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1 **From mycoheterotrophy to mutualism: mycorrhizal specificity and functioning in**

2 ***Ophioglossum vulgatum* sporophytes**

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21 **Summary**

- 22 • Mycorrhizal functioning in the fern *Ophioglossum* is complex and poorly understood.
23 It is unknown whether mature *O. vulgatum* sporophytes form mutualistic associations
24 with fungi of the Glomeromycota and with what specificity. Are green sporophytes
25 able to ‘repay’ fungal carbon invested in them by mycorrhizal partners during the
26 initially heterotrophic gametophyte and early sporophyte stages of the lifecycle?
- 27 • We identified fungal partners of *O. vulgatum* sporophytes using molecular techniques
28 and supplied them with ³³P-orthophosphate and *O. vulgatum* sporophytes with ¹⁴CO₂.
29 We traced the movement of fungal-acquired nutrients and plant-fixed carbon between
30 symbionts and analysed natural abundance ¹³C and ¹⁵N isotope signatures to assess
31 nutritional interactions.
- 32 • We found fungal specificity of *O. vulgatum* sporophytes towards a mycorrhizal
33 fungus closely related to *Glomus macrocarpum*. Our radioisotope tracers revealed
34 reciprocal carbon-for-phosphorus exchange between fern sporophytes and fungal
35 partners, despite competition from surrounding vegetation. Monocultures of *O.*
36 *vulgatum* were enriched in ¹³C and ¹⁵N, providing inconclusive evidence of
37 mycoheterotrophy when experiencing competition from the surrounding plant
38 community.
- 39 • We show mutualistic and specific symbiosis between a eusporangiate fern and fungi
40 of the Glomeromycota. Our findings suggest a ‘take now, pay later’ strategy of
41 mycorrhizal functioning through the lifecycle *O. vulgatum*, from mycoheterotrophic
42 gametophyte to mutualistic above-ground sporophyte.

43

44 **Key words:** Competition, mycoheterotrophy, mycorrhiza, Ophioglossaceae, *Ophioglossum*
45 *vulgatum*, pteridophyte, specificity, symbiosis

46

47 **Introduction**

48 The symbiosis between plants and arbuscular mycorrhizal fungi dates back more than 450
49 million years to the colonisation of the land by plants (Read *et al.*, 2000; Bonfante & Selosse,
50 2010). To better understand the role of mycorrhizal fungi in land plant evolution, there is
51 increasing interest in resolving relationships between plants and their fungal partners to
52 determine how these associations may have changed both across the land plant phylogeny
53 and functionally through coevolution (Bidartondo *et al.*, 2004; Merckx & Bidartondo 2008;
54 Arnold *et al.*, 2010; Merckx *et al.*, 2012; Merckx *et al.*, 2013). Advances in molecular and
55 physiological ecology have provided new insights into the evolutionary history of the
56 symbiosis in the major clades of plants and fungi (Wang *et al.*, 2010; Bidartondo *et al.*, 2011,
57 Field *et al.*, 2012, 2014). However, important knowledge-gaps regarding the fungal partners
58 of plants in key nodes of the land plant phylogeny remain (Fig. 1). In particular, neither the
59 identity of mycorrhizal fungi nor their functional roles have been determined in the widely
60 distributed (Singh *et al.*, 2009) basal euphyllophyte (“true-leaved plant”) genus
61 *Ophioglossum*, thought to have evolved prior to the break-up of Gondwana (Parris, 2001).

62 In common with >1,000 species of lycophytes and ferns, the subterranean gametophyte
63 generations of *Ophioglossum* are achlorophyllous, nourished with organic carbon and
64 nutrients via mycorrhizal fungi (Boullard, 1979; Leake, 1994; Winther & Friedman, 2007).
65 This form of nutrition, termed mycoheterotrophy, has evolved many times in land plants
66 (Leake, 1994; Bidartondo, 2005; Merckx & Freudenstein, 2010) with examples ranging from
67 a liverwort, to lycopods, ferns and angiosperms (Fig. 1). The initial developmental stages of
68 *Ophioglossum* sporophytes are also achlorophyllous (Bruchmann, 1904) and
69 mycoheterotrophic. However, mature sporophytes consist of a characteristic blade-like green
70 photosynthetic shoot (the trophophore) (Fig. S1a), often accompanied by an epiphyllous
71 fertile sporophore, in which the functional role of mycorrhiza has not been investigated.
72 Because of the life-stage changes from heterotrophy to autotrophy mycorrhizal functioning in
73 *Ophioglossum* is both complex and poorly understood.

74 *Ophioglossum vulgatum* L. (Fig. S1a), is one of the most widespread and abundant grassland
75 species in the Ophioglossaceae (GRIN taxonomic database). Sporophytes of *O. vulgatum*
76 are initially subterranean, achlorophyllous and colonised by aseptate fungi that form irregular
77 hyphal swellings in the plant tissues (Bruchmann, 1904; Boullard, 1979). These fungi must
78 provide the main carbon and nutrient supplies required to enable development of the
79 underground root axis from which shoots develop (Bruchmann, 1904).

80 Recent studies of achlorophyllous gametophyte and photosynthetic sporophyte generations of
81 lycopods (*Lycopodium* and *Huperzia*) and ophioglossoid ferns (*Botrychium*) have revealed
82 specificity and intergenerational fidelity in their arbuscular mycorrhizal fungal associates
83 (AMF) (Winther & Friedman, 2007, 2008, 2009). This suggests carbon invested by AMF
84 partners in supporting a mycoheterotrophic gametophyte and early subterranean sporophyte
85 may be repaid by established green sporophytes. Such ‘take now, pay later’ mycorrhizal
86 functioning has been suggested as the basis of fungal specificity and overall mutualism
87 through the mycoheterotrophic-to-autotrophic life stages of many green-leaved orchids
88 (Cameron *et al.*, 2008). However, experimental evidence for photosynthate ‘pay back’ to
89 fungal symbionts of any of the lower tracheophytes is currently lacking. Studies of
90 mycorrhiza specificity and functioning in these plants are of particular interest for species
91 like *O. vulgatum* that often inhabit plant species-rich permanent grasslands that host a highly
92 diverse community of AMF ranging from 24 to more than 70 phylotypes (Vandenkoornhuys
93 *et al.*, 2002; Johnson *et al.*, 2004; Dumbrell *et al.*, 2011).

94 Intergenerational fungal specificity opens the possibility of intergenerational carbon subsidy
95 from green-leaved sporophytes to achlorophyllous gametophytes via a shared fungal partner,
96 a form of ‘parental nurture’ suggested by Leake *et al.* (2008). This contrasts with the
97 suggestion that stabilization of mutualistic interactions in AM symbioses with autotrophs
98 involves the plants providing organic carbon strictly in proportion to the nutrients delivered
99 by the fungus (Fitter, 2006; Kiers *et al.*, 2011). Under the latter model of mutualism in the
100 chlorophyllous sporophyte, any fungal ‘reward’ for carbon investment in the gametophyte
101 and early stages of sporophyte establishment would be conditional upon ongoing nutrient
102 demand by the plant and its supply from the fungus. However, tight regulation of carbon-for-
103 nutrient exchange is not universal but represents only one position along the mutualism-
104 parasitism continuum model of mycorrhizal symbiosis proposed by Johnson *et al.* (1997;
105 Johnson & Graham, 2013).

