

1 **The impact of dung on inter- and intraspecific competition of**
2 **temperate grassland seeds**

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16 **Abstract**

17 *Questions:* In temperate grasslands, seeds of numerous dry-fruited plant species are dispersed
18 via ingestion and subsequent defecation by grazing animals. Depending on the herbivore species
19 and season, dung pats may contain a large assemblage of conspecific or heterospecific seeds
20 competing for space, light and nutrients in the space-limited environment of an individual dung
21 pat. In an environment rich in nutrients, such as herbivore dung, the outcome of inter- and
22 intraspecific competition might differ from situations where nutrients are limiting. Additionally,
23 dung pats being small and spatially isolated habitats with very specific conditions may impact
24 competitive interactions as well. Besides the plant-soil interactions on competition known from
25 literature, the specific quality and structure of dung pats might provoke more complex
26 interactions between different seed densities and species combinations.

27 *Methods:* We conducted a greenhouse competition experiment using three common perennial
28 grassland species. *Agrostis stolonifera*, *Trifolium pratense* and *Trifolium repens* were used in two-
29 species combinations with different proportions of each species and in monocultures. Seeds
30 were sown in three seed densities (50, 150 and 250 seeds) and the effects of cattle and horse
31 dung on establishment, growth and flowering were tested.

32 *Results:* Interactions, most probably attributable to interspecific competition, differed between
33 species mixtures. Seeds sown in polycultures generally emerged sooner, but the resulting
34 seedlings had lower relative growth rates compared with seeds sown in monocultures. Increased
35 biomass was measured for each species when growing in polycultures while evidence for
36 intraspecific competition was found in monocultures. *T. pratense* developed relatively more
37 flowers when plants were growing in polycultures compared with monocultures. Few effects of
38 seed densities were found, although higher seed densities led to lower establishment success in
39 both monocultures and polycultures. Adding dung generally increased the time needed to

40 emerge, relative growth rates and flowering, but decreased establishment success in
41 monocultures.

42 *Conclusions:* Both seed density and the presence of dung shape the post-dispersal fate of seeds.
43 While high seed densities imply a cost due to lower germinability, the nutritive environment of
44 dung acts as a compensation resulting in faster growth and an increased investment in
45 reproductive tissues.

46

47 **Keywords:** dispersal, dispersal success, dung, endozoochory, grassland species, interspecific
48 competition, intraspecific competition, plant competition, post-dispersal seed fate

49 **Running head:** Seedling competition in dung

50 Introduction

51 Seed dispersal in general, and specifically endozoochory, has the potential to be advantageous to
52 plants (1) by reducing mortality near the mother plant caused by density-dependent factors ('escape
53 hypothesis') (Connell 1971, Janzen 1970); (2) by facilitating the establishment of new populations
54 ('colonization hypothesis') (Howe & Smallwood 1982, Cain *et al.* 2000); and (3) by transporting seeds
55 towards micro sites characterized by the specific narrow range of environmental conditions which
56 favour establishment ('directed dispersal hypothesis') (D'hondt *et al.* 2012, Cavallero *et al.* 2012,
57 Wenny 2001). In the past decades, numerous studies measured the endozoochorous dispersal
58 potential of a wide array of dry-fruited grassland species (e.g., Malo & Suarez 1995b, Pakeman *et al.*
59 2002, Pakeman *et al.* 1999, Bakker & Olff 2003, Cosyns *et al.* 2005a & D'hondt & Hoffmann 2011),
60 but few studies examine the next steps in plant establishment. The decisive step in seed dispersal
61 depends on the post-dispersal success of seeds and includes the processes of secondary seed
62 dispersal, seed predation, germination, establishment, growth and the outcome of competitive
63 processes (Wang & Smith 2002, Nathan & Muller-Landau 2000, Traveset *et al.* 2001).

64 Seeds dispersed through endozoochory end up being deposited in a dung environment which can
65 alter germination success and the subsequent establishment, growth and flowering (Milotić &
66 Hoffmann 2016a, Ramos-Font *et al.* 2015). Fresh dung contains high concentrations of growth
67 promoting macronutrients and essential trace minerals (Traveset *et al.* 2001, Haynes & Williams
68 1993, Lupwayi *et al.* 2000). However, it must be noted that only a fraction of this nutrient load is
69 inorganic and readily available for plant uptake (Jørgensen & Jensen 1997). Furthermore, both the
70 dung C: N: P stoichiometry and the rates of dung decomposition and nutrient release are closely
71 related with herbivore diet and digestive physiology (Sitters *et al.* 2014). Factors such as humidity
72 (Dickinson & Craig 1990, Dickinson *et al.* 1981), presence of dung fauna (Lovell & Jarvis 1996, Sitters
73 *et al.* 2014), chemical composition of dung (Ouédraogo *et al.* 2004) and vegetation type at the
74 deposition site (Shepherd *et al.* 2000) define the rate at which dung mineralizes in natural

75 ecosystems. Besides the high nutrient content of dung, low concentrations of phytotoxic compounds
76 have been measured in fresh dung which may alter the activity of enzymes regulating germination
77 rate and seedling growth (Berendse & Möller 2009, Farnsworth 2008, Luna & Moreno 2009,
78 Marambe *et al.* 1993). The specific moist conditions within dung pats may have a considerable
79 impact on the early life stages of plants. The moisture content of dung pats is closely related to the
80 decomposition stage and weather conditions (Dickinson *et al.* 1981) and may even greatly differ
81 between various parts of the dung pat (Underhay & Dickinson 1978). As the concentration of
82 inorganic nutrients increases and the phytotoxic compounds gradually disappear from decomposing
83 dung, the nutritive environment of the dung pat is most useful in the growth phase of plants.
84 Furthermore, as most seeds contain a reserve of mineral and organic nutrients to nourish the
85 embryo in its initial stages of establishment (Fenner and Thompson, 2005), the poor nutrient
86 availability of fresh dung is expected to be of minor importance in the first life stages of plants. In
87 addition to the effects of dung, precipitation may play a significant role in germination and seedling
88 growth (Tjelele *et al.* 2015).

89 Dung pats often contain a high concentration of germinable seeds belonging to a wide range of
90 species. In temperate dune grasslands, Cosyns *et al.* (2005a) for example found an average 463
91 seedlings per litre of cattle or horse dung (summer sampling) belonging to a total of 31 species. Seed
92 density clearly varies with seed availability in the environment, herbivore type and size, and season
93 (Cosyns *et al.* 2005a, Malo & Suarez 1995b) and a high variability of seed concentrations and species
94 combinations have been found in dung. Seed dispersers may therefore not only affect plant fitness
95 by the act of dispersal itself, but by the different combinations and densities they deposit seeds in
96 dung (Loiselle 1990).

97 Despite the presumption that post-dispersal competition for resources modifies the establishment
98 success of seedlings, post-dispersal competition is rarely quantified in endozoochory studies. As
99 competing plants deprive each other of resources such as space, light, water and nutrients,

100 germination in an environment rich in nutrients, such as herbivore dung, might alter the outcome of
101 the competition process compared to a situation where nutrients are limiting. Furthermore, complex
102 interactions might exist between different seed densities, species combinations and the quality and
103 structure of dung. Previous research measured increasing interspecific competition with artificial
104 fertilisation levels (Gu *et al.* 2012), and faster germination times for seeds sown in highly competitive
105 environments (Orrock & Christopher 2010). The specific environmental conditions in dung pats might
106 therefore trigger a shift in species co-existing patterns which may locally result in an increase of
107 small-scale species richness (Cosyns *et al.* 2006).

108 To contribute to improved understanding of at least some aspects of zoochorous dispersal, we
109 conducted a competition experiment using three perennial grassland species with a comparable seed
110 size (see Bruun & Poschlod 2006 and D'hondt & Hoffmann 2011). In an attempt to disentangle the
111 effects of different species combinations and seed densities in the presence of dung with the effects
112 of digestive processes in the gut, undigested seeds were used in an experiment in a controlled
113 greenhouse environment. Two clover species (*Trifolium pratense* and *Trifolium repens*) with short
114 germination times (with mean germination times of respectively 1.9 ± 0.2 and 1.5 ± 0.0 days measured
115 for seeds of the same seed batch in Milotić & Hoffmann 2016c) and one grass species (*Agrostis*
116 *stolonifera*) with moderate germination times (10.1 ± 1.8 days in Milotić & Hoffmann 2016c) were
117 used in two-species combinations with different proportions of each species and in monocultures.
118 Three seed densities were used to assess the effects of different competition levels on the
119 establishment, growth, flowering and biomass of the experimental species. We studied the effects of
120 adding dung of cattle and horses; two herbivore species with a different digestion strategy
121 (respectively ruminants and hindgut fermenters) and which are commonly used in temperate
122 European nature management.

