leaf redness as a visible lure to attract prey. In this study, it is proposed that should the primary role of leaf redness be as a mechanism for prey

attraction, i.e. a measure of plant investment in botanical carnivory, then the prediction can be made that as root N availability decreases, leaf anthocyanin content per unit area increases ('N deficiency' hypothesis) - carnivorous plants demonstrate phenotypic plasticity in terms of investment in carnivory in response to root N and light availability (Ellison and Gotelli, 2002; Thorén *et al.*, 2003; Millett *et al.*, 2012). Thus, as a result of increased leaf anthocyanin content under N-deficient environments, redder leaves would be predicted to attract a larger number or size class of prey (as above).

There is a deficit of rigorous testing of the N deficiency hypothesis using carnivorous plants. The results of a study using *Nepenthes rafflesiana* by Moran and Moran (1998) suggest leaf redness to be an adaptive physiological response to plant N deficiency, in this case via prey denial:- prey-deprived pitchers were significantly redder than those with access to prey (*t*-test, $p < 0.05$). However, there are a number of limitations to this study; the availability of root N and light to the plants throughout the experimental period were not measured, and therefore these potentially confounding factors were unaccounted for. The potential adaptive role of anthocyanins to N deficiency is further supported by Ichiishi *et al.* (1999), who report the intensity of leaf redness of *Dionaea muscipula* and *Drosera spatulata* to be inversely proportional to the concentration of root N available to the plants. Whilst this study provides evidence that leaf redness increases as root N availability decreases, plants were kept in air-tight aquariums under constant light intensity of 3500 lux, and thus these results do not accurately represent how the species would respond *in-situ*.

Of the photoprotection and prey attraction hypotheses, the latter is the most disputed (Gloßner, 1992; Cresswell, 1993; Bennett & Ellison, 2009; Pavlovič *et al.*, 2014). In a controlled *ex-situ* study, *Drosera capensis* plants possessing red or white tentacles showed no significant difference in the capture rate of fruit flies (Pavlovič *et al.*, 2014). The results of prey attraction studies using *Sarracenia purpurea* indicate scent and/or pitcher size/height to significantly influence prey capture rate, but not colour (Cresswell, 1993; Bennett and Ellison, 2009). A prey attraction study *in-situ* using *Nepenthes rafflesiana* found pitcher colour (blue, yellow, and UV) and scent to account for the greatest number of captured prey (Moran, 1996). However there were limitations to the validity of the results: the abundance and order composition of background invertebrate population was not determined, red wavelengths of pitcher reflected light were not measured, nor pitcher anthocyanin content determined. A study exploring the influence of colour (green- or red-painted plants) on prey capture in *Nepenthes ventricosa* found red-painted plants to capture significantly greater numbers of prey than green-painted plants (paired *t*-test, $t = 2.98$, $p < 0.01$), and red-painted plants to capture significantly greater number of Dipterans than green-painted plants (paired *t*-test, $t = 3.25$, $p < 0.01$) (Schaefer & Ruxton, 2008). While these results provide support for the prey attraction hypothesis, there are several limitations to the design of the study; (i) *Nepenthes ventricosa* is a tropical species endemic to the Philippines, and therefore study plants situated adjacent to a freshwater pool in Germany were not

exposed to a typical background invertebrate population, which is predominantly ants; (ii) only Dipterans were captured in sufficient numbers by the plants to allow statistical comparison between treatment types.

In summary, the functional role of leaf redness in carnivorous plants is yet unconfirmed. This study utilises the carnivorous plant species of *Dionaea muscipula*, *Drosera rotundifolia* and *Pinguicula grandiflora* to determine whether light availability or root N availability (or both) influence leaf redness and leaf anthocyanin content. *Drosera rotundifolia* and *Dionaea muscipula* originate from the Caryophyllales, a lineage also encompassing the carnivorous families of the Drosera (*ca.* 194 species) and the Nepenthaceae (*ca.* 140 species), and the monotypic genera of Aldrovanda (aquatic waterwheel) and *Dionaea* (Ellison and Gotelli, 2009). *Dionaea muscipula* (Venus' flytrap) is a herbaceous rosette perennial that utilises an active 'snap-trap' mechanism of prey capture (Forterre *et al.*, 2005) (Table 27). *Drosera rotundifolia* (round-leaved sundew) is a short-lived, herbaceous perennial that utilises an adhesive, 'flypaper' mechanism of prey capture (Crowder *et al.*, 1990) (Table 27). In contrast to *D. muscipula* and *D. rotundifolia*, *Pinguicula grandiflora* (European butterwort) originates from the Lamiales, a lineage also comprising the carnivorous genera of Genlisea (corkscrew plants) and Utricularia (bladderworts) (Ellison & Gotelli, 2009). This species is an herbaceous perennial and utilises an adhesive, 'flypaper' mechanism of prey capture (Table 27).

Anthocyanins may accumulate in the leaves of *D. muscipula*, *D. rotundifolia* and *P. grandiflora* (Mendez *et al.*, 1999; Brockington *et al.*, 2011), however only *D. muscipula* and *D. rotundifolia* are reported to possess the ability to vary leaf colour from green to red (Joel *et al.*, 1985; Ichiishi *et al.*, 1999). These species were therefore selected in order to explore whether contrasting evolutionary lineage and/or mechanism of prey capture influences the functional role of anthocyanin in carnivorous plants. The utilisation of a 2x2 factorial design of light (+/- shade) and root N availability (+/- N) will enable the two hypotheses (photoprotection vs. N deficiency) for the functional role of anthocyanins in carnivorous plants to be tested.

Table 27 Ecological distributions and typical habitat characteristics of the carnivorous plants *Dionaea muscipula*, *Drosera rotundifolia* and *Pinguicula grandiflora*.

Habitat characteristic	<i>Dionaea muscipula</i>	<i>Drosera rotundifolia</i>	<i>Pinguicula grandiflora</i>
Geographical distribution	Narrow range – endemic to N. and S. Carolina, S.E. USA, only (Roberts & Oosting, 1958; Schulze <i>et al.</i> , 2001)	Wide range – found throughout Europe, Scandinavia, N. America, and S.E. Asia (Crowder <i>et al.</i> , 1990).	Mid range – disjunct European distribution, inc. UK, Ireland, France, Spain, and Switzerland (Steiger, 1998).
Biogeographic element	Temperate, N. American Atlantic region.	Boreo-temperate (in conifer and broadleaf zones), circumpolar (in Europe, Asia and N. America) (Hill <i>et al.</i> , 2004).	Temperate (broad-leaf forest zone), oceanic (in Atlantic zone of Europe, not or scarcely reaching east to Sweden, Germany or S. Spain) (Hill <i>et al.</i> , 2004).
Habitat description	Typically found growing in extremely nutrient-poor, wet sandy and peaty soils of bogs and wet pine savannahs (Adamec, 1997; Schulze <i>et al.</i> , 2001).	Typically found growing in peat or <i>Sphagnum</i> hummocks in bogs, valley mires, fens and heaths (Crowder <i>et al.</i> , 1990; Hill <i>et al.</i> , 2004).	Typically found growing in bogs, bog pools, valley mires, and heaths (Hill <i>et al.</i> , 2004).
Light requirement	Heliophyte to facultative heliophyte (Hájek and Adamec, 2010). Ellenberg's indicator value for light unavailable (UK <i>spp.</i> only).	Heliophyte. Ellenberg's indicator value for light = 8 (light-loving plant; rarely found where relative illumination in summer is less than 40%) (Hill <i>et al.</i> , 2004).	Heliophyte to facultative heliophyte. Prefers open habitats with protection from full sun (Legendre, 2000). Ellenberg's indicator value for light = 7 (generally growing in well-lit places, but also occurring in partial shade) (Hill <i>et al.</i> , 2004).
Moisture requirement	Ellenberg's indicator value for moisture unavailable (UK <i>spp.</i> only). Requires constantly wet soil, preferring access to free-flowing water (Steiger, 1998).	Ellenberg's indicator value for moisture = 9 (wet-site indicator, often on water-saturated, badly aerated soils) (Hill <i>et al.</i> , 2004).	Ellenberg's indicator value for moisture = 8 (in between constantly moist or damp soils and water-saturated soils) (Hill <i>et al.</i> , 2004).
Nitrogen requirement	Ellenberg's indicator value for N unavailable (UK <i>spp.</i> only).	Ellenberg's indicator value for N = 1 (extremely infertile sites; N value = 84) (Hill <i>et al.</i> , 2004).	Ellenberg's indicator value for N = 2 (in between the range of extremely infertile and more or less infertile; N value = 323) (Hill <i>et al.</i> , 2004).

5.1.1 Hypotheses

This study aims to clarify the functional role of leaf redness in the carnivorous plant species of *Dionaea muscipula*, *Drosera rotundifolia* and *Pinguicula grandiflora* by testing the photoprotection and N deficiency hypotheses.

If the hypothesis that leaf redness is a photoprotective response to light availability (hypothesis 1) is correct, the following predictions should be supported by the data:-

- Leaf colour is significantly redder upon exposure to high light availability than to low light availability.
- Leaf anthocyanin content per unit area is significantly greater upon exposure to high light availability than to low light availability.
- Plant stress response, as measured by F_v/F_m , is significantly greater when plants are exposed to low light availability than to high light availability.

If the alternative N deficiency hypothesis is correct, the following predictions should be supported by the data:-

- There is a significant negative correlation between root N availability and (i) leaf redness, (ii) leaf anthocyanin content per unit area.
- Plant stress response, as measured by F_v/F_m , is significantly greater when plants are exposed to low root N availability than to high root N availability.
- Plant investment in prey capture is significantly greater upon exposure to low root N availability than to high root N availability.

5.2 Methods

5.2.1 Experimental design

This experiment was conducted from May to September 2012 (plants' active growth season) at the greenhouse of the Department of Geography, Loughborough University, UK, under ambient conditions.

Three species of carnivorous plant that vary by evolutionary lineage or mechanism of prey capture were chosen as the study species:- *Dionaea muscipula* (Venus' flytrap), *Drosera rotundifolia* (round-leaved sundew), and *Pinguicula grandiflora* (large-flowered butterwort) (Ellison & Gotelli, 2009). The plants were subjected to two treatments in a randomised, factorial design: the addition of inorganic nutrients to the growth medium (no N and high N) and light availability (no shade and shade). *Drosera rotundifolia* plants were collected during July 2011 from Cors Fochno, Ceredigion (52°30'09N, 04°00'57W), an ombrotrophic peat bog receiving a relatively low background N deposition of *ca.* 8.0 kg N ha⁻¹ yr⁻¹ (APIS, <http://www.apis.ac.uk/>, accessed 21.04.2014). Remaining plants were sourced from commercial plant suppliers during April 2012 (South West Carnivorous Plants, Devon (*P. grandiflora*); Triffid Nurseries, Suffolk (*D. muscipula*)).

Fifteen replicate plants per species were used in each treatment group, giving a total of 120 plants. Prior to the start of experimental treatment, plants were planted individually into 6 x 6 x 5.5 cm black plastic pots, which were large enough for the leaves and root systems of the plant species. The composition of the growth medium varied between species in order to replicate optimal rooting micro-environment requirements:- 100% *Sphagnum* moss peat (*D. rotundifolia*); 3:1 of *Sphagnum* moss peat: horticultural sand (*P. grandiflora*); and 1:1 of *Sphagnum* moss peat: horticultural perlite (*D. muscipula*). *Sphagnum* peat was sieved < 2 mm prior to use. Horticultural sand and perlite were rinsed using deionised water prior to use.

The pots were randomly distributed on 12 large trays, 15 plants per tray, with three plants of each species on each tray; making three tray replicates per experimental treatment type which were randomised with regard to treatment. Half of the plants were placed under screens consisting of fine-meshed white voile fabric, blocking out approximately 50-60% of ambient light compared to unshaded plants, measured with a handheld light meter (SKP 200 PAR Quantum Sensor, Skye Instruments Ltd., Wales, UK). Each screen shaded one experimental tray. The growth medium of the plants was kept moist by capillary uptake of deionised water, with a water depth of 3 cm maintained in each tray. Half of the plants were allocated to a complete nutrient treatment with nitrogen (NH₄NO₃) concentration of *ca.* 5 mM (NPK = 5 : 1.28 : 1.9; Long Ashton NH₄NO₃ type formula (Hewitt, 1966)) and the other half to identical complete nutrient treatment but with NH₄NO₃ omitted. At weekly intervals, one litre of the

appropriate nutrient solution was applied to each tray and left standing for a 24 hour period (Karlsson *et al.*, 1991; Thorén *et al.*, 2003). Nutrient solution was then removed, trays rinsed and re-filled with deionised water. As nutrient solution reached the rhizosphere via capillary action and diffusion, nutrient availability to the rhizosphere of the plants may have been influenced by the composition of the rooting medium (Karlsson *et al.*, 1991). However, it was not possible to grow all species on one standardised growth medium.

Prey availability to all plants was minimised by the usage of artificial sticky traps in the greenhouse prior to and throughout the growth season, however prey capture by the plants occurred occasionally. Moss growth was minimised by gently scraping the substrate surface of each plant cell as required. The positions of each experimental tray and of each pot within the trays were rotated randomly at weekly intervals throughout the course of the experiment. At weekly intervals, light availability to the plants, recorded as percentage of ambient light available to the plants, was measured by taking five light measurements at the corners and centre of each experimental tray (under the screen where appropriate), and ten light measurements outside the greenhouse. Mean light availability to each plant was calculated as the percentage of the mean light intensity per tray of the mean ambient light intensity outside the greenhouse. Temperature (actual, max / min (°C)) in the greenhouse was recorded continuously at 10 minute intervals using an electronic data logger (TinyTag 'Plus 2' (TGP-4017), Gemini Data Loggers Ltd., West Sussex, UK) throughout the course of the experiment. Humidity was maintained generally between 60-70% by the placing of large trays filled with deionised water on the greenhouse floor.

5.2.2 Sampling protocol and measurements

In early May 2012, plant growth variables, investment in carnivory, and leaf colour were measured prior to the start of experimental treatment. Repeat measurements of variables of plant growth and investment in carnivory were taken at four-weekly intervals throughout the course of the experiment until plants were harvested in September 2012. Leaf colour was measured at two further survey points at the middle (July 2012) and towards the end of the experiment (late August 2012).

Growth measurements comprised rosette / plant base diameter (mm), number of leaves, cross-sectional diameter of each leaf (mm), number of inflorescences, length of flower stem (mm), and the number of seed pods. Leaf area (mm²) was calculated as follows:- ten randomly selected leaves were taken from non-survey plants of each species at regular time intervals throughout the growth season. Each leaf was scanned using a flatbed colour scanner, and the digital image adjacent to a transparent ruler uploaded into image processing software (ImageJ, v.1.47, <http://rsbweb.nih.gov/ij/index.html>), where actual leaf area (mm²) was calculated (O'Neal *et al.*, 2002). Estimated leaf area (mm²) was

calculated by applying the equation of an appropriate shape to the cross-sectional leaf radii measurements. Linear regression was conducted to quantify, and assess the statistical significance of the relationship between estimated and actual leaf area. Leaf area was calculated by correcting for the discrepancy between estimated leaf area and actual leaf area through application of the linear regression equation.

Leaf stickiness is considered a reliable indicator of investment in prey capture for species possessing 'flypaper' trap morphologies (Zamora *et al.*, 1998; Thorén *et al.*, 2003), thus measurements were taken for *D. rotundifolia* and *P. grandiflora*. A 1 x 1 cm piece of filter paper was attached to a dynamometer, the filter paper pressed lightly to the upper surface of a randomly selected, mature leaf, and the force (N) required for the leaf to separate from the filter paper measured (Thorén *et al.*, 2003). In species possessing separate traps and petioles, such as *D. muscipula*, the trap to petiole ratio is considered an appropriate measurement of plant investment in carnivory (Ellison & Gotelli, 2002; Farnsworth & Ellison, 2008). Thus, cross-sectional diameter length measurements (mm) of the petioles of *D. muscipula* plants were taken at each survey session in order to calculate petiole area (mm²) per plant.

The spectral reflectance of one randomly selected, mature leaf (attached) per plant was measured in a dark room using a UV-Vis fibre optic miniature spectrometer (USB 2000+ model, Ocean Optics Ltd., Florida, USA) and accompanying spectrometric software (SpectraSuite, Ocean Optics Ltd., Florida, USA). Leaf spectrometry data was calibrated using SpectraSuite by dark and light reference files generated from the measurement of the reflected light spectrum of black and white reference cards for every tray of 15 plants.

In order to quantify leaf redness, the Commission Internationale de l'Eclairage (CIE) L*a*b* (CIELAB) colour space parameter of CIELAB a* (CIE, 1978), a measurement of colour variation on a red to green scale where increasingly positive values represent redder leaves and increasingly negative values represent greener leaves, was used (equation in Chen *et al.*, 2011).

In order to evaluate the influence of experimental treatment on the photochemical efficiency within the leaves, chlorophyll fluorescence was measured four weeks prior to the end of the experiment using a portable chlorophyll fluorescence monitoring system (PEA system, Hansatech Instruments Ltd., Norfolk, UK). Measurements were taken from one healthy leaf from three randomly selected plants per species per tray. For *D. muscipula*, measurements were also taken of the corresponding petioles. Following a dark adaptation period of 30 minutes, F_0 (minimal chlorophyll fluorescence yield in the dark-adapted state) was measured. F_m (maximal chlorophyll fluorescence yield in the dark-adapted state) was obtained by exposing the leaf to a saturation light pulse ($>3000 \mu\text{mol}^{-2} \text{s}^{-1}$). Variable to maximal fluorescence ratio (F_v/F_m), reflecting the efficiency of Photosystem II (Roháček, 2002), was calculated as $(F_m - F_0)/F_m$ (Genty *et al.*, 1989).

In early September 2012 prior to plant harvest, leaf pigment content (anthocyanins, carotenoids, and chlorophyll (Chl_a, Chl_b)) was measured. One healthy leaf was removed from three randomly selected plants per species per experimental treatment type, and fresh mass (mg) measured. For *D. rotundifolia* and *P. grandiflora*, separate leaves from each plant were used for the determination of (i) leaf Chl_a, Chl_b, and carotenoid content, and (ii) leaf anthocyanin content. For *D. muscipula*, each trap was dissected along the midrib and one of the two symmetrical lobes used for each analysis. Leaf area (mm²) was measured and units converted to m². Extractant solutions were prepared of acetone / Tris buffer (80:20 volume, pH 7.8) for the determination of leaf Chl_a, Chl_b, and carotenoid content, and acidified methanol (methanol / HCl / deionised water; 90:1:1 vol:vol:vol) for the determination of leaf anthocyanin content (Sims & Gamon, 2002). Each leaf sample was placed in a clean mortar, a small amount of the appropriate extractant added, and the leaf ground using a pestle. The homogenised mixture was transferred to a graduated cylinder, the mortar / pestle rinsed with fresh extractant and transferred to the cylinder, and the mixture made up to a final volume of 4 ml. The mixture was transferred to a centrifuge tube, and spun at the highest setting for three minutes.

Using a spectrophotometer (UV-1650PC system, Shimadzu Corporation, Kyoto, Japan) and accompanying UV Probe software (v. 2.20, Shimadzu Corporation, Kyoto, Japan), the absorbance of the supernatant was measured at the wavelengths of 470, 537, 647, and 663 nm for the determination of leaf Chl_a, Chl_b, and carotenoid content, and at the wavelengths of 529 and 650 nm for the determination of leaf anthocyanin content (Sims & Gamon, 2002). The concentrations (µmol ml⁻¹) of Chl_a, Chl_b and carotenoids in the supernatant were calculated using Equations 11, 12, and 10 respectively of Sims and Gamon (2002). For each pigment, the unit of measurement for concentration was converted to mmol ml⁻¹, and multiplied by the total volume of extractant (ml) in order to calculate to pigment concentration per leaf (mmol leaf⁻¹). Leaf pigment content per unit area (mmol mm⁻²) was then calculated by dividing the leaf pigment concentration per leaf (mmol leaf⁻¹) by leaf area (m²).

For the determination of leaf anthocyanin content, corrected anthocyanin absorbance was calculated using Equation 14 of Sims and Gamon (2002). The concentration of anthocyanin in the extractant solution (mg/l) was calculated as follows, assuming a mean molecular weight for anthocyanin of 207.24724 g/mol (National Center for Biotechnology Information, U.S. National Library of Medicine, Maryland, USA) and a molar absorption coefficient for anthocyanin of 30,000 l mol⁻¹ cm⁻¹ (Murray & Hackett, 1991):

$$c = (A/e \times l) \times MW \times DF \times 1000$$

Where:-

c = concentration of anthocyanin in the extractant (mg/l)

A = corrected anthocyanin absorbance

e = molar absorptivity of anthocyanin ($\text{l mol}^{-1} \text{cm}^{-1}$)

l = path length (cm) (= diameter of cuvette that the light passed through the solution)

MW = mean molecular weight of anthocyanin (g/mol)

DF = dilution factor

The units of measurement for anthocyanin concentration were converted to mmol by dividing the anthocyanin concentration (mg/l) by the mean molecular weight for anthocyanin (g/mol). Leaf anthocyanin content per unit area (mmol m^{-2}) was then calculating by dividing anthocyanin concentration (mmol) by the leaf area dissolved in the extractant (m^2).

Plants were harvested from 11-15 September 2012 and dissected into constituent parts as follows: flower stalk and seed pods, leaves, main stem, current growth season's roots, and previous growth season's roots. For *D. muscipula*, leaves were further divided into current growth season's traps and petioles, and previous growth season's traps and petioles. Fresh mass (mg) measurements of plant parts were taken. Plant material was dried to a constant weight by placing in a forced-air oven at 70 °C for 72 hours (Campbell & Plank, 1992), and weighed to obtain dry mass (mg) measurements.

5.2.3 Data analyses

Repeated measures General Linear Model (GLM) and univariate analysis of variance (ANOVA), Pearson's correlation, and linear and curve estimation regression were used to test the predictions of the study.

Four-way repeated measures GLM ANOVAs were conducted to explore the influence of light, N, species, and time on each plant physical characteristic measured throughout the active growth season. Where data did not conform to the assumption of homoscedascity, data were \log_{10} , ln or square root transformed as appropriate prior to analysis. Data for each physical characteristic at survey session 0 (pre experimental treatment) were used as a covariate. Post-hoc comparisons were conducted using Fisher's Least Significant Difference (LSD) (0.05 significance level).

Curve estimation regression analysis (linear, logarithmic, quadratic, cubic, and power) was performed to identify the best fit relationship between the CIELAB a^* score of red-green leaf colour and each of the following pigments:- leaf anthocyanin content per unit area (mmol m^{-2}), leaf total chlorophyll content per unit area (mmol m^{-2}), and leaf carotenoid content per unit area (mmol m^{-2}). The following statistically significant cubic relationships possessed the best fit between the CIEa* score and each pigment:- anthocyanin ($F_{(3,26)} = 57.620$, $P < 0.001$, $R^2 = 0.869$); total chlorophyll ($F_{(3,26)} = 39.809$, $P < 0.001$, $R^2 = 0.821$), and carotenoids ($F_{(3,26)} = 72.331$, $P < 0.001$, $R^2 = 0.893$). Thus, natural logarithm transformation was performed to all variables to permit the use of linear regression analyses.

Multiple linear regression analysis was performed to explore the influence of the leaf pigment content per unit area of anthocyanin, total chlorophyll, and carotenoid on the CIELAB a* score of leaf colour for all plant species combined (*D. muscipula*, *D. rotundifolia* and *P. grandiflora*). The model was statistically significant overall ($F_{(3,29)} = 50.677$, $P < 0.001$, $R^2 = 0.854$), however the collinearity tolerance score of the predictor variable of leaf carotenoid content of 0.020 was substantially less than 0.1, indicating that this variable should be excluded from further analysis due to the strong collinearity with the predictor variables of leaf anthocyanin and total chlorophyll content. Upon splitting the data by species, the regression models did not reach statistical significance (*D. muscipula*:- $F_{(3,6)} = 0.954$, $P = 0.515$, $R^2 = 0.488$; *D. rotundifolia*:- $F_{(3,10)} = 1.771$, $P = 0.240$, $R^2 = 0.431$; *P. grandiflora*:- $F_{(3,11)} = 0.829$, $P = 0.514$, $R^2 = 0.237$). Further multiple linear regression analysis exploring the relationship between leaf anthocyanin content per unit area and leaf total chlorophyll content per unit area on the CIELAB a* score of leaf colour found the model to be statistically significant overall ($F_{(2,29)} = 76.499$, $p < 0.001$, $R^2 = 0.850$). However, the predictor variable of leaf total chlorophyll content did not reach statistical significance at the 95% confidence level ($t = 1.617$, $p = 0.118$), therefore leaf chlorophyll content was therefore excluded from further analyses. Upon splitting the data by species, the models did not reach statistical significance (*D. muscipula*:- $F_{(2,6)} = 1.855$, $p = 0.269$, $R^2 = 0.481$; *D. rotundifolia*:- $F_{(2,10)} = 0.035$, $p = 0.965$, $R^2 = 0.009$; *P. grandiflora*:- $F_{(2,11)} = 0.737$, $p = 0.505$, $R^2 = 0.141$). Thus, linear regression was performed to explore the relationship between leaf anthocyanin content per unit area and the CIELAB a* of leaf colour.

