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*Improved persistence of red clover (*Trifolium pratense* L.) increases the protein supplied by red clover/grass swards grown over four harvest years*

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1 **Improved persistence of red clover (*Trifolium pratense* L.) increases the protein**
2 **supplied by red clover/grass swards grown over four harvest years**

3

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12 **Abstract**

13 UK livestock agriculture can significantly reduce its protein imports by increasing the
14 amount of forage based protein grown on-farm. Forage legumes such as red clover
15 (*Trifolium pratense* L.) produce high dry matter yields of quality forage but currently
16 available varieties lack persistence, particularly under grazing. To assess the impact
17 of red clover persistence on protein yield, diploid red clover populations selected for
18 improved persistence were compared with a range of commercially available
19 varieties. All populations were grown over four harvest years in mixed swards with
20 either perennial ryegrass (*Lolium perenne* L.) or perennial plus hybrid ryegrass (*L.*
21 *boucheanum* Kunth). Red clover and total sward dry matter (DM) herbage yields
22 were measured in Years 1-4, red clover plant survival in Years 3 and 4 and herbage
23 protein (CP) yield and concentration in Years 2 and 4. In general, red clover DM yield
24 in year 4 (3.4 t ha⁻¹) was lower than in year 1 (13.9 t ha⁻¹) but the red clover
25 populations differed in the extent of this decline. Differences in the persistence of the
26 red clover populations in terms of plant survival and yield were reflected in the
27 contribution of red clover to the total sward yield in Year 4, which ranged from 61%
28 for the highest yielding population, AberClaret, to 11% in the lowest yielding, Vivi..
29 Increased red clover DM yield was reflected in a greater CP yield (protein weight per
30 unit area), which ranged from 1.6 t ha⁻¹ year⁻¹ to 2.9 t ha⁻¹ year⁻¹ in Year 2 and from
31 1.1 t ha⁻¹ year⁻¹ to 1.9 t ha⁻¹ year⁻¹ in Year 4. CP concentration (protein weight per unit
32 herbage weight) of all of the red clover populations was within a range considered
33 suitable for ruminant production. The implication of these results for the future use of
34 red clover in sustainable grassland systems is discussed.

35 **Keywords:** *Trifolium pratense*, yield, persistence, protein yield, variety

36

37 1. Introduction

38

39 Increasing the amount of forage based protein that can be grown “on-farm” is
40 one way of addressing the UK’s protein deficit and increasing the efficiency of
41 livestock production (Abberton and Marshall, 2005). Forage legumes such as white
42 (*Trifolium repens* L.) and red clover (*T. pratense* L.) are important species for
43 sustainable livestock production systems because in mixture with grasses they can
44 fix, on average, 150 kg N ha yr⁻¹, some of which subsequently becomes available to
45 the companion grass (Pirhofer-Walzl *et al.*, 2012). They produce high yields of good
46 quality forage with a crude protein (CP) content of 18-19% (Frame *et al.*, 1997),
47 which, in turn, leads to high voluntary intakes and improvements in livestock
48 performance (Lüscher *et al.*, 2014). Recent studies also show that red clover
49 contains high levels of the enzyme polyphenol oxidase (PPO) in comparison with
50 other forage legumes (Winters and Minchin, 2005). PPO has beneficial effects in
51 improving nitrogen utilization in ruminants (Lee *et al.*, 2006) and in protecting lipids
52 from degradation, both ‘in silo’ as well as in the rumen, leading to a higher output of
53 polyunsaturated fatty acids (PUFA) in ruminant products (meat and milk) (van Ranst
54 *et al.*, 2011).

55

56 Despite these beneficial attributes of red clover, its use may be limited by its
57 lack of persistence in swards. For example, under a typical UK management of 3
58 conservation cuts per year followed by a late autumn grazing, red clover tends to
59 persist for only two to three years, after which dry matter (DM) yields decline (Frame,
60 2005). One challenge to improving the persistence of forage legumes is a lack of
61 scientific consensus on how persistence is defined (Phelan *et al.*, 2014). In this study

62 we considered two aspects of persistence in red clover swards. The first of these was
63 the number of individual red clover plants surviving in swards after three or more
64 years (Choo, 1984). In contrast to the other major temperate forage legume, white
65 clover, which has a stoloniferous growth habit and is therefore able to spread
66 vegetatively in swards, red clover plants grow from apical meristems from the crown
67 tissue, which means that poor plant survival has a significant impact on DM yield
68 under managements that do not allow regeneration from seed *i.e.* red clover cannot
69 replace plants that die. Secondly, we analysed the capacity of red clover based
70 swards to produce 'functionally relevant' forage yield in the second and fourth
71 growing seasons (Taylor and Quesenberry, 1996) *i.e.* an amount of CP sufficient to
72 meet the nutritional requirements of animals ingesting the conserved forage. Efficient
73 ruminant production requires forages with a CP concentration of 100 to 170g kg⁻¹ DM
74 (Phelan et al., 2014) and the CP concentration of red clover silage has been
75 measured as 171-181 g kg⁻¹ DM, depending upon growth stage (Dewhurst 2013).
76 The maintenance of DM and CP yield is therefore crucial to red clover's agronomic
77 value as a source of protein grown on-farm, and explains why persistence is a
78 common breeding target of red clover breeding programmes in the UK (Marshall *et*
79 *al.*, 2012) and in other countries (Herrmann *et al.*, 2008; Taylor, 2008; Riday 2010).