106 There has been convergent evolution of mycoheterotrophic protocorm structures in lower
107 tracheophyte gametophytes and the mycoheterotrophic seedlings of higher plants like orchids
108 (Leake *et al.*, 2008). In the case of *O. vulgatum*, the underground early stages of sporophyte
109 ontogeny and anatomy closely parallel those of the mycoheterotrophic seedling stages of
110 members of the angiosperm genera *Monotropa* (Leake *et al.*, 2004) and *Pyrola* (Hashimoto *et*
111 *al.*, 2012; Hynson *et al.*, 2013a) along with those of the family Orchidaceae (see Fig. S1a-b),
112 in which germination leads to the formation of a simple branched root system from which

113 shoots later arise by adventitious buds, often only after several growing seasons underground.
114 Sprouting from detached roots has been reported in *O. vulgatum* (Bruchmann, 1904;
115 Wardlaw, 1953), providing a means of asexual reproduction and possible reversion to
116 mycoheterotrophy when roots are detached from photosynthetic shoots, a frequent trait of
117 mycoheterotrophic plants.

118 It may be possible to assess the extent to which green-leaved plants, such as trophophore-
119 bearing *O. vulgatum*, are partially mycoheterotrophic through measurements of their tissue
120 ^{13}C and ^{15}N natural abundance as these isotopes are often enriched in fully mycoheterotrophic
121 plants (Gebauer & Meyer, 2003; Cameron & Bolin, 2010; Hynson *et al.*, 2013b). However,
122 the extent of relative ^{13}C and ^{15}N enrichment of AM plants with putative and established
123 mycoheterotrophy has proved inconsistent (Courty *et al.*, 2011; Hynson *et al.*, 2013b).

124

125 Mycorrhizal colonization of *O. vulgatum* sporophytes has been reported many times, with
126 exquisite line drawings of hyphae in roots by Bruchmann (1904) and degenerated arbuscule-
127 like structures by Boullard (1979), as well as detailed light and electron micrographs of
128 vesicles and coils by Schmidt and Oberwinkler (1996), all of which are consistent with Paris-
129 type AMF (Zhang *et al.*, 2004). However, the fungal symbiont(s) in *O. vulgatum* have not
130 been identified and their functional roles are unknown for the green trophophore-bearing fern
131 sporophytes.

132 This study aims to address the following questions:

- 133 1. Do *O. vulgatum* sporophytes form mycorrhizal associations with AMF, and if so, with
134 what specificity?
- 135 2. Are mycorrhizal associations in the photosynthetic sporophyte generation mutualistic,
136 i.e. based on exchange of plant-carbon for fungal-acquired nutrients?
- 137 3. To what degree are fern shoots supported (via mycoheterotrophy) or out-competed by
138 surrounding vegetation within mixed communities?

139

140 **Materials and methods**

141 *Plant material and fungal identification*

142 Sporophytes of a natural population of *O. vulgatum* were collected as turf monoliths 30 cm
143 (w) x 40 cm (l) x 20 cm (d) sampled from across an area of base-rich grassland of

144 approximately 100 m x 20 m at the margins of mature deciduous woodland at Treborth
145 Botanic Gardens, Bangor, UK in July 2009 (Fig. S2). The vegetation community consisted
146 of grasses and forbs, dominated by *Plantago lanceolata*, *Ranunculus repens*, *Trifolium*
147 *pratense*, *Agrostis capillaris*, *Festuca rubra*, and *Rubus fruticosus*. Weeding created
148 experimental mesocosms comprising three community types: *O. vulgatum* in monoculture, *O.*
149 *vulgatum* within a mixed community of grasses and forbs, and the mixed community
150 vegetation only. Care was taken to ensure each turf contained ten *O. vulgatum* shoots and
151 regular weeding maintained the composition of each community. Each mesocosm was
152 replicated four times, giving a total of 12 experimental turfs. We created a further four plant-
153 free soil monoliths in which all plants were removed to provide controls for diffusion rates in
154 our ³³P and ¹⁴C isotope tracer studies described later.

155 We inserted soil-filled, cylindrical plastic cores (20 mm diameter, 90 mm length) with two
156 windows cut out from 30 mm below the top of the tube in a position below the soil surface
157 when the cores are inserted vertically, with 25 mm remaining above ground, covered by 35
158 μ m pore-size nylon mesh (Johnson *et al.*, 2001). The mesh was fine enough to exclude roots
159 while being coarse enough to allow fungal hyphal ingrowth (Fig. S3). The cores were filled
160 with native soil from the turfs mixed with 5 % v/v of sand-sized basalt grains added to
161 increase AM hyphal proliferation (Field *et al.*, 2012). We inserted ten cores vertically into
162 each turf regularly spaced with one further, glass wool-filled core inserted centrally for
163 below-ground gas sampling.

164 The mesocosms were maintained under controlled glasshouse conditions designed to provide
165 conditions similar to early summer (see SI) at the Arthur Willis Environment Centre,
166 University of Sheffield, UK. Turfs were maintained for three months to allow plant and
167 fungal acclimation, community stabilisation and colonisation of the mesh cores by fungi. In
168 late October 2009, the mesocosms were moved outdoors for vernalization for three months
169 over the winter. At the beginning of February 2010, the turfs were returned to the glasshouse
170 to induce early emergence of the *O. vulgatum* sporophytes, which commenced in the first
171 weeks of March 2010 (Fig. S2).

172 Roots were randomly sampled from individual *O. vulgatum* plants from six different
173 mesocosms and cleared and stained for quantification of mycorrhizal colonisation following
174 Trouvelot *et al.* (1986) (details in SI). In parallel, fungal DNA was extracted and sequenced
175 for a further six *O. vulgatum* root samples taken from plants removed from separate turfs in
176 July 2009 (Fig. S4) following the protocols of Desirò *et al.*, 2013 (details in SI).

177 Representative DNA sequences have been deposited in GenBank (accession numbers
178 KJ952225-6, KM065416-20).

179 *Quantification of reciprocal plant-fungal ^{14}C for ^{33}P exchange*

180 Plant-to-fungus C transfer and fungus-to-plant ^{33}P assimilation was quantified using methods
181 adapted from Johnson *et al.* (2001, 2002) and Field *et al.* (2012, 2014). Following emergence
182 of *O. vulgatum* shoots in March/April 2010, 1.4 MBq of ^{33}P (specific activity 148 GBq
183 mmol^{-1}) as 38 μl of $\text{H}_3^{33}\text{PO}_4$, comprising 312 ng ^{33}P (Perkin Elmer, UK) in total were added to
184 each turf, including plant-free soil monoliths, via a perforated central capillary tube in each of
185 the ten mesh-windowed cores established the previous year.

186 After 28 d the turfs were prepared for ^{14}C labelling. Anhydrous lanolin was applied to the
187 rim of each mesh windowed core and the top sealed with a plastic cap. The glass wool-filled
188 core in each turf was sealed with a rubber septum (SubaSeal, Sigma) to allow non-destructive
189 monitoring of below-ground $^{14}\text{CO}_2$ respiration. The turfs remained in the greenhouse but were
190 each enclosed in a gas-tight transparent Perspex chamber into which 2 MBq of $^{14}\text{CO}_2$,
191 comprising 13.7 μg ^{14}C , was released from $\text{NaH}^{14}\text{CO}_3$ by addition of 2 ml 10% lactic acid
192 soon after sunrise (07:00 h). After 1 h, and every 4 h thereafter, we sampled 1 ml of the
193 headspace air in the Perspex chambers above each turf through a rubber septum (SubaSeal,
194 Sigma) using a syringe. Similarly, a sample of 1 ml of air from the glass-wool filled mesh
195 cores was taken after 1 h and every 2 h thereafter. We ceased air sampling and removed turfs
196 from the labelling chambers following a peak in $^{14}\text{CO}_2$ detected in below-ground gas samples
197 occurring between 14 and 16 h post- $^{14}\text{CO}_2$ release. This time-point indicates maximum flux
198 of ^{14}C -labelled photosynthates to symbionts and other soil micro-organisms through inclusion
199 of the isotope in below-ground respired CO_2 . The air samples were injected through a
200 SubaSeal into gas-evacuated vials containing 10 ml CarboSorb (Perkin Elmer, Beaconsfield,
201 UK). Permafluor scintillation cocktail (Perkin Elmer, Beaconsfield, UK) was then added (10
202 ml) and ^{14}C measured by liquid scintillation counting (Packard Tri-Carb 3100TR, Isotec,
203 UK).