Rosette area (cm²) was selected as a non-destructive measure of plant size throughout the growth season, and was used with plant mortality (proportion of dead plants (%)) to explore the impact of experimental treatment, and an unplanned heat and herbivory stress event in July, on plant vigour. Leaf colour was quantified by calculation of the CIELAB a* score. As some plants possessed negative CIELAB a* scores, CIELAB a* values were converted to positive integers by the addition of 25 to raw values in order to permit logarithmic and power curve estimation. Leaves which showed signs of senescence, and possessed extreme outlying CIELAB a* scores, were excluded from further analyses (n = 6; 1 *D. rotundifolia*, 5 *D. muscipula*).

All statistical analyses were conducted using SPSS Statistics version 21 (IBM, Chicago, USA).

5.3 Results

Table 28 Results of four-way (species, N, light, time) repeated-measures GLM for characteristics of three species of carnivorous plant (*Dionaea muscipula*, *Drosera rotundifolia* and *Pinguicula grandiflora*) subjected to one of four contrasting N and light treatment combinations (2 x 2 factorial design: + N, + shade; + N, - shade; - N, + shade; -N – shade). Presented are degrees of freedom (*df*), *F* and *P* values from the analyses of rosette area (A_R), total leaf area (A_L) (excluding petioles in *D. muscipula*), relative growth rate (RGR) calculated as the proportional change in rosette area compared with the pre-treatment rosette area (%), CIELAB a^* score of leaf colour on a red to green continuum (CIELAB a^*), and stickiness per unit leaf area (S_L) as a measure of plant relative investment in prey capture (*D. rotundifolia* and *P. grandiflora* only). Effect abbreviations as follows: L = light, N = nitrogen, S = species, *t* = time. Significant effects at $P < 0.05$ are highlighted in bold.

Effect	A_R^a			A_L^a			RGR ^b			CIELAB a^{*a}			S_L^b		
	<i>df</i>	<i>F</i>	<i>P</i>	<i>df</i>	<i>F</i>	<i>P</i>	<i>df</i>	<i>F</i>	<i>P</i>	<i>df</i>	<i>F</i>	<i>P</i>	<i>df</i>	<i>F</i>	<i>P</i>
L	1, 167	<1	0.513	1, 121	<1	0.651	1, 168	<1	0.558	1, 120	9	0.004	1, 76	13	0.001
N	1, 167	6	0.015	1, 121	12	0.001	1, 168	9	0.003	1, 120	2	0.206	1, 76	69	<0.001
S	2, 167	63	<0.001	2, 121	80	<0.001	2, 168	72	<0.001	2, 120	229	<0.001	1, 76	70	<0.001
<i>t</i>	2, 342	1	0.396	3, 329	6	0.001	2, 396	183	<0.001	1, 120	<1	0.707	3, 228	4	0.012
L x <i>t</i>	2, 342	3	0.077	3, 329	1	0.650	2, 396	6	0.001	1, 120	12	0.001	3, 228	1	0.534
L x N	1, 167	1	0.265	1, 121	<1	0.834	1, 168	1	0.253	1, 120	1	0.917	1, 76	1	0.823
L x S	2, 167	1	0.898	2, 121	<1	0.646	2, 168	<1	0.839	2, 120	4	0.027	1, 76	1	0.251
N x S	2, 167	1	0.744	2, 121	1	0.371	2, 168	2	0.199	2, 120	2	0.226	1, 76	<1	0.353
N x <i>t</i>	1, 342	10	<0.001	3, 329	10	<0.001	2, 396	17	<0.001	1, 120	3	0.110	3, 228	48	<0.001
S x <i>t</i>	4, 342	17	<0.001	5, 329	41	<0.001	5, 396	24	<0.001	2, 120	2	0.110	3, 228	12	<0.001
L x N x S	2, 167	<1	0.999	2, 121	1	0.365	2, 168	1	0.523	2, 120	1	0.506	1, 76	1	0.349
L x N x <i>t</i>	2, 342	<1	0.915	3, 329	<1	0.974	3, 396	<1	0.966	1, 120	2	0.118	3, 228	<1	0.973
L x S x <i>t</i>	4, 342	5	0.001	5, 329	2	0.134	5, 396	5	<0.001	2, 120	4	0.014	3, 228	<1	0.919
N x S x <i>t</i>	4, 342	2	0.144	5, 329	5	<0.001	5, 396	4	0.004	2, 120	1	0.255	3, 228	10	<0.001
L x N x S x <i>t</i>	4, 342	2	0.054	5, 329	1	0.187	5, 396	3	0.008	2, 120	1	0.304	3, 228	1	0.508

^a Data were Log_{10} transformed before analysis.

^b Data were square root transformed before analysis.

5.3.1 Plant environment throughout the active growth season

Light availability to shaded plants (mean \pm 1 S.E. = $9.9 \pm 2.3 \times 10 \mu\text{mol sec}^{-1} \text{m}^{-2}$) was significantly lower than the light availability to unshaded plants ($17.1 \pm 4.3 \times 10 \mu\text{mol sec}^{-1} \text{m}^{-2}$) (repeated measures GLM, $F_{(1,10)} = 8.200$, $P = 0.017$). Light availability to plants varied significantly across the active growth season ($F_{(12,120)} = 21.716$, $P < 0.001$); light intensity peaked at 23-29 June, 14-21 July, and 11-19 August (Fig. 33).

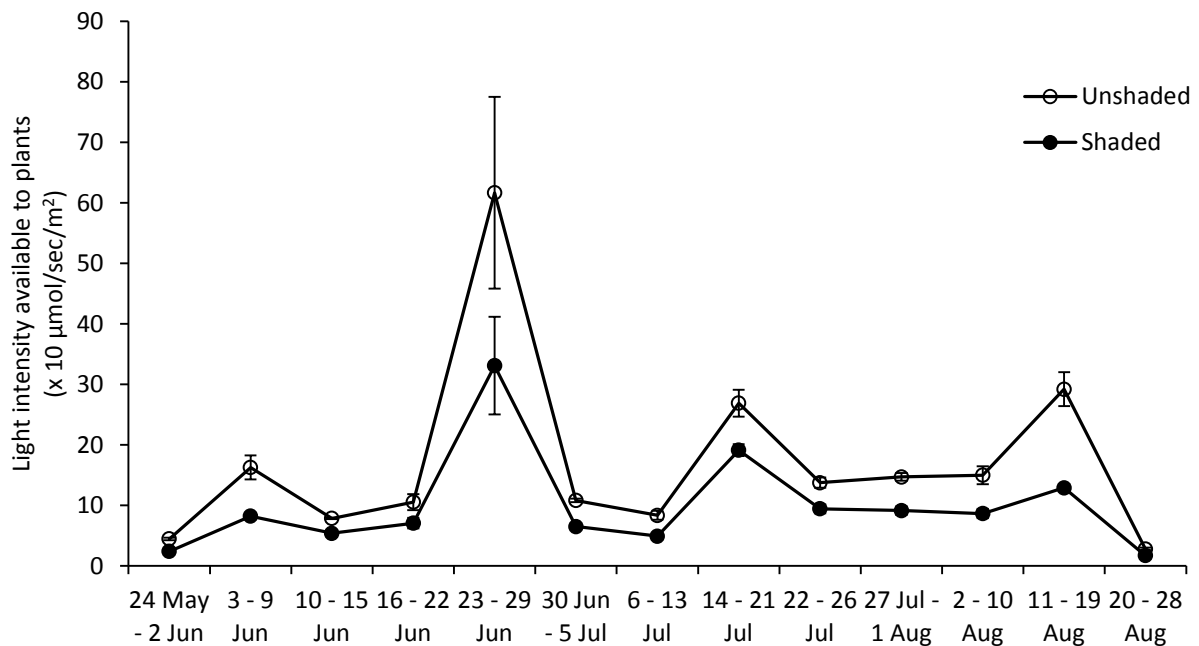


Figure 33 Light availability to plants throughout the active growth season. Presented is the mean \pm 1 S.E. for light intensity ($\times 10 \mu\text{mol sec}^{-1} \text{m}^{-2}$) available to the plants per light treatment type per week. Dot fill represents the experimental light treatment applied to the plants (filled = shaded; unfilled = unshaded).

Plants were subjected to heat stress from 22 to 26 July 2012 (Fig. 34); the maximum daily greenhouse temperature (mean \pm 1 S.E.) peaked at $33.9 \pm 1.1 \text{ }^\circ\text{C}$ and the mean daily greenhouse temperature peaked at $23.1 \pm 0.6 \text{ }^\circ\text{C}$. Plants were subjected to additional stress caused by aphid herbivory during this time period.

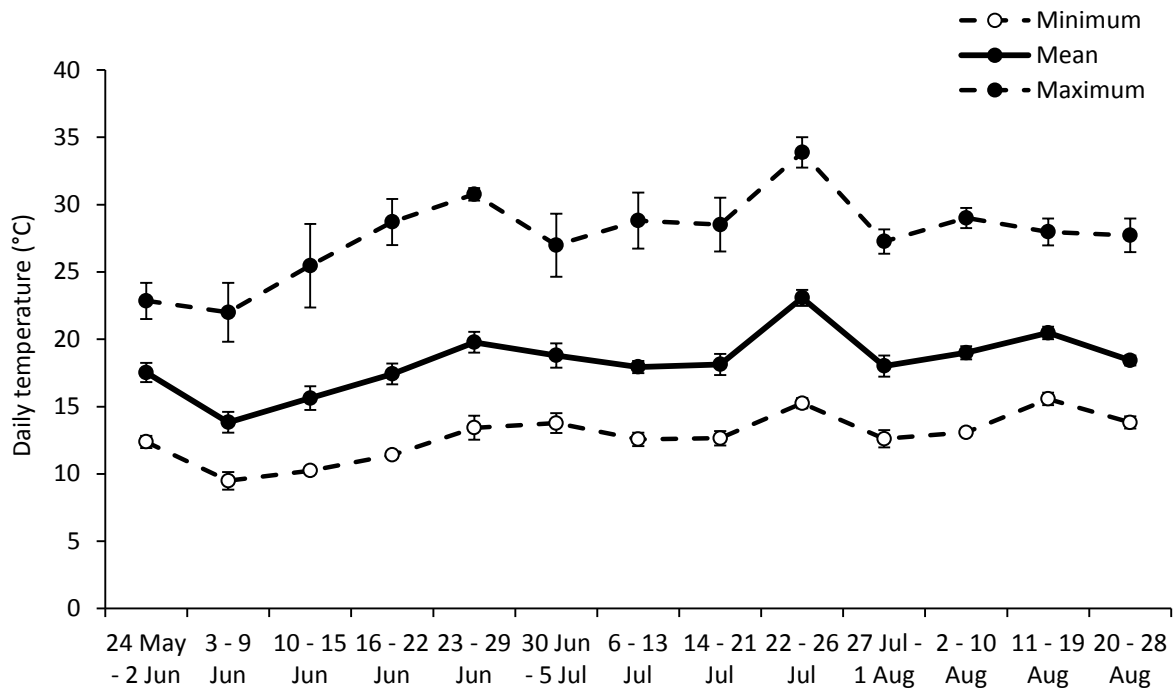


Figure 34 Mean daily greenhouse temperatures throughout the active growth season of *Dionaea muscipula*, *Drosera rotundifolia* and *Pinguicula grandiflora*. Presented are the mean \pm 1 S.E. for: ambient daily maximum, mean, and minimum greenhouse temperatures throughout the plants' active growth season.

5.3.2 Plant size

The results of repeated measures GLM of rosette area vs. light (shaded, unshaded), N, species and time found the main effect of N reached statistical significance (Table 28); plants receiving N were significantly larger than plants not receiving N. There was a significant interaction effect of N x time; plants receiving N during August and September possessed significantly larger rosette areas than plants not receiving N during the same time period.

The main effect of species type reached statistical significance (Table 28). In order of decreasing rosette area: *P. grandiflora*, *D. muscipula*, and *D. rotundifolia*. The interaction effect of species x time was statistically significant both within-species and between-species (Table 28). Within each species, rosette area was significantly larger during June and July than during August and September, reflecting the detrimental influence of a heat / herbivory stress event during late July 2012 (Figs. 35(a), 35(b), 35(c)). Between species, *D. rotundifolia* showed over twice the level of sensitivity to the stress event compared to the other species; rosette area decreased by a magnitude of -66.9% between the time period of June – July and August – September. *D. muscipula* and *P. grandiflora*

showed a similarly low level of sensitivity to the stress event; with a magnitude of decrease in rosette areas of -24.6% and -23.1% respectively.

The interaction effect of light x species x time reached statistical significance (Table 28). Specifically, *D. rotundifolia* and *P. grandiflora* plants subjected to shading possessed substantially larger rosette areas following the stress event in August compared with unshaded plants (Figs. 35b), 35(c)). In contrast, *D. muscipula* plants receiving unshaded treatment possessed larger rosette areas than shaded plants in August following the stress event, and unshaded plants continued to thrive with larger rosette areas in September than shaded plants (Fig. 35(a)). The rosette area of *D. rotundifolia* plants decreased in September regardless of light treatment. Unshaded *P. grandiflora* plants showed an increase in rosette area in September compared to August, whereas shaded plants showed a decrease in rosette area in September compared to August.

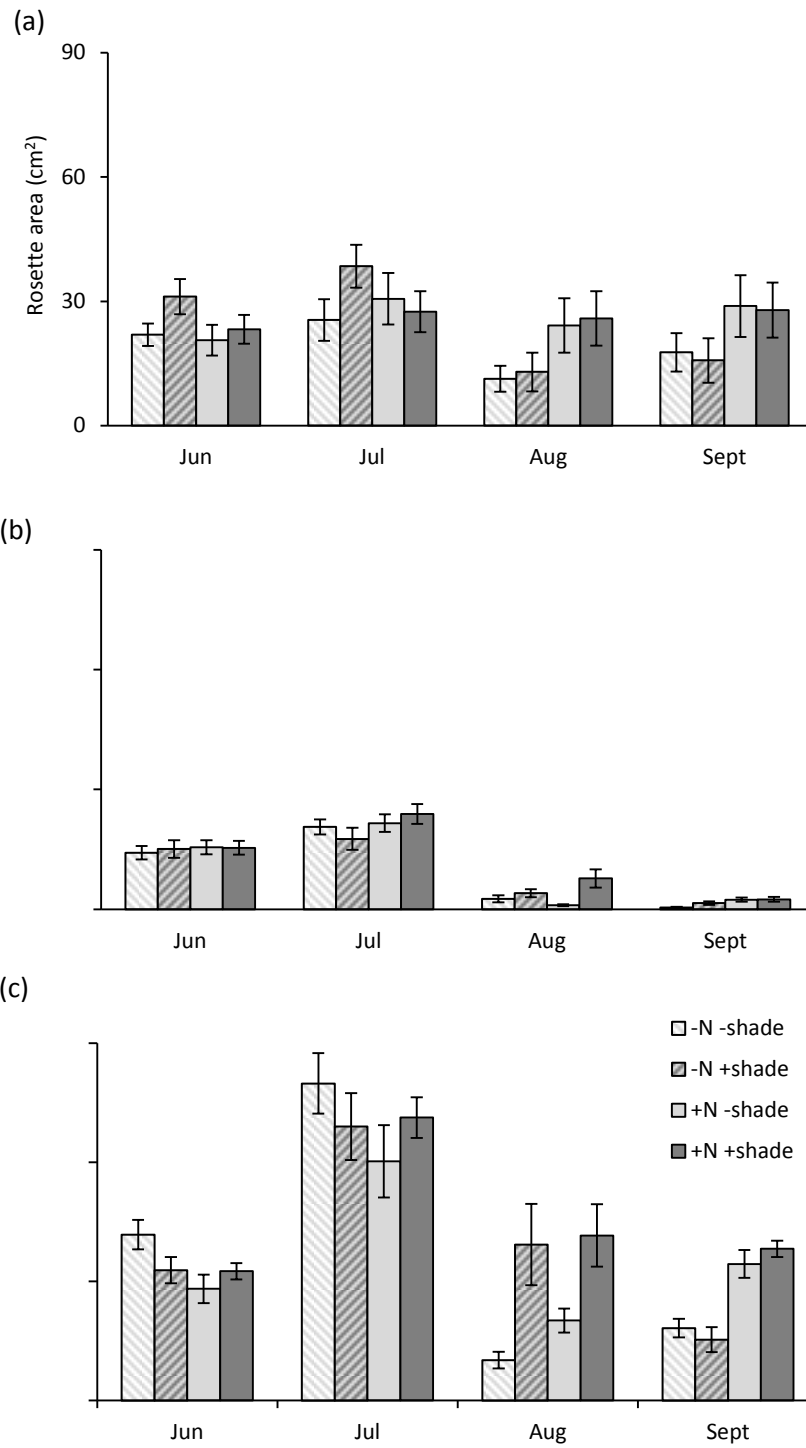


Figure 35 Plant size of three carnivorous plant species throughout the active growth season. Presented is the mean \pm 1 S.E. rosette area for: (a) *Dionea muscipula*; (b) *Drosera rotundifolia*; (c) *Pinguicula grandiflora*. Bar fill at each survey month illustrates the four experimental treatments.

5.3.3 Exploring the influence of a stress event on plant mortality

A large increase in mortality occurred following the heat / herbivory stress event for all plant species (Fig. 36). Between species, *Drosera rotundifolia* was the most sensitive and *P. grandiflora* was the least sensitive to stress.

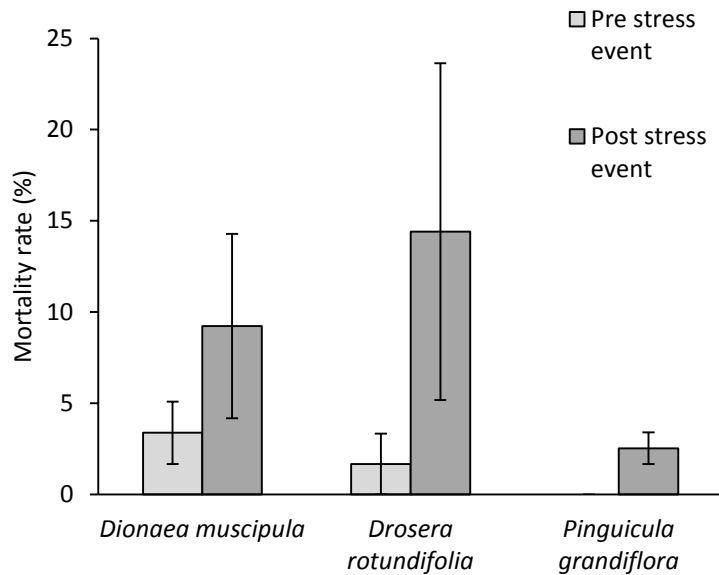


Figure 36 Influence of a stress event on plant mortality rate (mean \pm 1 S.E.) for three species of carnivorous plant.

Experimental treatment type influenced the post-stress mortality rates of plants, and this varied between species. For *D. muscipula* and *P. grandiflora*, plants exposed to the experimental treatment of -N +shade exhibited the highest mortality rate compared to other treatment types (Figs. 37(a) and 37(c)). For *P. grandiflora*, only plants subjected to -N treatment suffered post stress event mortality, with the largest mortality rate for plants subjected to the experimental treatment of -N +shade (Fig. 37(b)).

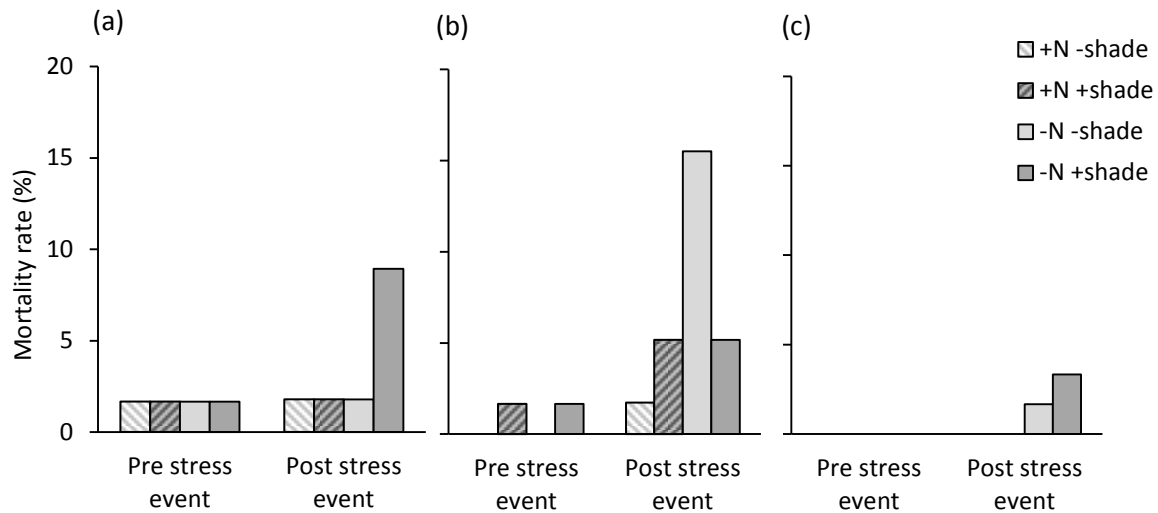


Figure 37 Influences of experimental treatment and a stress event on the mortality rates of three species of carnivorous plant. Presented are the pre-stress event and post-stress event mortality rates per experimental treatment for:- (a) *Dionaea muscipula*; (b) *Drosera rotundifolia*; (c) *Pinguicula grandiflora*.

5.3.4 Plant relative growth rates

The main effect of species reached statistical significance (Table 28); listed in order of decreasing RGR:- *P. grandiflora*, *D. muscipula* and *D. rotundifolia*. The interaction effect of species x time reached statistical significance; the RGR of all species peaked in July with *D. rotundifolia* showing the largest magnitude and *D. muscipula* showing the smallest magnitude of RGR decline in August following the heat / herbivory stress event.

The interaction effect of light x N x species x time reached statistical significance (Table 28). For *D. muscipula*, RGR was uninfluenced by experimental treatment type during June and July, however plants subjected to +N +shade underwent a significantly higher RGR in August compared to other experimental treatments, and plants subjected to -N +shade underwent significantly lower RGR in September compared to other treatments (Fig. 38(a)). For *D. rotundifolia*, RGR was uninfluenced by experimental treatment type at each month (Fig. 38(b)). For *P. grandiflora*, experimental treatment type exerted no significant influence on RGR throughout the growth season except in September, where plants subjected to -N treatment underwent significantly lower RGR than plants subjected to +N treatment (Fig. 38(c)).

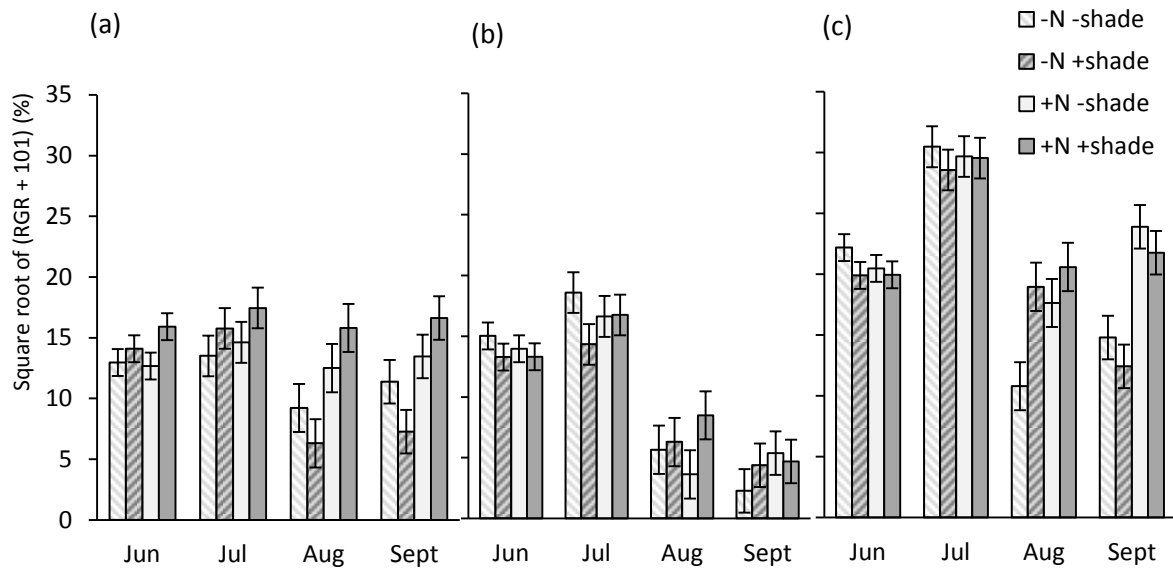


Figure 38 Influence of root N and light availability throughout the plants' active growth season on the relative growth rates (RGRs) of (a) *Dionaea muscipula*, (b) *Drosera rotundifolia*, (c) *Pinguicula grandiflora*. RGR is defined as the proportional change in rosette area between each month and the measurement taken prior to the start of experimental treatment. Data presented are the mean \pm 1 S.E. of the square root of (RGR +101) per experimental treatment (-N -shade, -N +shade, +N -shade, +N +shade) per species.