123 In this study we seek an answer to the following questions:

- 124 1. How are inter- and intraspecific competition processes shaping the post-dispersal seed fate
125 of different combinations of two early germinating legumes and one slower germinating
126 grass species?
- 127 2. To what extent are these competitive processes affected by seed density over a gradient of
128 low, medium and high seed density?
- 129 3. How does the dung of two herbivores with distinct digestive strategies affect seedling
130 establishment and growth?
- 131

132 **Materials and methods**

133 *Selected species and seed quality test*

134 Three perennial species were used as model species in the experiments: *Agrostis stolonifera* L.,
135 *Trifolium pratense* L. and *Trifolium repens* L. All selected species are common in temperate European
136 grasslands and have been found emerging from dung samples (e.g., Cosyns *et al.* 2005a) and were
137 able to germinate in controlled feeding trials (e.g., Cosyns *et al.* 2005b, D'hondt & Hoffmann 2011).
138 Seeds have been purchased in a webshop specialised in seeds of wild plants grown in a temperate
139 climate (Berkshire, UK, www.herbiseed.com) as it was practically unfeasible to sample such large
140 quantities of wild seeds in their natural habitat. As such, seeds of a high and homogeneous quality
141 were used in the experiments, although eventual maternal effects were not completely ruled out
142 using this approach. Seed quality was tested in a standardised lab environment in a germination trial
143 during 60 days. For each species, 5 replicates were used and per replicate 50 seeds were sown in
144 Petri dishes filled with 1% water agar substrate. Newly germinated seeds were counted daily during
145 the first 30 days and once every two days in the last part of the trial. Mean time to germination
146 (MTG) (Ranal & Garcia De Santana 2006) and the proportion of germinated seeds (germinability)
147 were derived from the count data.

148 *Experimental design*

149 The establishment, biomass, growth and flowering of the selected species were assessed in three
150 dung treatments: cattle and horse dung and a dung-free control treatment. Furthermore, different
151 densities were used: 50 (low density), 150 (medium density) and 250 seeds (high density) and in
152 different combinations of monocultures and polycultures in order to assess different levels of inter
153 and intraspecific competition. Experimental seed densities were derived from the densities of seeds
154 found germinating in cattle and horse dung in previous research conducted in temperate dune
155 grasslands (Cosyns 2004). Polycultures consisted of 2 species which were sown in either equal (50%-
156 50%) or unequal (20%-80% or 80%-20%) proportions of the total seed density of 50, 150 or 250 seeds

157 per replicate (Table 1). All possible combinations of species mixtures, dung treatments and seed
158 densities were made. Round plastic plant pots (diameter: 15 cm, height: 16 cm) with drainage holes
159 were filled with a 1:1 mixture of sand and compost. The compost was homogenised and mixed
160 before usage and contained nutrients for the first 6-8 weeks of plant growth (1.25 kg/m³ with N-P-K
161 concentrations of 14-16-18) according to the manufacturer (www.structural.be). Planting pots were
162 put in an unheated greenhouse. Temperature and relative humidity ranged between 15.2 °C and 24.7
163 °C, and 79.6 % and 96.7 % during the experiment. In order to reduce the influence of the specific
164 micro-climate at the edges, plastic rings (diameter: 9 cm, height: 5 cm) were inserted in the centre of
165 the plant pots. In each ring, intact seeds were sown either mixed with cattle or horse dung or on top
166 of the substrate in the control treatment. Each combination of dung treatments, seed densities and
167 species mixtures was replicated five times for each of the three species with a total of 540 pots in the
168 entire experiment (Table 1). Fresh dung was collected from 10-15 stabled cattle and horses fed on a
169 similar diet of pellet feed and hay in order to keep contamination with wild seeds minimal. The
170 sampled dung was mixed prior to application in order to obtain homogeneous cattle and horse dung
171 samples. In the dung treatments, rings were filled with 2 cm of either cattle or horse dung after
172 which the seeds were evenly mixed with the dung layer, while in the control treatment seeds were
173 put on top of the substrate. In addition to these control pots which served as a measure for the
174 effects of dung on plant establishment and growth and contained seeds, blank pots were installed as
175 a quality control measure. These blank pots had either a top layer of dung or bare substrate and the
176 emergence of any seedlings was monitored as a control for seed contamination originating from the
177 sand-compost mix, dung or surroundings. Experimental and blank pots were put in a complete
178 randomised design in order to minimise the impact of location-based environmental fluctuations.
179 Plant pots were watered manually on a daily basis and no artificial light was used. The experiment
180 started on May 30th 2012 when all species were sown and ended after 100 days. Emergence timing
181 was monitored by counting emerged seedlings every two days during the first two weeks of the
182 experiment and weekly thereafter. Height and flowering (if applicable) were measured after 40, 70

183 and 100 days. The height of the tallest individual of each species per pot was measured by lifting up
184 the hanging parts or tillers. Flowering was expressed as the sum of the number of flower buds,
185 flowers and fruits of all plants of each species present in each pot. After 100 days, plant
186 establishment was measured by counting the number of plants present at that moment in each pot
187 with a distinction between juvenile, flowering and non-flowering adult plants. Plants were harvested
188 and separated into below- and above-ground parts and dried to a constant weight at 65 °C after
189 which biomass was measured.

190 *Data analysis*

191 Emergence timing was analysed using a Cox proportional hazard model with dung type, seed density,
192 competing species and a competing species: proportion interaction term as covariates.

193 In each census interval, relative growth rates (RGR) were derived from the height of the tallest
194 individual of each species per pot:

$$RGR = \frac{\ln(H_t) - \ln(H_{t-\Delta t})}{\Delta t}$$

195 where H_t is the maximum height at time t , $H_{t-\Delta t}$ is the height in the previous interval and Δt is the
196 length of the time interval (Hunt 1982). The effects of dung types and seed densities on RGR were
197 assessed using repeated measures ANOVAs with a proportion: competing species interaction term.
198 RGR was \log_{10} transformed and Tukey posthoc tests were applied.

199 Establishment success in monoculture pots was expressed as the percentage of the sown seeds that
200 established to juvenile plants and flowering or non-flowering adults at the end of the 100-day
201 experiment. Two-way ANOVAs were used to analyse the effects of dung addition and seed densities
202 for each tested plant species. If needed to meet the assumptions for parametric tests, establishment
203 success logit transformed. Significantly differing dung types and seed densities were identified using
204 Tukey posthoc tests.

205 Competition between species in polycultures was expressed as relative neighbour effect (RNE)
206 (Markham & Chanway 1996). Two sets of RNE values were calculated using relative establishment
207 success or individual biomass in polycultures and monocultures as follows:

$$208 \quad RNE = \frac{(M_{mono} - M_{poly})}{x}$$

209 with M_{mono} : mean establishment success or harvested biomass in monocultures, M_{poly} : mean
210 establishment success or harvested biomass in polycultures and $x = M_{mono}$ if $M_{mono} > M_{poly}$; $x = M_{poly}$ if
211 $M_{poly} > M_{mono}$

212 RNE values range between -1 and 1, with positive values indicating interspecific competition and
213 negative values indicating facilitation between species (Weigelt & Jolliffe 2003). Differences in RNE
214 between dung types, seed densities and competing species were assessed with ANOVAs with a
215 proportion: competing species interaction term. *t*-tests were used to identify whether RNE differed
216 significantly from 0.

217 The bivariate relationships between root and shoot biomass and between the average biomass of
218 individual plants and flower counts were analysed using standardised major axis regressions (SMA)
219 with plant species, dung types, competing species, seed densities and proportions as fixed factors.
220 SMA is an alternative for classical regression techniques and is useful in situations where both the *x*
221 and *y* dimensions are subjected to measurement errors (Warton *et al.* 2006). First, the data were
222 tested for common slopes between species, dung types or seed densities. If the slopes did not differ,
223 we tested for common elevations (or *y*-intercepts) between dung types as the lines fitted to the dung
224 types may both represent a shift along their common slope and/or a shift in elevation (Warton *et al.*
225 2012). Models were tested for normality and residual distribution and if needed, data were \log_{10}
226 transformed.

227 All analyses were performed using R version 3.3.1. (R Core Team 2016). The 'survival' package version
228 2.38-3 (Therneau, 2015) and the 'smatr' package version 3.4-3 (Warton *et al.* 2012) were used for
229 respectively survival analyses and SMA regressions.

230 **Results**

231 In the seed quality test interspecific differences in germination timing were found ($F_{2,12}= 95.624$,
232 $p<0.001$) as *Agrostis stolonifera* (MTG (se): 10.1 (1.8) d) needed more time compared to *Trifolium*
233 *pratense* and *Trifolium repens* (MTG (se): respectively 1.9 (0.2) and 1.5 (0.0) d). Also, the proportion
234 of germinated seeds after 60 days differed between species ($F_{2,12}: 191.001$, $p<0.001$) with the lowest
235 germinability for *A. stolonifera* (22.0 (3.6) %) while nearly all *T. repens* seeds germinated (97.2 (0.4)
236 %) and 88.8 (2.6) % of the seeds of *T. pratense*.

237 In the greenhouse experiments, all tested species emerged later when sown in dung ($z=-5.087$,
238 $p<0.001$) (Fig. 1). Seed densities generally did not affect emergence timing ($z=-0.370$, $p= 0.711$),
239 although *T. pratense* emerged slightly faster in high densities compared to low seed density ($z=$
240 2.403 , $p= 0.016$). All species emerged later when sown in polycultures ($z= -2.329$, $p= 0.020$) and in
241 most cases, emergence was faster in case seeds were dominant in a mixture ($z= 6.158$, $p<0.001$). No
242 seedlings emerged in the blank pots which served as a proxy for the measurement of seed
243 contamination.