80

81 Previous studies on red clover persistence have considered the potential of
82 exploiting variation in morphological traits such as the development of adventitious
83 roots (Montpetit and Coulmann, 1991) and stolons (Smith and Bishop, 1993).
84 However, the most direct way to breed for persistence involves exposing plants to
85 their target environment and selecting for survival (Taylor and Quesenberry, 1996). In
86 the red clover breeding programme at IBERS, Aberystwyth the focus has been on

87 identifying factors contributing to the poor persistence of red clover in swards and
88 applying this information to the selection of improved red clover varieties that
89 combine high forage yields with greater persistence. Two approaches to selection for
90 persistence have been applied: (1) previous studies in spaced plants showed that
91 crown diameter is the morphological characteristic most associated with plant
92 mortality (Ortega and Rhodes, 1996), and a number of selections in the breeding
93 programme were made on that basis (indirect selection); (2) survivor plants were
94 collected at the end of long term variety evaluation experiments that were carried out
95 for up to four years. These survivor plants were subsequently subject to a single
96 round of polycrossing to produce a number of selection lines (direct selection).

97

98 This paper describes a field experiment undertaken to quantify the effects of
99 differences in red clover persistence on sward DM production and CP yield over a
100 four year period, using new red clover varieties with greater crown diameter
101 (developed by indirect selection), survivor populations selected for improved
102 persistence (developed by direct selection) and a range of current commercially
103 available varieties. To avoid confusion, all red clover germplasm (selection lines and
104 varieties) used in this study is referred to collectively as 'populations'.

105

106 In this study the red clover populations were grown in binary mixtures with two
107 companion grass treatments. In the UK, red clover is usually sown in mixtures with
108 Italian (*Lolium multiflorum* Lam.) or hybrid ryegrass (*L. boucheanum* Kunth)
109 (Abberton and Marshall, 2005), species that are high yielding but relatively short lived
110 in comparison with perennial ryegrass (*L. perenne* L.). However, breeding red clover
111 varieties that are more persistent will lead to greater longevity of mixed grass/red

112 clover swards, beyond the current two or three years. This may necessitate the
113 inclusion of the more persistent perennial ryegrass in such mixtures. No published
114 information is currently available on the performance of red clover when sown with
115 different companion grasses over more than three harvest years, so the effect of
116 adding perennial ryegrass to hybrid ryegrass as a companion grass treatment was
117 analysed here.

118

119 **2.0 Materials and methods**

120

121 **2.1 Plant material and site**

122 The experiment was established in July 2008 at IBERS, Aberystwyth, UK (52°
123 4'N, 4° 0'W) on soil of the Rheidol series (well drained fine loamy soil over gravel)
124 with a pH value of 6.1.

125

126 Twelve red clover populations (Table 1), were sown in mixtures either with the
127 tetraploid hybrid ryegrass cv. AberEcho on its own ('companion grass treatment 1')
128 or with a mixture of the intermediate heading perennial ryegrass cv. AberDart and cv.
129 AberEcho ('companion grass treatment 2'). The red clover populations comprised
130 eight commercial varieties in use in the UK at the time of sowing, including two
131 (AberChianti and AberClaret) developed at IBERS and possessing increased crown
132 diameter, a trait linked with improved persistence, plus four survivor populations
133 selected from long term cutting and grazing trials carried out at IBERS experimental
134 sites at Aberystwyth and Bronydd Mawr Upland Research centre at Brecon, Wales.

135

136 **2.2 Experimental design**

137 A randomized complete block (RCB) design was used. Each treatment
138 combination (12 red clover populations with two companion grass treatments) was
139 replicated three times, giving 72 experimental plots in total. Plots (5m x 1m) were
140 sown by broadcasting seed. In both companion grass treatments, red clover was
141 sown at a seed rate of 7.5 kg ha⁻¹. In 'companion grass treatment 1' hybrid ryegrass
142 was sown at 35 kg ha⁻¹, and at 22.4 kg ha⁻¹ in 'companion grass treatment 2' together
143 with perennial ryegrass at 12.6 kg ha⁻¹. Thus, the total seed rate for the grass
144 component was the same in both companion grass treatment types. Plots were
145 lightly topped in the establishment year and three harvests taken in each of the
146 following four harvest years. In the autumn of each harvest year the plots were mob
147 grazed with sheep to a sward height of 4cm.

148

149 The grass and red clover components of sward DM yield were quantified in
150 three cuts per year in 2009 (Year 1: 20 May, 8 July, 24 August), 2010 (Year 2: 27
151 May, 19 July, 6 September), 2011 (Year 3: 31 May, 20 July, 12 September) and 2012
152 (Year 4: 31 May, 2 August, 10 October). Plots were cut with a Haldrup forage
153 harvester to a height of 5cm. The harvested fresh weight was measured on each plot
154 and grass and red clover content determined on a 300g subsample. DM yields were
155 calculated after drying the subsample in a forced draught oven at 80°C for 18 h.

156

157 The density of red clover plants in each plot was recorded in spring of Years 3
158 and 4. Two 0.25m² quadrats were placed at random in each plot and the number of
159 red clover plants in each quadrat was recorded.