5.3.5 Plant dry mass parameters

Table 29 Results of 3-way (light, N, species) univariate ANOVAs for dry mass characteristics of three species of carnivorous plant (*Dionaea muscipula*, *Drosera rotundifolia* and *Pinguicula grandiflora*) subjected to one of four contrasting N and light treatment combinations (2 x 2 factorial design: + N, + shade; + N, - shade; - N, + shade; -N – shade). Presented are degrees of freedom (*df*), *F* and *P* values from the analyses of:- total dry mass per plant (g), DM; leaf mass fraction, LMF; root mass fraction, RMF. Effect abbreviations as follows: L = light, N = nitrogen, S = species. Significant effects at $P < 0.05$ are highlighted in bold.

Effect	DM ^a			LMF ^a			RMF ^a		
	<i>df</i>	<i>F</i>	<i>P</i>	<i>df</i>	<i>F</i>	<i>P</i>	<i>df</i>	<i>F</i>	<i>P</i>
L	1, 173	<1	0.648	1, 173	<1	0.801	1, 173	<1	0.886
N	1, 173	22	<0.001	1, 173	2	0.156	1, 173	10	0.002
S	2, 173	21	<0.001	2, 173	12	<0.001	2, 173	43	<0.001
L x N	1, 173	1	0.239	1, 173	<1	0.891	1, 173	4	0.046
L x S	2, 173	1	0.562	2, 173	2	0.104	2, 173	1	0.510
N x S	2, 173	5	0.012	2, 173	8	0.001	2, 173	9	<0.001
L x N x S	2, 173	<1	0.779	2, 173	3	0.036	2, 173	1	0.470

^a Data were square root transformed before analysis.

The main effect of species on the total dry mass per plant reached statistical significance (Table 29); in order of decreasing mass:- *Dionaea muscipula* (mean \pm 1 S.E. of total dry mass per plant = 0.278 ± 0.028 g), *Pinguicula grandiflora* (0.196 ± 0.017 g), *Drosera rotundifolia* (0.080 ± 0.004 g). The main effect of N and the interaction effect of N x species reached statistical significance (Table 29); plants receiving +N treatment were significantly heavier (mean \pm 1 S.E. of total dry mass per plant = 0.222 ± 0.021 g) than plants receiving –N treatment (0.147 ± 0.013 g). The magnitude of the difference between N treatments varied significantly between species (Fig. 39); *P. grandiflora* showed the largest difference:- +N plants were 49.0% heavier on average than –N plants, followed by *D. muscipula* (26.1%) and *D. rotundifolia* (17.9%).

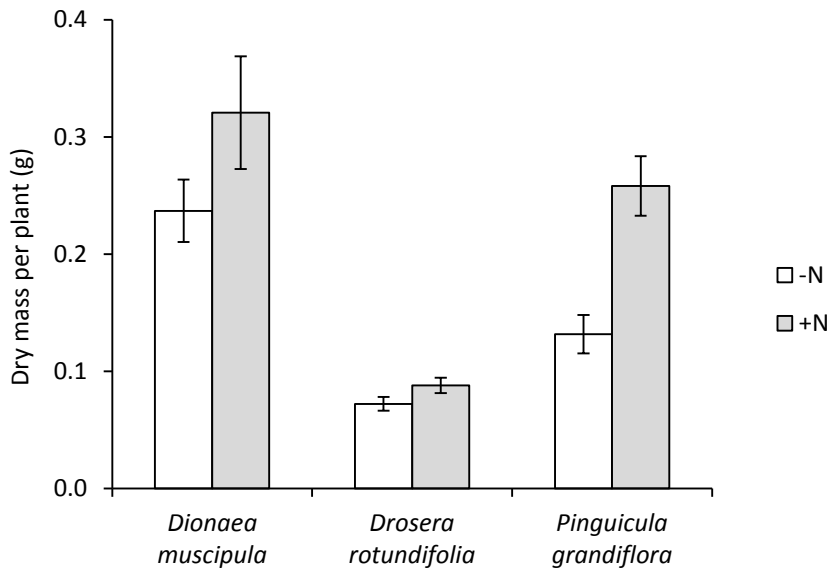


Figure 39 Influence of root N availability on the total plant dry mass (mean \pm 1 S.E.) of *Dionaea muscipula*, *Drosera rotundifolia* and *Pinguicula grandiflora*.

Dionaea muscipula and *P. grandiflora* allocated the largest proportions of mean plant dry mass to leaves, whereas *D. rotundifolia* allocated the largest proportion of mean plant dry mass to reproductive structures (Fig. 40). *Dionaea muscipula* and *P. grandiflora* allocated approximately threefold the proportion of mean plant dry mass to the stem compared with *D. rotundifolia*.

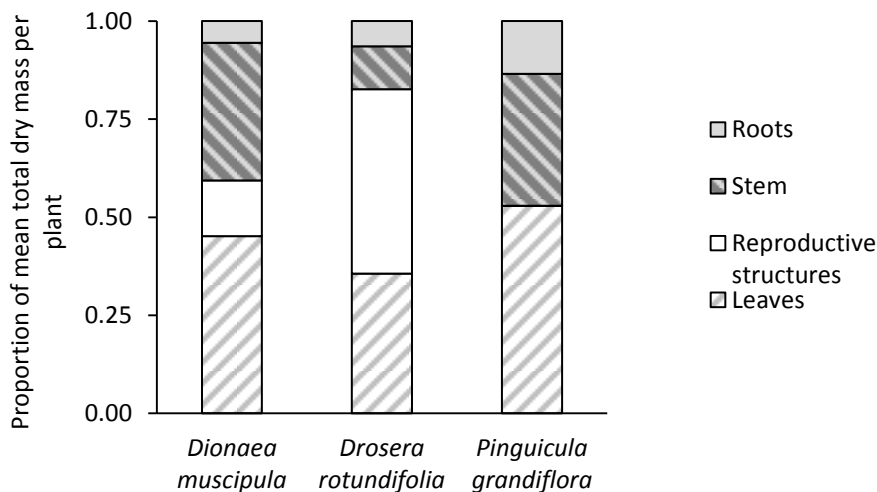


Figure 40 Influences of root N and light availability on the proportional allocation of dry mass to plant parts by *Dionaea muscipula*, *Drosera rotundifolia* and *Pinguicula grandiflora* plants.

The effect of species on leaf mass fraction (LMF) reached statistical significance (Table 29); *P. grandiflora* possessed the highest LMF and *D. rotundifolia* possessed the lowest LMF. *Pinguicula grandiflora* plants receiving +N treatment possessed significantly higher LMF than plants receiving –N treatment (Fig. 41). The interaction effect of N x light x species reached statistical significance. For *D. muscipula* plants receiving –N treatment, unshaded plants possessed significantly higher LMF than shaded plants, whereas light treatment exerted no significant effect on the LMF of plants receiving +N treatment. For *P. grandiflora*, plants receiving +N -shade treatment possessed significantly higher LMF than plants receiving –N treatment. For *D. rotundifolia*, plants receiving –N +shade treatment possessed significantly higher LMF than plants receiving +N –shade treatment.

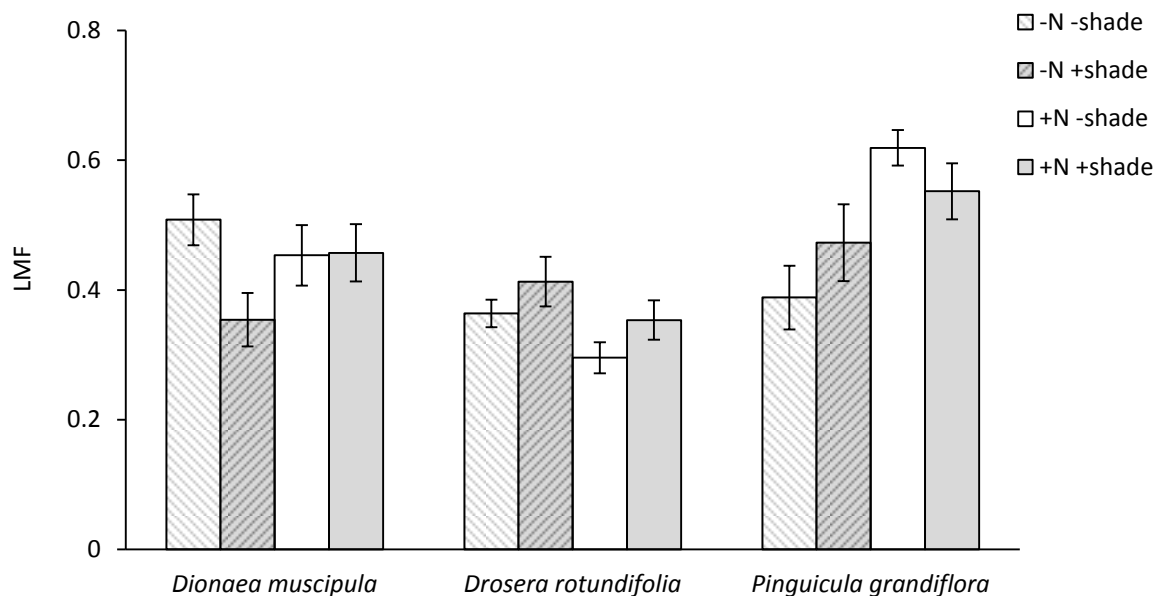


Figure 41 Influences of root N and light availability on the Leaf Mass Fraction (LMF) (mean \pm 1 S.E.) of *Dionaea muscipula*, *Drosera rotundifolia* and *Pinguicula grandiflora* plants.

The effect of species on root mass fraction (RMF) reached statistical significance (Table 29); *P. grandiflora* possessed the highest RMF, and *D. muscipula* and *D. rotundifolia* possessed joint lowest RMF. The main effect of N reached statistical significance; plants receiving -N treatment possessed higher RMF than plants receiving +N treatment. For *P. grandiflora*, plants receiving +N treatment possessed significantly higher RMF than plants receiving –N treatment, and light treatment exerted no significant influence (Fig. 42). For *D. muscipula* and *D. rotundifolia*, N and light availability exerted no significant influence on RMF, but marginally lower RMF was observed for *D. muscipula* plants

receiving +N +shade treatment compared with other treatment types, and marginally lower RMF was observed for *D. rotundifolia* plants receiving -N -shade treatment than other treatment types.

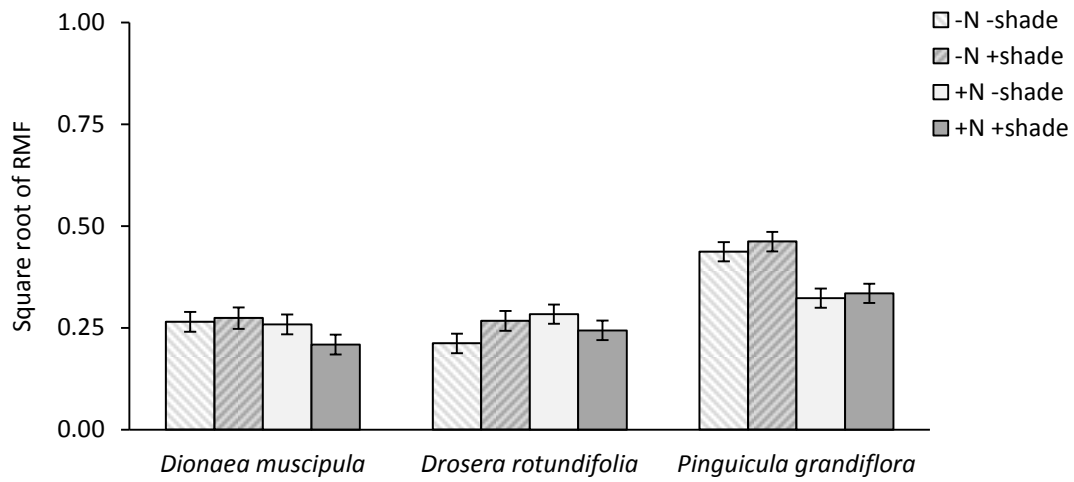


Figure 42 Influences of root N and light availability on the Root Mass Fraction (RMF) (mean \pm 1 S.E.) of *Dionaea muscipula*, *Drosera rotundifolia* and *Pinguicula grandiflora* plants.

5.3.6 Exploring the relationship between leaf redness and leaf anthocyanin content

Leaf anthocyanin content was strongly and positively correlated with leaf redness (Pearson's correlation coefficient, r , (two-tailed):- $r_{(30)} = 0.914$, $P < 0.001$) (Fig. 43).

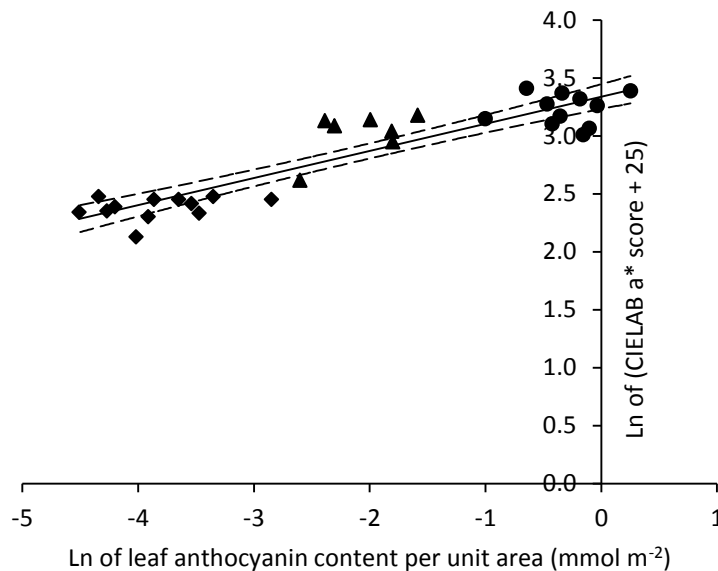


Figure 43 The relationship between leaf anthocyanin content per unit leaf area and leaf redness (CIELAB a* score of leaf colour on a red to green continuum). Presented are the linear regression equation and 95% confidence intervals for $y = 0.234x + 3.339$, where y = leaf CIELAB a* score (Ln of (CIELAB a* + 25)), and x = Ln of leaf anthocyanin content per unit leaf area ($F_{(1,29)} = 142.192$, $P < 0.001$, $R^2 = 0.835$).

5.3.7 Exploring the influence of light availability on leaf traits and plant stress

The leaves of *D. rotundifolia* and *D. muscipula* were predominantly red but possess the ability to vary to green, whereas the leaves of *P. grandiflora* remained green throughout the active growth season. The prediction of leaf colour to be significantly redder upon exposure to high light availability than to low light availability was supported for *Dionaea muscipula* and *Drosera rotundifolia* in September; the interaction effect of species x light x time was statistically significant (Table 28). For *D. muscipula*, the leaves of unshaded plants became redder over time, whereas there was no change in leaf redness of shaded leaves over time (Fig. 44). For *D. rotundifolia*, there was no significant change in leaf redness for unshaded leaves over time, whereas shaded leaves became significantly less red over time. There was no significant effect of light availability on leaf colour of *P. grandiflora*, however leaves became significantly greener over time. The main effect of species reached statistical significance (Table 28); in order of decreasing leaf redness:- *D. muscipula*, *D. rotundifolia* and *P. grandiflora*.

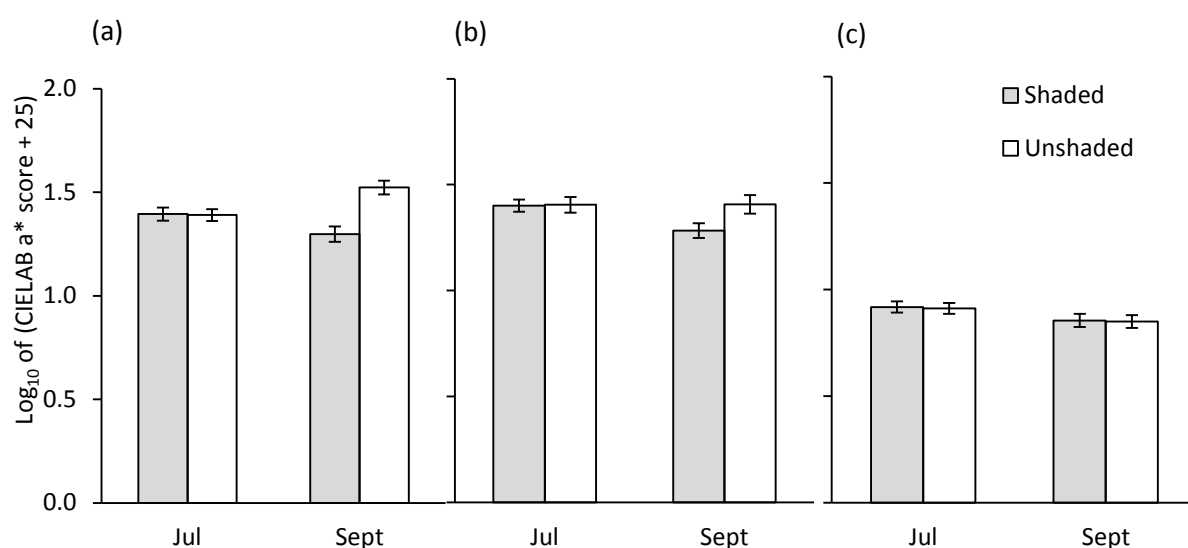


Figure 44 The relationship between light availability and the CIELAB a* red-green score of leaf colour for three carnivorous plant species exposed to shaded or unshaded experimental treatment across the active growth season. Presented are the mean ± 1 S.E. of \log_{10} of (leaf CIELAB a* score +22) per light treatment per species for: (i) *Dionaea muscipula*, (ii) *Drosera rotundifolia*, (iii) *Pinguicula grandiflora*.

Table 30 Results of 3-way (light, N, species) univariate ANOVAs for stress characteristics of three species of carnivorous plant (*Dionaea muscipula*, *Drosera rotundifolia* and *Pinguicula grandiflora*) subjected to one of four contrasting N and light treatment combinations (2 x 2 factorial design: + N, + shade; + N, - shade; - N, + shade; -N – shade). Presented are degrees of freedom (*df*), *F* and *P* values from the analyses of the level of plant stress of leaves ($(F_v/F_m)_L$) (all species; refers to the traps of *D. muscipula*) and of the level of plant stress of petioles ($(F_v/F_m)_P$) (*D. muscipula* only). Effect abbreviations as follows: L = light, N = nitrogen, S = species. Significant effects at $P < 0.05$ are highlighted in bold.

Effect	$(F_v/F_m)_L$			$(F_v/F_m)_P$		
	<i>df</i>	<i>F</i>	<i>P</i>	<i>df</i>	<i>F</i>	<i>P</i>
L	1, 83	2	0.202	1, 28	3	0.110
N	1, 83	1	0.309	1, 28	<1	0.852
S	2, 83	4	0.024	n/a	n/a	n/a
L x N	1, 83	1	0.279	1, 28	5	0.034
L x S	2, 83	1	0.539	n/a	n/a	n/a
N x S	2, 83	2	0.132	n/a	n/a	n/a
L x N x S	2, 83	<1	0.873	n/a	n/a	n/a

The leaves of unshaded *D. muscipula* and *D. rotundifolia* plants exhibited higher F_v/F_m values, and therefore greater efficiency of photosystem II, than the leaves of shaded plants (Fig. 45). The leaves of shaded *P. grandiflora* plants exhibited higher F_v/F_m values than the leaves of unshaded plants, however the difference between light treatments was marginal. The difference in F_v/F_m between shaded and unshaded plants did not reach statistical significance (Table 30). The main effect of species reached statistical significance; in order of decreasing F_v/F_m (mean \pm 1 S.E.):- *P. grandiflora*: 0.967 ± 0.008 ; *D. rotundifolia*: 0.961 ± 0.011 ; *D. muscipula*: 0.935 ± 0.009).

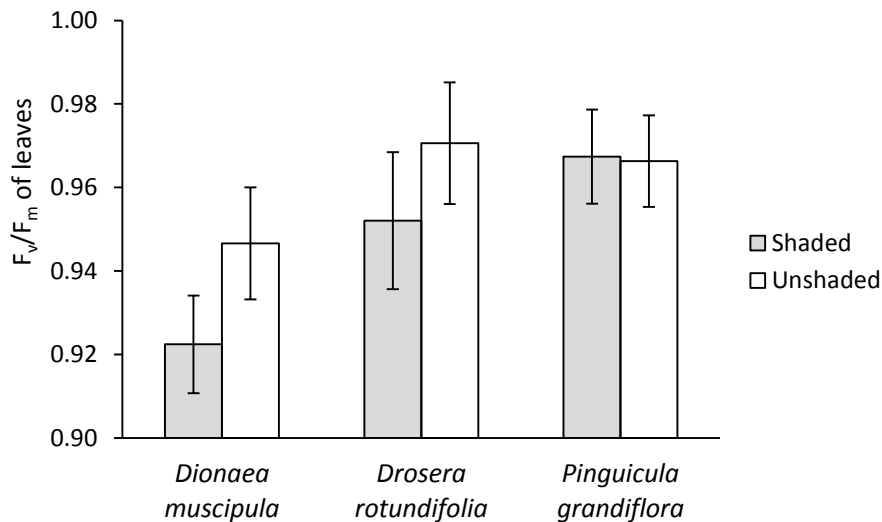


Figure 45 Influence of light availability on photosystem II stress (F_v/F_m) (mean \pm 1 S.E.) of the leaves of *Dionaea muscipula*, *Drosera rotundifolia* and *Pinguicula grandiflora* plants.

Table 31 Results of two-way univariate ANOVAs exploring the influence of N and light availability on investment in prey capture by *Dionaea muscipula*, as measured by the trap to petiole (T:P) ratio by dry mass. Plants received one of four contrasting N and light treatment combinations (2 x 2 factorial design: + N, + shade; + N, - shade; - N, + shade; -N – shade). Presented are degrees of freedom (*df*), *F* and *P* values from the analyses of the trap to petiole ratio by dry mass for: all leaves (current and previous growth seasons' combined), T:P; current growth season's leaves only, T:P_{new}; previous seasons' leaves only, T:P_{old}. Effect abbreviations as follows: L = light, N = nitrogen. Significant effects at $P < 0.05$ are highlighted in bold.

Effect	T:P			T:P _{new}			T:P _{old}		
	<i>df</i>	<i>F</i>	<i>P</i>	<i>df</i>	<i>F</i>	<i>P</i>	<i>df</i>	<i>F</i>	<i>P</i>
L	1, 56	<1	0.581	1, 51	<1	0.714	1, 52	1	0.445
N	1, 56	<1	0.702	1, 51	<1	0.752	1, 52	<1	0.924
L x N	1, 56	<1	0.596	1, 51	1	0.425	1, 52	4	0.040

The main effects of light and N, and the interaction effect of light x N, on the T:P ratio of all leaves, and of current season's leaves, failed to reach statistical significance (Table 31). For previous growth season's leaves, the interaction effect of light x N reached statistical significance (Table 31); leaves of plants receiving -N -shade treatment possessed the highest T:P ratio of the experimental treatment combinations (Fig. 46). A surprising result is evident; leaves of plants receiving +N -shade treatment possessed the lowest T:P ratio of the experimental treatment combinations.