244 For all tested species relative growth rate (RGR) was higher when seeds were sown in cattle or horse
245 dung compared to the control treatment. *Trifolium pratense* and *T. repens* grew respectively 3 and
246 1.5 times faster when sown in cattle dung compared to horse dung ($F_{2,256}= 26.631$, $p<0.001$ and
247 $F_{2,238}= 15.148$, $p<0.001$ respectively). RGR of *A. stolonifera* and *T. repens* decreased significantly over
248 time. While seed densities and different species combinations did not differ in RGR, monocultures
249 consistently had a higher RGR compared to polycultures irrespective of the species that were
250 competing (Table 2).

251 In monocultures, the presence of dung resulted in lower establishment success, especially when
252 seeds were sown in cattle dung (both *Trifolium* species) and overall establishment success decreased
253 with increasing seed densities (Fig. 2, Appendix 1, Appendix S1). Significantly more seeds of the
254 *Trifolium* species emerged compared to *A. stolonifera* ($F_{2,132}: 40.624$; $p<0.001$). Establishment success

255 of *A. stolonifera* and *T. pratense* seedlings in polycultures was altered by interspecific competition
256 effects while the establishment of *T. repens* was not affected by the presence of competing species
257 when sown in 50%-50% combinations with *A. stolonifera* (Fig. 3). Relative neighbour effects (RNE)
258 were affected by the presence of dung, seed densities and competing species (Appendix, Appendix
259 S2). More specifically, individual plant biomass was relatively higher in polycultures for all tested
260 species. Furthermore, in most cases, differences between species combinations were found (Fig. 4,
261 Appendix, Appendix S2). In *A. stolonifera* and *T. pratense* intraspecific competition was stronger
262 when seeds were sown in horse dung compared to the control and cattle dung treatments while
263 dung addition did not have any effect on *T. repens* RNE (Appendix, Appendix S2).

264 Species generally differed in root: shoot biomass allocation and *T. repens* invested more in
265 aboveground biomass (Fig. 5, Appendix, Appendix S3 and A 4). The addition of cattle dung resulted in
266 a lower root: shoot ratio in both *Trifolium* species compared to the control treatment. Furthermore,
267 the root: shoot ratio was higher in monocultures compared to polycultures for *T. pratense* and *T.*
268 *repens*. Proportionally more flowers developed on *A. stolonifera* in monocultures than in
269 combination with *T. pratense* while the opposite effect was found for *T. pratense* as significantly
270 more flowers appeared in polycultures compared to monocultures. Plants grown in cattle (both
271 *Trifolium* species) and horse (*T. repens*) dung developed more flowers compared to the control
272 treatment and relatively fewer flowers appeared in high densities of *T. pratense* (Fig. 5, Appendix,
273 Appendix S5).

274 **Discussion**

275 Adding dung generally increased the time to emergence, which is in line with the findings of previous
276 research (Miller 1995, Meyer & Witmer 1998, Ramos-Font *et al.* 2015) and has been ascribed to the
277 rather unfavourable environment of fresh dung. Animal excrements may contain toxic compounds
278 with adverse effects on seed survival and germination (Marambe *et al.* 1993, Welch 1985). In some
279 cases, dung facilitates fungal and bacterial growth with a mortal effect on seeds (Meyer & Witmer
280 1998, Traveset *et al.* 2007) while in other cases dung was found to protect seeds from being attacked
281 by parasites and predators (Fragoso *et al.* 2003). On the other hand, once germinated, the growth of
282 young seedlings is in some cases promoted in the dung environment, most probably due to the
283 fertilising effect of this micro-environment (Traveset *et al.* 2001, Milotić & Hoffmann 2016a).

284 Furthermore, both the structural and chemical composition of dung is known to vary between
285 herbivore species (e.g., between ruminants and non-ruminants (Holter 2016)) or with diet (e.g.,
286 brown bear dung composed of animal vs. vegetal material (Traveset *et al.* 2001)). According to Holter
287 (2016), ruminant dung generally consists of smaller particles and has a higher C: N ratio while the
288 water content is in the same order of magnitude. In our experiment, a hard crust developed soon
289 after application of cattle dung while the appearance of the used horse dung remained structurally
290 unchanged during the whole experimental period. The slower emergence rate and lower
291 establishment success of seedlings in cattle dung might be related with this dry and hard top layer
292 (Grellier *et al.* 2012). The structural composition and water potential of soils are further
293 determinants of plant health and are known to differ greatly between soil types (MacDonald 1994).
294 Plant growth is closely related to the availability of macro and micro nutrients and toxic elements in
295 the soil environment (Mengel & Kirkby 1978). Therefore, the differing nutrient concentrations in
296 ruminant and non-ruminant dung could result in a species-specific response in plant development
297 (Dai 2000, Jørgensen & Jensen 1997). As fresh dung contains relatively high levels of toxic
298 compounds and most nutrients are bound in organic compounds that need to be mineralized before

299 becoming available for plant uptake, dung might have adverse effects on the early life stages of
300 plants (Cosyns et al. 2005, Ramos-Font et al., 2015). Furthermore, dung may also promote the
301 growth of fungi and bacteria which may damage seeds and inhibit germination and seedling survival
302 (Clark & Wilson 2003, Traveset et al. 2007).

303 As high seed densities generally have a negative impact on plant establishment, a selection pressure
304 can be expected for tactics that overcome this competition, e.g., early germination or induced
305 dormancy (Loiselle 1990, Murray 1998). Orrock and Christopher (2010) measured shorter
306 germination times when intraspecific competition was greater in a bird-dispersed shrub and early
307 germinated plants grew larger and had higher growth rates as they had more time to grow without
308 competitors. Likewise examples of the advantages of early germination can be found in both
309 intraspecific (e.g., Black & Wilkinson 1963) and interspecific (e.g., Bergelson & Perry 1989) seed
310 mixtures while other species might postpone germination until the subsequent season in highly
311 competitive environments (Turkington *et al.* 2005). In a lab experiment, Linhart (1976) measured an
312 increased germination rate in *Trifolium* and *Agrostis* species when sown in high densities. Although
313 we should note that we used different methods and species, we did not measure any effect of seed
314 density on germination timing. Overall, high seed densities led to a reduced establishment success in
315 monocultures which may be attributed to intraspecific competition or to the mechanism of
316 autotoxicity that results in an inverse correlation between seed abundance and germination success
317 (Barnea *et al.* 1992, Loiselle 1990, Murray 1998). Plants could be able to escape sibling competition in
318 high densities through an adaptation of the proportion of dormant seeds and seeds that germinate
319 instantaneously (Cheplick 1992, Hyatt & Evans 1998).

320 The functional equilibrium theory (Brouwer 1962) postulates that the allocation of biomass shifts
321 towards roots when belowground resources (e.g., nutrient level and water) are low whereas shoot
322 biomass increases in case aboveground resources (e.g., CO₂ and light) are limiting. Doing so, plants
323 optimise their ability to compete for limiting resources (Poorter & Nagel 2000). Our results partly

324 support this theory, as both *Trifolium* species indeed had a higher root biomass in the control
325 treatment. Nevertheless, few interactions between dung treatments and seed densities were found
326 which is in contrast with the differing root: shoot biomass allocation in *Plantago lanceolata* with
327 varying seed densities and nutrient levels found by Berendse and Möller (2009) or the increased
328 belowground competition Aerts *et al.* (1991) found in fertilised heathland plants.

329 In all tested species individual plant biomass was relatively higher in polycultures which suggests that
330 intraspecific competition has stronger effects than interspecific competition. Higher biomass of
331 species in polycultures has been previously found in natural ecosystems and increases with
332 increasing nutrient levels (Gu *et al.* 2012, Li & Watkinson 2000). On the other hand, lower
333 establishment of *A. stolonifera* and *T. pratense* has been found in polycultures, so rather than
334 producing more biomass spread over a large number of individuals, the average biomass per
335 individual was greater in polyculture stands. In monocultures, both *Trifolium* species invested more
336 in belowground biomass which suggests that intraspecific competition is harsher than interspecific
337 competition in our species mixtures. Furthermore, through a symbiotic relationship with soil bacteria
338 of the genus *Rhizobia* in their root nodules both *Trifolium* species are able to bind atmospheric
339 nitrogen into ammonium and gain a competitive advantage. In grass-clover mixtures, Nesheim and
340 Boller (1991) noted an increased nitrogen fixation when clovers were growing in combination with
341 grasses whereas Ledgard and Steele (1992) measured reduced nitrogen fixation when N fertiliser was
342 used. In our experiments, we indeed measured a higher allocation to *Trifolium* root biomass in the
343 control treatments. The presence of dung following endozoochorous dispersal might, therefore,
344 change the competitive interactions of species mixtures, affect the development of root mutualists
345 or act as a potential source of inoculum. Furthermore, the reproductive effort decreases with
346 increasing seed density in *T. pratense* which could be related to the fact that individual plants are
347 smaller in high seed densities and can, therefore, allocate a smaller proportion of their biomass in
348 reproductive tissues (Weiner 1988). Our results suggest that the presence of dung and competition
349 affects endozoochorous dispersal success. However, we should keep in mind that we conducted our

350 experiment in a standardised greenhouse environment which differs from the natural situation. As
351 we directly put dung on the substrate we did not account for competition by already established
352 vegetation. Large herbivores deposit a rather large quantity of dung per defecation and their dung
353 often smothers and kills the existing vegetation at the deposition site and consequently creates a gap
354 for seedling establishment (Traveset 1998, Williams & Haynes 1995). In a dung deposition
355 experiment in temperate dune grasslands, Cosyns *et al.* (2006) measured an increase in small-scale
356 species richness as species co-existing patterns shifted from a dominance by monocotylous species
357 towards a more diverse assemblage of dicotylous and monocotylous species. Therefore, dung pats
358 not only seem to trigger inter- and intra-specific competition processes between their seed load, but
359 also create safe sites in the existing vegetation enabling the germination and establishment of
360 endozoochorously dispersed seeds.