204 Once the ^{14}C detected in the glass-wool filled mesh windowed cores had peaked, we removed
205 the soil-filled cores and placed them into individual airtight containers with 2 ml 2M KOH to
206 trap respired $^{14}\text{CO}_2$. Cores were incubated with fresh 2M KOH every 2 h for a further 6 h. We
207 transferred 1 ml of each KOH sample used to trap $^{14}\text{CO}_2$ into a scintillation vial containing 10

208 ml Ultima Gold scintillation cocktail and radioactivity was measured by liquid scintillation
209 (Packard Tri-carb 3100TR, Isotech).

210 We removed above-ground vegetation from each turf and divided the samples into *O.*
211 *vulgatum* and ‘plant community’ tissue samples. We sieved the soil to extract and separate
212 the *O. vulgatum* and plant community roots. For the plant-free soil monoliths the bulk soil
213 outside the mesh cores was sieved, homogenized and subsampled for ^{33}P determination to test
214 for diffusion and non-plant-mycorrhiza mediated transfer of isotope from the cores to the
215 surrounding soil.

216 All plant and soil samples were freeze-dried, weighed and homogenised into a powder using
217 a Yellowline A10 Analytical Grinder (IKA, Germany). Samples underwent acid-digestion
218 before mixing with Emulsify-Safe (Perkin Elmer, Beaconsfield, UK) and ^{33}P activity
219 measured by liquid scintillation counting (details in SI). The ^{33}P content in plant tissues was
220 calculated by following the equation in Cameron *et al.* (2007) after decay correction and
221 scaled to plant biomass to give total plant ^{33}P (see SI).

222 To quantify ^{14}C present within each sample, between 10 and 100 mg of freeze-dried
223 homogenized sample were placed into individual Combusto-Cones (Perkin Elmer), oxidised
224 in a Model 307 Packard Sample Oxidiser, (Isotech, UK) with the $^{14}\text{CO}_2$ trapped in 1:1 v/v
225 Carbosorb: Permafluor scintillation cocktail and counted (Packard Tri-Carb 3100TR,
226 Isotech). Total C fixed by the plants and made available to AM fungal networks within soil
227 cores (hereafter termed ‘fungal carbon’) was determined by calculating the difference in ^{14}C
228 found in the soil cores in vegetated turfs compared to those in the plant-free soil monoliths.
229 The calculated quantity of ^{14}C allocated from the plants into the mesh-windowed cores as
230 fungal carbon, after correction using the plant-free controls, was expressed as a function of
231 total volume of atmospheric CO_2 in the labeling chamber and the proportion of the supplied
232 $^{14}\text{CO}_2$ label fixed by the plants (Cameron *et al.*, 2008) (see SI).

233 *Natural abundance ^{13}C and ^{15}N enrichment and total % N*

234 Relative abundances of ^{13}C and ^{15}N and tissue %N were determined using Isotope Ratio Mass
235 Spectrometry. Between 2 and 5 mg of freeze-dried homogenised root and shoot tissues of *O.*
236 *vulgatum* and community plants from all experimental turfs ($n = 3$ for each tissue type in
237 each turf) were weighed out into 6 x 4 mm tin capsules (Sercon Ltd, UK) and analysed using
238 a continuous flow Isotope Ratio Mass Spectrometer (PDZ 2020 IRMS, Sercon Ltd, UK).
239 Data are expressed as δ values, which were calculated using the following equation:

240 $\delta^{13}\text{C}$ or $\delta^{15}\text{N} = 1000[(R_{\text{sample}} / R_{\text{standard}}) - 1]$

241 Where R = molar ratio of $^{13}\text{C}/^{12}\text{C}$ or $^{15}\text{N}/^{14}\text{N}$ of the sample or standard (Hynson *et al.*,
242 2013b). Air was used as the reference standard for ^{15}N and commercially available poplar
243 leaf standards for ^{13}C after every 10 samples analysed. The detector was regularly calibrated
244 to commercially available reference gases.

245

246 *Statistical analyses*

247 The effect of monoculture of *O. vulgatum* versus growth in grassland community, and the
248 effects on the community of the presence or absence of *O. vulgatum* on biomass, $\delta^{13}\text{C}$ and
249 $\delta^{15}\text{N}$, %N, ^{33}P uptake and ^{14}C allocation were tested using ANOVA with post-hoc Tukey
250 testing where appropriate. Data were checked for normality and homogeneity of variance.
251 Where assumptions for ANOVA were not met, data were transformed using Log_{10} and
252 arcsine-square-root transformations as indicated in Table 1. All statistics were calculated in
253 Minitab v12.21 (Minitab Inc., US). Different letters in figures denote statistical significance
254 (following transformation where necessary, see Table 1) where $P < 0.05$ and error bars show
255 ± 1 standard error.

256

257 **Results**

258 *Mycorrhizal colonisation and fungal identification.*

259 *Ophioglossum vulgatum* roots ($n = 6$ plants) showed a 100% frequency of mycorrhizal
260 colonisation (Table S1) with both coils and arbuscules being present (Fig. S5). The mean
261 colonisation intensity was just over 40% of root segments examined. Arbuscule abundance
262 was low, with a mean of 7% of root segments examined. The six *O. vulgatum* sporophytes
263 contained a fungal associate from the Glomeraceae that corresponded closely with the
264 published sequences for *Glomus macrocarpum*, one also harboured a member of
265 Diversisporaceae (Fig. 2; GenBank accession numbers KJ952225-6, KM065416-20).

266 *Biomass*

267 *Ophioglossum vulgatum* roots and shoots had 4 and 8.5 times greater biomass respectively
268 when grown in monoculture than in the plant community turfs (Fig. 3a, Table 1) even though
269 the mesocosms contained identical numbers of *O. vulgatum* shoots (Fig. S4). The

270 surrounding plant community showed no difference in root or shoot biomass whether *O.*
271 *vulgatum* sporophytes were present or had been removed from the turf (Fig. 3a).

272 The root:shoot biomass ratio of *O. vulgatum* more than doubled when the ferns were grown
273 within mixed community turfs compared to monocultures, and in the community it was 22
274 times higher than the surrounding vegetation (Fig. 3b). There was no significant difference in
275 root:shoot biomass ratio of the community vegetation whether the ferns were present or
276 removed.

277

278 *Plant-to-fungus carbon transfer into soil compartments.*

279 *Ophioglossum vulgatum* monocultures supplied the fungal networks within soil cores with
280 just over half the amount of carbon provided by the grassland community turfs, although the
281 shoot biomass of monoculture *O. vulgatum* was only 1/8th of that of the community (Fig. 3a
282 and 4a). Removal of *O. vulgatum* had no effects on total fungal C allocated into the soil cores
283 by the plant community.