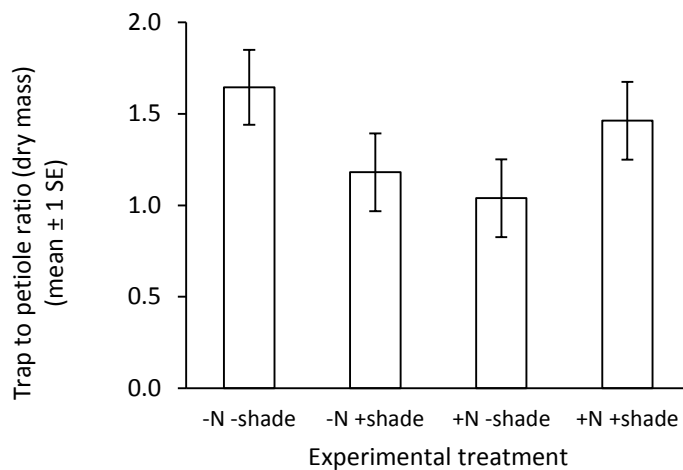


Figure 46 Influences of light and root N availability on relative investment in prey capture by previous seasons' leaves of *Dionaea muscipula*, as measured by the trap to petiole ratio by dry mass. Presented is the mean ± 1 S.E. trap to petiole ratio by dry mass per experimental treatment type.

Unshaded *D. rotundifolia* and *P. grandiflora* plants possessed stickier leaves than shaded plants (Table 27; Fig. 47).

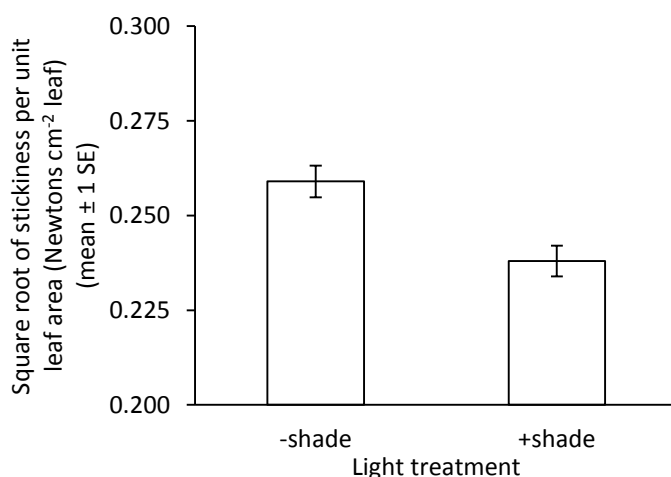


Figure 47 Influence of light availability on relative investment in prey capture by *Drosera rotundifolia* and *Pinguicula grandiflora*. Presented is the mean ± 1 S.E. of stickiness per unit leaf area (Newtons cm⁻² leaf) per light treatment.

5.3.8 Exploring the influence of N availability on leaf traits and plant stress

Leaf redness was not influenced by root N availability or any interaction effects incorporating N (Table 28).

The interaction effect of light x N reached statistical significance for the F_v/F_m data of the petioles of *D. muscipula* (Table 30). Specifically, upon exposure to an unshaded environment, +N plants exhibited higher F_v/F_m values than -N plants, whereas upon exposure to a shaded environment, +N plants exhibited lower F_v/F_m values than -N plants (Fig. 48).

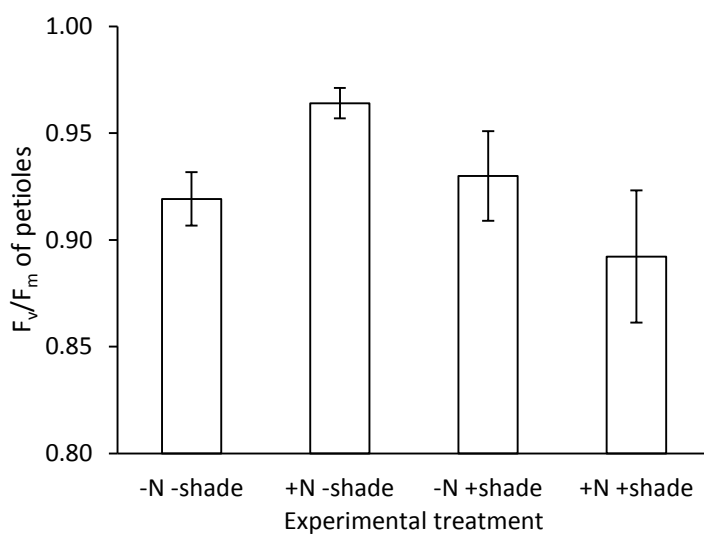


Figure 48 Influences of root N and light availability on photosystem II stress (F_v/F_m) (mean \pm 1 S.E.) of the petioles of *Dionaea muscipula*.

The leaves of plants exposed to -N treatment were significantly stickier than the leaves of plants exposed to +N treatment (Fig. 49). The magnitude of this effect was larger for *P. grandiflora* than for *D. rotundifolia* during August. The leaves of +N plants increased in stickiness between June and July, after which stickiness declined. The leaves of *D. rotundifolia* plants subjected to -N treatment increased in stickiness throughout the growth season. The leaves of *P. grandiflora* plants subjected to -N treatment increased in stickiness until August, after which stickiness declined. The main effect of species and the interaction effect of species x time reached statistical significance (Table 28); *P. grandiflora* leaves were stickier than *D. rotundifolia* leaves, with the magnitude of this effect largest during July.

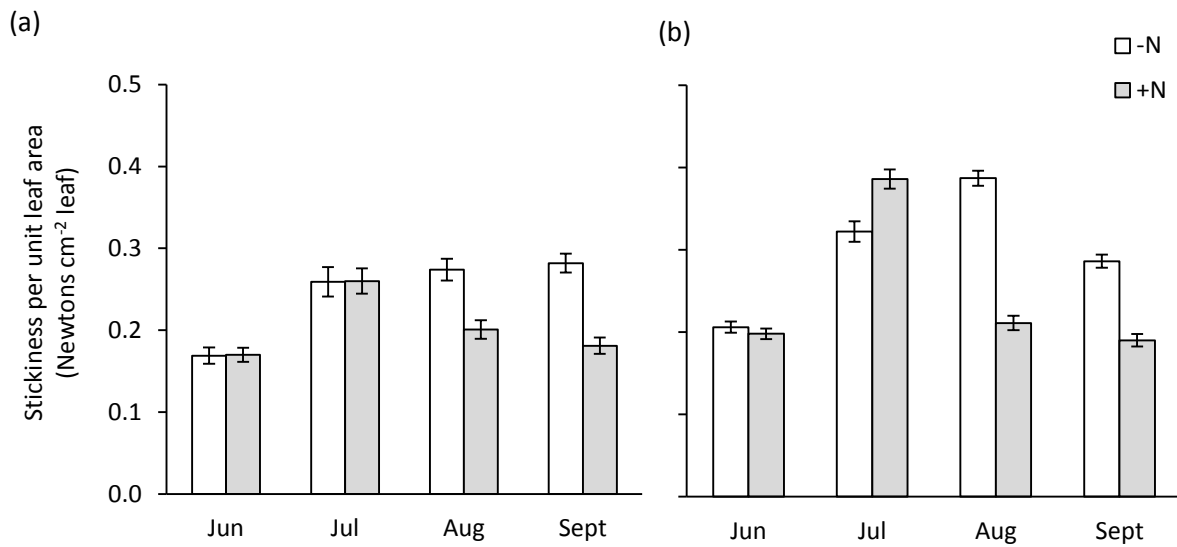


Figure 49 Influence of root N availability on stickiness per unit leaf area as a measure of relative investment in prey capture by two species of carnivorous plant throughout the active growth season. Presented are the mean \pm 1 S.E. of stickiness per unit leaf area (Newtons cm⁻² leaf) (square root-transformed) for: (a) *Drosera rotundifolia*; (b) *Pinguicula grandiflora*.

5.4 Discussion

In this study, the influence of light and root N availability on morphological and physiological characteristics of three carnivorous plant species with contrasting prey capture mechanism or evolutionary lineage was investigated. Research foci consisted of: (i) the testing of Givnish's cost-benefit model for the evolution of botanical carnivory (Givnish *et al.*, 1984) by exploring whether plant investment in carnivory is influenced by root N and light availability in ecological time; (ii) the testing of two competing hypotheses to propose the functional role of anthocyanins in carnivorous plants; anthocyanin accumulation as a photoprotective response to high light availability, or as a stress response to root N deficiency.

Differences in plant fitness and ecophysiological responses to root N and light availability between the plant species used in this study reflect inherent variation in life strategy, ecological niche, and evolutionary history. *Pinguicula grandiflora* plants exhibited the highest level of plant fitness; plants were largest in size and exhibited the highest RGR of the three species. The plants also showed the highest level of stress tolerance, exhibiting the lowest mortality rate both pre- and post- stress event, indicating stronger resilience and higher adaptive ability to range of environmental conditions than the remaining species. Light availability failed to significantly influence RGR throughout the active growth season, supporting the species' classification as a facultative heliophyte (Hill *et al.*, 2004). Of the three plant species, *Pinguicula grandiflora* plants allocated the largest proportion of total plant dry mass to roots and exhibited the largest growth response to root N availability, indicating that root N availability is more important than light availability for growth. The relatively vigorous growth habit of *P. grandiflora*, along with the species' tolerance of shade, provides support for this species to possess a higher competitive ability than *D. muscipula* and *D. rotundifolia*.

Drosera rotundifolia plants exhibited the lowest fitness of the study species; plants were smallest in size, lowest in dry mass, RGR, and tolerance level to stress - exhibiting joint highest pre-stress event mortality and highest post-stress mortality. In June and July, unshaded *D. rotundifolia* plants exhibited higher RGR than shaded plants, whereas the RGR of *D. muscipula* was higher under shaded conditions and the RGR of *P. grandiflora* was uninfluenced by light availability, suggesting *D. rotundifolia* to be the most light-demanding of the species. This result is indicative of the species' nature as a primary coloniser of bare peat and disturbed surfaces (Crowder *et al.*, 1990). The species also showed the highest allocation of total dry mass to reproductive structures of the three species. These results are indicative of the species' life history as an r-strategist, where the traits of high investment in seed production, early maturity and small size maximise the species' probability of success (Pianka, 1970); *D. rotundifolia*, as with most *Drosera spp.*, is a poor competitor and struggles

to compete with co-habitant vascular plants for resources (Wilson and Keddy, 1986; Svensson, 1995). Of the study species, *Drosera rotundifolia* plants allocated the smallest proportion of total plant dry mass to roots, indicating evolutionary adaptation to extremely nutrient deficient environments. This observation supports the literature of the underdeveloped root structure of *D. rotundifolia* is well documented, where carbon may be preferentially allocated to leaves for prey capture (Bruzzese *et al.*, 2010).

Dionaea muscipula possessed the largest dry mass per plant of the study species, but a smaller rosette area than *P. grandiflora*, the larger mass relative to rosette area probably due to the relatively high leaf mass area (LMA) of *D. muscipula* traps compared to the traps of other carnivorous plant species (Karagatzides and Ellison, 2009). Whilst the effect of light and root N availability on the RGR of *D. muscipula* plants in June and July failed to reach statistical significance, plants receiving +N treatment exhibited higher RGR than plants receiving –N treatment in August and September, suggesting that plants became more N-demanding following the stress event in late July. This result may be explained by the relatively high N and C costs associated with new trap growth to replace those damaged following the stress event; the payback time of trap construction for *D. muscipula* is one of the longest of all carnivorous plants (Karagatzides and Ellison, 2009).

The leaf stickiness of *D. rotundifolia* and *P. grandiflora* plants receiving –N treatment was significantly greater than the leaf stickiness of plants receiving +N treatment, and the leaf stickiness of unshaded plants was significantly greater than the leaf stickiness of shaded plants, indicating that these species are phenotypically plastic in terms of investment in botanical carnivory in response to N and light availability. These results provide support for Givnish's cost-benefit model; investment in carnivory, in this case via leaf mucilage production, is only of net marginal benefit to the plant upon exposure to a nutrient-deficient but high light environment; as root N availability increases or light availability decreases, the net benefit to the plant decreases (Givnish *et al.*, 1984). This result supports those of earlier studies using *D. rotundifolia in-situ* (Millett *et al.*, 2012) and *ex-situ* in a greenhouse environment (Thorén *et al.*, 2003), and of an *ex-situ*, outdoors study of *Pinguicula vallisneriifolia* (Zamora *et al.*, 1998). Whilst *D. rotundifolia* and *P. grandiflora* are derived from separate evolutionary lineages of the angiosperm phylogeny, this result provides support for the convergent evolution of adaptive physiological traits that maximise plant fitness in response to similar historical resource constraints (Darwin, 1859; Grime, 1977; Ellison and Gotelli, 2009).

For *Dionaea muscipula*, evidence was found of phenotypic plasticity in terms of investment in carnivory (as measured by the trap to petiole (T:P) ratio by dry mass) in response to N and light availability, but the nature of the response differed to those of *D. rotundifolia* and *P. grandiflora*. Specifically, the influence of the main effects of N and light on the T:P ratios of all leaves, current

season's leaves, and previous seasons' leaves failed to reach statistical significance. However, evidence of phenotypic plasticity of investment in carnivory in response to N and light was found through the significant interaction effect of light x N on the T:P ratio of previous seasons' leaves. Leaves of plants receiving -N -shade treatment possessed the highest T:P ratio of the experimental treatment combinations, suggesting growth to be N-limited under high light availability. This result provides further support for Givnish's cost/benefit model; investment in carnivory is only of net benefit to the plant when light availability is not limiting.

A somewhat surprising result was discovered:- upon exposure to low light availability, *D. muscipula* plants receiving +N treatment showed greater relative growth allocation to traps than plants receiving -N treatment. A possible explanation may be found by comparison of the between-species differences in resource allocation trade-offs as a consequence of variation in morphological and physiological leaf traits of the carnivorous plants used in this study. *Drosera rotundifolia* and *P. grandiflora* possess leaves with identical dual functions; the provision of nutrients (via the capturing of prey) and the provision of energy via photosynthesis. For *D. muscipula*, however, energy acquisition via photosynthesis is obtained from the morphologically distinct leaf structures of petioles and traps, with traps serving the additional function of nutrient acquisition via prey capture. Further to evidence that indicates traps to be less photosynthetically efficient than petioles (Hájek and Adamec, 2010), it may be predicted that upon exposure to +N treatment under low light availability, plant growth would be limited by light availability, therefore resource allocation would shift to the more photosynthetically-efficient petioles to maximise energy gain. However, this was not the case. *Dionaea muscipula* plants receiving +N +shade treatment exhibited the highest RGR of the experimental treatment combinations throughout the growth season, and the magnitude of the difference in RGR was largest in September, prior to dry mass measurement. Thus, it is possible that as a consequence of increased plant N demand following high investment in growth, plants receiving +N +shade treatment became co-limited by N and light, thus shifting growth allocation to traps capable of performing dual functions of nutrient and energy acquisition.

An alternative explanation may be offered; the increased RGR of plants is likely to have increased the intensity of shading to previous seasons' leaves by new growth, leaving the petioles, situated lower down the leaf stalk than the traps, more vulnerable to a greater relative degree of shading than the traps. Thus, previous seasons' leaves may have allocated greater relative growth to traps to maximise energy gain; the maximum photosynthetic rate per unit mass of traps under higher light availability may have exceeded that of the petioles despite differences in net maximum photosynthetic rate. However, this possible explanation is perhaps less probable than the former, as evidence from the literature suggests petioles to possess significantly lower light compensation

points (I_c) than traps (Table 32) (Hájek and Adamec, 2010). Therefore, in order for net energy gain to be greater for traps than petioles, light availability would be required to be lower than *ca.* $6.2 \mu\text{mol m}^{-2} \text{s}^{-1}$ to the petioles but greater than *ca.* $7.5 \mu\text{mol m}^{-2} \text{s}^{-1}$ to the traps, assuming a leaf temperature (mean ± 1 S.D.) of 23 ± 1 °C (Table 32) (Hájek and Adamec, 2010).

Table 32 Basic photosynthetic characteristics of *Dionaea muscipula*, *Drosera rotundifolia* and *Pinguicula vulgaris* (in the absence of data for *P. grandiflora*) collated from the literature. Data presented are: light-saturated maximum area-based photosynthetic rate, $P_{N\text{max (area)}}$ (mean ± 1 S.E.), light saturation point, I_s (mean ± 1 S.E.), light compensation point, I_c (mean ± 1 S.E.), ambient (a) or leaf (L) temperature during photosynthetic measurement (mean ± 1 S.D.), number of plant replicates (n) and the plant growth environment (G = greenhouse, O = outdoors) during the course of the study.

Species	Plant part	$P_{N\text{max (area)}}$ ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	I_s ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	I_c ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	Temp. (°C)	n	Growth environment	Source
<i>Dionaea muscipula</i>	Traps	3.04 ± 0.20	182 ± 5	7.5 ± 0.3	23 ± 1 (L)	5	G	Hájek & Adamec, 2010
	Petioles	4.03 ± 0.38	231 ± 24	6.2 ± 1.1	23 ± 1 (L)	5	G	Hájek & Adamec, 2010
<i>Drosera rotundifolia</i>	Leaves	2.28 ± 0.16	-	-	24 ± 2 (a)	6	G	Bruzzese <i>et al.</i> , 2010
	Leaves	2.12 ± 0.20	-	-	24 ± 2 (a)	6	O	Bruzzese <i>et al.</i> , 2010
	Leaves	1.56 ± 0.29	182 ± 21	35.6 ± 5.1	23 ± 1 (L)	5	O	Hájek & Adamec, 2010
	Leaves	2.1 ± 0.1	-	-	<i>ca.</i> 18	10	O	Méndez & Karlsson, 1999
<i>Pinguicula vulgaris</i> ^a	Leaves	2.3 ± 0.2^b	-	-	<i>ca.</i> 18	30	O	Méndez & Karlsson, 1999
	Leaves	2.0 ± 0.1^c	-	-	<i>ca.</i> 18	8	O	Méndez & Karlsson, 1999

^a Data for photosynthetic characteristics of *Pinguicula grandiflora* are unavailable in the literature. Data for *P. vulgaris* are presented as a substitute for *P. grandiflora*; both species are homophyllous and temperate, with similar circumboreal distributions.

^b Plants collected from sub-alpine heathland at Abisko, Sweden (68° 21' N, 18° 49' E).

^c Plants collected from a poor fen at Katerjåkk, Sweden (68° 27' N, 18° 10' E).

The results of this study show that influence of total chlorophyll content per unit area and carotenoid content per unit area on leaf redness failed to reach statistical significance, indicating that anthocyanin alone is responsible for leaf redness. This relationship is not always the case with non-carnivorous vascular plant species; results of a study using *Quintinia serrata*, a New Zealand tree

species possessing leaves that are polymorphic in terms of anthocyanin content and distribution, showed that whilst anthocyanin concentration, and not location, was the primary factor influencing leaf optical properties, a significant negative correlation was found between the reflectance of red wavelengths and chlorophyll content ($r = -0.64$, $P < 0.001$) (Neill and Gould, 1999).

The significant increase in the redness of unshaded leaves compared to shaded leaves of *D. muscipula* and *D. rotundifolia* indicates that leaf anthocyanin induction occurs as an ecophysiological response to high PAR availability. Whilst there is a deficit of previous research exploring the influence of PAR availability on leaf anthocyanin accumulation using carnivorous plants, similar results are reported for many non-carnivorous vascular plant species (Paiva *et al.*, 2003; Hughes *et al.*, 2005; Lightbourn *et al.*, 2007; Albert *et al.*, 2009). For *Galax urceolata*, an evergreen herb possessing leaves showing a transient colour variation from green to red as a response to high winter light availability, found the anthocyanin content of high-light leaves to increase linearly with light intensity (Hughes *et al.*, 2005).

Pinguicula grandiflora, however, contained very low anthocyanin content per unit area compared to the other plant species, and anthocyanin content was uninfluenced by light availability. This result may be partly explained by the differences in physiology and environmental requirements between *P. grandiflora* and the remaining carnivorous plant species used in this study. The widely distributed but geographically disjunct populations of *P. grandiflora* reflect its relatively narrow micro-habitat niche; the species requires a constant, or near constant, high level of air humidity and shows intolerance to large diurnal fluctuations in temperature (Steiger, 1998). The leaves are relatively large and thin compared to the other study species, with the adaxial surfaces covered in holes, called secretory gaps, through which mucilage is excreted (Legendre, 2000). These physiological characteristics are highly vulnerable to desiccation-induced structural damage as a result of fluctuating air temperatures; whilst the species can thrive in full sun, it prefers light shade (Legendre, 2000). Further support for the shade tolerant nature of *P. grandiflora* was found in the results of this study; no significant difference in plant stress, as measured by the efficiency of photosystem II (F_v/F_m), was found between light treatments. Thus, it may be predicted that during the evolutionary history of *P. grandiflora*, low light availability drove selective pressure to favour the survival of individuals that showed the adaptive trait of relatively lower investment in the energetically costly process of anthocyanin synthesis that was required to a lesser extent under shaded conditions compared to high light availability (Darwin, 1859), leading to the evolution of the current, relatively low leaf anthocyanin content levels that are unresponsive to light availability.

The main effect of light on leaf anthocyanin content per unit area reached statistical significance. However, leaves of unshaded *D. muscipula* and *D. rotundifolia* plants were significantly redder than

the leaves of shaded plants in September only, towards the end of the active growth season and after the unplanned heat / herbivory stress event. Several options offer a potential explanation for this result; (i) the difference in light intensity between light treatments may have only been great enough to induce significantly different leaf anthocyanin contents during September; (ii) *D. muscipula* and *D. rotundifolia* may exhibit a substantial time lag in the accumulation of leaf anthocyanin in response to light; (iii) anthocyanin accumulation occurred as a physiological response to multiple abiotic and biotic stress factors as a result of the heat / herbivory stress event in late July.

Options (i) and (ii) are less probably explanations for the above result; re. option (i), no substantial change in the difference between mean light intensities available to shaded and unshaded plants during August (prior to September measurement) was observed compared with the difference between light treatments during earlier months (Fig. 33). Regarding option (ii), scant data of the response time of anthocyanin production using carnivorous plants currently exists at a finer time scale other than accumulation observed after the following experimental treatment periods:- *Dionaea muscipula* and *Drosera spatulata*, 4 months (Ichiishi *et al.*, 1999), *Pinguicula vulgaris*, ca. 2 months (Méndez *et al.*, 1999). However, results of studies using non-carnivorous vascular plants capable of synthesising leaf anthocyanin indicate that photocontrol of the anthocyanin biosynthetic pathway induces production within the first few hours following exposure to high light intensities (Duke *et al.*, 1976; Taylor and Briggs, 1990). Thus, it may be predicted that anthocyanin production in the leaves of carnivorous plants is unlikely to be delayed until the near the end of the active growth season.

Regarding option (iii), vascular plants have been shown to respond interactively to multiple combined or successive environmental stresses than to individual stresses alone, often either amplifying or ameliorating the response to subsequent stresses following an initial stress event (Niinemets, 2010; Atkinson and Urwin, 2012). In addition, leaf anthocyanin accumulation has been shown to be a versatile stress response to many abiotic and biotic factors other than light, including intense heat and herbivory (Close and Beadle, 2003, and refs. therein; Gould, 2004, and refs. therein). Thus, it is plausible that rapid anthocyanin accumulation during August, prior to the September plant measurement session, represented a multiple stress response to high light availability, aphid herbivory and heat stress.

The occurrence of the unplanned heat /herbivory stress event prior to the September plant measurement session leaves ambiguity as to whether anthocyanin accumulation occurred as a direct response to light availability for two reasons:- (i) results of plant stress measurement, conducted after the stress event but prior to the September plant measurement session, show that the influence of light on leaf photochemical efficiency (F_v/F_m) values failed to reach statistical

significance; however unshaded leaves were less stressed than shaded leaves; (ii) it is unknown as to whether the timing of the rapid accumulation of anthocyanin in the leaves of unshaded plants between August and September plant measurement sessions occurred prior to or after plant stress measurement.

If anthocyanin accumulation occurred prior to plant stress measurement, this suggests that anthocyanin accumulation in the leaves of unshaded plants caused a greater reduction in stress relative to that of the leaves of shaded plants. This is perhaps an unlikely explanation based on the literature exploring the response of anthocyanin accumulation to light in carnivorous plants:- the results of a study exploring the influence of UV-B radiation and light availability on photoinhibition of *Pinguicula vulgaris* found that whilst UV-B-treated, anthocyanin-rich leaves experienced lower level of stress compared to control plants under high light availability, anthocyanin-rich and control plants exposed to low light availability were less stressed than anthocyanin-rich and control plants exposed to high light availability (Méndez *et al.*, 1999).

If anthocyanin accumulation occurred after plant stress measurement, this suggests that the heat / herbivory stress event exerted a greater relative level of stress on shaded plants compared with unshaded plants, and that anthocyanin production is not induced by herbivory. As heat stress is likely to have been substantially higher to unshaded plants, increased stress experienced by shaded plants is likely to have occurred as a result of differences in aphid herbivory feeding pressure between light treatments. Temperature is widely acknowledged to be the primary abiotic factor influencing the life stage development, fecundity and mortality of aphid *spp.*; species-specific high temperature thresholds and exposure period lengths are linked with increased mortality, developmental delay, and decreased fecundity (Taylor, 1981; Morgan *et al.*, 2001; Özder and Sağlam, 2013). Thus it is plausible that the aphid population showed a feeding preference for plants growing in shaded environments, causing these plants to exhibit reduced levels of photo efficiency compared with plants growing in unshaded environments.