160

161 The nitrogen (N) content of dried, milled samples of the total mixture (grass
162 and red clover) from all plots harvested in Years 2 and 4 was determined by a rapid
163 combustion method using a LECO FP- 428 analyses (Leco Corp., St. Joseph, MI,
164 USA) and the CP content was calculated using the formula $CP = N \times 6.25$.

165

166 **2.3 Meteorological data**

167 Weather conditions during the growing season (March-October) of the
168 establishment year and the subsequent four harvest years are presented in Table 2,
169 together with the 25-year average.

170

171 **2.4 Statistical analysis**

172 Data for annual red clover and total (red clover + grass) DM yields from each
173 plot were analysed by ANOVA in each growing season according to the RCB design,
174 and also over the four growing seasons as repeated measures. Red clover means
175 were compared within growing seasons using the Student-Newman-Keuls method for
176 multiple comparisons. The stability of annual red clover DM yield over the four
177 growing seasons was examined using modified joint regression analysis (Digby,
178 1979) as implemented in procedure RJOINT of the statistical package GenStat
179 (Payne et al, 2015). The stability (i.e. sensitivity) estimate for a population
180 characterized the average linear relationship between the means for that population
181 in each year and the overall site means. The stability estimates for all populations
182 were standardized to have mean +1 (i.e. slope > 1 = more sensitive (less stable) than
183 average and slope < 1 = less sensitive (more stable) than average).

184

185 Plant densities and CP content and yield compared between treatments
186 across two growing seasons were analysed by repeated measures ANOVA.
187 Bonferroni adjusted pairwise multiple comparisons were made between years within
188 red clover populations. These analyses were carried out using GenStat (Payne et al.,
189 2015).

190

191 **3. Results**

192

193 **3.1 Meteorological conditions**

194 Growing seasons in the establishment year (2008) and year 4 (2012) were
195 wetter than average (100.6mm and 108.1mm respectively), and the growing season
196 in Years 1 (718.0mm) and 3 (65.2mm) drier than average (81.5mm) (Table 2).
197 Growing seasons in Years 1 and 3 were slightly warmer than average but in all other
198 years conditions were comparable with the 25-year average for this location. The
199 minimum temperature in harvest years 1 (9.1°C) and 3 (9.0°C) was higher than
200 average (8.1°C) and Years 1 (16.1°C), 2 (15.8°C) and 3 (16.5°C) had a higher
201 average maximum temperature than the 25 year mean (15.4°C).

202

203 **3.2 Dry matter yield**

204 Repeated measures analysis over years showed that annual total yield and
205 red clover yield were significantly influenced by red clover population ($P<0.001$), year
206 ($P<0.001$) and a year x population interaction ($P<0.001$). Results for annual total and
207 red clover DM yields are presented in Tables 3a and 3b respectively. There was a
208 significant difference between grass treatments in Year 1, in which the addition of
209 perennial ryegrass in grass treatment 2 resulted in significantly higher average total

210 yield (17.2 vs. 18.0 t ha⁻¹ in grass treatments 1 and 2 respectively; P<0.008).
211 However, this was the only year in which grass treatment had a significant effect on
212 total yield. By contrast, the identity of the red clover population had a strong effect on
213 total yield in all years. Mixtures containing AberClaret or Milvus were highest yielding
214 in Year 1 (P<0.001); Milvus or Milvus (S) in Year 2 (P<0.001); and AberClaret or
215 Milvus (S) in Year 3 (P<0.001). For total yield in Year 4, the means comparisons test
216 at P<0.05 did not detect any differences between red clover populations even though
217 the effect of red clover was highly significant (P=0.007). This may have been due to
218 grouping of the means (Thomas, 1973). In order to identify differences between the
219 populations the experiment-wise type I error rate was reduced. In absolute terms,
220 the highest total yields in Year 4 were in mixtures containing Milvus (S), AberChianti
221 and Pavo and the lowest yielding was Vivi (S). In all years, total yields in mixtures
222 containing the population Vivi were the lowest yielding. There was no interaction
223 between grass treatment and red clover population in terms of annual total yield in
224 any year. Although mean total yields decreased over time, they were still
225 agronomically acceptable in Year 4 (17.6, 15.9, 12.7 and 10.4 t ha⁻¹ in Years 1, 2, 3
226 and 4 respectively).

227

228 Average annual red clover yield was significantly affected by grass treatment
229 in Year 1 only, when it was higher in mixtures containing perennial ryegrass (13.4
230 and 14.2 t ha⁻¹ in grass treatments 1 and 2 respectively; P<0.05). As observed for
231 total sward yield, the yield of the red clover component differed significantly between
232 populations in all years. In Year 1 the highest red clover yields were measured in
233 Milvus and AberClaret (P<0.001); Milvus was again the highest yielding in Year 2
234 (P<0.001), while AberClaret and Milvus (S) were highest in Year 3 and Year 4

235 (P<0.001). Population Vivi was consistently low yielding in all years, although by Year
236 4 many of the other red clover populations were equally low yielding. The average
237 red clover yield was very high in Years 1 and 2, but decreased substantially in Years
238 3 and 4 (13.8, 14.6, 8.6 and 3.4 t ha⁻¹, respectively). Milvus (S) Varieties developed
239 by both indirect (AberClaret) and direct (Milvus (S)) selection strategies produced
240 high red clover DM yields in Years 3 and 4 compared to the commercially available
241 red clover varieties.