361 Throughout the experiment we used a 2 cm thick layer of dung, which should enable the germination
362 of the rather large seeded test species (see e.g., Grundy *et al.* 2003), but in natural situations cattle
363 dung pats are frequently thicker than 2 cm (Malo & Suarez 1995a) and horses often exhibit latrine
364 behaviour by which both dung and dispersed seeds becomes aggregated (Edwards & Hollis 1982).
365 Furthermore, due to practical considerations, intact seeds were used while ingested seeds undergo a
366 sequence of mechanical, thermal and chemical processes (Milotić & Hoffmann 2016b). A decreased
367 germination success has been observed after gut passage in many species (e.g., Cosyns *et al.* 2005b).
368 In a gut passage experiment using cattle, D'hondt and Hoffmann (2011) found an increased
369 germination success of *T. pratense* while *A. stolonifera* seeds were less likely to germinate after gut
370 passage. On the other hand, both of our tested *Trifolium* species had a lower germinability after
371 being fed to horses and sheep (Cosyns *et al.* 2005b). Therefore, our results should rather be
372 interpreted as the outcome of one particular step in the complex process of endozoochory, and we
373 can assume that an even lower germination success would have been found when using ingested
374 seeds.

375 We conclude that the variable concentrations and species combinations in dung pats may trigger
376 processes of inter- and intraspecific competition and hence define the post-dispersal fate of
377 endozoochorously dispersed seeds. While high seed densities imply a cost due to lower germinability
378 and establishment, the nutritive environment of dung acts as a compensation in the later life stages
379 of plants resulting in faster growth and an increased investment in reproductive tissues in
380 competitive superior species.

381

382 **Author's contribution**

383 Tanja Milotić and Maurice Hoffmann conceived and designed the experiment and wrote the paper,
384 Tanja Milotić did all practical and analytical work.

385

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390 and drug-free dung, Yves Israel for cutting to size plastic tubes and three anonymous reviewers for
391 their constructive comments on an earlier version of this manuscript.

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578

579 **Supporting information**

580 **Appendix S1** - Two-way ANOVA results with establishment success in monocultures as measured
581 variable and dung type and seed density as nominal variables. Establishment success is expressed as
582 the percentage of sown seeds that established to visible plants at the end of the experiment and was
583 analysed for each size class. Significant results are written in bold.

584 **Appendix S2** - ANOVA results with RNE based on establishment success and biomass as measured
585 variable and dung type, seed density and competing species as nominal variables and a nested
586 proportion: competing species term.

587 **Appendix S3** - Likelihood ratios and p-values for the SMA regressions between root and shoot
588 biomass and between flower count and total biomass by species with dung types, seed densities and
589 competing species as factors.

590 **Appendix S4** - Standardized major axis (SMA) regression coefficients between root and shoot
591 biomass by species and factor. Only factors which differ significantly after a likelihood test are listed
592 (see **Appendix S3**). Factor levels marked with different letters differ significantly ($p < 0.05$) after
593 multiple comparisons with Šidák correction.

594 **Appendix S5** - Standardized major axis (SMA) regression coefficients between flower counts and total
595 biomass by species and factor. Only factors which differ significantly after a likelihood test are listed
596 (see **Appendix S3**). Factor levels marked with different letters differ significantly ($p < 0.05$) after
597 multiple comparisons with Šidák correction.

598 **Tables**599 **Table 1** - Experimental scheme with all treatment combinations of dung types, seed densities and species combinations.

600 Numbers indicate the number of seeds of the focal species (in bold) and competing species (between brackets). Each

601 combination was replicated 5 times.

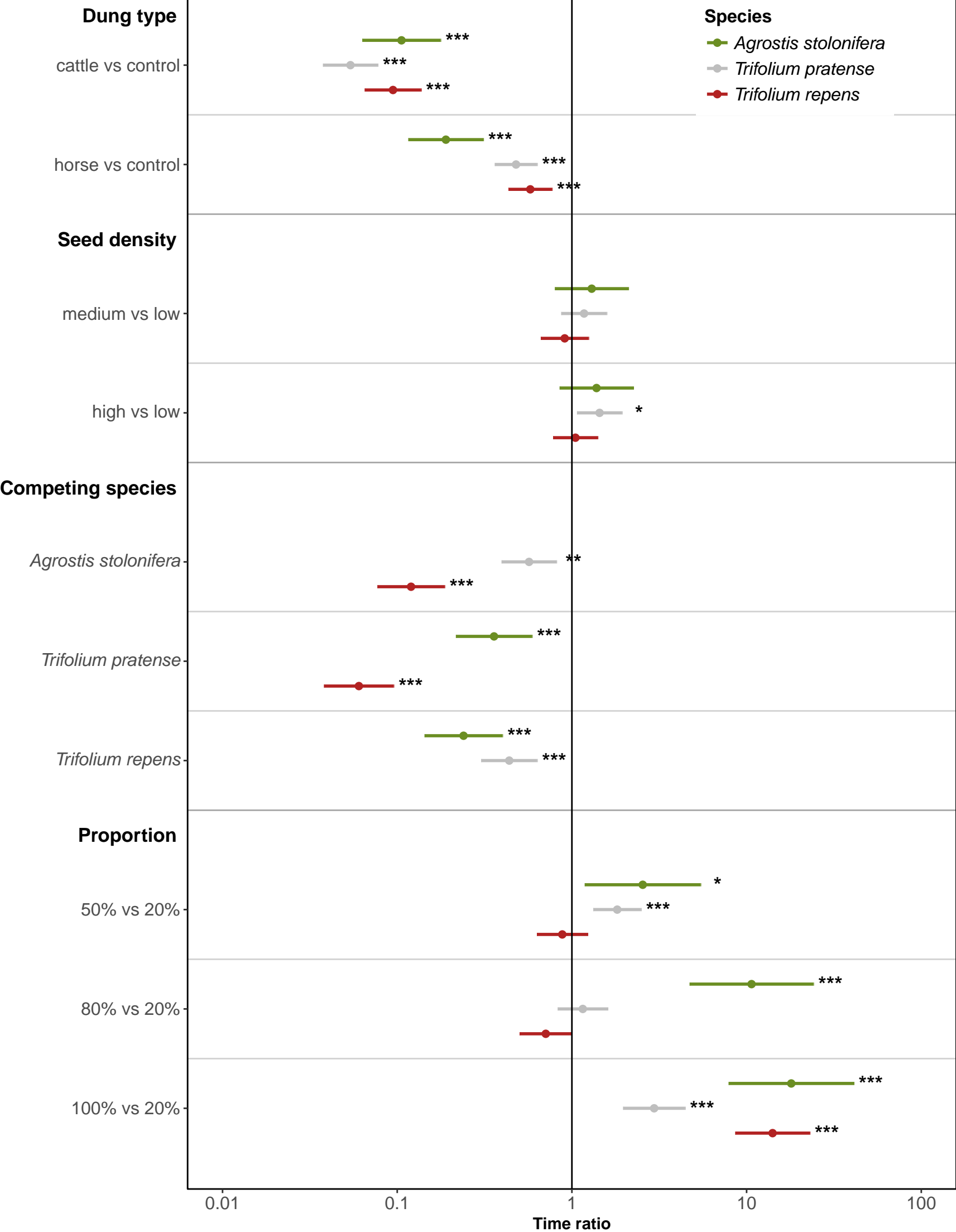
Dung type	Seed density	Competing species	Focal species		
			<i>Agrostis stolonifera</i>	<i>Trifolium pratense</i>	<i>Trifolium repens</i>
control, cattle or horse	low	<i>Agrostis stolonifera</i>	50 (0)	10 (40), 25 (25) or 40 (10)	10 (40), 25 (25) or 40 (10)
		<i>Trifolium pratense</i>	10 (40), 25 (25) or 40 (10)	50 (0)	10 (40), 25 (25) or 40 (10)
		<i>Trifolium repens</i>	10 (40), 25 (25) or 40 (10)	10 (40), 25 (25) or 40 (10)	50 (0)
	medium	<i>Agrostis stolonifera</i>	150 (0)	30 (120), 75 (75) or 120 (30)	30 (120), 75 (75) or 120 (30)
		<i>Trifolium pratense</i>	30 (120), 75 (75) or 120 (30)	150 (0)	30 (120), 75 (75) or 120 (30)
		<i>Trifolium repens</i>	30 (120), 75 (75) or 120 (30)	30 (120), 75 (75) or 120 (30)	150 (0)
	high	<i>Agrostis stolonifera</i>	250 (0)	50 (200), 125 (125) or 200 (50)	50 (200), 125 (125) or 200 (50)
		<i>Trifolium pratense</i>	50 (200), 125 (125) or 200 (50)	250 (0)	50 (200), 125 (125) or 200 (50)
		<i>Trifolium repens</i>	50 (200), 125 (125) or 200 (50)	50 (200), 125 (125) or 200 (50)	250 (0)