The main effect of N and all interaction effects incorporating N on leaf redness failed to reach statistical significance, indicating that anthocyanin does not accumulate as an ecophysiological response to root N deficiency in *D. muscipula*, *D. rotundifolia* and *P. grandiflora*. Thus, this result does not provide support for the N deficiency hypothesis. This result contrasts with the results of Ichiishi *et al.*, 1999, which showed the absorption maxima of leaf redness of *D. muscipula* and *Drosera spathulata* to decrease in response to increasing root N availability in the form of NH_4NO_3 and KNO_3 . However, the rigour of the results of this study are limited; plants were grown on ½ MS agar medium in air-tight tanks exposed to a continuous light intensity of 3500 lux and thus replication of the plants' growth environment under ambient, *in-situ* conditions was not achieved.

5.5 Conclusions

The carnivorous plant species selected for this study vary in terms of overall fitness, ecological requirements, and tolerance to abiotic and biotic stress; *Pinguicula grandiflora* exhibited highest plant fitness, thrived under shaded and unshaded environments, and showed the highest tolerance to stress; *D. muscipula* exhibited a mid-level of fitness and tolerance to stress, showing signs of N-limitation following the stress event, and *D. rotundifolia* exhibited lowest fitness and required an unshaded environment in order to thrive. Results show all species to be phenotypically plastic in terms of investment in carnivory in response to N and light availability, providing evidence in ecological time to support Givnish's energetic cost/benefit model for the evolution of botanical carnivory. The nature of the ecophysiological response of investment in carnivory of *D. muscipula* to N and light availability varied from the responses of the remaining study species – the interaction effect of N x light significantly influenced the trap to petiole ratio of *D. muscipula*. This result provides evidence in ecological time to suggest that the evolutionary diversification of leaf structure from a single photosynthetic and prey capture leaf to separate trap and petiole from a *Drosera*-like ancestor of the Caryophyllales (Heubl *et al.*, 2006) is responsible for different resource allocation strategy between carnivory and photosynthesis compared with that of *D. rotundifolia* and *P. grandiflora*. However, ambiguity of the results caused by the influence of an unplanned plant stress event warrants further investigation to determine whether this result is a direct response to light alone, or an indirect, interactive stress response of light with additional abiotic and biotic variables.

Results of investigation into the influence of root N and light availability on the leaf anthocyanin content of the study plant species has indicated that anthocyanin accumulates in response to light availability in *D. muscipula* and *D. rotundifolia*, whereas the leaf anthocyanin content of *P. grandiflora* was very low and unresponsive to light. Thus, the photoprotection hypothesis is supported by the results for *D. muscipula* and *D. rotundifolia*. These results offer potential insight into the variation in historical environmental resources driving the evolution of leaf functional traits; low levels of anthocyanin of *P. grandiflora* (and many other *Pinguicula* species) suggest that potentially damaging high light intensity was not a positive selective pressure acting on the Lamiales, but that high light availability may have driven the selection of plants showing an adaptive response in terms of anthocyanin accumulation during evolution of the Caryophyllales.

The results of this study do not support the alternative theory for the functional role of anthocyanins of the N deficiency hypothesis; the influence of root N availability on leaf redness failed to reach statistical significance. This result suggests that leaf colour is unlikely to be synthesised primarily for the role of prey attraction, but further research is required to determine whether leaf redness as a

result of high light availability may indirectly influence the capture rate and species composition of captured prey.

5.7 References

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Chapter 6: Exploring the influence of ambient temperature and prey availability on root N uptake by *Drosera rotundifolia*

6.1 Abstract

The trait of botanical carnivory is widely considered to be of net benefit to carnivorous plants through increased growth and reproductive investment, and thin evidence for increased rates of photosynthesis. It is also indicated to be of benefit to the plant through the stimulation of root N uptake by prey capture; however the influence of ambient warming on prey N and root N uptake by carnivorous plants is not reported in the literature.

In this study, the influence of manipulated increase in ambient temperature of *ca.* 2 °C and prey availability on prey N and root N uptake by the carnivorous plant *Drosera rotundifolia* were investigated *in-situ* at an estuarine lowland ombrotrophic bog in the UK. A ¹⁵N tracer solution ([¹⁵N]nitrate as NH₄¹⁵NO₃, 98% APE) was applied to the vegetation surrounding *D. rotundifolia* plants at the start of the experiment in order to use tissue δ¹⁵N content as a quantitative measure of root N uptake by *D. rotundifolia*. Prey availability was manipulated by the application of mass-standardised *Drosophila melanogaster* flies to the plants. The uptake efficiency of prey N by *D. rotundifolia* plants from applied insects was estimated as the percentage difference in N content between experimental (partially digested) and reference flies.

Root N uptake by *Drosera rotundifolia* plants was uninfluenced by prey addition and vegetation warming treatment. *Drosera rotundifolia* plants displayed a mean prey N uptake efficiency of 49% at one day following prey application, increasing to 70% at four days. Prey N uptake efficiency was uninfluenced by prey retention time and vegetation warming treatment.

The statistical significance of results presented in this study are likely to be limited by small sample sizes (n = 3 plant replicates per experimental treatment). Despite statistically insignificant effects of prey availability and warming treatment on prey N and root N uptake, marginal differences in predicted directions were found, warranting the need for further investigation using larger sample sizes.

6.2 Introduction

Predicted rises in global mean surface temperatures of *ca.* 0.2 °C per decade until 2100 as a result of increasing greenhouse gas concentrations (IPCC, 2013) are expected to exert substantial and complex influences on the functioning and health of terrestrial ecosystems, particularly in northern latitudes (Melilo *et al.*, 1993; Shaver *et al.*, 2000). At the plant community level, substantial evidence in the literature indicates that temperature increases in the range of 0.3 – 6.0 °C generally stimulate plant productivity and therefore vegetation biomass, leading to changes in community structure and dynamics across a wide range of ecosystem types (Rustad *et al.*, 2001; Gornish and Tylianakis, 2013). At the plant physiological level, increases in ambient temperature at northern latitudes are linked to increased rates of photosynthesis and therefore increased growth (Hughes, 2000) and higher tissue C: N ratios (Day *et al.*, 2008), and increased winter respiration rates (Myneni *et al.*, 1997). Warming also directly influences plant phenological events, e.g. by extension of the active growth season (Myneni *et al.*, 1997) and advancement of the onset of flowering (Fitter and Fitter, 2002). Despite the wealth of studies exploring the influence of increased ambient temperature at the ecosystem and plant community level, there is a deficit of research investigating the influence of warming on root N uptake by plant species. Understanding the influences of temperature on root N uptake is essential due to the underpinning control of root N uptake on C and N processes at the plant, community and ecosystem level (Bassirrad, 2000).

Carnivorous plants endemic to the northern hemisphere are ideal model systems for exploring the influence of warming on N acquisition due to their predominant geographical restriction to relatively cold and nutrient-limited peatlands and due to their heterotrophic nature; N uptake is possible via the roots and from insect prey (Darwin, 1875). As with most angiosperms, root N uptake in carnivorous plants increases as root N availability increases, e.g. *Drosera rotundifolia* plants growing in the site receiving a relatively high N deposition load of 11.3 kg N ha⁻¹ yr⁻¹ obtained 67% of the plant N budget via the roots, whereas plants growing in the site receiving a relatively low N deposition load of 1.9 kg N ha⁻¹ yr⁻¹ obtained 43% of plant N via the roots (Millett *et al.*, 2012). In addition, interactions are reported to occur between prey N and root N uptake in carnivorous plants (Pate and Dixon, 1978; Aldenius *et al.*, 1983; Karlsson and Carlsson, 1984; Hanslin and Karlsson, 1996; Adamec, 2002). For example, the results of a study by Adamec (2002) show that prey N uptake by *Drosera capillaris*, *D. aliciae* and *D. spathulata* stimulates the increased uptake of root N by an average of 63% compared with control plants under greenhouse conditions. For *Drosera rotundifolia*, root N uptake is more than doubled for *in-situ* plants fed supplemental prey (1-2 *Drosophila* flies per plant per week over an experimental period of 4-6 weeks) compared with control plants with access to background insects only (Hanslin and Karlsson, 1996). These results

provide evidence of another positive benefit of the trait of carnivory to plants in addition to those of increased growth, increased investment in reproduction, and weak evidence for increased rates of photosynthesis (Ellison and Gotelli, 2009), thus providing evidence to support Givnish's cost/benefit model for the evolution of botanical carnivory (Givnish *et al.*, 1984).

The influence of warming on interactions between prey and root N uptake by carnivorous plants is not yet reported in the literature. Tentative, indirect evidence suggests that increased ambient temperatures may increase prey N uptake efficiency:- results of a study (Hanslin and Karlsson, 1996) exploring the influence of prey capture level on root N and prey N uptake by four carnivorous plant species growing *in-situ* or in the greenhouse found that greenhouse plants showed prey N uptake efficiencies of 40-50% compared with *in-situ* plants (30-40%), however the authors acknowledge that the potentially confounding factor of the absence of rainfall to greenhouse plants (some prey may have been washed off the leaves of *in-situ* plants) cannot be discounted. The results of a recent biochemical study support this tentative evidence:- the production of proteinases used for prey digestion by *Drosera capensis* increased as ambient temperature was increased (Takahashi *et al.*, 2009). As a consequence, it may be predicted that the magnitude of root N uptake following prey addition would be larger for warmed plants compared with control plants.

This study utilises *in-situ* *Drosera rotundifolia* plants growing in experimental and control open-top chambers situated on an estuarine lowland ombrotrophic bog in the UK to investigate the influence of a manipulated increase in ambient temperature of *ca.* 2 °C on parameters of prey N and root N uptake. *Drosera rotundifolia* is selected as the model carnivorous plant species due to its geographical limitation to nutrient-limited peatlands in northern latitudes (Crowder *et al.*, 1990). Specifically, the following aims are addressed:

1. To test the hypothesis that *Drosera rotundifolia* plants growing in warmed plots display increased magnitudes of prey N uptake efficiencies than plants growing in unwarmed plots.
2. To test the hypothesis that *Drosera rotundifolia* plants growing in warmed plots display increased magnitudes of root N uptake efficiencies than plants growing in unwarmed plots.
3. To test the hypothesis that prey addition to *Drosera rotundifolia* plants stimulates root N uptake.
4. To test the hypothesis that *Drosera rotundifolia* plants growing in warmed plots are larger in size than plants growing in unwarmed plots.
5. To test the hypothesis that *Drosera rotundifolia* plants growing in warmed plots invest more heavily in reproduction than plants growing in unwarmed plots.

6. To explore the influence of ambient warming treatment and the time period that flies were retained on *Drosera rotundifolia* plants on prey digestion.

To achieve Aims 1, 3 and 6, mass-standardised fruit flies were added to *D. rotundifolia* plants *in-situ* and removed in 24 hour increments after 1-4 days following the start of the experiment; control plants did not receive prey. The efficiency of prey N uptake by *D. rotundifolia* plants was calculated as the percentage difference in N content between digested flies and reference flies. To measure the influence of warming and prey addition on root N uptake efficiency of *D. rotundifolia* (Aims 2 and 3), the $\delta^{15}\text{N}$ of *D. rotundifolia* plants was determined using $\delta^{15}\text{N}$ stable isotope analysis following the application of ^{15}N -labelled NH_4NO_3 tracer solution to *Sphagnum* surrounding each plant. The $\delta^{15}\text{N}$ and C:N ratio of *D. rotundifolia* plants was compared with those of surrounding *Sphagnum* to provide context. To test the influence of ambient warming treatment on plant size (Aim 4), plant dry mass was measured at the end of the experiment. The influence of warming treatment on reproductive investment by *D. rotundifolia* plants (Aim 5) was explored by measuring the number of inflorescences for experimental and control plants throughout the course of the experiment. To achieve Aim 6, the C:N ratio and dry mass of flies were compared between (a) experimental flies and reference flies that were not applied to the plants, and (b) experimental treatment type (warming status / prey retention time for flies applied to *D. rotundifolia* plants).

The results of this study therefore offer an insight into how carnivorous plants may respond ecophysiologicaly, particularly in terms of prey N and root N uptake, to projected temperature increases as a result of climate change.

6.3 Methods

The fieldwork element of this study was conducted during July 2011 at Cors Fochno, an estuarine lowland raised bog situated in Ceredigion, Mid Wales (Table 33). This research study was completed in collaboration with the PEATBOG project, a European Research Association BiodivERsa programme funded research project investigating the impact of nitrogen pollution and climate change on the health of peatlands (see <http://www.sste.mmu.ac.uk/peatbog/> for further details).

Table 33 Location, climatic and environmental characteristics of Cors Fochno ombrotrophic raised bog, UK. Table (a) summarises site characteristics for 2006 to 2011 (where possible), this time period representing the maximum potential life span of survey *Drosera rotundifolia* plants; Table (b) summarises site characteristics for 2011 during which fieldwork was undertaken.

(a)

Location	Mean annual precipitation (mm yr ⁻¹) ^a	Mean temperature January / July (°C) ^a	Mean growing season length (d) ^b	Growing season average temperature (°C) ^b	N deposition (kg N ha ⁻¹ yr ⁻¹) ^c
52°30'09N, 04°00'57W	1381	3.5/14.7	320	11.6	8.0

^a Based on observed meteorological data from KNMI Climate Explorer (<http://climexp.knmi.nl>) (accessed 08.09.2014). Data are mean values for 2006 - 2011 inclusive.

^b Growing season is the number of days with mean temperature $\geq 5^{\circ}\text{C}$. Data are mean values for 2011 – 2012 inclusive (earlier years unavailable). Based on observed meteorological data from automatic weather stations at the site; data accessed from Countryside Council for Wales.

^c Modelled N deposition data from APIS (<http://www.apis.ac.uk/>) (accessed 21.04.2014). Data are mean values for 2010-2012 inclusive (earlier years unavailable).

(b)

Annual precipitation (mm yr ⁻¹) ^a	Temperature January / July (°C) ^a	Growing season length (d) ^{a,b}	Growing season average temperature (°C) ^{a,b}
1008	3.7/15.0	324	12.1

^a Data values for 2011 only. Based on observed meteorological data from automatic weather stations at the site; data accessed from Countryside Council for Wales.

^b Growing season is the number of days with mean temperature $\geq 5^{\circ}\text{C}$.

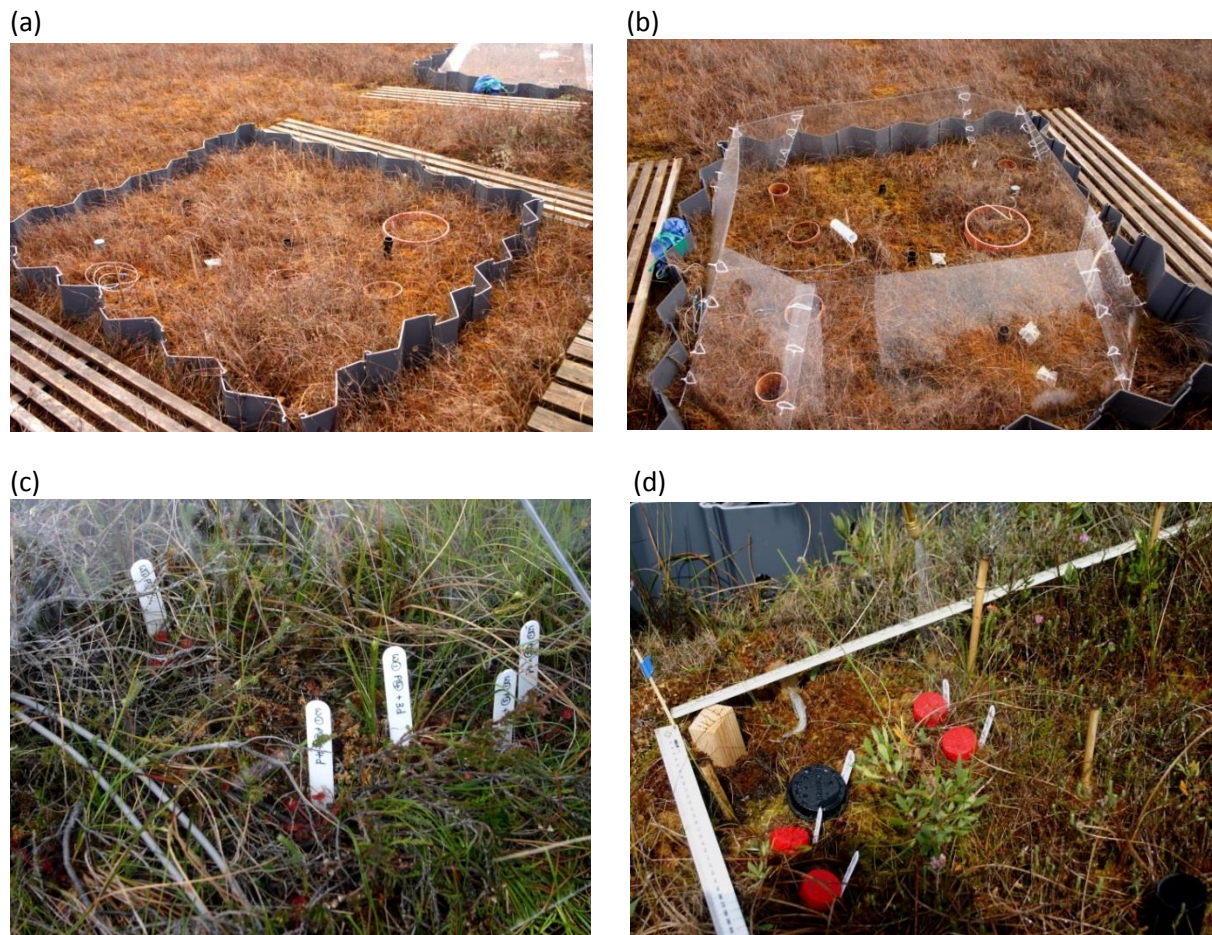
6.3.1 Preparation of mass-standardised insect prey

The Dipteran species *Drosophila melanogaster* (average length 2-3 mm) was used as the representative prey species of *Drosera rotundifolia*, as (i) adult Diptera have been found to constitute 74.4% of the taxonomic prey composition of *D. rotundifolia* used by a previous study (Crowder *et al.*, 1990); (ii) the size class of this species falls within the ≤ 4.4 mm size range of 87.1% of prey caught by *D. rotundifolia* used by a previous study (Crowder *et al.*, 1990). One hundred standardised flies were selected from a population raised to adulthood on complete nutrient medium and euthanized by freezing shortly before usage (Hanslin & Karlsson, 1996). The fresh mass of each fly was measured, and flies individually stored in labelled sterile vials. Flies were stored in the freezer (*ca.* -18 °C) to minimise tissue decomposition during the short time frame between euthanasia and the commencement of the fieldwork. Thirty of the 100 standardised flies were reserved for the determination of the mean $\delta^{15}\text{N}$, $\delta^{13}\text{C}$, N and C content per fly.

6.3.2 Fieldwork and sample collection

The fieldwork element of this study utilised experimental plots of the PEATBOG Work Package 2 research study (BiodivERsa European Research Association funded collaborative research project, http://www.sste.mmu.ac.uk/peatbog/work_packages/field_manipulations/) at Cors Fochno (Plates 5(a), 5(b)). This study utilised the passive warming chambers and control plots (three replicates of each), all constructed by the PEATBOG team in randomised locations in spring 2009. All plots contained natural abundances of *D. rotundifolia*. The passive warming chambers raised temperature above ambient by $\sim 2^\circ\text{C}$, and consist of hexagonal open-top chambers (OTCs) (0.5 m height x 2 m length x 2 m width) constructed from transparent polycarbonate (Plates 5(a), 5(b)). Within each plot, five *D. rotundifolia* plants were randomly selected from those (i) with an identical number / order of previously captured insect prey (no prey if possible); (ii) within a 1 m² area of vegetation (for purposes of ¹⁵N tracer application methodology employed by the PEATBOG team), and (iii) within 30 cm of the OTC perimeter, to facilitate fine-scale measurement (Plate 5(d)). The location and individual I.D. of each selected plant was marked with a small plastic label adjacent to each plant (Plate 5(c)).

Plate 5 Experimental set-up of passive warming and control chambers at Cors Fochno, and the application of ^{15}N -labelled solution to the vegetation by the PEATBOG team. Presented are:- (a) control (unwarmed) chamber; (b) passive warming chamber; (c) labelled *Drosera rotundifolia* plants located in one of the warmed chamber replicates; (d) spray application of ^{15}N tracer solution to 1m^2 quadrat of vegetation in each chamber, showing the use of plastic lids over the survey *D. rotundifolia* plants to prevent leaf ^{15}N absorption during application.



The following morphological measurements were taken prior to $\delta^{15}\text{N}$ tracer application:- rosette diameter (average of two perpendicular cross-section measurements) (mm), number of leaves (n), leaf length (mm), leaf width (mm), leaf area (using leaf length and width measurements) (mm^2) and number of inflorescences. If selected *D. rotundifolia* plants possessed previously captured prey, prey size class (length in mm) and prey order were recorded for each plant. Prey previously captured by the survey *D. rotundifolia* plants were removed and labelled for identification in the laboratory.

The ^{15}N tracer solution ($[^{15}\text{N}]$ nitrate as $\text{NH}_4^{15}\text{NO}_3$ (product number 366536, Sigma-Aldrich Company Ltd., Gillingham, Dorset, UK; 98% APE) was applied to a 1m^2 quadrat of vegetation within each

chamber (where the experimental *D. rotundifolia* plants were situated) by the PEATBOG team and the date and time of application recorded (Plate 5(c)) (Aims 2 and 3). The ^{15}N tracer solution was applied to the quadrats prior to prey addition to *D. rotundifolia* plants to prevent the flies being washed off the leaves. Small plastic lids were placed over each survey *D. rotundifolia* plant to prevent the direct absorption of the $\delta^{15}\text{N}$ tracer solution through the leaves (Plate 5(d)), and removed immediately afterwards. Within each survey quadrat, the following treatments to the *D. rotundifolia* plants were immediately applied following ^{15}N addition (Aims 1, 3 and 6):-

- Control (no prey addition, plant collection after 4 days).
- Prey addition, plant removal after 1 day.
- Prey addition, plant removal after 2 days.
- Prey addition, plant removal after 3 days.
- Prey addition, plant removal after 4 days.

For *D. rotundifolia* plants receiving prey treatment, one *D. melanogaster* fly was placed onto a healthy leaf of each plant, selecting newly developed leaves of approximately equal leaf area between plants. The corresponding fresh mass of the fly applied to each *D. rotundifolia* plant was noted (as previously recorded). The selected prey addition time frames prior to plant collection reflect findings from previous literature that suggest (i) *D. rotundifolia* leaves that have captured insect prey then open up and release the chitinous insect remains at one to two weeks following prey capture (Crowder *et al.*, 1990); (ii) the digestive enzymes acid phosphatase and chitinase are secreted by *D. rotundifolia* leaves between 24 - 96 h and 5 – 72 h respectively following prey capture (McNally *et al.*, 1988; Matušíková *et al.*, 2005).

Survey *D. rotundifolia* plants were removed after the appropriate time frame had elapsed, and the date/time of plant collection recorded. Approximately 5 cm³ of *Sphagnum* surrounding each survey *D. rotundifolia* plant was removed, and *Sphagnum* samples pooled per quadrat following the collection of the final survey plant. Plant material was stored in sealable plastic bags prior to the measurement of fresh and dry mass in the laboratory. Each *D. melanogaster* fly was removed from each survey plant immediately following plant collection, and placed into a labelled, sterile vial. In the laboratory, the fresh mass of each fly was recorded. Flies were dried in a forced air oven at 70°C for 12 hours, or until no change in mass was evident, and the dry mass recorded for each specimen (Aims 1 and 6).

6.3.3 Enriched ^{15}N isotopic determination

Drosera rotundifolia and insect samples were ground to a fine powder using a pestle and mortar, and *Sphagnum pulchrum* samples using a ball mill (Retsch MM200, Retsch, Haan, Germany) to ensure sample homogeneity (Baker & Thompson, 1992). Samples of individual standardised reference *D. melanogaster* flies and of partially digested flies were prepared for $\delta^{15}\text{N}$ stable isotope analysis. Where the dry mass of individual flies did not meet the minimum sample mass requirement for stable isotope analysis, flies were pooled (by identical experimental treatment where applicable).