242

243 Yields of the grass component (averaged over grass treatments and red
244 clover populations) were 3.8, 1.4, 4.1 and 7.1 t ha⁻¹ in Years 1, 2, 3 and 4
245 respectively. There was no effect of grass treatment on annual grass yield except in
246 Year 3 when the yield of grass treatment 2 was higher than grass treatment 1
247 (P<0.05). Over time there was an inverse relationship between annual grass and red
248 clover yield, so that as the yield of the latter decreased the contribution of the grass
249 to total yield increased. The relationship between DM yields in Year 1 against
250 subsequent yields in Years 2, 3 and 4 is shown in Figure 1. There was a significant
251 positive relationship in all cases, indicating that performance in Year 1 was a good
252 predictor of the performance in later years. The red clover and total yields of the 12
253 red clover populations were also broadly similar in Years 1, 2 and 3, as the slopes of
254 the fitted lines in Year 1 compared with yield in Years 2 and 3 were similar. The
255 comparison of yields in Years 1 and 4 however, showed a greater relative drop in
256 performance in total yield in comparison with red clover yield, as the fitted lines
257 diverged and the total yield exhibited a smaller slope than that of red clover. Figure 1
258 also shows the relative change in total and red clover yield over time. In a
259 comparison of Year 1 vs Year 2, the slopes of both yields were not significantly

260 different from 1, indicating there was no difference between red clover populations in
261 performance between those years in either total or red clover yield. There was,
262 however, a drop in performance in Year 3 as the slope of the fitted lines become
263 significantly ($P < 0.05$) less than 1, and a bigger drop again in Year 4 for both total and
264 red clover yields, as the slope of each fitted line was significantly ($P < 0.001$) less than
265 1.

266

267 **3.3 Stability of red clover yield over growing seasons**

268 The stability of red clover yield of each population over 4 years is shown in
269 Figure 2. The closer the sensitivity estimates were to 1 the more similar the
270 population stability was to the overall mean stability. Populations with sensitivities > 1
271 had less stable yields than the mean, and those with sensitivities < 1 were more
272 stable than the mean. Milvus tended to be the least stable population. Yield in Vivi
273 was more stable ($P < 0.05$) than that in Vivi (S), Aa4577, Merviot, Amos, Pavo, Milvus
274 and AberChianti.

275

276 **3.4 Plant density**

277 Analysis of plant density (count m^{-2}) in Years 3 and 4 (mean of the plant
278 density data from May and September counts in each year) as repeated measures in
279 each year showed significant effects of red clover population, year and the red clover
280 population x year interaction. There was a significant effect ($P < 0.001$) of population
281 on the density of red clover plants in both years (Table 4). In Years 3 and 4
282 respectively, plant density was highest in mixtures containing Britta (S1) (85.4 and
283 66.3) and lowest in Vivi (24.5 and 16.7). However, only the populations Amos and
284 Vivi (S) showed a significant decrease in plant density between Years 3 and 4

285 (P<0.05), based on Bonferroni adjusted pairwise multiple comparisons between red
286 clover populations. Effects of companion grass treatment, the interaction of grass
287 treatment with red clover population and year, and the interaction between grass
288 treatment, red clover population and year were not significant (P=0.725, 0.363, 0.129
289 and 0.088 respectively). The relationship between plant density and annual red
290 clover DM yield in Years 3 and 4 was analysed using linear regression. The
291 correlation coefficient for the regression in Year 3 was not significant, but became
292 significant in Year 4 ($r = 0.72$; 10 df; $P<0.01$). The regression equation for this
293 relationship was: Annual clover yield (t DM ha⁻¹) = 0.089 (± 0.0275) x Plant density
294 (count m⁻²) - 0.47 (± 1.298) ($R^2=0.462$, $P=0.009$, $rsd=1.68$).

295

296 **3.5 Forage quality**

297 Annual CP yield (t CP ha⁻¹ year⁻¹) and the CP concentration of the harvested
298 herbage (g CP kg⁻¹ DM) of the mixtures in Years 2 and 4 were analysed as repeated
299 measures with Bonferroni adjusted multiple comparisons between years and within
300 red clover population. Values are shown in Figure 3 (a) and (b).

301

302 CP yield of the harvested herbage was higher in Year 2 than Year 4 (2.55 vs.
303 1.39 t ha⁻¹, s.e.d. 0.048, $P<0.001$) with no effect of grass treatment (1.96 and 1.98 t
304 ha⁻¹ for treatments 1 and 2 respectively, s.e.d. 0.038, $P=0.520$), but a significant red
305 clover population x year interaction (Figure 3a) (s.e.d. 0.165 between years within
306 populations otherwise 0.149; $P<0.001$). All red clover populations showed a
307 decrease ($P<0.05$) in CP yield between Years 2 and 4 except Vivi which had its
308 lowest CP yield in Year 2.

309

310 CP concentration of the harvested herbage was higher in Year 2 than in Year
311 4 (161.1 vs 129.7 g kg⁻¹ DM, s.e.d. 1.37, P<0.001) and, averaged over years, was
312 higher in grass treatment 1 than treatment 2 (147.5 vs. 143.3 g kg⁻¹ DM, s.e.d. 1.15,
313 P<0.001) (data not shown), but there was no interaction between grass treatment
314 and either red clover population or year. There was a significant interaction between
315 red clover population and year (P<0.001) due to a significant decrease in CP
316 concentration between Years 2 and 4 in all populations except Milvus (S), Milvus,
317 AberChianti, AberClaret and Pavo, *i.e.* those populations that maintained a higher
318 percentage contribution of red clover to total annual yield between these years. CP
319 concentration of the red clover populations ranged from 150.3 to 174.3g kg⁻¹ DM in
320 Year 2, and from 103.4 to 152.3g kg⁻¹ DM in Year 4 (Figure 3 b). The strong effect of
321 the percentage red clover contribution to total mixture DM on the CP concentration of
322 the herbage is illustrated in Figure 4.