284 Carbon allocation to fungus in the mesh-windowed cores per unit of shoot biomass, was more
285 than four times higher for monocultures of *O. vulgatum* than for the plant community turfs,
286 (Fig. 4b, Table 1) irrespective of whether the latter contained *O. vulgatum* or not.

287 Shoots of the grassland community retained just over double the amount of new
288 photosynthate (¹⁴C + ^{12/13}C) found in *O. vulgatum* shoots grown in monoculture. The
289 suppressed shoots of *O. vulgatum* grown in the community turfs contained negligible
290 amounts of new photosynthate (Fig. 4c). In monoculture, *O. vulgatum* allocated three-fold
291 more photosynthate to its roots than the grassland community, and the community-suppressed
292 ferns showed negligible photosynthate allocation to roots (Fig. 4c, Table 1).

293 The concentration of new photosynthate in *O. vulgatum* monoculture shoots was between
294 three and four-fold higher than in the shoots of the plant community [two-sample t (4 df) = -
295 6.47, $P = 0.0029$], and 17-fold higher than in the fern when grown in the community turfs
296 [two-sample t (3 df) = 9.39, $P = 0.0026$] (Fig. 4d, Table 1). Similarly, new photosynthate
297 concentration in roots of monocultures of the fern exceeded substantially those of the turfs
298 [two-sample t (3 df) = 6.89, $P = 0.0063$] and the suppressed ferns in the community turfs
299 [two-sample t (3 df) = 7.31, $P = 0.0053$] (Fig. 4d).

300

301 *Fungus ³³P uptake from mesh-windowed soil cores and transfer to plants*

302 We were unable to detect any ³³P transfer from mesh-walled cores to bulk-soil in the plant-
303 free mesocosms, indicating that transfer of ³³P to the plants in the vegetated mesocosms was
304 driven by plant-fungal associations. The uptake of ³³P into *O. vulgatum* shoots was strongly
305 affected by competition from other plants, falling from 4 ng when grown in monoculture to
306 less than 0.06 ng when grown in the grassland community (Fig. 5a, Table 1). In monoculture
307 *O. vulgatum* took up ~30% of the total ³³P that was assimilated by the grassland community
308 on its own, whereas when grown in direct competition with the community, the fern took up
309 < 0.7% of the ³³P gained by neighbouring plants. Removal of *O. vulgatum* had no effect on
310 ³³P uptake into shoots of the community plants (Fig. 5a).

311 ³³P concentration in shoots of *O. vulgatum* grown in monoculture was 2.4 times higher than in
312 the shoots of the plant community without the fern. In the plant community-grown *O.*
313 *vulgatum* shoot ³³P concentrations declined by nearly an order of magnitude (8.6) to become
314 no longer significantly different to that of shoots of the surrounding plants (Fig. 5b).

315

316 *Plant N concentration and natural abundance ¹⁵N and ¹³C*

317 Overall, *O. vulgatum* shoots had higher N concentrations than community shoots, being 43%
318 higher than the community shoot concentrations when grown separately, and 83% higher
319 than the community values when grown together [two-sample t (10 df) = 3.35, P = 0.012].
320 Root %N in *O. vulgatum* was consistently higher than in the both root and shoots of the
321 community plants, but this was not statistically significant (Fig. 6a, Table 1). Monoculture
322 and community-grown *O. vulgatum* showed no significant differences in shoot or root %N,
323 and there was no effect of removal of the fern on the %N in the community plant shoots or
324 roots (Fig. 6a).

325 *Ophioglossum vulgatum* shoots and roots had greater ¹⁵N abundance than the grassland
326 community plants (Fig. 6b). The mean ¹⁵N abundance in *O. vulgatum* shoots grown in
327 monoculture was higher than that of community plants by $\delta^{15}\text{N}$ values of 3.2 ‰ (two-sample
328 t (10 df) = 5.53, P = 0.0003), although there was no significant difference between fern $\delta^{15}\text{N}$
329 values when it was grown in monoculture or within a mixed community. Overall, *O.*
330 *vulgatum* root tissues had greater abundance of ¹⁵N than the grassland community plant roots
331 $\delta^{15}\text{N}$ values by 1.6 ‰, (two sample t (13 df) = 2.52, P = 0.026). There was no significant

332 difference in root ^{15}N abundance between ferns grown in a mixed community or those grown
333 in monoculture (Fig. 6b, Table 1).

334 *Ophioglossum vulgatum* shoots contained a greater abundance of ^{13}C when grown in
335 monoculture compared to the shoots of the community turfs [two-sample t (7 df) = 4.04, P =
336 0.0049], and compared to the fern grown in the plant community (Fig. 6c, Table 1). ^{13}C was
337 more abundant in *O. vulgatum* grown within mixed community turfs than in the community
338 with which it was growing, but it was not different from the shoots of the community from
339 which it had been excluded the previous year. There was no significant difference between
340 the $\delta^{13}\text{C}$ values in the plant communities with, and without, *O. vulgatum* present (Table 1).
341 Root tissue ^{13}C abundance showed similar responses to the shoots, but the differences were
342 not significant (Fig. 6c).

343

344 **Discussion**

345 *Do O. vulgatum* sporophytes form mycorrhizal associations with AMF, and if so, with what
346 specificity?

347 Our study provides the first definitive evidence that photosynthetic sporophytes of a
348 eusporangiate fern in the genus *Ophioglossum* form mutualistic mycorrhizal associations with
349 a fungus in the Glomeromycota. Field-collected roots of *Ophioglossum vulgatum* were all
350 colonised by a mycorrhizal symbiont closely related to *Glomus macrocarpum* (Fig. 2 and Fig.
351 S5). The fungus formed hyphal coils and arbuscules throughout the root cortex, consistent
352 with previous reports indicating ‘Paris-type’ AMF colonization in the sporophyte of *O.*
353 *vulgatum* (Russow, 1872; Bruchmann, 1904; Boullard, 1976; Schmidt and Oberwinkler 1996;
354 Zhang *et al.*, 2004).

355

356 *Glomus macrocarpum* is widely distributed throughout grassland, herbaceous and woodland
357 habitats, being especially abundant in association with *Acer* tree species (Klironomos &
358 Kendrick 1996). While there are a number of reports of this fungal species increasing host
359 plant biomass production and phosphorus uptake (Nandini & Tholkappian, 2012), it is also
360 the causal agent of tobacco stunt disease (Guo *et al.* 1994). Large reproductive structures
361 formed by *G. macrocarpum* and stunting of some host plants suggest that colonisation by this
362 fungus can place a high carbon demand on host, potentially representing a substantial carbon
363 source for the mycoheterotrophic gametophytes of *O. vulgatum*. Moreover, *G. macrocarpum*

364 is a derived species of *Glomus* (Glomeraceae) (Fig. 2), a feature shared with the fungal
365 symbionts of members of the sister Ophioglossaceae genus *Botrychium* (Winther &
366 Friedman, 2007). Both *B. lanceolatum* and *B. crenulatum* have specialised on a narrow range
367 of Glomeromycota fungi (Merckx *et al.*, 2012). The specialisation of some ferns on these
368 closely related fungal taxa suggests that plant and fungal partners have co-evolved through
369 phylogenetic tracking of the partnership over millions of years, as has been shown in the
370 highly AM-specific mycoheterotroph genus *Afrothismia* (Burmanniaceae) in which more
371 recently diverged members of this genus associate with Glomeromycete fungi that have
372 diverged from a specific fungal partner of the ancestral plants (Merckx & Bidartondo, 2008).
373