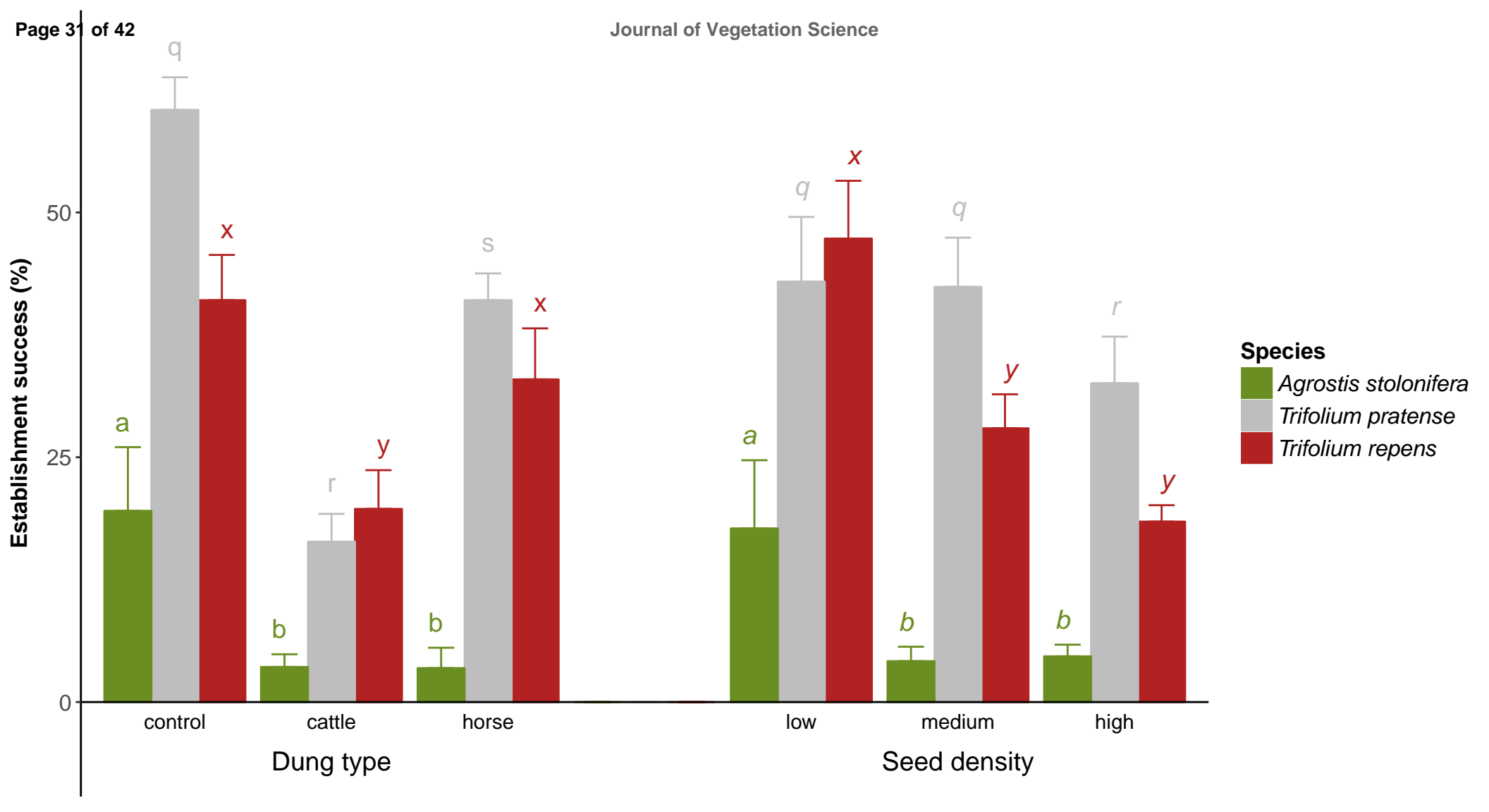
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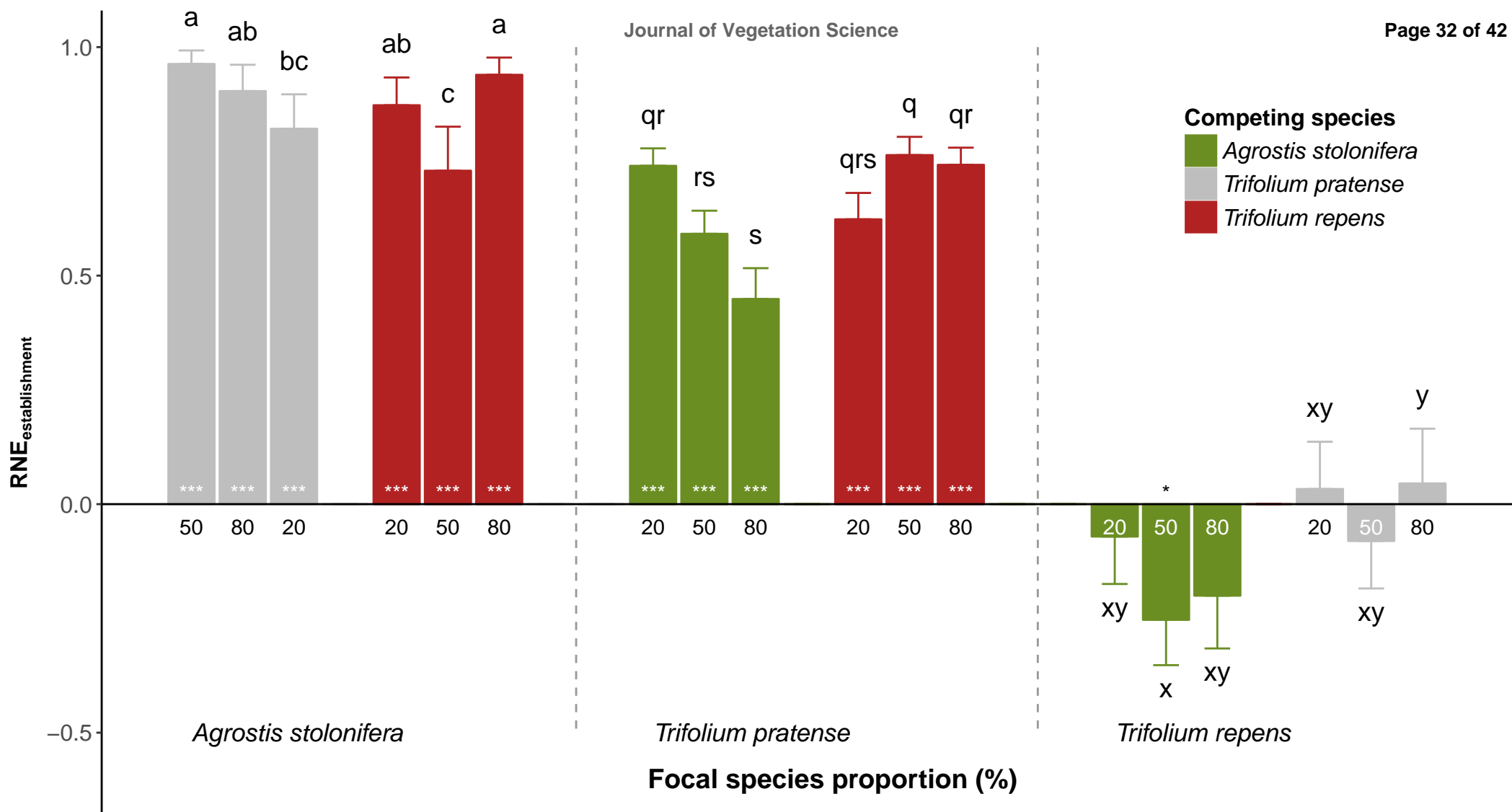
603 **Table 2** - Repeated measures ANOVA results in order to evaluate the effect of dung, seed density, competing species and
 604 species proportion on relative growth rate (RGR).

species	factor	df	F-value	p
<i>Agrostis stolonifera</i>	dung	2	11.704	<0.001
	seed density	2	0.488	0.616
	time	1	4.591	0.036
	competing species	2	19.785	<0.001
	proportion	4	0.803	0.600
	dung: seed density	4	0.697	0.040
	dung: time	2	3.374	0.345
	density: time	2	1.081	0.933
<i>Trifolium pratense</i>	dung	2	26.631	<0.001
	seed density	2	1.115	0.330
	time	1	0.007	0.933
	competing species	2	193.693	<0.001
	proportion	4	1.084	0.365
	dung: seed density	4	2.566	0.039
	dung: time	2	11.952	<0.001
	density: time	1	10.061	0.002
<i>Trifolium repens</i>	dung	2	15.148	<0.001
	seed density	2	0.162	0.851
	time	1	9.910	0.002
	competing species	2	24.325	<0.001
	proportion	4	0.489	0.744
	dung: seed density	4	0.679	0.607
	dung: time	1	8.058	0.005