323

324

325 **4. Discussion**

326

327 **4.1 Variation in dry matter yield**

328 Typically in the UK, red clover performs well for two years in mixed swards,
329 but its subsequent decrease in DM yield has a negative impact on the amount of
330 protein supplied by the sward. Analysis of the impact of red clover persistence on
331 forage DM yield and CP yield is therefore important for evaluating the dynamics of
332 forage quality in red clover-based swards, and also for establishing targets for
333 genetic improvement programmes. To investigate this we analysed the performance
334 of red clover germplasm selected with an *a priori* expectation of improved

335 persistence, namely, four populations (Britta (S1), Milvus (S), Britta (S2) and Vivi (S))
336 developed using direct selection for DM yield persistence, plus two commercial
337 populations (AberChianti and AberClaret) derived from crosses carried out at IBERS,
338 Aberystwyth using germplasm selected for the larger crowns associated with red
339 clover plant persistence (indirect selection). The primary objective of this experiment
340 was to compare the persistence (using the criteria outlined in the Introduction) of
341 these populations with that of five red clover populations that are currently
342 commercially available in the UK.

343

344 Red clover yields of all the populations declined in the third and fourth harvest
345 year compared with Year 1. The decline in the total sward yield over time was less
346 evident, as mixtures with a lower red clover yield showed a concomitant increase in
347 grass yield in compensation. As shown in other studies (e.g. Ford and Barrett, 2011),
348 there were clear differences here in the persistence of the red clover populations in
349 terms of both plant survival and maintenance of DM yield. These differences were
350 reflected in the contribution of red clover yield to the total sward yield in Year 4 which
351 ranged from 61% for the highest yielding population, AberClaret, to 11% in the lowest
352 yielding, Vivi. Despite the differences in red clover yield, however, all of the
353 populations demonstrated the capacity to produce swards containing functional
354 levels of CP in Year 4 i.e. herbage CP concentration never fell below the level
355 required for efficient ruminant production (Phelan *et al.*, 2014), although in some
356 mixtures CP concentration had declined to a level that was approaching the lower
357 end of the acceptable range for ruminant production.

358

359 **4.2 Stability of red clover yield over harvest years**

360 Regression values (slope) >1 describe populations with less yield stability over
361 years, whilst those with slope values <1 show above- average stability compared to
362 the overall mean stability. This approach has been used in previous studies to
363 quantify the adaptation of varieties to environment in plant breeding programmes
364 (Finlay and Wilkinson, 1963) and to analyse the stability of seed yield of accessions
365 of forage species when multiplied in different environments (Hinton Jones et al.,
366 2007). In the current study, this approach has been combined with yield data to
367 provide some indication of the yield stability of different populations over years. The
368 results confirmed that, although not significantly different, AberClaret and Milvus (S)
369 were the most stable varieties in terms of producing high red clover yields over the
370 four years, while Milvus and Merviot were the least stable of the higher-yielding
371 varieties.

372

373 **4.3 Variation in plant survival**

374 The overall decline in red clover yield over the duration of the experiment was
375 a consequence of poor plant survival, and this deficiency was greater in some
376 populations than others. Plant density in Year 4 showed a good correlation with red
377 clover yield, and there were significant differences between populations in plant
378 survival. The highest red clover yields in Year 4 were produced by populations Milvus
379 (S) and AberClaret. Milvus (S) was produced through direct selection from survivors
380 of long term field trials that included Milvus, whilst AberClaret was derived from
381 crosses specifically aimed at increasing crown diameter (indirect selection). Evidence
382 from the current experiment suggests that both strategies could be used to improve
383 persistence of red clover. Recent studies have shown that persistence in red clover is
384 also positively correlated with length of the longest stem of each plant, which may

385 therefore be useful as another candidate trait for indirect selection (Herrmann *et al.*,
386 2008).

387

388 **4.4 Contribution of red clover to total dry matter yield and protein yield**

389 Differences in plant persistence between the populations had an impact on the
390 red clover content of the total DM yield and on the CP yield of the mixtures. There
391 was a strong temporal effect on the contribution of red clover to the total annual yield
392 such that it was 86% for the best red clover population in Year 1 and nearly 60% in
393 Year 4. In the lowest yielding red clover populations this contribution was closer to
394 11% in Year 4. However, despite this six-fold difference in red clover content, in Year
395 4 the total DM yield of all mixtures only ranged from 9.3 to 11.4 t ha⁻¹. Thus, mixtures
396 with low red clover DM yields had a proportionally higher grass DM yield. It is
397 possible that in these mixtures increased mortality over time in red clover resulted in
398 more physical spaces being available to fill for the grass component. However,
399 previous results have shown a decrease in ryegrass plant density due to the vigorous
400 growth of red clover (Eriksen *et al.*, 2012). Similar reductions in tiller formation of
401 ryegrass when grown in mixtures with tall legumes were found by Roscher *et al.*,
402 (2008). In the latter study the depression of ryegrass yield occurred despite a very
403 low content of red clover in the seed mixture (1kg ha⁻¹) which was considerably lower
404 than the 7.5 kg ha⁻¹ used in the present study.