374 The apparent specificity of *O. vulgatum* is consistent with the high fungal specificity in the
375 sister genus *Botrychium* where it is linked to intergenerational gametophyte-sporophyte
376 fidelity (Winther & Friedman, 2007), and contrasts to many other fern species which
377 typically host several species of Glomeromycota fungi (West *et al.*, 2009). Fungal specificity
378 and intergenerational fidelity is documented in several lycopods (see Fig. 1) including
379 *Lycopodium clavatum* and *Huperzia* spp. (Winther & Friedman, 2008). In these cases,
380 fidelity is maintained, even though there is no fungal colonisation across the gametophyte-
381 sporophyte tissue junctions (Ligrone *et al.*, 1993; Winther & Friedman, 2007, 2008). In the
382 *Botrychium* species studied by Winther & Friedman (2007), and in *Psilotum nudum* (Winther
383 & Friedman, 2009; Fig. 1), the mycoheterotrophic gametophytes show extreme fungal
384 specificity, each plant typically only associated with a single fungal partner. However, the
385 green shoot stage of sporophyte generations often host several mycorrhizal fungi in its roots
386 in addition to retaining the specific partner of the gametophytes. The single *O. vulgatum* root
387 that contained a fungus from the Diversisporaceae in addition to *G. macrocarpum*, may be an
388 example of this, but a larger number of plants across a number of sites would need to be
389 sampled to resolve this issue.

390

391 We were unable to find or raise any *O. vulgatum* gametophytes to test for intergenerational
392 fidelity in its AMF. Assuming this specificity occurs at all developmental stages, we concur
393 with the previously published notion that parental nurture may occur from photosynthetic
394 sporophyte-to-mycoheterotrophic gametophyte via interconnecting fungal partners, as
395 suggested for members of the Lycopodiaceae and Ophioglossaceae (Winther & Friedman,
396 2008; Leake *et al.*, 2008).

397

398 *Are mycorrhizal associations in the photosynthetic sporophyte generation mutualistic, i.e.*
399 *based on exchange of plant-carbon for fungal-acquired nutrients?*

400 Using isotope tracers, we clearly demonstrate the reciprocal exchange of plant-carbon for
401 fungal-acquired nutrients (N and P) between the green sporophyte of *O. vulgatum* and its
402 fungal partner, *G. macrocarpum*. This life-stage dependent trophic switch from heterotrophic
403 juvenile to autotrophic adult is not uncommon and has been observed across a diverse array
404 of tracheophytes including most orchids (Leake and Cameron 2010). The substantial carbon
405 allocation to the external mycorrhizal mycelium in our *O. vulgatum* monocultures provides
406 the first experimental evidence that carbon invested by a fungus in supporting the
407 heterotrophic juvenile life stage of a eusporangiate fern may be repaid with photosynthates
408 later.

409
410 In this ‘take now, pay later’ form of mutualism, fungal partners may have to wait many years
411 before they are repaid for their investment of carbohydrate in the plants (Cameron et al. 2008;
412 2006). This contrasts with the view that mycorrhizal mutualism in plant communities is
413 stabilized by a ‘biological market’ in which plant photosynthate is exchanged for fungal-
414 acquired nutrients in a tightly coupled pay and reward system. This is conceptualized as
415 involving either preferential photosynthate supply to fungal partners that are delivering the
416 greatest amounts of growth-limiting nutrients (Fitter, 2006; Kiers *et al.*, 2011), or fungal
417 partners preferentially allocating nutrients to host plants supplying the greatest amount of
418 photosynthate (Fellbaum *et al.*, 2012; Fellbaum *et al.*, 2014). Clearly, in plants that for part
419 of their lifecycles do not photosynthesise, during which time they are mycoheterotrophic, the
420 biological market model of mutualism cannot be operational (Selosse & Rousset, 2011). We
421 hypothesise that high fungal specificity and fidelity through mycoheterotroph-to-autotroph
422 plant lifecycles, stabilizes the symbiosis through selection for net overall fitness benefits for
423 both partners over their lifetimes. However, such a symbiosis does leave the fungi vulnerable
424 to exploitation in cases where the plants do not repay them in full as in complete
425 mycoheterotrophs (Leake & Cameron 2010).

426
427 *To what degree are fern shoots supported (via partial mycoheterotrophy) or out-competed by*
428 *surrounding vegetation within mixed communities?*

429 The major impact of the plant community is to partially suppress both trophophore and root
430 biomass, together with reducing ³³P uptake by *O. vulgatum*. Moreover, under competition

431 from the grassland community the root:shoot ratio of *O. vulgatum* increased, likely reflecting
432 reduced shoot growth in response to light, water or most likely, nutrient limitation. Together,
433 these observations demonstrate the superior competitive ability of the community vegetation
434 compared to this fern.

435

436 Previous work has shown the efficiency of phosphorus exchange for photosynthates between
437 plants and AM fungi increases with the evolution of increasing plant complexity from
438 liverworts to a fern and angiosperm (Field *et al.*, 2012). This is consistent with the results of
439 the present study. In nature, *O. vulgatum* often occurs in mixed communities adjacent to
440 woodlands where there is likely to be at least some grazing pressure. We did not simulate
441 grazing or mowing of the community, consequently the herbaceous plant community grew
442 taller, and probably produced denser shade than in the woodland margin site from which the
443 *O. vulgatum* turfs originated. The fern trophophore has a small photosynthetic area, simple
444 vasculature and limited root surface area, so is poorly adapted to compete for light, water and
445 nutrients with the more structurally advanced angiosperm community. Although we did not
446 measure it directly in this investigation, low rates of photosynthesis are reported in *O.*
447 *vulgatum* (Löhr, 1968). We expect this to also be the case in the shaded examples within
448 mixed community turfs presented here.

449

450 Partial mycoheterotrophy is typically accompanied by a suite of distinguishing physiological
451 characteristics, including elevated tissue ^{13}C and ^{15}N contents. Such traits have been seen in
452 green-leaved orchids as well as in mycoheterotrophic orchid protocorms and fully
453 mycoheterotrophic adult orchids (Gebauer & Meyer, 2003; Leake & Cameron, 2010; Hynson
454 *et al.*, 2013b; Stöckel *et al.*, 2014;) along with some mycoheterotrophic plants associated with
455 AM fungi (Cameron & Bolin 2010; Merckx *et al.* 2010; Courty *et al.* 2011). Under
456 suppression by the plant community, we expected that if the sporophyte were able to revert to
457 partial mycoheterotrophy, this would be reflected in the stable isotope abundances of ^{15}N and
458 ^{13}C compared to *O. vulgatum* grown in monoculture. In contrast to our expectations, the ^{13}C
459 abundance of fern shoots was higher when grown in monoculture and there were no
460 differences in ^{13}C in roots or ^{15}N in both roots and shoots of fern monocultures versus the
461 ferns grown in the grassland community (Fig. 6d). However, the fern consistently contained
462 higher concentrations of these isotopes than the mixed-species community, most likely
463 resulting from high rates of carbon allocation belowground to their mycorrhizal partner and
464 to storage, both causing preferential respiratory losses of ^{12}C , and storage of N resulting in

465 enrichment in ^{15}N . When grown in the turf, *O. vulgatum* trophophores have $\delta^{13}\text{C}$ values
466 closer to those of the community plants, either as a result of reduced allocation of C to below-
467 ground storage (Fig. 4c-d) or mycoheterotrophic carbon gain from surrounding vegetation.
468 Unfortunately, our experimental design does not allow us to resolve the extent to which either
469 (or both) processes are involved and this issue warrants further investigation.