Plant and invertebrate material were pre-weighed into tin capsules and analysed for $\delta^{15}\text{N}$ at the NERC Life Science Mass Spectrometry Facility, East Kilbride, Scotland (Aim 3). Nitrogen isotopes were analysed by continuous flow using a Thermo Scientific DELTA V Plus isotope ratio mass spectrometer (Thermo Scientific, Bremen, Germany) interfaced with a Costech ECS 4010 elemental analyser (Costech Instruments, Milan, Italy). Two in-house standards were run every ten samples for quality assurance. All data are reported with respect to the international standard of atmospheric N_2 for $\delta^{15}\text{N}$. Results were reported in δ notation as the deviation from standards in parts per thousand (‰), where $\delta X = [(R_{\text{sample}} - R_{\text{reference}}) / R_{\text{reference}}] \times 1000$, and $R = ^{15}\text{N} : ^{14}\text{N}$, and $X = ^{15}\text{N}$. Precision was 0.2 ‰ for $\delta^{15}\text{N}$. Total N and C contents of plant and invertebrate material were also obtained during the $\delta^{15}\text{N}$ analysis (Aim 2, 3 and 6).

6.3.4 Data analyses

The uptake efficiency of prey N (absorbed N_{prey}) from standardised flies applied to *Drosera rotundifolia* plants was calculated as the percentage difference in N content between experimental (digested) and reference flies (Dixon *et al.*, 1980; Adamec, 2002) (Aim 1). Hypotheses relating to prey N uptake efficiency (Aim 1) and root N uptake efficiency (Aims 2 and 3) were tested using ANOVA. Other hypotheses concerning plant size (Aim 4), reproductive investment (Aim 5) and parameters of prey digestion (Aim 6) were tested using ANOVA or independent-sample *t*-tests. Post-hoc comparisons were conducted using Fisher's Least Significant Difference (LSD) ($P < 0.05$ significance level). Data were \log_{10} -transformed prior to analysis where data did not conform to the assumptions of homoscedascity. All statistical analyses were conducted using SPSS Statistics version 21 (IBM, Chicago, USA).

6.4 Results

Table 34 Results of 2-way (warming status of survey quadrats, time) univariate ANOVAs for parameters of N of *Drosera rotundifolia* and co-occurring *Sphagnum pulchrum*. Warming statuses of survey quadrats: unwarmed (control), warmed; time = prey retention time (0-4 days) or time period following ^{15}N tracer application (1-4 days). Presented are degrees of freedom (*df*), *F* and *P* values for the percentage uptake efficiency of prey N by *D. rotundifolia* from mass-standardised flies (absorbed N_{prey}), $\delta^{15}\text{N}$ and the C : N ratio. Significant effects at $P < 0.05$ are highlighted in bold.

	<i>Drosera rotundifolia</i>									<i>Sphagnum pulchrum</i>					
	Absorbed N_{prey} (%)			$\delta^{15}\text{N}^1$			C : N ratio ¹			$\delta^{15}\text{N}$			C : N ratio		
	<i>df</i>	<i>F</i>	<i>P</i>	<i>df</i>	<i>F</i>	<i>P</i>	<i>df</i>	<i>F</i>	<i>P</i>	<i>df</i>	<i>F</i>	<i>P</i>	<i>df</i>	<i>F</i>	<i>P</i>
Warming status (WS)	1, 22	0.131	0.722	1, 27	0.7	0.428	1, 27	2.5	0.135	1, 23	<0.1	0.990	1, 23	<0.1	0.877
Time	3, 22	1.083	0.386	4, 27	0.9	0.487	4, 27	1.3	0.307	3, 23	0.6	0.648	3, 23	1.1	0.388
WS x time	3, 22	0.955	0.439	4, 27	0.4	0.813	4, 27	0.2	0.927	3, 23	0.1	0.955	3, 23	0.1	0.979

¹ Data were \log_{10} -transformed before analysis.

6.4.1 Prey N uptake by *Drosera rotundifolia*

The uptake efficiency of prey N by *D. rotundifolia* plants showed marginal incremental increases from 49.0 % to 69.8 % during the four days following prey addition, with the largest uptake occurring between days 1 and 2 during this time period (Fig. 50(a)). However, prey retention period failed to exert a statistically significant effect on the uptake efficiency of prey N (Table 34). Warmed *D. rotundifolia* plants displayed marginally lower prey N uptake efficiencies than unwarmed plants, however the main effect of vegetation warming treatment, and the interaction effect of vegetation warming treatment and prey retention period, on the uptake efficiency of prey N to reach statistical significance (Table 34; Fig. 50(b)) (Aim 1).

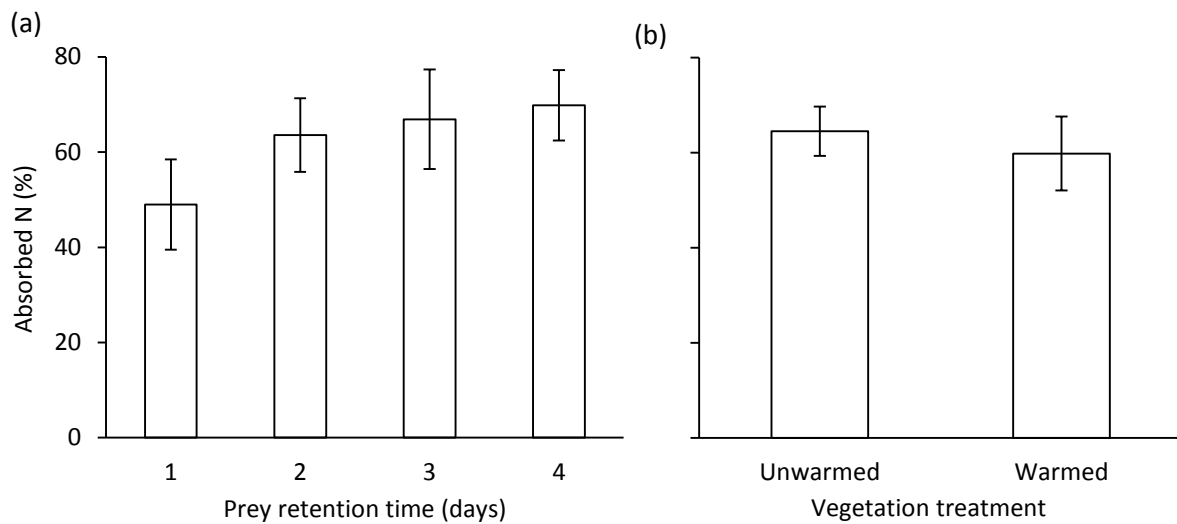


Figure 50 Influence of a *ca.* 2 °C ambient temperature increase and prey retention time period on the prey N uptake efficiency of *Drosera rotundifolia* plants growing in unwarmed and warmed plots. Presented are the influences of (a) prey retention time and (b) vegetation warming treatment on the mean \pm 1 S.E. of prey N uptake efficiency of *D. rotundifolia*. Bars with different letters in Fig. (a) are significantly different from each other (Fisher's least significant difference, $P < 0.05$).

6.4.2 Root N uptake by *Drosera rotundifolia*

Drosera rotundifolia plants subjected to warming treatment were marginally $\delta^{15}\text{N}$ -enriched (mean \pm 1 S.E. = 173.0 ± 3.6 ‰) compared with unwarmed plants (145.4 ± 31.3 ‰), however warming treatment type and the retention time of *Drosophila* prey on leaves exerted no statistically significant influence on plant $\delta^{15}\text{N}$ (Fig. 51(a); Table 34) (Aim 2). Similarly, warming treatment type and time period since ^{15}N tracer application exerted no statistically significant influence on the $\delta^{15}\text{N}$ of co-occurring *Sphagnum pulchrum* (Fig. 51(b); Table 34).

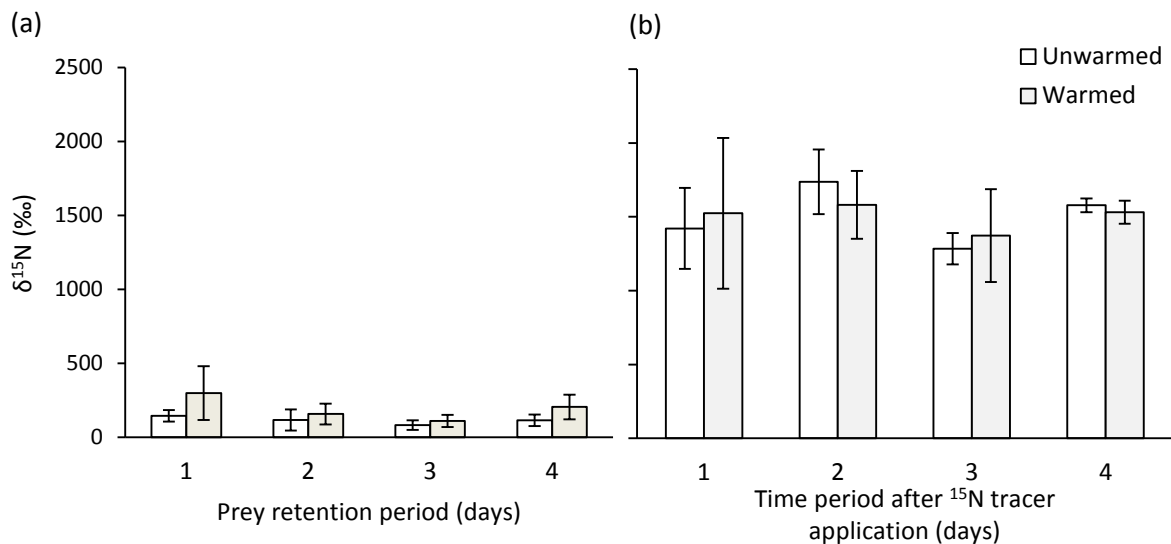


Figure 51 Influences of warming status and time on the $\delta^{15}\text{N}$ of co-occurring *Drosera rotundifolia* and *Sphagnum pulchrum* present in experimental quadrats of unwarmed and warmed chambers, to which ^{15}N tracer was applied. Presented are the mean ± 1 S.E. of $\delta^{15}\text{N}$ for (a) *Drosera rotundifolia*; (b) *Sphagnum pulchrum*. Warming statuses: warmed, unwarmed. Time: prey retention period on each *Drosera rotundifolia* plant (days) or time period after ^{15}N application (days). Bars with different letters are significantly different from each other (Fisher's least significant difference, $P < 0.05$).

6.4.3 Tissue C : N ratios of *Drosera rotundifolia* and *Sphagnum pulchrum*

ANOVA results show that warming treatment type and the retention time of *Drosophila* prey on leaves exerted no statistically significant influence on the C : N ratio of *D. rotundifolia* (Fig. 52(a); Table 34). Similarly, warming treatment type and time period since ^{15}N tracer application exerted no statistically significant influence on the C : N ratio of co-occurring *Sphagnum pulchrum* (Fig. 52(b); Table 34).

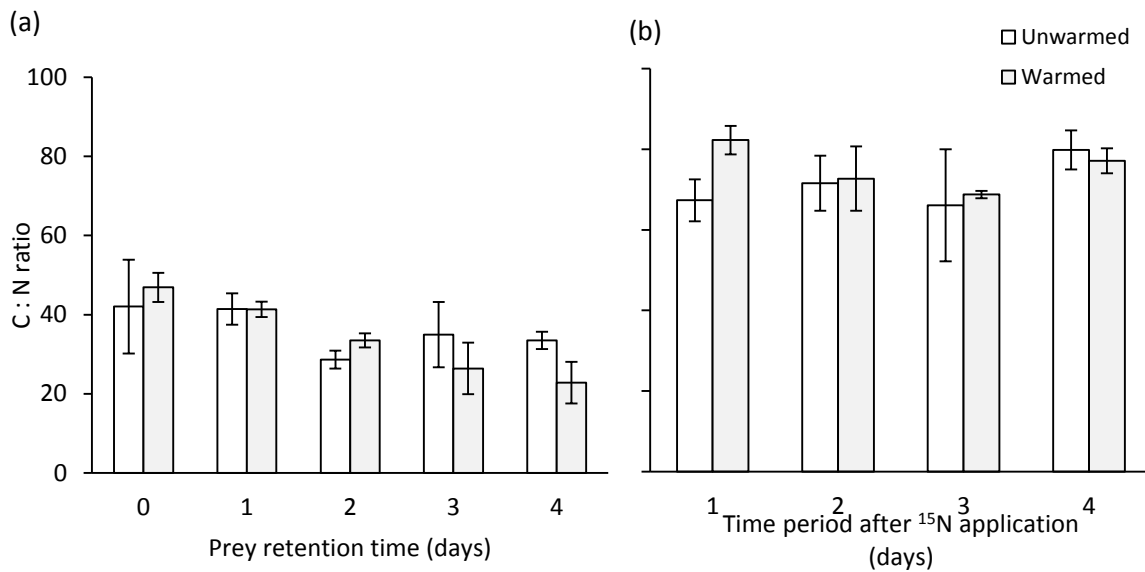


Figure 52 Influences of warming status and time parameters on the C : N ratio (by weight) of co-occurring *Drosera rotundifolia* and *Sphagnum pulchrum* present in experimental quadrats of unwarmed and warmed chambers, to which ¹⁵N tracer was applied. Presented are the mean ± 1 S.E. of the C : N ratio for (a) *Drosera rotundifolia*; (b) *Sphagnum pulchrum*. Warming statuses: warmed, unwarmed; time: prey retention time period on each *Drosera rotundifolia* plant (days) or time period after ¹⁵N application (days). Bars with different letters are significantly different from each other (Fisher's least significant difference, $P < 0.05$).

6.4.4 Plant size and reproductive investment

Table 35 Physiological characteristics of *Drosera rotundifolia* plants at two ombrotrophic bogs in the UK that vary by N deposition input. Presented are the mean \pm 1 S.E. for: total dry mass per plant (mg), mass; number of inflorescences per plant (n). Significant effects at $P < 0.05$ are highlighted in bold.

		Mass	Influences
Unwarmed	Mean	10.75	0.73
	SE	1.58	0.38
Warmed	Mean	15.44	1.13
	SE	2.95	0.46
Independent samples t-test results ¹		0.176	0.508

¹ Comparing differences between warming treatments.

Drosera rotundifolia plants that were subjected to warmed environment were marginally heavier and produced marginally more inflorescences than plants subjected to unwarmed environment (Fig.s 53(a), 53(b)), however the effect of warming treatment on plant dry mass (Aim 4) and the number of inflorescences (Aim 5) failed to reach statistical significance (Table 35).

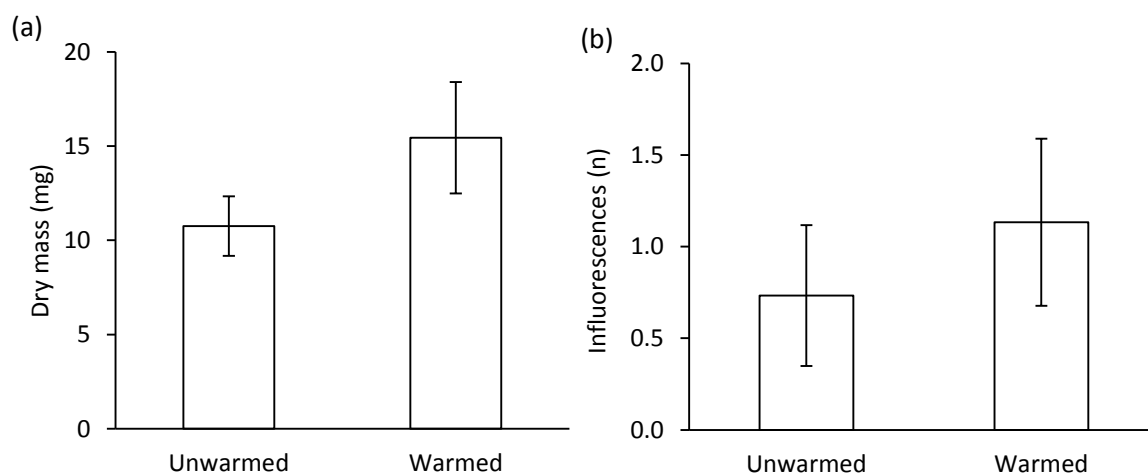


Figure 53 Influence of warming status on the dry mass and number of inflorescences of *Drosera rotundifolia* plants growing in experimental quadrats of unwarmed and warmed chambers, to which ¹⁵N tracer was applied. Presented are the mean \pm 1 S.E. for: (a) dry mass per plant; (b) number of inflorescences per plant. Vegetation warming status = warmed, unwarmed.

6.4.5 Prey digestion by *Drosera rotundifolia*

Table 36 Physiological characteristics of *Drosophila melanogaster* prey fed to *Drosera rotundifolia* plants growing in experimental plots at Cors Fochno that differ by ambient warming treatment. Presented are the following Tables: (a) mean \pm 1 S.E. for:- dry mass per fly (mg), mass; C : N ratio per milled *D. melanogaster* sample (pooled where necessary) for the independent samples *t*-test output for testing the influence of fly experimental status on the dry mass and C : N ratio of flies; (b) results of 2-way (vegetation warming status of survey quadrats, time) univariate ANOVA for the dry mass of *D. melanogaster* flies. Vegetation warming statuses of survey quadrats: unwarmed (control), warmed; time = prey retention time on *D. rotundifolia* plant: 1, 2, 3 or 4 days. Presented are degrees of freedom (*df*), *F* and *P* values for $\delta^{15}\text{N}$ and the C : N ratio; (c) mean \pm 1 S.E. for the C : N ratio per milled *D. melanogaster* sample (pooled where necessary) for the independent samples *t*-test output for testing the influence of vegetation warming status on the fly C : N ratio. Significant effects at *P* < 0.05 are highlighted in bold.

(a)

Experimental status		Mass	C : N ratio
Reference	Mean	0.374	3.65
	SE	0.023	0.03
Experimental	Mean	0.141	5.40
	SE	0.017	0.52
Independent samples <i>t</i> -test results ¹		<0.001	0.011

(b)

Effect	<i>df</i>	Mass	
		<i>F</i>	<i>P</i>
Warming status (WS)	1, 22	0.131	0.722
Time	3, 22	1.083	0.386
WS x time	3, 22	0.955	0.439

(c)

Warming status		C : N ratio
Unwarmed	Mean	4.94
	SE	0.18
Warmed	Mean	5.86
	SE	1.04
Independent samples <i>t</i> -test results ¹		0.414

¹ Comparing differences between vegetation warming treatments.

Experimental *Drosophila melanogaster* flies that were fed to *Drosera rotundifolia* plants growing in warmed and unwarmed plots were significantly lighter than reference flies (Fig. 54(a); Table 36(a)) (Aim 6). Fly dry mass decreased marginally as prey retention time on *D. rotundifolia* plants increased from one to four days (Fig. 54(b)). However, the main effects of fly retention time and warming treatment on fly dry mass failed to reach statistical significance (Table 36(b)) (Aim 6). Experimental flies contained significantly higher C : N ratios than reference flies (Fig. 54(c); Table 36(a)) (Aim 6). Of the experimental flies, those fed to plants growing in warmed plots contained marginally higher C : N ratios than those fed to plants growing in unwarmed plots, however the effect of warming treatment on fly C : N ratio failed to reach statistical significance (Fig. 54(d); Table 36(c)) (Aim 6).

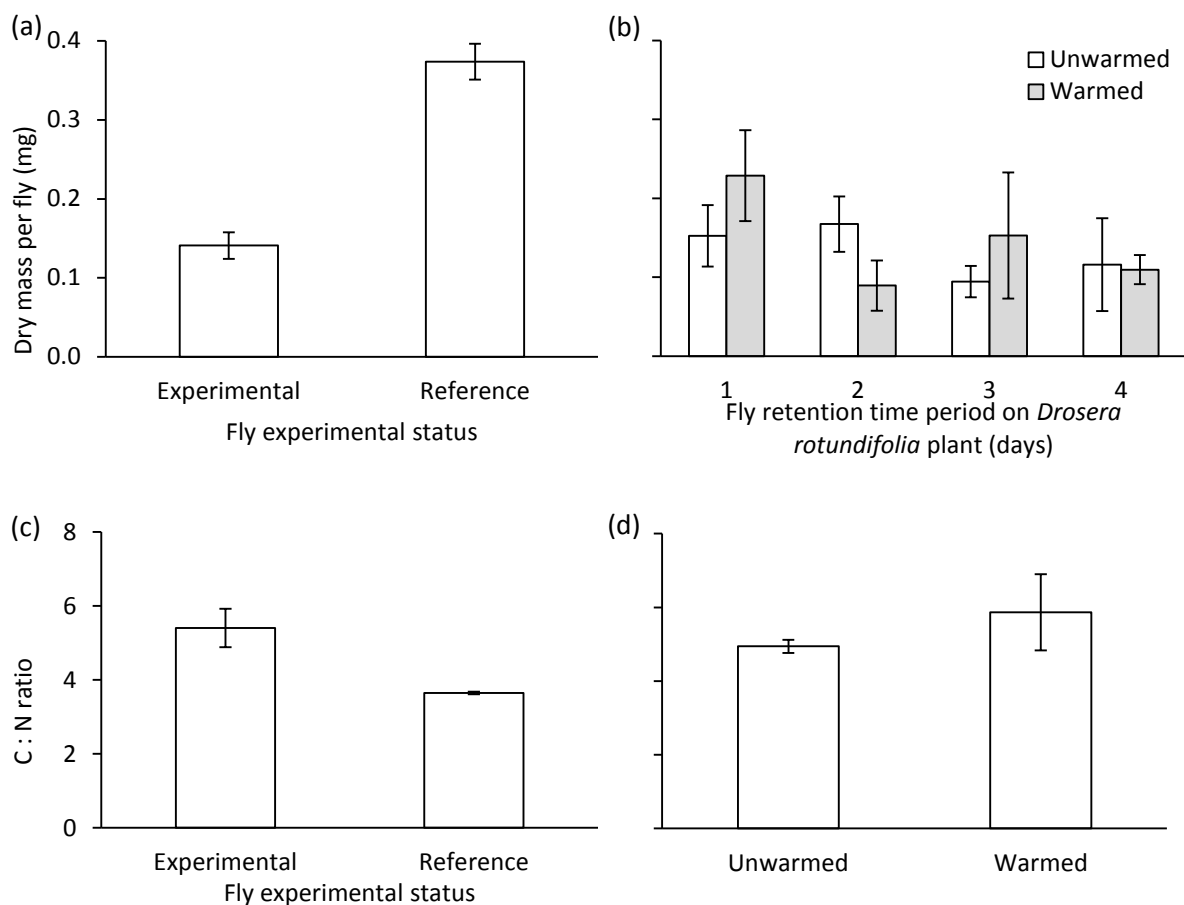


Figure 54 Influences of fly experimental status, vegetation warming status and fly retention time on physiological characteristics of *Drosophila melanogaster* flies fed to *Drosera rotundifolia* plants growing in warmed and unwarmed survey plots at Cors Fochno. Presented are the mean \pm 1 S.E. for: (a) dry mass per fly for experimental and reference flies; (b) dry mass per fly for 2 x 4 treatment of vegetation warming status (warmed, unwarmed) and prey retention period on *D. rotundifolia* plants (1 – 4 days); (c) C : N ratio of experimental and reference flies; (d) C : N ratio of flies fed to *D. rotundifolia* plants growing in warmed and unwarmed plots. Bars with different letters in Fig. (b) differ significantly from each other (Fisher's least significant difference, $P < 0.05$).

6.5 Discussion

The results of this study showing $\delta^{15}\text{N}$ of *Drosera rotundifolia* plants to be uninfluenced by prey retention time suggest that prey addition does not stimulate root N uptake. This is a somewhat surprising result; previous studies indicate that the experimental addition of prey or foliar nutrients stimulates root N uptake in *D. rotundifolia* and other carnivorous plant species (Pate and Dixon, 1978; Aldenius *et al.*, 1983; Karlsson and Carlsson, 1984; Hanslin and Karlsson, 1996; Adamec, 2002). Three possible explanations are proposed for this result. Firstly, perhaps the most plausible explanation, is that any possible patterns in the data may have been obscured by the limited number of replicates ($n = 3$ plants per treatment type), as well as substantial within-treatment variability in $\delta^{15}\text{N}$; *D. rotundifolia* plants in warmed plots were marginally more $\delta^{15}\text{N}$ -enriched than plants in unwarmed plots. Secondly, it may be possible that the application of a single *Drosophila melanogaster* fly (*ca.* 0.4 mg dry mass) to each plant was below the minimum prey mass threshold required to stimulate root N uptake in *Drosera rotundifolia*; results of a study by Adamec (2002) found the addition of two flies to each plant (*ca.* 0.6 mg in total) triggered significant increases in root N uptake by *D. rotundifolia* plants compared with plants with no prey added. Thus, these results combined suggest that the minimum threshold of prey dry mass required to stimulate root N uptake in *D. rotundifolia* lies within the range of 0.4 – 0.6 mg. Thirdly, it may be possible that an increase in root N uptake following prey addition was undetected if the surge occurred before the removal of the first plants at 24 h following prey addition; whilst the results of biochemical studies indicate that digestive enzyme activity secreted by the leaves peaks at 24 – 96 h following prey capture (McNally *et al.*, 1988; Matušíková *et al.*, 2005), acid phosphatase and chitinase are secreted as early as 5 h following prey capture (Matušíková *et al.*, 2005). Indeed, *D. rotundifolia* plants in this study absorbed 49% of prey N and were most $\delta^{15}\text{N}$ -enriched at 24 h following prey addition than at longer time periods, suggesting that root N uptake may have been stimulated within the first 24 hour period following prey addition. However, it should be noted that these plants were only marginally more $\delta^{15}\text{N}$ -enriched than control plants with no prey added at 24 h following prey addition, warranting the need for further investigation.