405

406 A key objective of this study was to ascertain the effect of persistence of the
407 red clover populations on the CP yield and CP concentration of the herbage
408 mixtures. The CP yield of the 12 red clover populations ranged from 1.6 t ha⁻¹ year⁻¹
409 to 2.9 t ha⁻¹ year⁻¹ in Year 2 and from 1.1 t ha⁻¹ year⁻¹ to 1.9 t ha⁻¹ year⁻¹ in Year 4,

410 reflecting differences in the proportion of red clover in the mixtures and highlighting
411 the importance of red clover persistence to the feed value of the sward. Such
412 differences in CP yield will have a significant impact on the carrying capacity of the
413 sward in terms of the number of livestock that can be supported and therefore on the
414 economic value of the sward to the livestock farmer. Ruminant production requires
415 forages with a CP concentration of 100 to 170g kg⁻¹ DM (Phelan et al., 2014) and the
416 CP concentration of red clover silage has been measured as 171-181 g kg⁻¹ DM,
417 depending upon growth stage (Dewhurst 2013). In the present study, the herbage CP
418 concentration in Year 2 of the 12 red clover populations, averaged over the three
419 cuts, ranged from 150.3 to 174.3 g kg⁻¹ DM and from 103.4 and 152.3 g kg⁻¹ DM in
420 Year 4. Although the CP concentration declined between Years 2 and 4 as the
421 proportion of red clover in the mixtures decreased, in Year 4, it was still in the
422 acceptable range for ruminant production. However for some of the red clover
423 populations CP concentration had declined to a level that was approaching the lower
424 end of the acceptable range for ruminant production.

425

426 **4.5 The effect of companion grass**

427 An additional objective here was to consider the role of the companion grass
428 species in the mixtures. Persistent red clover varieties may produce high DM yields
429 for up to 4 or 5 years, significantly extending the life of the red clover based leys, and
430 this may require the use of more persistent perennial ryegrass varieties than the high
431 yielding, short lived grass species currently used for 2 year leys. The present study
432 compared 2 grass treatments; tetraploid hybrid ryegrass cv. AberEcho on its own
433 ('companion grass treatment 1') or with a mixture of the intermediate heading
434 perennial ryegrass cv. AberDart and cv. AberEcho ('companion grass treatment 2').

435 AberDart, the perennial ryegrass variety used in the study, has a high WSC content,
436 which is known to also improve silage quality (Conaghan *et al.*, 2008). In the current
437 study, the grass DM yield was not generally influenced by grass treatment, though
438 over time there was an inverse relationship between annual grass and red clover
439 yield, so that as the yield of the latter decreased the contribution of the grass to total
440 yield increased. Further work is needed to consider the interaction between red
441 clover variety, ley duration and type of management e.g. persistence under grazing,
442 as this may have a greater impact on the choice of companion grass species than
443 the management system included in this study. This is particularly important if the
444 proposed management system will involve some longer and more severe grazing,
445 and if it includes an element of sheep grazing in each growing season, as in this
446 study, or cattle grazing, as in other studies (Ford and Barrett, 2011).

447

448 The results presented here also have implications for official variety testing
449 systems. The current UK red clover variety testing system evaluates variety
450 performance over two harvest years. Our data show that although the major
451 differences between populations in persistence and yield were not apparent until the
452 third and fourth harvest year, performance of the populations in year 1 was a good
453 indicator of their performance in year 4. . This suggests that testing beyond two years
454 may not be necessary to identify appropriate varieties that are suitable for longer
455 leys.

456

457 **5. Conclusion**

458

459 Increasing the longevity of red clover based swards is increasingly attractive to
460 livestock farmers, but lack of persistence of red clover has a significant impact on the
461 yield and quality of mixed red clover/grass swards. Evidence from this field
462 experiment carried out over four harvest years shows that inadequate plant
463 persistence can significantly reduce red clover DM yields and CP yield. New
464 germplasm, selected for improved persistence, can maintain a higher red clover yield
465 into the fourth harvest year, and the yield decline between harvest Years 1 and 4 is
466 considerably less than in other commercially available varieties. Selection for
467 improved persistence was reflected in improved plant survival, and consequently
468 higher DM yield in the third and fourth harvest years, which also resulted in a higher
469 CP yield per hectare in comparison with unselected material.

470

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475

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557

558 **Figure legends**

559 Figure 1. The relationship between total (grass + clover) yield and red clover DM
560 yield (t ha^{-1}) in Year 1 with yields in Years 2, 3 and 4 of 12 red clover varieties grown
561 in a field experiment at IBERS, Aberystwyth. Data are averaged over two companion
562 grass treatments.

563 Figure 2. Sensitivity estimates for annual red clover yield (t DM ha^{-1}) for 12 red
564 clover varieties over 4 years (vertical bars indicate 95 per cent simultaneous
565 confidence intervals). The Y-axis is stability (sensitivity) coefficient for annual red
566 clover yield from modified joint regression analysis (Digby, 1979) and X-axis is mean
567 annual red clover yield over 4 years. The dotted line represents overall mean
568 sensitivity.