605

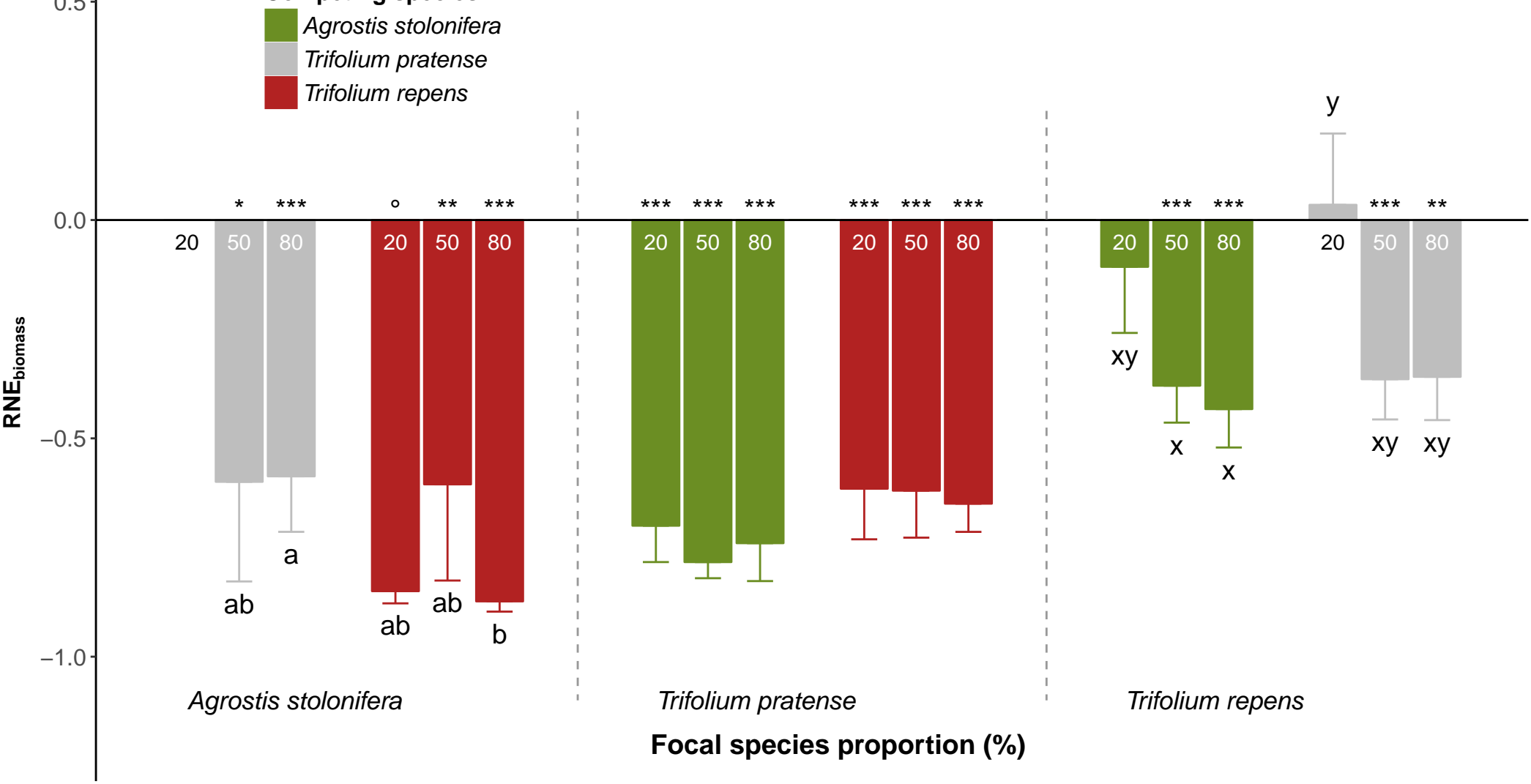


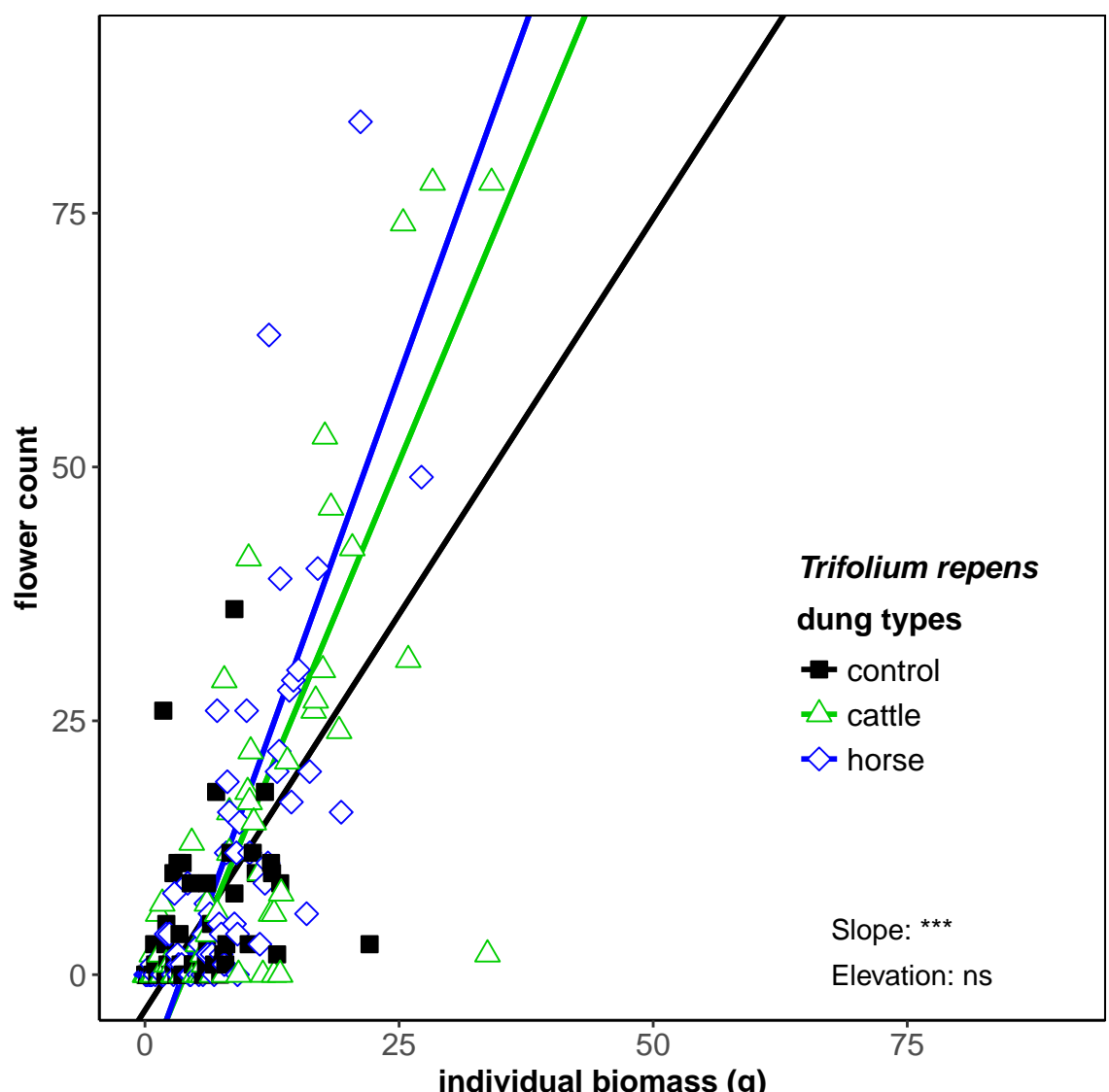