470

471 *Conclusions and perspectives:*

472 This study represents the first assessment of mycorrhizal functioning in a eusporangiate fern
473 revealing the association is mutualistic in mature sporophytes. The symbiosis appears to
474 operate on a ‘take-now, pay-later’ basis (Cameron *et al.*, 2008) with fully mycoheterotrophic
475 subterranean gametophytes and early achlorophyllous sporophyte stages (Bruchmann, 1904;
476 Boullard, 1979; see Fig. 1), followed by the photosynthetic trophophore of the sporophyte
477 participating in bidirectional exchange of organic carbon-for-nutrients with a fungus closely
478 related to *Glomus macrocarpum*. Whether the sporophyte then reverts to mycoheterotrophy
479 during its below-ground dormancy period from mid-summer until early spring of the
480 following year requires further investigation.

481

482 Our findings support the hypothesis that other early-diverging tracheophytes with
483 mycoheterotrophic gametophytes that retain the same fungal partners as in their sporophytes,
484 are also likely to operate this form of mutualism. This strengthens the view that specificity
485 and fidelity may be important in stabilizing the benefits of mutualistic interactions for
486 mycoheterotroph-to-autotrophic lifecycle transitions in plants. Our findings provide clear
487 evidence of the strength of competitive interactions and suppression of the growth of *O.*
488 *vulgatum* sporophytes by angiosperms, but we were unable to establish the extent to which
489 sporophytes are mycoheterotrophic when the trophophore is not present. Further
490 experimental work is required to resolve the relative carbon contributions of ophioglossoid
491 ferns to shared mycorrhizal networks and whether this carbon contributes to the
492 establishment of the subterranean gametophyte and initial stages of the sporophyte
493 generations.

494

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 500 comments that have improved the manuscript.

501

502 **Tables**

503 **Table 1. Summary of differences in resource allocation and acquisition (*F* ratio from**
 504 **ANOVA) in root and shoot tissues of *Ophioglossum vulgatum* and community**
 505 **vegetation.** All two-way ANOVA have 3, 24 d.f., and 3, 15 d.f. for one-way ANOVA. **P* <
 506 0.05, ***P* < 0.01, ****P* < 0.001; post-hoc Tukey test (*n* = 4). Where assumptions for ANOVA
 507 were not met, data were transformed as indicated in the table.

	Turf treatment	Tissue type	Turf x tissue
Dry mass (g) (<i>log</i> (10) transformed)	37.59 ***	0.04	12.51 ***
Root : shoot (g) (<i>log</i> (10) transformed)	13.05 ***		
Fungal C / above-ground plant biomass (ng g ⁻¹)	7.70 *		
Fungal C (ng)	4.43 *		
Total ¹⁴ C in plant tissues (ng) (<i>log</i> (10) transformed)	41.72 ***	28.72 ***	9.60 ***
[¹⁴ C] in plant tissues (ng g ⁻¹) (<i>arcsine square root transformed</i>)	268.36 ***	127.20 ***	19.24 ***
³³ P in shoot tissues (ng) (<i>arcsine square root transformed</i>)	58.98 ***		
[³³ P] in plant tissue (ng g ⁻¹) (<i>arcsine square root transformed</i>)	11.51 **		
Plant tissue % N	7.48 **	12.19 **	3.78 *
δ ¹⁵ N in shoots	11.20 ***	1.93	1.42
δ ¹³ C in shoots	10.63 ***	22.95 ***	4.10 *

508

509 **Figure legends**

510 **Figure 1.** Evolution of mycoheterotrophy in land plants. Lineages where myco-heterotrophy
511 is present indicated with bold branches, dashed branches show clades with myco-
512 heterotrophic gametophytes. Text in brackets indicates genus/families within clades
513 displaying myco-heterotrophic properties. Clades with arbuscular mycorrhizas are indicated
514 with an asterisk (adapted from Bidartondo, 2005 and Field *et al.*, 2012)

515 **Figure 2.** Molecular identification of fungal symbionts within root tissues of *O. vulgatum*
516 sporophytes showing phylogenetic placement within AM fungi. Taxonomy follows Schüßler
517 *et al.* (2011). Maximum likelihood tree produced in MEGA v.5, evolutionary model T92 + G
518 + I with 10,000 bootstrap replicates.

519 **Figure 3 (a)** Total dry mass of above-ground and below-ground plant tissues in experimental
520 turfs (g). (*n* = 4) **(b)** Root:shoot dry mass ratios of plant tissues in experimental turfs (*n* = 4),
521 ± 1 S.E. Letters show statistical significance, where *P* < 0.05 (ANOVA, Tukey post-hoc, data
522 log(10) transformed to meet assumptions for ANOVA). Note that the letter codes in (a)
523 compare both root and shoot biomass.

524 **Figure 4 (a)** Total carbon allocated by plant assemblages to below-ground mycorrhizal
525 networks per gram of above-ground plant biomass (ng g⁻¹) **(b)** Total carbon allocated by plant
526 assemblages to mycorrhizal fungal networks within turfs (ng) **(c)** Total new photosynthate
527 (ng) within plant tissues following the ¹⁴C labelling period **(d)** Tissue concentration of new
528 photosynthate following the ¹⁴C labelling period (ng g⁻¹). Error bars show ± 1 S.E. Different
529 letters denote statistical significance, where *P* < 0.05 (ANOVA, Tukey post-hoc, data
530 transformed using log(10) and arcsine-square-root transformations where assumptions of
531 ANOVA were not met) *n* = 4.

532 **Figure 5 (a)** Total ³³P assimilation into above-ground tissues of plants in experimental turfs
533 (ng) **(b)** ³³P concentration (ng g⁻¹) of above-ground *O. vulgatum* and mixed community plant
534 tissues (*n* = 4). Error bars show ± 1 S.E. Different letters indicate where *P* < 0.05 (ANOVA,
535 Tukey post-hoc. Data arcsine-square-root transformed where assumptions for ANOVA not
536 met).

537 **Figure 6 (a)** Total tissue % N of plants within turfs **(b)** δ¹⁵N enrichment of plant tissues
538 within turfs **(c)** δ¹³C enrichment in above-ground biomass for *O. vulgatum* and mixed
539 community plants in experimental turfs **(d)** δ¹³C vs. δ¹⁵N enrichment of plant tissues (*n* = 4).