569 Figure 3. a) Crude protein yield ($\text{t ha}^{-1}\text{year}^{-1}$) and b) crude protein concentration (g kg
570 $^{-1}$ DM) in Years 2 (black columns) and 4 (shaded columns) of 12 red clover varieties
571 grown in mixed swards with a grass companion. Yields are from 3 cuts in each
572 harvest year. Measurements were made on total (grass + clover) forage samples,
573 and data were averaged over two companion grass treatments. Crude protein yield:
574 Year x Variety $P < 0.001$ s.e.d. 0.149 (83 df) and within variety s.e.d. 0.165 (44 df);
575 Crude protein content: Year x Variety $P < 0.001$ s.e.d. 0.149 (83 df) and within variety
576 s.e.d. 0.165 (44 df) Grass $P < 0.001$ – grass treatment 1 147.5 v grass treatment 2
577 143.3, s.e.d. 1.15.

578 Figure 4. The effect of % red clover contribution to annual total DM on CP
579 concentration (g kg^{-1} DM) in the herbage in Year 2 (●) and Year 4 (○).
580 $Y = 0.5322(0.0253)x + 112.85$ (1.75) $\text{rsd} = 9.80$, $r = 0.873$, ($P < 0.001$)

581

583 Table 1 Red clover (*Trifolium pratense* L.) populations included in the field
584 experiment.

585

Population	Country of origin	Description
Britta (S1)	UK	Survivor ex. Britta
Britta (S2)	UK	Survivor ex. Britta
Milvus (S)	UK	Survivor ex. Milvus
Vivi (S)	UK	Survivor ex Vivi
AberChianti	UK	Variety selected for strong crowns
AberClaret	UK	Variety selected for strong crowns
Amos (tetraploid)	Czech Republic	Variety
Britta	Sweden	Variety
Merviot	Belgium	Variety
Milvus	Switzerland	Variety
Pavo	Switzerland	Variety
Vivi	Sweden	Variety

Table 2 Meteorological data (rainfall (mm), average minimum and maximum temperature (°C), at IBERS, Aberystwyth in 2008, 2009, 2010, 2011 and 2012, with 25 year (1988-2012) mean for comparison.

	Rainfall (mm)						Average minimum temperature (°C)						Average maximum temperature (°C)					
	2008	2009	2010	2011	2012	25 year mean	2008	2009	2010	2011	2012	25 year mean	2008	2009	2010	2011	2012	25 year mean
March	107.8	40.4	78.6	24.2	22.6	76.0	3.6	3.5	1.9	2.4	4.7	3.1	9.5	10.4	10.1	11.5	12.8	9.5
April	62.8	48.6	34.0	20.2	93.2	60.8	5.1	5.4	4.0	6.8	3.9	4.2	12.0	13.7	13.6	16.5	10.9	12.0
May	22.0	99.6	59.6	62.4	73.8	60.4	9.5	8.2	5.5	9.2	7.4	6.9	15.1	15.3	14.9	15.6	15.8	15.1
June	65.4	31.0	40.4	96.4	184.0	74.5	9.9	10.8	10.1	9.3	10.5	9.7	17.5	19.1	19.3	17.4	16.6	17.5
July	98.8	152.0	149.8	65.2	89.4	79.1	13.0	12.9	13.6	10.6	11.7	11.9	19.0	18.7	18.8	18.7	17.6	19.0
August	117.6	51.2	54.4	45.4	108.0	90.1	13.6	13.1	11.9	11.6	13.4	11.7	19.0	19.1	17.8	18.2	19.5	19.0
September	102.0	50.2	118.2	118.2	138.4	97.1	9.3	9.9	10.7	12.0	9.5	9.8	17.1	17.2	17.5	18.0	15.9	17.1
October	228.6	104.4	96.6	89.2	155.6	114.2	7.8	8.8	7.1	10.2	6.9	7.4	14.2	15.4	14.7	15.8	13.5	14.2
<i>AVERAGE</i>	<i>100.6</i>	<i>71.8</i>	<i>78.9</i>	<i>65.2</i>	<i>108.1</i>	<i>81.5</i>	<i>8.9</i>	<i>9.1</i>	<i>8.1</i>	<i>9.0</i>	<i>8.5</i>	<i>8.1</i>	<i>15.4</i>	<i>16.1</i>	<i>15.8</i>	<i>16.5</i>	<i>15.3</i>	<i>15.4</i>

Table 3 a) Annual total (grass + clover) yield (t DM ha⁻¹) of 12 red clover populations grown in plots with hybrid ryegrass (Treatment 1) or a hybrid/perennial ryegrass mixture (Treatment 2) over 4 harvest years. Yield is based on 3 cuts in each harvest year.