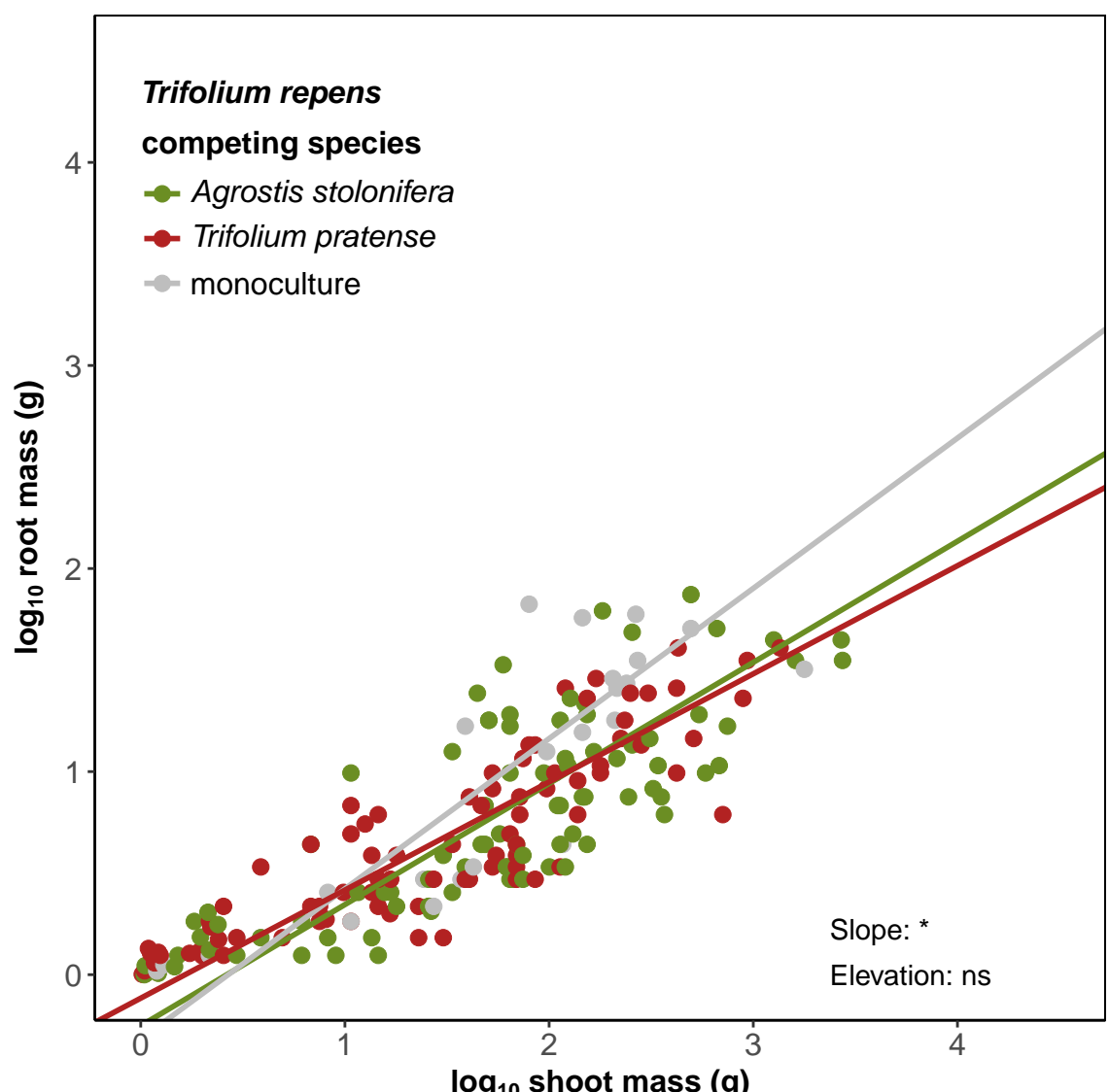
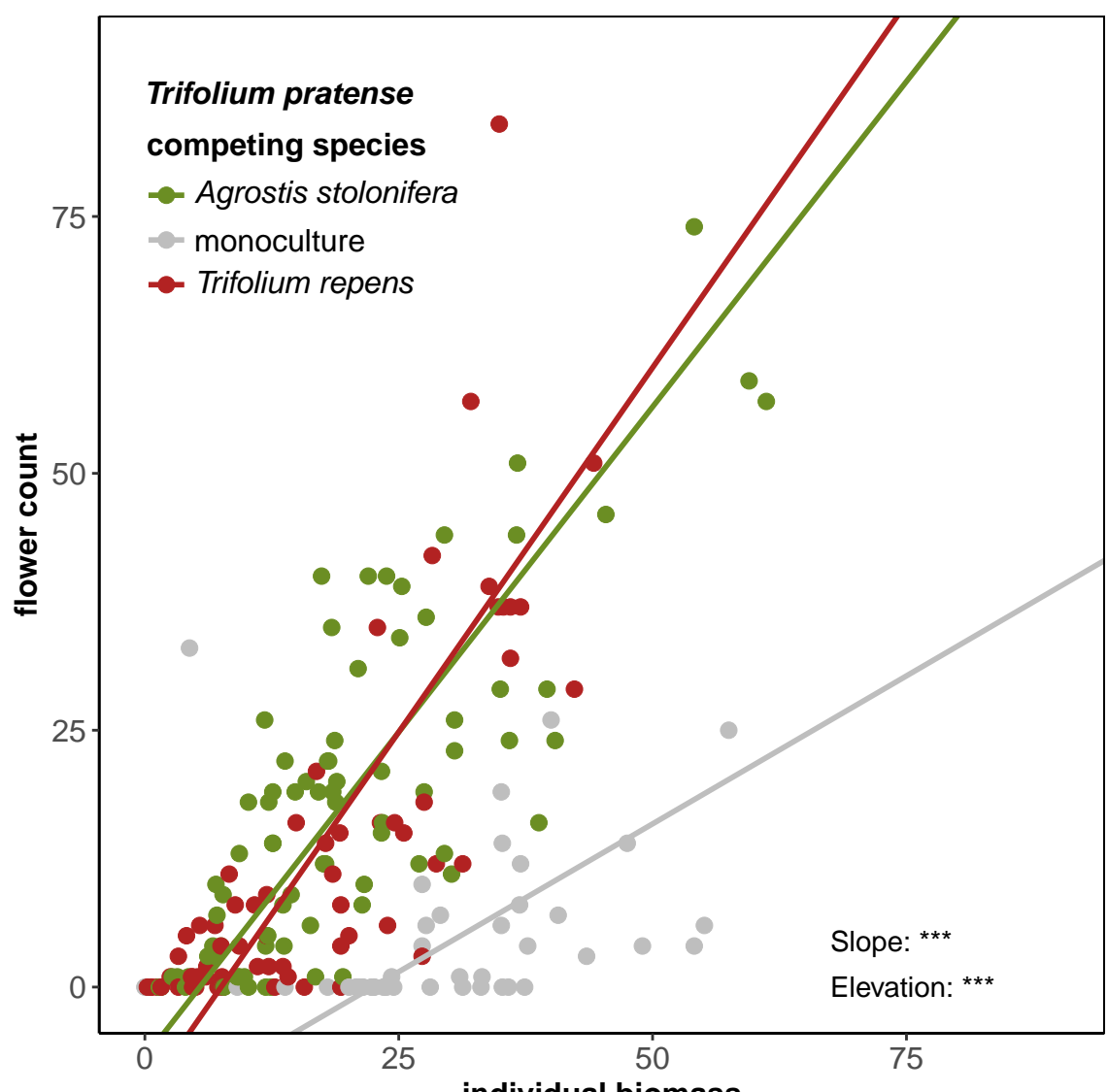
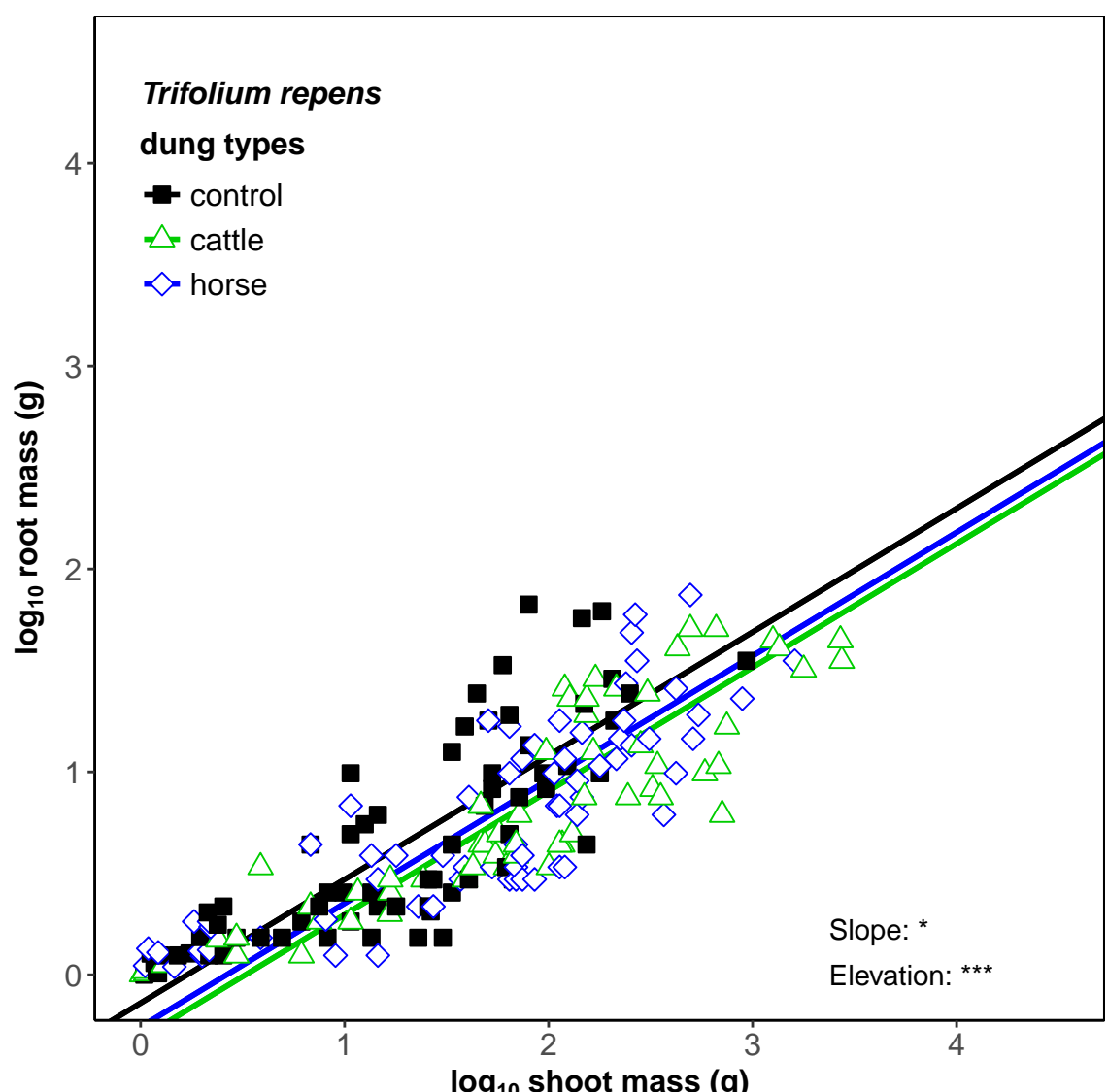