540 In all panels, error bars show ± 1 S.E. Different letters denote statistical significance, where P
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542

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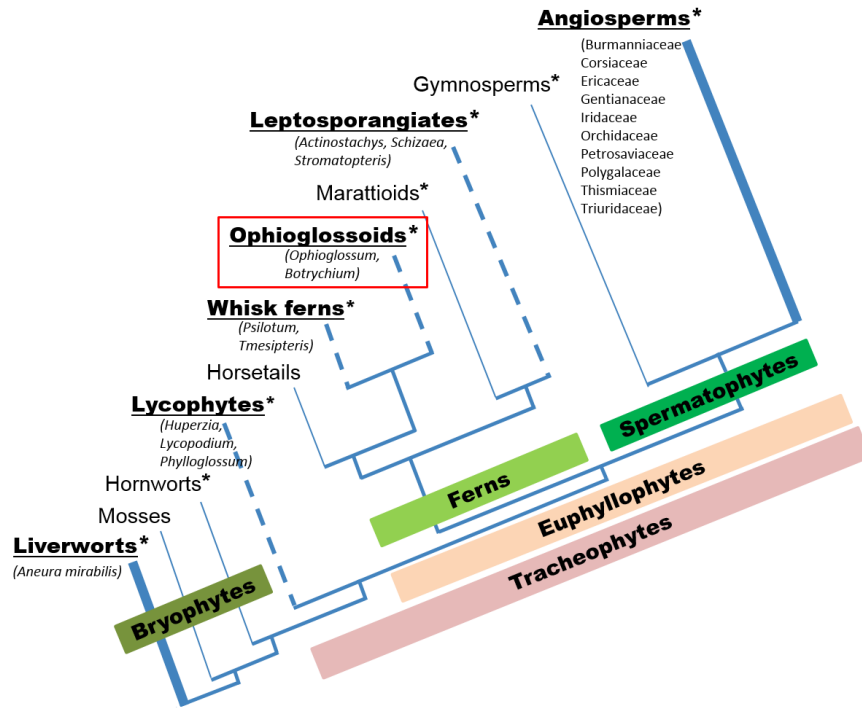
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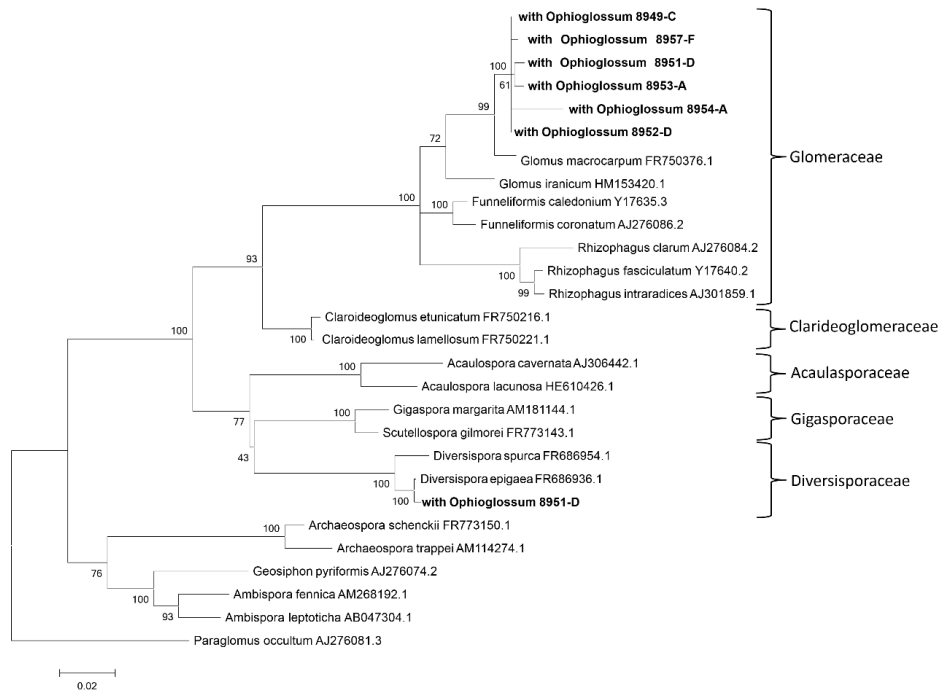
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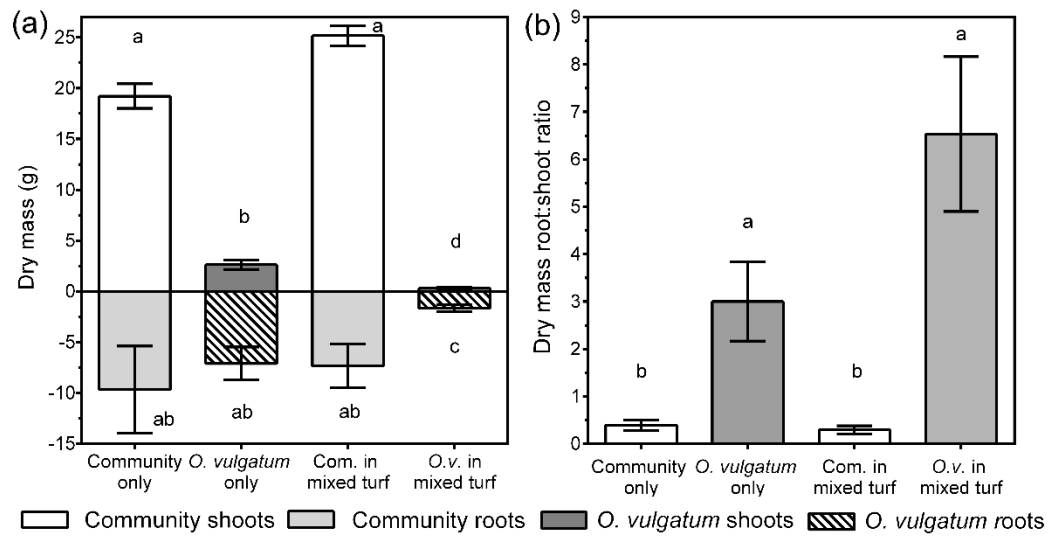
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753 **Figure 1**

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759 **Figure 2**

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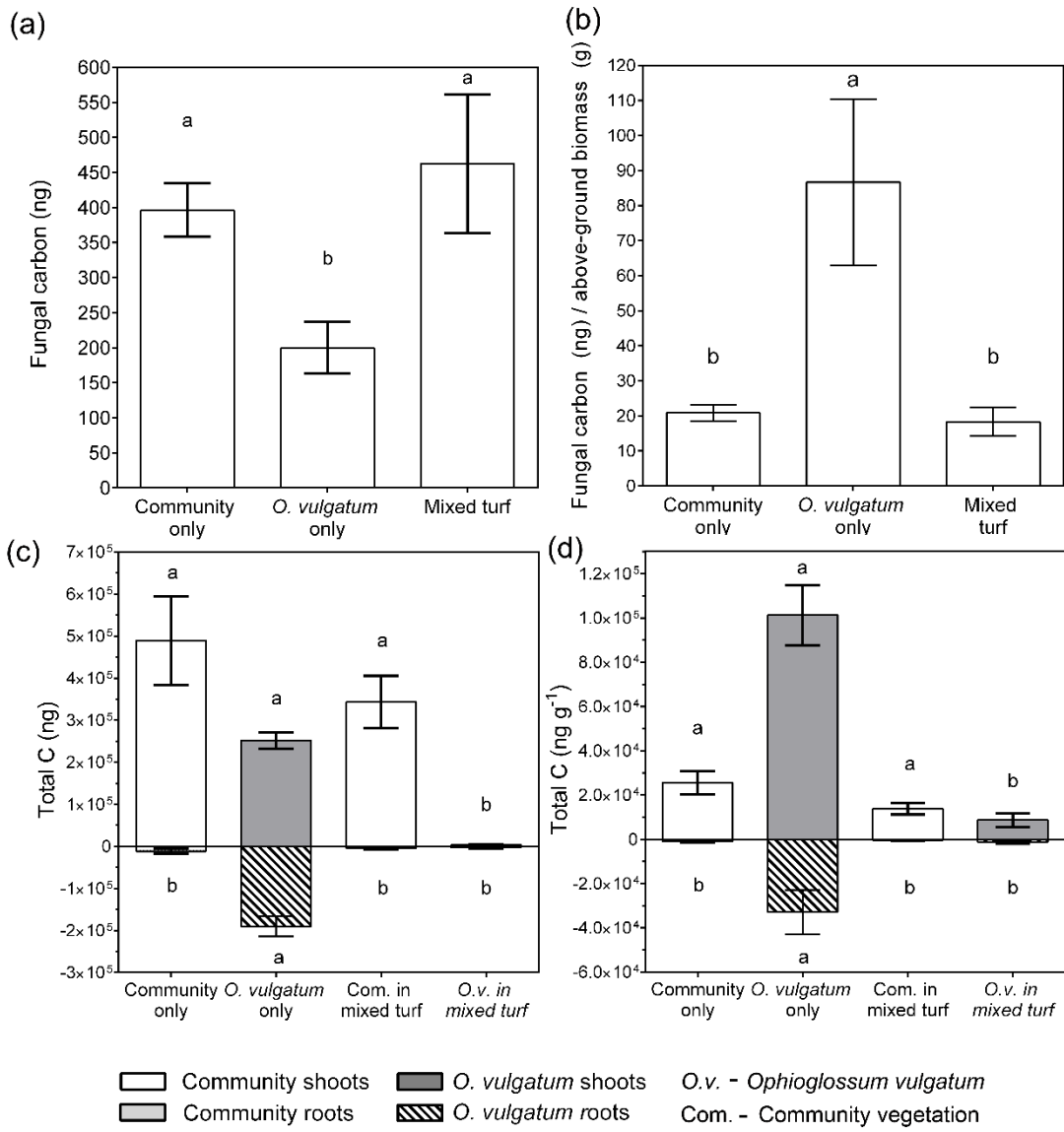
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783 **Figure 3**