Clover Population	Year 1		Mean	Year 2	Year 3	Year 4
	Treatment 1	Treatment 2		Mean	Mean	Mean
Britta (S1)	15.0	17.9	16.5 ^e	16.2 ^{bcd}	12.8 ^{cde}	10.2 ^{ab}
Britta (S2) 60	15.2	15.4	15.3 ^e	12.5 ^e	10.7 ^{ef}	10.8 ^{ab}
Milvus (S)	19.4	20.5	19.9 ^{bc}	19.0 ^b	16.5 ^a	11.8 ^a
Vivi (S)	15.6	16.1	15.9 ^e	15.4 ^{de}	11.2 ^{def}	9.3 ^b
AberChianti	20.2	18.6	19.4 ^{bcd}	18.2 ^{bc}	14.2 ^{bcd}	11.4 ^{ab}
AberClaret	21.0	23.0	22.0 ^a	17.7 ^{bc}	16.5 ^a	11.1 ^{ab}
Amos	18.8	18.3	18.6 ^{de}	16.1 ^{bcd}	12.2 ^{cdef}	9.7 ^{ab}
Britta	14.5	15.1	14.8 ^e	13.9 ^{de}	10.4 ^{ef}	9.9 ^{ab}
Merviot	17.5	18.3	17.9 ^d	16.0 ^{bcd}	11.2 ^{def}	9.5 ^b
Milvus	20.8	21.3	21.1 ^{ab}	19.3 ^b	13.1 ^{cde}	10.4 ^{ab}
Pavo	19.2	20.1	19.6 ^{bcd}	18.1 ^{bc}	14.6 ^{bc}	11.4 ^{ab}
Vivi	9.6	11.3	10.4 ^f	9.1 ^f	9.1 ^f	9.4 ^b
<i>Mean</i>	17.2	18.0		15.9	12.7	10.4
Prob	Grass	0.008		0.063	0.584	0.483
	Clover	<0.001		<0.001	<0.001	0.007
	Grass.Clover	0.148		0.556	0.242	0.446
s.e.m.	Grass	0.19		0.31	0.31	0.22
	Clover	0.47		0.75	0.75	0.53
	Grass.Clover	0.67		1.06	1.06	0.75

^{a-e}; Means with differing superscripts differ (P<0.05); df for within years = 46
df for within years = 46

Table 3 b) Annual red clover yield (t DM ha⁻¹) of 12 red clover populations grown in plots with hybrid ryegrass (Treatment 1) or a hybrid/perennial ryegrass mixture (Treatment 2) over 4 harvest years. Yield is based on 3 cuts in each harvest year.

Clover Population	Year 1		Mean	Year 2	Year 3	Year 4
	Treatment 1	Treatment 2		Mean	Mean	Mean
Britta (S1)	12.4	13.1	12.8 ^{de}	14.7 ^{abc}	8.3 ^{dc}	2.1 ^c
Britta (S2)	11.4	11.4	11.4 ^e	10.6 ^d	5.0 ^e	2.0 ^c
Milvus (S)	14.2	17.0	15.6 ^{abc}	17.5 ^{ab}	12.7 ^a	6.9 ^a
Vivi (S)	12.6	12.2	12.4 ^{de}	14.1 ^{bc}	7.4 ^{cde}	1.7 ^b
AberChianti	16.4	15.2	15.8 ^{abc}	17.2 ^{ab}	11.2 ^{ab}	5.2 ^b
AberClaret	17.3	17.8	17.6 ^a	16.5 ^{ab}	12.8 ^a	6.8 ^a
Amos	13.4	14.8	14.1 ^{cd}	14.8 ^{abc}	8.5 ^{cd}	1.9 ^c
Britta	11.0	11.6	11.3 ^e	12.2 ^{dc}	5.7 ^{de}	1.9 ^c
Merviot	13.9	15.4	14.6 ^{bcd}	14.6 ^{abc}	6.9 ^{cde}	1.3 ^c
Milvus	17.4	18.7	18.1 ^a	18.1 ^a	9.4 ^{bc}	4.3 ^b
Pavo	16.0	18.0	17.0 ^{ab}	17.0 ^{ab}	11.1 ^{ab}	5.0 ^b
Vivi	5.3	5.9	5.6 ^{ff}	7.5 ^e	5.0 ^e	1.0 ^c
Mean	13.4	14.2	..	14.6	8.66	3.4
Prob	Grass	0.050		0.245	0.439	0.979
	Clover	<0.001		<0.001	<0.001	<0.001
	Grass.Clover	0.774		0.406	0.278	0.470
s.e.m.	Grass	0.28		0.34	0.29	0.18
	Clover	0.68		0.82	0.70	0.45
	Grass.Clover	0.96		1.16	0.99	0.64

^{a-f}; Means with differing superscripts differ (P<0.05); df for within years = 46

Table 4. Number of red clover plants m⁻² in Years 3 and 4, averaged over two companion grass treatments. Data are derived from the mean of duplicate 0.25m² quadrats and observations in May and September.

Clover Variety/Selection line	Year		Mean
	Year 3	Year 4	
Britta (S1)	85.4	66.3	75.9
Britta (S2) Aa 4560	57.3	42.5	49.9
Milvus (S)	77.0	66.0	71.5
Vivi (S)	66.0 ^a	28.8 ^b	47.4
AberChianti	61.8	64.8	63.3
AberClaret	62.7	57.7	60.2
Amos	47.3 ^a	20.5 ^b	33.9
Britta	55.8	46.2	51.0
Merviot	34.5	20.5	27.5
Milvus	45.0	49.0	47.0
Pavo	55.8	47.3	51.6
Vivi	24.5	16.7	20.6
<i>Mean</i>	<i>56.1</i>	<i>43.9</i>	
Probability	Clover	<0.001	
	Year	<0.001	
	Year x Clover	0.020	
s.e.m.	Year x Clover	6.09 (84 df)	
	within Clover	5.32 (46 df)	

^{a, b}; indicates a significant change in plant density between years based on Bonferroni adjusted pairwise multiple comparisons within red clover population