Vegetation warming treatment also exerted no statistically significant effect on *Drosera* $\delta^{15}\text{N}$, indicating that ambient temperature does not influence root N uptake in *D. rotundifolia*. As increased root N uptake following prey addition did not occur, or was undetected, this result is not surprising. However, *D. rotundifolia* plants in warmed plots were marginally more $\delta^{15}\text{N}$ -enriched than plants in unwarmed plots, which along with the limitations of replicate numbers as detailed above, highlights the need for further research. The results showing the C: N ratio of *D. rotundifolia* to be uninfluenced by vegetation warming treatment is surprising; the literature indicates that,

generally speaking across a wide range of N-limited ecosystems, manipulated increases in ambient temperature of 0.3 – 6.0 °C increase plant productivity, and therefore lead to higher tissue C: N ratios (Rustad *et al.*, 2001; Day *et al.*, 2008). For *Sphagnum pulchrum*, the $\delta^{15}\text{N}$ of mosses growing in warmed plots were not significantly different from the $\delta^{15}\text{N}$ of mosses growing in unwarmed plots, indicating that ambient temperature does not influence the amount of $\delta^{15}\text{N}$. This result is to be expected; *Sphagnum* walls are single-celled and thus absorb atmospheric- and precipitation-derived N directly (Van Breemen, 1995).

The C: N ratio of *S. pulchrum* also did not differ significantly between warming treatments, indicating that temperature does not influence the amount of N held in the capitula, however it remains unknown whether the C: N ratio of the senescent part of the moss (via internal transport of N (Van Breemen, 1995)) is affected. The substantial $\delta^{15}\text{N}$ enrichment of *S. pulchrum* compared with co-occurring *D. rotundifolia* plants indicates that root N uptake is very low in *D. rotundifolia* plants in comparison with the direct absorption of $\text{NH}_4^{15}\text{NO}_3$ solution by *S. pulchrum*, and / or that *S. pulchrum* only releases a small fraction of the $\text{NH}_4^{15}\text{NO}_3$ solution into the rhizosphere of vascular plants. Tentative support for these possible explanations exists in the literature:- the root system of *D. rotundifolia* is sparse and weak (Adlassnig *et al.*, 2005), carnivorous plants uptake relatively low amounts of root N in comparison with non-carnivorous vascular plants and bryophytes (*cf.* Thorén *et al.*, 2003), and the retention ability of precipitation-derived N by *Sphagnum spp.* exposed to low N deposition levels (e.g. Cors Fochno) is very high, via their functional role as ecosystem engineers (Lamers *et al.*, 2000; Aldous, 2002). These explanations may also account for the substantially lower C : N ratios of *D. rotundifolia* plants compared with co-occurring *S. pulchrum*.

Results showing that vegetation warming treatment failed to exert a statistically significant influence on plant size and the number of inflorescences were surprising; increases in ambient temperature are widely reported to increase biomass and therefore productivity (*cf.* Rustad *et al.*, 2001), and to advance flowering seasons (Fitter and Fitter, 2002; Aerts *et al.*, 2004). However, the marginally larger (if statistically insignificant) size and number of inflorescences of plants growing in warmed plots compared with plants growing in unwarmed plots, along with the limitations of the small number of replicates of this study, warrants the need for further investigation.

Experimental flies were significantly lighter and contained significantly higher C: N ratios than reference flies, showing that prey digestion by the plants occurs within 4 days following prey addition. This result is further supported by the uptake efficiency of prey N data which shows that nearly three-quarters of prey N is absorbed by *D. rotundifolia* within the first four days following prey capture. This result contrasts with the results of a study by Hanslin and Karlsson (1996) which show *in-situ* *D. rotundifolia* plants to display a prey N uptake efficiency of 35% to prey captured from

the background invertebrate population. These differences may be explained by potential contrasts in root N availability between sites; as the proportion of prey-derived N decreases as root N availability increases (Millett *et al.*, 2012), it may be plausible that investment in digestive enzyme production may decrease (and therefore prey N uptake efficiency decreases) as root N availability increases, or these differences may be explained by the effects of supplemental prey added to plants in this study.

Results showing the main effect of fly retention time to fail to exert a statistically significant influence on the uptake efficiency of prey N are surprising, and indicate the inappropriateness of the time interval period of 1 day selected for the study; nearly half of prey N was absorbed by *D. rotundifolia* plants during the first 24 hours following prey addition. This result warrants experimental replication but with the time interval amended to higher frequencies during the first 24 hours following prey addition (e.g. 1 h, 2 h, 4 h, 8 h, 16 h, etc.). The result showing the absorption efficiency of prey N by *D. rotundifolia* to be uninfluenced by vegetation warming treatment is also surprising; tentative, indirect evidence from the results of a study by Hanslin and Karlsson (1996) exploring the influence of prey capture level on root N and prey N uptake by four carnivorous plant species found that greenhouse plants showed prey N uptake efficiencies of 40-50% compared with *in-situ* plants (30-40%), however the potentially confounding influence of the absence of rainfall to greenhouse plants may offer an explanation for this difference in result. Biochemical evidence also contrasts with the result from this study:- proteinase relative activity of the related species of *Drosera capensis* has been demonstrated to respond positively to ambient temperature increases, with activity rising steeply at around 30 °C and peaking at *ca.* 53 °C (Takahashi *et al.*, 2009), however this relatively high optimum temperature is likely to be indicative of the species' physiological adaptation to the Mediterranean climate of the species' geographic range (Cape, South Africa).

6.6 Conclusions

The failure of ambient vegetation warming treatment and prey addition to exert statistically significant effects on parameters of prey N and root N uptake by *Drosera rotundifolia* plants, and the contrast of the results of this study with those reported in the literature, are likely to reflect the severe limitations imposed on the statistical power of the analyses completed by the small sample sizes ($n = 3$ plant replicates per experimental treatment) of this study, rather than hypotheses being proven to be unsupported. The marginal (if statistically insignificant) differences in parameters of prey N and root N uptake in keeping with the hypotheses of this study adds further weight to the need for experimental replication using larger sample sizes. The removal of experimental (prey added) and control (no prey added) *D. rotundifolia* plant replicates at each time interval following ^{15}N tracer application would enable the influence of prey availability on prey N and root N uptake over time to be explored.

6.7 References

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Chapter 7: Discussion

The unique, adaptive trait of carnivory enables carnivorous plants to survive, and thrive, in extreme environments that are predominantly characterised by nutrient-deficiency, anoxia and waterlogging. This research addresses several unanswered questions relating to the N nutrition and patterns and processes of prey capture of *Drosera rotundifolia* that strengthen our understanding of how this carnivorous plant responds ecophysiologicaly to changes in resource availability and provide an insight into why the trait of botanical carnivory has evolved independently across many lineages of flowering plants. Carnivorous plants are particularly sensitive to changes in root N availability and are therefore are considered reliable indicators of N deposition. Understanding the physiological processes and strategies underlying the responses of carnivorous plants to changes in root N availability therefore assists with predicting the ecophysiological responses underlying how N-limited plant communities may respond to sustained levels of relatively high N deposition inputs.

7.1 Comparison of the life history strategy of *Drosera rotundifolia* with co-occurring plant species

Drosera rotundifolia possesses adaptive traits, growth characteristics and resource allocation trade-offs that are indicative of the plant's life history strategy as a stress-tolerant ruderal (Grime *et al.*, 2007). Results show that *D. rotundifolia* contains one of the highest tissue N concentrations of co-occurring plant and bryophyte species that frequently inhabit ombrotrophic bogs and obtains 26-49% of the total N budget from prey, illustrating that insect capture provides a successful nutrient acquisition strategy in sunny but severely nutrient-limited environments. This adaptive trait enables this relatively small, weakly competitive forb to acquire sufficient N for survival, growth and reproduction in co-existence with larger, faster-growing, stress-tolerant competitors such as *Calluna vulgaris*, *Erica tetralix* and *Eriophorum vaginatum*. Results also show that plant reliance on carnivory is phenotypically plastic in response to root N availability, enabling a prompt ecophysiological response to spatial and temporal variation in resource availability in order to maximise N uptake. This plasticity in resource acquisition strategy enables the plant to co-exist with other plant species that also show plasticity in reliance on an alternative N source, such as the mycorrhizal species of *C. vulgaris* and *E. tetralix*. The relatively large proportional investment in reproductive structures and shorter life span of *D. rotundifolia* in comparison with co-occurring plant species is characteristic of the plant's ruderal strategy; *D. rotundifolia* is a primary coloniser of bare peat and therefore these characteristics enable the plant to exploit the temporarily favourable conditions of full light and reduced interspecific competition for resources by reproducing quickly (Grime, 1977). The result of increased SLA in response to decreased root N availability illustrates the stress-tolerator aspect of

the plant's strategy, where resources are preferentially allocated towards leaf conservation and therefore preservation of plant N upon exposure to unfavourable conditions. Therefore, it is the combination of life history, strategy and phenotypic plasticity in ecophysiological responses that enable *D. rotundifolia* to thrive in an extreme environment.

The comparison of N use between co-occurring plant and bryophyte species growing at two sites that vary by N deposition input as presented in Chapter 3 offers an insight into how ombrotrophic bog communities may respond to long-term inputs of relatively high N deposition. Results showing all species to contain higher tissue N concentrations at the high N deposition site compared with the low N deposition site indicate that N inputs absorbed directly by *Sphagnum* capitula are released into the rhizosphere of vascular plants at the former site and are therefore available for uptake by plants. This result corresponds with those of Lamers *et al.* (2000) which show that the 'N filtering' ability of *Sphagnum* fails at N deposition inputs above the threshold of $18 \text{ kg N ha}^{-1} \text{ yr}^{-1}$, therefore indicating that *S. fuscum* loses the ability to act as an 'ecosystem engineer' (Svensson, 1995).

Results showing that the magnitude of increase in tissue N concentration per species between plants growing at Cors Fochno and plants growing at Whixall Moss indicates that some species possess strategies and adaptations that enable them to better utilise additional root N. *Drosera rotundifolia* underwent the highest magnitude of tissue N concentration increase, suggesting that the adaptations of a shallow root system (Crowder *et al.*, 1990) and micro-habitat preference for living amongst *Sphagnum* species (Svensson, 1995) provide the benefit of first access to N released by *Sphagnum* before it seeps into the lower rhizosphere and becomes available to deeper rooting plant species. *E. vaginatum* and *C. vulgaris* contained the second highest magnitude of tissue N concentration increase of the species, therefore it may be predicted that upon exposure to sustained high N deposition inputs these deeper rooting species would utilise adaptive strategies of high growth rates, relatively long life spans and high rates of nutrient resorption efficiency to out-compete *r*-selected species such as *D. rotundifolia*. Indeed, results of a four-year study exploring the influence of experimental addition of NH_4NO_3 equivalent to five levels of N deposition between $0\text{-}40 \text{ kg N ha}^{-1} \text{ yr}^{-1}$ on ombrotrophic bog vegetation found that *D. rotundifolia* populations exposed to $>20 \text{ kg N ha}^{-1} \text{ yr}^{-1}$ suffered approximately 80 % greater mortality than populations exposed to additions of $0\text{-}10 \text{ kg N ha}^{-1} \text{ yr}^{-1}$, and that these higher mortality rates were attributed to the detrimental effects of increased growth of the taller graminoid *E. vaginatum* which outcompeted *D. rotundifolia* for light (Redbo-Torstensson, 1994). Therefore, between-species differences in N use in response to increasing N deposition may lead to changes in the nature of, and strength of interactions between co-existing plant species for resource acquisition, resulting in changes to species' relative abundance and ultimately to changes in community structure.

7.2 Patterns and processes of prey capture by *Drosera rotundifolia*

7.2.1 Relative investment in prey capture

Results from Chapter 2 shows that relative investment in prey capture by *D. rotundifolia*, measured as leaf stickiness, is phenotypically plastic in response to root N availability; as root N availability decreases, relative investment in prey capture (and therefore the carbon content of the mucilage) increases. This result corresponds with those of previous studies exploring investment in prey capture by *D. rotundifolia* (Thorén *et al.*, 2003; Millett *et al.*, 2012), and provides additional support for the carbon/nutrient balance (CNB) hypothesis (Bryant *et al.*, 1983) and the energetic cost/benefit model for the evolution of botanical carnivory (Givnish *et al.*, 1984). Results from Chapter 5 show that the same pattern is observed with *Pinguicula grandiflora*, but only after the stress event. Therefore, these results suggest that N-deficient plants were more vulnerable to stress than plants with N available. Therefore, further research is required to assess whether the patterns found by Thorén *et al.* (2003) are generalizable to other lineages of carnivorous plants such as Lentibulariaceae.

7.2.2 Diet and dietary strategy

Results exploring the patterns and processes of prey capture by *D. rotundifolia* indicate that the species is a dietary generalist, where the spectrum of captured prey represents random samples from the population of potential prey. This result provides partial support for the evolutionary 'predictable prey capture hypothesis' proposed by Müller *et al.* (2004) which proposes that species with relatively complex traps have more predictable and frequent prey capture as a result of dietary specialism than species with relatively simple traps, which have less predictable and frequent prey capture as a result of dietary generalism. This result corresponds with those of a meta-analysis of the diet of *D. rotundifolia* by Ellison and Gotelli (2009) which indicates that species possessing adhesive traps (*Pinguicula* spp. and *Drosera erythrorhiza*) are dietary generalists. These results therefore provide an insight into the relationship between prey capture mechanism and dietary strategy, however further research addressing the dietary strategy of species with relatively complex traps is required to fully test the proposals of the predictable prey capture hypothesis.

The comparison of the results presented in this thesis with those of earlier studies exploring the feeding ecology of *D. rotundifolia* is hampered by substantial differences in methodology between studies which influence the conclusions made; one study suggests dietary generalism (Jennings *et*

al., 2010), one suggests dietary specialism (Volkova *et al.*, 2010), and some are inconclusive (Thum, 1986; Foot *et al.*, 2012). Results of studies exploring feeding ecology of other adhesive trap species of the *Pinguicula* genus are also mixed, with about equal support for dietary generalism (Zamora, 1990, 1995) compared with dietary specialism (Antor and García, 1994; Alcalá and Domínguez, 2003), and some mixed (Karlsson *et al.*, 1987). Therefore, further research is needed to clarify the feeding ecology of carnivorous plants with adhesive traps. Whilst between-species differences in dietary strategy may be possible, variation in the results for *D. rotundifolia* highlight the influence of methodology on determining the dietary strategy; with results influenced by or the strength of conclusion limited by factors such as the lack of consideration for potential prey, leaf stickiness and/or prey size, and variation in the potential appropriateness of methods used to survey background populations of potential prey to the plants. Therefore future studies exploring the feeding ecology of carnivorous plants would benefit from consideration of these factors.

Results of the comparison of the diet of *D. rotundifolia* between sites show that N deposition influences the quantity and size class of prey captured, but not the quality (order composition) of prey; as N deposition decreases, the total prey mass and mean prey size per plant increase. These results are likely to be explained by phenotypic plasticity in investment in prey capture by the plant in response to root N availability; as N deposition and therefore root N availability decreases, leaf stickiness increases. Therefore, these results indicate a positive correlation between leaf stickiness per unit area and leaf prey retention capacity per unit area; invertebrates are more likely to be captured by stickier leaves than less sticky leaves, however the potential caveat that leaf stickiness was measured at a different growth season to that of the captured prey survey is acknowledged. This indicated relationship corresponds with the results of a study exploring the feeding ecology of *Pinguicula vallisneriifolia* (Zamora, 1995), which found that leaves in sunny habitats had a higher prey retention capacity for experimentally placed large flies and captured larger mean size of background prey than leaves in shaded habitats, however leaf stickiness per plant was not directly measured. Positive correlations between prey size and trap stickiness are reported for spider webs (Nentwig, 1982); upon exposure to identical web adhesivity, average prey escape time decreased with increasing prey mass. Therefore, it appears likely that stickier leaves have higher prey retention capacities than less sticky leaves and therefore capture larger prey, however further research is required to clarify the relationships between these leaf traits and prey size.

7.3 N nutrition of *Drosera rotundifolia*

Results presented in Chapters 2 and 4 show that the reliance of *D. rotundifolia* on the trait of carnivory decreases as root N availability increases. This evidence for phenotypically plasticity corresponds with the results of a study of the N nutrition of *D. rotundifolia* at three sites that vary by N deposition input by Millett *et al.* (2012), and indicates that the trait becomes increasingly less beneficial to the plant as root N availability increases. Results show the influence of root N availability on prey capture processes and N nutrition of *D. rotundifolia*, and the interrelatedness of growth parameters and leaf traits (Fig. 55). As root N availability increases, uptake of root N increases as this N source is less energetically costly to the plant to uptake than prey N. As a result, plants grow larger, total leaf area per plant increases, and investment in prey capture decreases. Leaves are less sticky but the total leaf area is larger, therefore the amount of mass-adjusted prey-derived N stays the same as for plants exposed to lower levels of root N availability (where leaves are stickier but total leaf area is smaller). Therefore, %N_{dfp} decreases as a consequence of the amount of mass-adjusted root N increasing as the amount of mass-adjusted prey N stays the same.

The amount of prey-derived N was statistically insignificant between sites following adjustment for plant mass, indicating that the total mass of prey captured per plant was the same between sites. This result is likely to be explained by dual influence of leaf stickiness and leaf area on the probability of prey capture success by the plant; whilst plants at Whixall Moss possessed less sticky leaves than plants at Cors Fochno, total leaf area (and therefore total potential prey capture area) was larger. These results indicate a possible resource allocation trade-off between leaf area and leaf stickiness; similar results are reported by Foot *et al.* (2014), which show a leaf size-area trade-off by *D. rotundifolia*, and highlight the complexity of resource allocation trade-offs for leaves that serve multiple functions of prey capture, photosynthesis and respiration.

Results showing plants at Cors Fochno to contain significantly higher %N_{dfp} and tissue C : N ratios than plants at Whixall Moss are consistent with those of earlier studies showing a positive correlation between tissue C : N ratio and %N_{dfp} or mass-adjusted N_{dfp} (Millett *et al.*, 2003, 2012) and indicate a positive benefit of prey capture to the plant. Results of this study therefore demonstrate the substantial influence of root N availability on phenotypic plasticity in terms of plant reliance on botanical carnivory, and provide support for the energetic cost-benefit model for the evolution of botanical carnivory (Givnish *et al.*, 1984) which proposes that carnivory is only of net benefit to the plant exposed to nutrient-limited, bright and waterlogged habitats as the photosynthetic costs of investment in carnivory are not exceeded by the energetic gain from prey N uptake in shady or dry habitats.

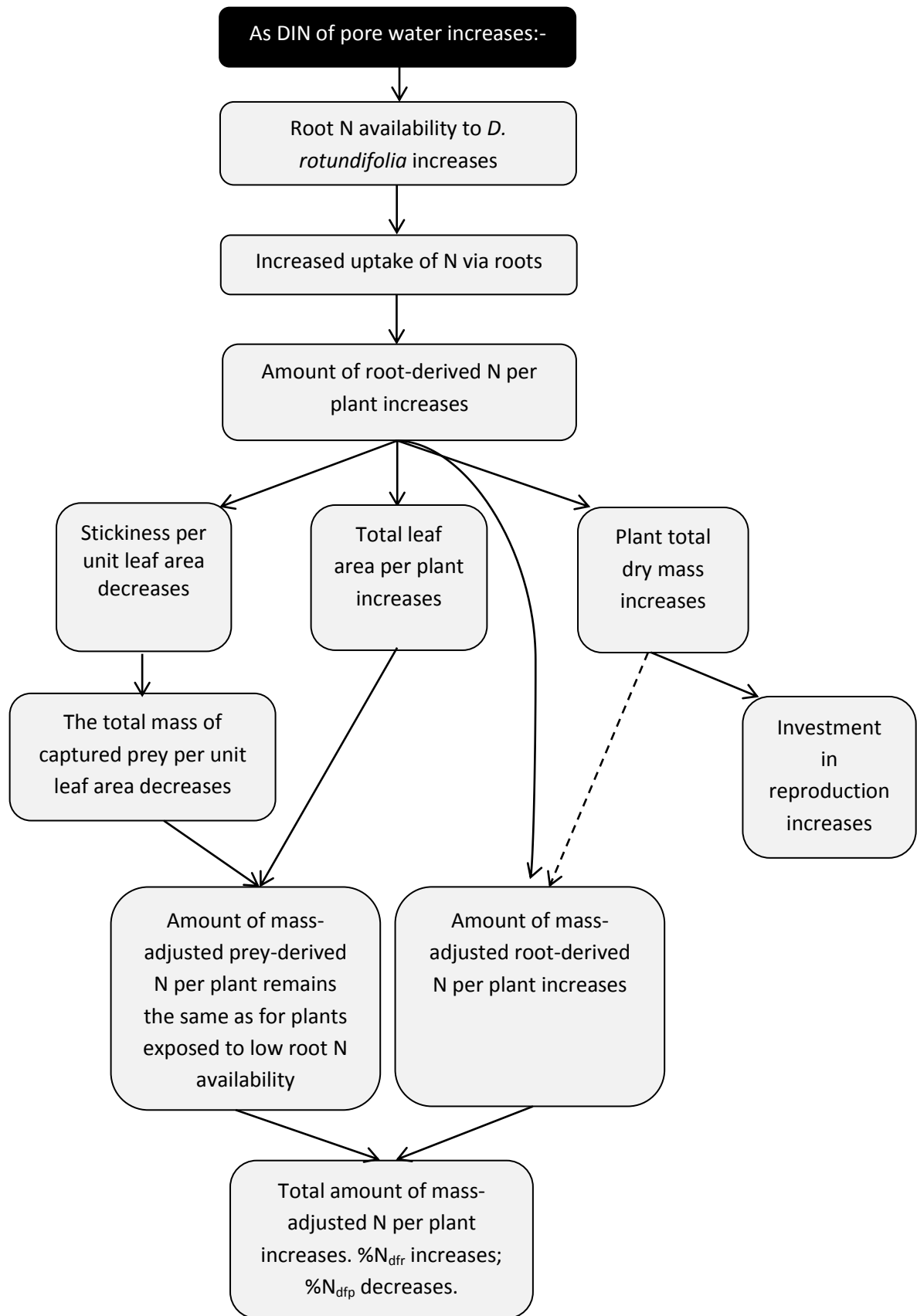


Figure 55 Flow diagram of the influence of root N availability on the N nutrition of *Drosera rotundifolia*, based on the results presented in this thesis.

7.4 Reducing uncertainty in the calculation of the proportion of prey-derived N of the total N budget of carnivorous plants

Results from the natural abundance study show that precision in the calculation of %N_{dfp} of carnivorous plants may be increased by: (i) incorporating the diet of the plant into the calculation of the variability of the prey $\delta^{15}\text{N}$ end-point; (ii) maximising the number of replicates (preferably at least ten) of each taxon of captured prey per survey plot; (iii) identifying prey and background invertebrates to as fine a taxonomic level as feasible. In the case of exploring the N nutrition of carnivorous plants, there is evidently a trade-off between the resources available for associated field/lab work versus the required precision level of %N_{dfp}; the total time associated with calculating weighted $\delta^{15}\text{N}_{\text{prey}}$ is much larger than the total time associated with calculating unweighted $\delta^{15}\text{N}_{\text{prey}}$. If less precise measures of %N_{dfp} for generalist carnivorous plant species are acceptable for satisfying the research objectives of future studies, time may be saved by excluding prey orders that constitute less than 5% of the plants' diet from the calculation of weighted $\delta^{15}\text{N}_{\text{prey}}$.