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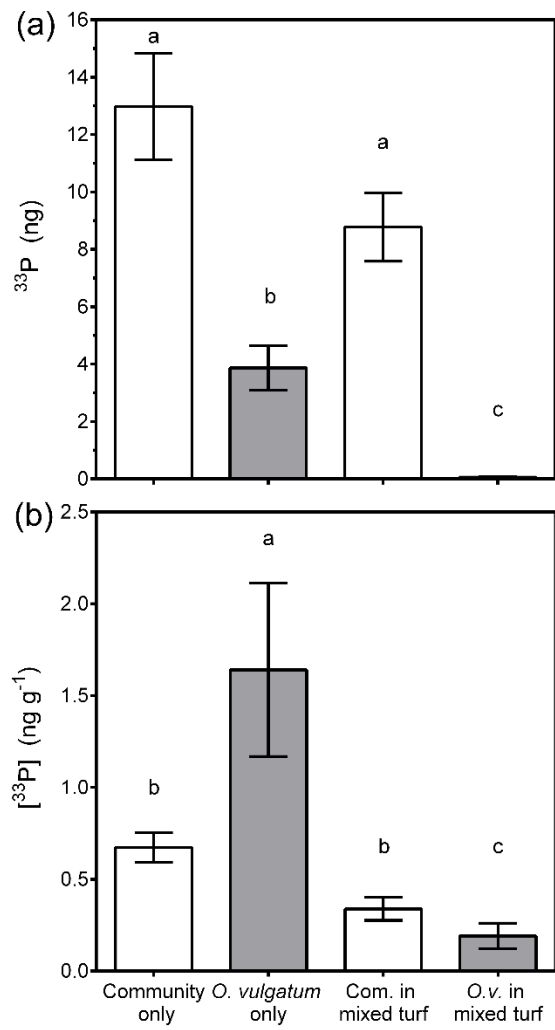
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794 **Figure 4**

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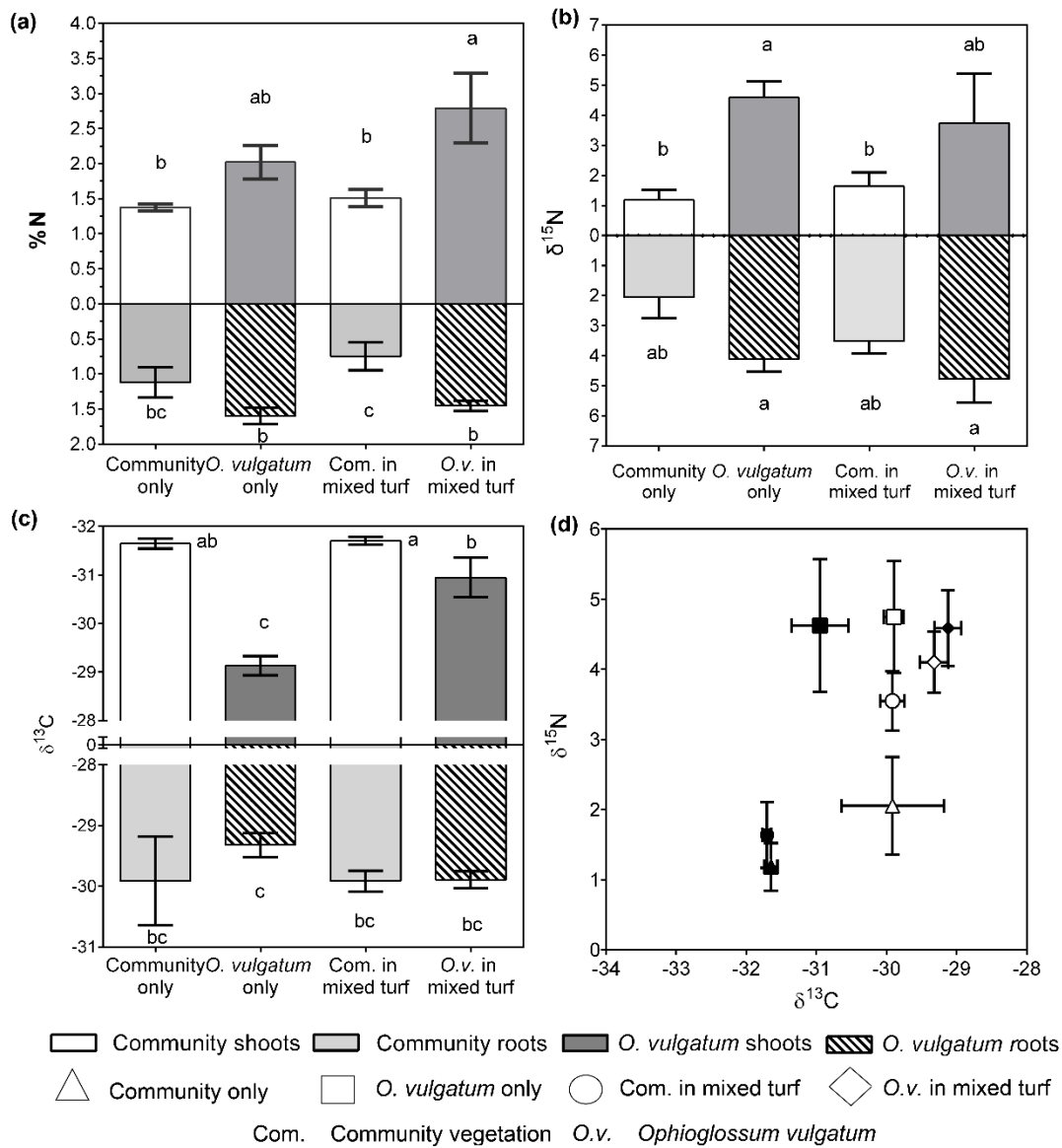
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 807 **Figure 5**
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