Incorporation of the diet of *D. rotundifolia* did not significantly influence the value of the prey $\delta^{15}\text{N}$ end-point and therefore the accuracy in %N_{dfp}. This result is mostly explained by the two most frequently captured prey orders captured by *D. rotundifolia* at each site, Diptera and Formicidae, possessing $\delta^{15}\text{N}$ values that differed by only ca. 2 ‰. For other generalist carnivorous plant species where the most frequently captured prey orders possess a wider range in $\delta^{15}\text{N}$ values, the incorporation of plant diet may lead to significant differences in $\delta^{15}\text{N}_{\text{prey}}$ compared with using unweighted prey $\delta^{15}\text{N}$ values. For example, the results of a study by Schulze *et al.* (2001) found that the diet of *Dionaea muscipula* plants growing in heathland and woodland in North Carolina, USA, consisted of predominantly ants ($\delta^{15}\text{N} = +6$ ‰) and grasshoppers ($\delta^{15}\text{N} = -4$ ‰), with plants with small leaves capturing a larger proportion of ants and plants with large leaves capturing a larger proportion of grasshoppers. Therefore, it is likely that incorporating plant diet would lead to significantly different values of $\delta^{15}\text{N}_{\text{prey}}$ between small and large leaved plants. Thus, future studies exploring the N nutrition of carnivorous plants would benefit from conducting a pilot study of the order composition of the diet of a small sample of carnivorous plants *in-situ*, and using the mean $\delta^{15}\text{N}$ and %N values for each prey order available from previous studies (using sites at similar N deposition inputs) to calculate estimated values for weighted and unweighted $\delta^{15}\text{N}_{\text{prey}}$. If significant differences between weighted and unweighted $\delta^{15}\text{N}_{\text{prey}}$ are found, the extra workload associated with the calculation of weighted $\delta^{15}\text{N}_{\text{prey}}$ for the main study would be justified.

The method of incorporating proportional contributions from level one contributors to the variability of the isotopic signature of a level two source, as presented in this thesis, is likely to be applicable to minimising uncertainty in the calculation of source proportions to a mixture for most multi-level

research using the one-isotope linear mixing model where one or both of the two sources are comprised of multiple contributors. One example of application to non-carnivorous plant ecology is calculating the plant reliance on mycorrhizal-derived N for legumes and *Pinus* spp. that undergo associative N fixation, where the $\delta^{15}\text{N}$ of the fungal symbiont is influenced by the $\delta^{15}\text{N}$ of many genera of resident N_2 -fixing bacteria (Paul *et al.*, 2007). At the broader community level, the weighting of the isotope of level one contributors should be applicable to food web studies using lower trophic levels of food webs (e.g. Galván *et al.*, 2011), however further research is required to assess the suitability of the method for food web data using more than two trophic levels.

Results obtained from exploring the influence of proxy type for the root N end-point of the mixing model show substantial variation in the $\delta^{15}\text{N}$ of plant and bryophyte species that co-exist with *D. rotundifolia*; these differences in $\delta^{15}\text{N}$ may be explained by variation in life history, rooting depth, physiological adaptations for nutrient acquisition and retention and mycorrhizal association. These between-species differences in proxy $\delta^{15}\text{N}$ exerted substantial influence on the value of $\%N_{\text{dfp}}$, showing that proxy choice directly influences the conclusion made regarding the reliance of *D. rotundifolia* on carnivory. In order to minimise this potential error source, care should be taken to select proxy(s) that are most closely representative of the carnivorous plant that has obtained 100% of N via the roots.

Some studies have chosen to minimise this potential error source by using the average $\delta^{15}\text{N}$ of as many co-occurring non-carnivorous plant species as possible, in order to minimise the influence of factors that alter $\delta^{15}\text{N}$ such as varying rooting depth (Shearer and Kohl, 1988; Schulze *et al.*, 1991, 1997, 2001). In this study, the mean $\delta^{15}\text{N}$ of *S. fuscum* and *E. vaginatum* was chosen as the root N proxy due to the similarity of $\delta^{15}\text{N}$ values to the lowest $\delta^{15}\text{N}$ value of *D. rotundifolia*, due to these species possessing maximum rooting depths and combined N uptake and assimilation strategies that are closest to that of *D. rotundifolia*, however it remains unknown how truly representative this proxy is of the root N end-point. Similarly, it remains unknown how truly representative the use of invertebrate $\delta^{15}\text{N}$ is of a carnivorous plant that has obtained 100% of N from prey; potential error may be incurred due to the differential fractionation of stable isotopes of N at different trophic levels of food webs, with consumer tissue usually $\delta^{15}\text{N}$ -enriched compared to that of corresponding prey species (DeNiro and Epstein, 1981; Minagawa and Wada, 1984). Therefore further research is required to quantify how closely representative $\delta^{15}\text{N}$ values of a wide range of plant and invertebrate proxies are of carnivorous plants that have obtained 100% of N via the roots or from prey respectively in order to justify future proxy choices.

7.5 Conclusions

The results from this research show several new lines of evidence relating to the ecophysiological responses of the carnivorous plant *Drosera rotundifolia* to changes in root N availability, and how these responses compare with those of co-occurring non-carnivorous plants found in ombrotrophic bogs and other species of carnivorous plant. These findings are of broad relevance in the fields of physiological ecology and evolutionary ecology, for both carnivorous and non-carnivorous plants. They also reveal information of potential importance for the management of ombrotrophic bogs under pressure from N deposition and climate change. Furthermore, experimental testing of a method for reducing uncertainty in the calculation of source proportions to a stable isotope mixture is of wider relevance to plant ecophysiology and community or ecosystem ecology research. The wider implications from the research outputs of this project are therefore relevant to a variety of potential stakeholders, as summarised below.

7.5.1 Recommendations for ombrotrophic bog management and policy making

The results of this research clearly show that N deposition is a key driver of environmental change in ombrotrophic bog systems. Increased N deposition inputs of 8.0 to 30.8 kg N ha⁻¹ yr⁻¹ trigger the release of atmospherically-derived N by *Sphagnum* into the rhizosphere, resulting in increased uptake of N by vascular plants. Furthermore, results show that as N deposition increases, the magnitude of change in N uptake and use differs between plant species with contrasting life history strategies. Specifically, high N-demanding functional types such as graminoids (e.g. *Eriophorum vaginatum*) absorb larger magnitudes of root N at high N deposition inputs than less competitive, r-strategist forbs such as *Drosera rotundifolia*, suggesting that the nature and strength of plant to plant interactions may change along N deposition gradient. These results therefore provide the missing evidence link at the ecophysiological level that underlies resultant shifts in the species composition and richness of ombrotrophic bog plant communities over time that are observed upon exposure to sustained inputs of chronic N deposition. Broadly, these shifts culminate in an increase in graminoid cover and a decline in *Sphagnum* and forb cover (Field *et al.*, 2014). Therefore, ombrotrophic bogs receiving sustained, chronic N deposition inputs are vulnerable to biodiversity loss and a reduction, or even reversal, in their capacity to store carbon.

These research outcomes are of high relevance to policy makers specialising in nitrogen pollution, biodiversity and climate change legislation and to managers of peatland bog systems. From a policy perspective, these results are relevant to current biodiversity and N pollution control legislation at national, European and international levels, such as the EU Habitats Directive, EU 2020 Biodiversity

Strategy, and the UN Convention on Long-Range Transboundary Air Pollution (CLRTAP). In terms of N deposition, the results of this research provide evidence to support the empirical critical load for raised and blanket bogs of 5-10 kg N ha⁻¹ yr⁻¹ as set by CLRTAP (Hall *et al.*, 2011). Critical loads for N deposition are not currently being met; 71.2 % of the UK's habitat area (including bogs) currently receiving nitrogen deposition inputs that exceed empirical critical loads set by CLRTAP (Hall *et al.*, 2011), and the EU's nitrogen dioxide (NO₂) pollution limit has been exceeded since 2010 and is forecast to continue to be exceeded for 40 out of 43 air quality zones of the UK until at least 2030 (DEFRA, 2014). Despite this infringement, the UK government has avoided associated penalties issued by the European Commission to date (Rincon, 2015). Therefore it is critical that strict enforcement of EU and international N deposition legislation is conducted as soon as possible to coerce the UK government to act on curtailing major UK domestic sources of N emissions.

Furthermore, there is a need for more detailed management policies to be incorporated into legislation relating to reducing the effects of N deposition inputs on peatland systems across Europe. Recommendations proposed by the BioDiversa research team to develop a network of early warning systems of the threat of N deposition in peatland bogs across Europe, and to create 'nitrogen pollution protection areas' in regions particularly vulnerable to N deposition (Dise, 2014) are supported by the results of this research. Indeed, Cors Fochno, as a pristine raised bog currently receiving relatively low N deposition inputs within the CLRTAP critical range, would be an ideal candidate UK site for the network of early warning systems. An ideal candidate area of the UK to be designated as a 'nitrogen pollution protection area' would be Shropshire and the part of Wales containing a proportion of Whixall Moss; Shropshire receives high N deposition inputs but contains over 60 peatland sites including Whixall Moss, the third largest bog in the UK at 948 ha (Shropshire Biodiversity Partnership, 2009).

Evidence from this research highlights the potential suitability of *Drosera rotundifolia* as a sensitive biological indicator of N deposition inputs in exceedance of the CLRTAP critical load range for bogs; interannual comparison of the relative abundances of *D. rotundifolia* per unit of land area between and within sites would provide a straight forward and cost-effective management strategy for assessing spatial and temporal changes in N deposition inputs and subsequent effects on plant community structure. Future work would benefit from quantifying the relationship between N deposition inputs and the percentage population decline of *D. rotundifolia*. These results would enable the setting of specific percentage population decline benchmarks as indicators of N deposition input ranges to bogs, such as those set for the liverworts *Odontoschisma denudatum* and *Anastrophyllum minutum* where population declines of 20% compared to baseline populations at

bogs receiving $0 \text{ kg N ha}^{-1} \text{ yr}^{-1}$ represent an incremental increase in N deposition input to $5\text{-}10 \text{ kg N ha}^{-1} \text{ yr}^{-1}$ (Hall et al., 2011; Stevens *et al.*, 2011).

At present, and over at least the next couple of decades, the vast majority of conservation teams managing ombrotrophic bogs in the UK face the extremely challenging situation of trying to minimise the effects of site N deposition inputs in exceedance of CLRTAP critical loads in order to meet biodiversity and climate change targets set by national, EU or international bodies, such as favourable SSSI or NNR statuses at the national level or Favourable Conservation Status of peatland habitat types covered by the EU Habitats Directive. Several management techniques may be implemented to reduce losses to the biodiversity and the carbon storage potential of an ombrotrophic bog due to N deposition inputs. The control of nitrophilous plant species with high magnitudes of root N uptake, such as *Eriophorum vaginatum*, would enable some of the excess N to be removed from the bog ecosystem. This could be achieved by removing the above-ground biomass of plants by targeted grazing or mowing regimes in areas that are dominated by nitrophilous plant species, or by the manual removal of individual plants. The creation of bare peat areas by scraping would encourage increased cover by *Sphagnum* and by vascular plant species that colonise recently disturbed peat such as *Drosera rotundifolia*, therefore maximising biodiversity through the maintenance of habitat heterogeneity. Whilst these measures may not represent cost-effective options for peat bog management in the long-term, they offer short-term solutions for minimising losses to the biodiversity and carbon storage potential of sites until future N deposition inputs fall within the range of CLRTAP empirical critical loads.

Carnivorous plants are extremely valuable to scientists as they act as model systems for exploring a wide range of ecological and evolutionary questions relating to plants. Therefore in addition to importance of the role that carnivorous plants play in maintenance of biodiversity and ecosystem function, it is critical to ensure the continued survival of these species for their value to scientific knowledge and advancement. In addition to the management techniques described above, teams managing peatlands that support populations of carnivorous plants can provide invaluable assistance towards this objective by collecting seeds for long-term *ex-situ* conservation; the team managing the Millenium Seed Bank (MSB) based at Kew Gardens encourage donations of seed harvested from wild plant populations. This is particularly important in the case of *Drosera rotundifolia* as seeds only form a short-term persistent seed bank; seeds remain viable in the peat layer for less than 5 years (Thompson *et al.*, 1997). *Drosera rotundifolia* is therefore more vulnerable to local extinction than co-occurring plant species with longer-term seed bank persistence. Furthermore, only four sets of *D. rotundifolia* seed have been submitted to the MSB from wild plant populations in the UK to date (Millenium Seed Bank Partnership, 2015). Storage of seed collected

from a large number of spatially isolated carnivorous plant populations would preserve a wide range of genetic diversity found within a particular species (Li and Pritchard, 2009), and provide a valuable propagation source for future (re)introductions of a species to newly created or recently restored peatlands throughout the world.

7.5.2 Carnivorous plant ecology research

The results of this research show several new insights relating to the physiological ecology and evolutionary ecology of *Drosera rotundifolia* and to the calculation of prey N nutrition of carnivorous plants that are relevant to researchers using any species of carnivorous plant to explore these research themes. The results of this research provide, for the first time, an integral picture of the patterns and processes of prey capture by *Drosera rotundifolia* and subsequent effects on plant N nutrition, and how these stages of botanical carnivory are influenced by N deposition. Results show that *Drosera rotundifolia* displays a high level of phenotypic plasticity in relative investment in prey capture in response to root N availability; leaves become stickier as N deposition decreases. However, plasticity in other traits, such as dietary strategy, in response to N deposition was not found. This evidence for the evolution of phenotypic plasticity for some plant functional traits influencing the N nutrition of carnivorous plants but not others is interesting and warrants further attention. The plasticity in relative investment in prey capture in response to changes in root N availability may be explained by the energetic cost-benefit model for the evolution of botanical carnivory (Givnish *et al.*, 1984), which proposes that the net marginal benefit of carnivory only outweighs the associated costs in sunny, wet and nutrient-deficient environments. The indicated dietary strategy of *D. rotundifolia* as a dietary generalist that utilises a passive mechanism for prey capture may be partially explained by the predictable prey capture hypothesis, which proposes that carnivorous plant species with more complex trapping mechanisms are likely to have more frequent and predictable prey captures than species with less complex trapping mechanisms (Müller *et al.*, 2004).

However, neither hypothesis offers an explanation as to why dietary strategy utilised by carnivorous plants has not also evolved to be phenotypically plastic in response to changes in N availability. Several possible explanations are as follows. Firstly, the potential nutritional benefits to the plant of capturing a prey taxon with a higher tissue C:N ratio than other taxa (e.g. Hymenoptera) may not be large enough to outweigh the additional energetic costs associated with the selective attraction, capture and digestion of that particular taxon. Secondly, the order composition and availability of background invertebrates during the evolutionary history of *D. rotundifolia*; relatively small

invertebrates (e.g. flies of 0.5-1.5 cm in length) may have always been readily available in the plants' micro-environment compared with other size classes and taxa of potential prey. Thirdly, evolutionary constraints may have restricted the development of plasticity in dietary strategy, such as a lack of genetic variation, associated genetic trade-offs or multivariate selection; albeit this option is less likely due to the wealth of evidence indicating that adaptive evolution is almost always limited by natural selection rather than genetic variation (Futuyma, 2010). Further research is required at the ecophysiological and genetic levels to build a complete picture in terms of the ecological consequences in terms of N nutrition and the species' evolutionary history. This is important as associated data will provide further insight into the drivers of the evolution of phenotypically plastic traits relating to the N nutrition of plants.

Results of this research correspond with the results of previous studies exploring the relationship between N deposition and plant reliance on carnivory (%N_{dfp}) by *Drosera rotundifolia*; as N deposition decreases, %N_{dfp} and tissue C:N ratio increase, providing further support for Givnish's energetic cost-benefit model for the evolution of botanical carnivory. Results from the study exploring increasing precision in the calculation of %N_{dfp} show that the incorporation of weighted prey $\delta^{15}\text{N}$ into the linear mixing model reduces SE(%N_{dfp}) for *Drosera rotundifolia* by ca. 24% compared with the use of unweighted prey $\delta^{15}\text{N}$. Furthermore, the method for calculating weighted prey $\delta^{15}\text{N}$ presented in Chapter 4 is applicable to calculations of %N_{dfp} and plant reliance on root-derived N for any species of carnivorous plant. This substantial reduction in SE(%N_{dfp}) arguably justifies the extra effort required in terms of fieldwork and lab analyses to determine the diet of the carnivorous plant, the %N of each prey taxon and the proportional mass contribution of each prey taxon to the total mass of captured prey in order to calculate weighted prey $\delta^{15}\text{N}$. Future research would benefit from quantifying the percentage reduction in SE(%N_{dfp}) obtained using weighted prey $\delta^{15}\text{N}$ compared with unweighted prey $\delta^{15}\text{N}$ for carnivorous plant species with a range of diets and dietary strategies, and using these data to re-evaluate recommendations resulting from %N_{dfp} calculations reported in the literature. Results showing that use of weighted prey $\delta^{15}\text{N}$ exerted a statistically insignificant but marginal decrease in the value of %N_{dfp} for *D. rotundifolia* compared with the use of unweighted prey $\delta^{15}\text{N}$ warrant further investigation using plants collected from a larger sample size of sites. This is important as data would clarify whether the influence of N deposition on values of %N_{dfp} for *D. rotundifolia* have been overestimated in the literature.

Results from Chapter 5 provide an insight into the functional role of leaf redness in carnivorous plants, and the potential factors driving the evolution of anthocyanins in carnivorous plant lineages. Evidence shows that leaf redness in *Drosera rotundifolia* and *Dionaea muscipula* serves a photoprotective role in response to potentially damaging levels of high light availability. This result

corresponds with the species' restriction to exposed, high light environments and suggests that high light availability acted as a positive selective pressure driving the evolution of foliar anthocyanins in these species. Results also suggest that foliar anthocyanin accumulation may represent a multiple stress response by *D. rotundifolia* and *D. muscipula* to a combination of high light availability, herbivory and heat stress. Further research is required to explore the potential multifunctional role of leaf redness in carnivorous plants in order to provide further insight into the benefits of anthocyanins to the plant, the relationship between leaf anthocyanin content and plant tolerance to environmental stressors and the abiotic and/or biotic factors that drive the evolution of leaf anthocyanin accumulation in carnivorous plants. Results showing that leaf redness does not play a primary role in prey attraction by *Drosera rotundifolia* correspond with results showing that leaf anthocyanin content is uninfluenced by root N availability. These results contest those reported by manipulative pigmentation studies using *D. rotundifolia*, and therefore provide new insight into the functional role of foliar anthocyanins in carnivorous plants and the prey capture strategy used by *D. rotundifolia*. These results provide further, but not conclusive, support for the dietary generalist strategy utilised by *D. rotundifolia*; future research is required to explore whether other mechanisms for prey attraction are utilised by the plant, such as UV reflectance or scent.

7.5.3 Ecology and plant science research in general

Results of this research illustrate that the trait of botanical carnivory and the phenotypic plasticity of *Drosera rotundifolia* lends this species as a model system for exploring a wide range of questions relating to plant ecology. Results presented in this research use *D. rotundifolia* to address a variety of questions centred around plant N nutrition that are relevant to researchers of evolutionary ecology and physiological ecology of plants, plant and community responses to N deposition and the use of multi-level, natural abundance linear mixing models, and to plant scientists working in the agricultural industry.

The acquisition of supplemental nutrients from captured invertebrate prey gives *D. rotundifolia* a competitive advantage in nutrient acquisition in comparison to co-occurring, non-carnivorous plants. The nature of botanical carnivory and high degree of phenotypic plasticity in the trait displayed by carnivorous plants is potentially of interest to plant scientists researching strategies to maximise nutrient acquisition by economically important plant species that are used for agriculture or biofuels. Over 30% of vascular plants possess glandular trichomes that capture prey (Wheeler and Krimmel, 2015) but which are classed as protocarnivorous, where only one or a few of the characteristics of the carnivorous trait are utilised. Invertebrate prey captured by these trichomes

subsequently fall to the soil around the plant and begin to decompose, eventually leading to the availability of prey-derived nutrients to the plant via root uptake. Species possessing glandular trichomes include economically important crops such as extra long staple cotton (*Gossypium barbadense*), potato (*Solanum tuberosum* L.), aubergine (*Solanum melongena*), tomato (*Solanum lycopersicum*) and tobacco (*Nicotiana tabacum*). Therefore there may be potential to maximise crop yields from these species under N-limited environments in the future by selectively breeding for increased plant relative investment in prey capture, resulting in increased reliance on %N_{dfp} absorbed via the roots and therefore providing the benefit of reducing environmental and financial costs associated with the conventional application of soil fertilisers.

The functional role(s) of foliar anthocyanins is a popular topic in plant ecology research; anthocyanins are indicated to be a versatile stress response to a wide range of environmental stressors, including visible light, UV radiation, herbivory and nutrient deficiency, however the functional role(s) remain under debate (Gould *et al.*, 2000). Understanding how and why leaf anthocyanin accumulates upon plant exposure to varying environmental conditions, and the factors driving the evolution of flavonoids, are of wider benefit to a wide variety of plant science research themes, such as photosynthetic performance, phenology and plant defence. Results showing leaf redness of the carnivorous plants *Drosera rotundifolia* and *Dionaea muscipula* to be a visible sign of a plant stress response to potentially damaging light levels may have wider implications for plant scientists. Both these species possess similar r-selected life history strategies that are characteristic of recently disturbed, open and sunny environments, and therefore these results suggest that leaf anthocyanin accumulation may have co-evolved with elements of this life history strategy. In order to build a more complete picture of the functional role(s) of foliar anthocyanins in angiosperms, future research would benefit from exploring the influence of light availability on foliar anthocyanin accumulation by other plant species found in ombrotrophic bogs that also show phenotypically plastic variation in leaf colour and by r-selected plant species with similar life history strategies found in other semi-natural habitats.

With global deposition inputs of reactive nitrogen projected to increase from 100 Tg N yr⁻¹ in 1995 to 200 Tg N yr⁻¹ by 2050 (Galloway *et al.*, 2008), there is an urgent need to further the understanding of N deposition effects on physiological processes at the species level and on interactions between organisms that drive shifts in community structure in order to develop evidence-based land management policies to minimise losses to biodiversity and ecosystem functioning. Results show that N deposition exerts a substantial influence on the N nutrition of co-occurring plants growing in ombrotrophic bogs and that the magnitude of change in N uptake and use varies between species according to plant functional type and life history strategy. These results therefore provide evidence

at the ecophysiological level that underlies shifts in community structure and composition in response to N deposition, and suggest that the nature and strength of interactions between plants may change along an N deposition gradient. Therefore these results are of wider value to research exploring plant community responses to N deposition in ombrotrophic bogs and other semi-natural ecosystems.

The results presented in this thesis also suggest wider implications of the ecophysiological responses of *Drosera rotundifolia* to N availability for the structure and abundance of the invertebrate community and for other organisms that also predate invertebrates. These findings are therefore of interest to researchers exploring invertebrate community structure and dynamics, trophic interactions and ecosystem structure. For example, the decrease in the size of prey captured by *D. rotundifolia* as N deposition increases is likely to alter the size distribution of the background invertebrate population, leading to changes in the strength and nature of interactions involving invertebrates and other organisms. Furthermore, if similar competitive relationships for invertebrate prey exist from dietary overlap between *D. rotundifolia* and co-occurring spiders as observed between *D. capillaris* and wolf spiders (Lycosidae) (Jennings *et al.*, 2010), it may be predicted that the strength of the competitive plant-spider interaction decreases with increasing N deposition. Changes to pairwise biotic interactions at lower trophic levels often combine to cause significant effects on entire communities (Tylianakis *et al.*, 2008). Future research would therefore benefit from attempting to quantify the relative influence of changes in prey capture by *D. rotundifolia* on the nature and strength of interactions between invertebrates and other organisms, and to determine the subsequent effects on invertebrate community structure and wider ecosystem structure and function.

Chapter 4 presents an experimentally tested method for reducing uncertainty in the calculation of proportional contributions of two stable isotope sources to a mixture using a single isotope, multi-level linear mixing model, which is therefore potentially useful to a wide range of ecological research including plant ecophysiology, community or ecosystem ecology and to research exploring the limitations and error associated with the use of stable isotope linear mixing models. One example of a multi-level model application in plant ecophysiology research is the calculation of the N nutrition of heterotrophic plants, such as plant reliance on ant-provided N and rain-derived N by epiphytes that display mutualisms with two or more ant species (Treseder *et al.*, 1995) or plant reliance on mycorrhizal-derived N and non mycorrhizal-derived N by mycorrhizal plants that display mutualisms with two or more fungal types. In the fields of community and ecosystem ecology, one example of potential method application is the determination of the diet composition of organisms occupying lower trophic levels, such as the feeding ecology of saltmarsh polychaetes (Galván *et al.*, 2011).

However, further research is required to explore whether the method presented is applicable for reducing uncertainty in source proportions to a stable isotope mixture for organisms occupying higher trophic levels of food webs where more complex trophic interactions between organisms occur.

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