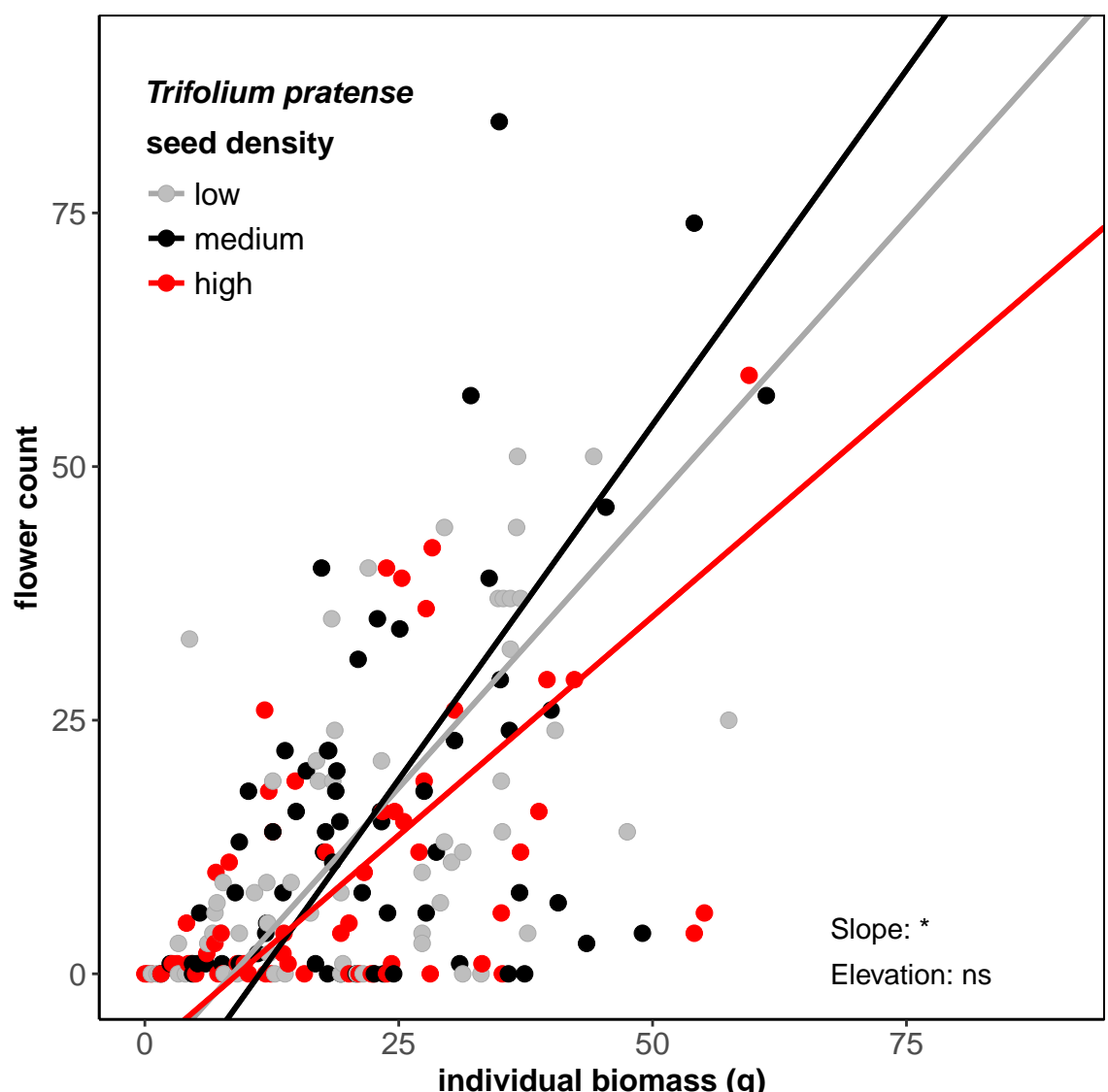
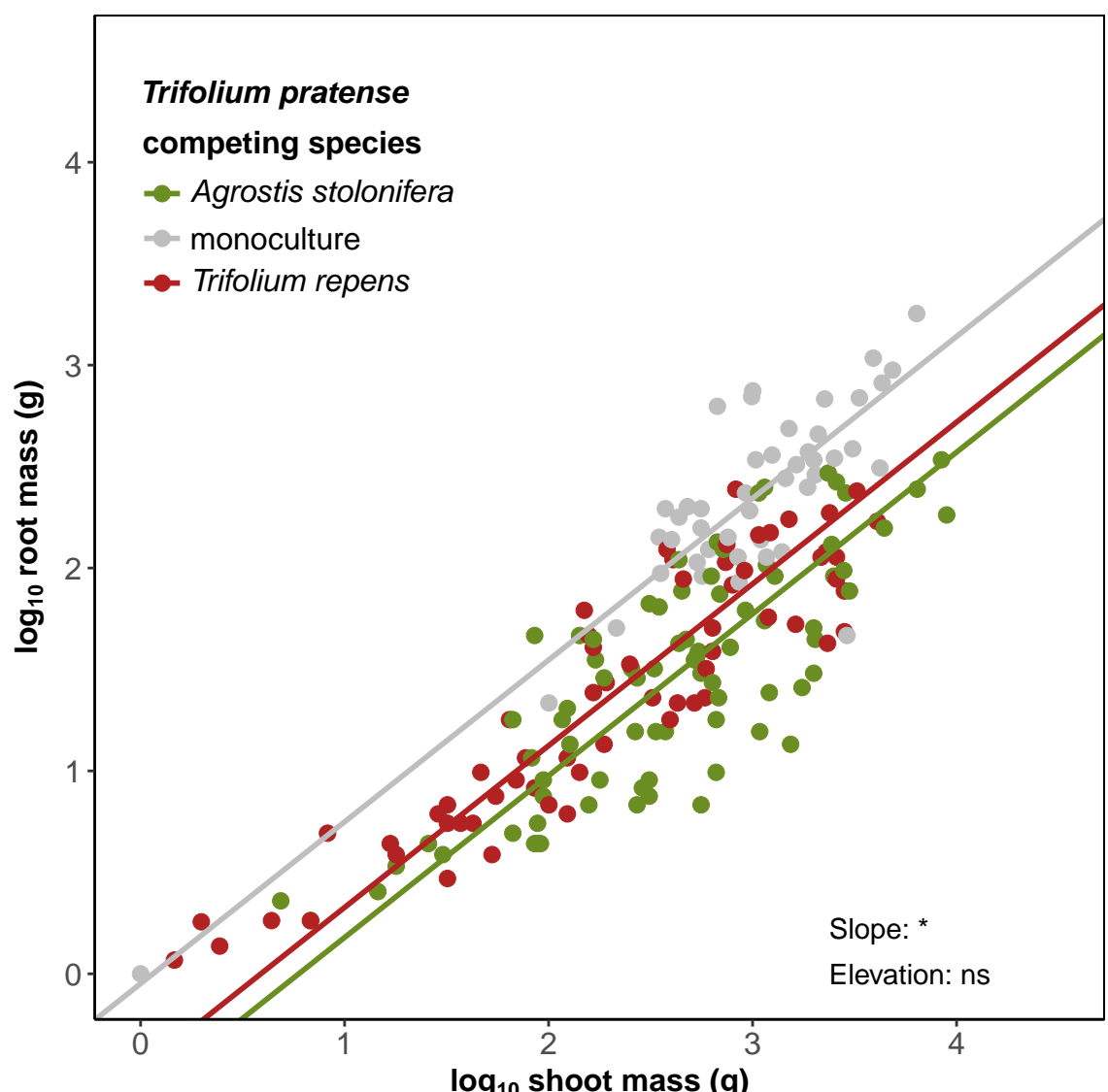
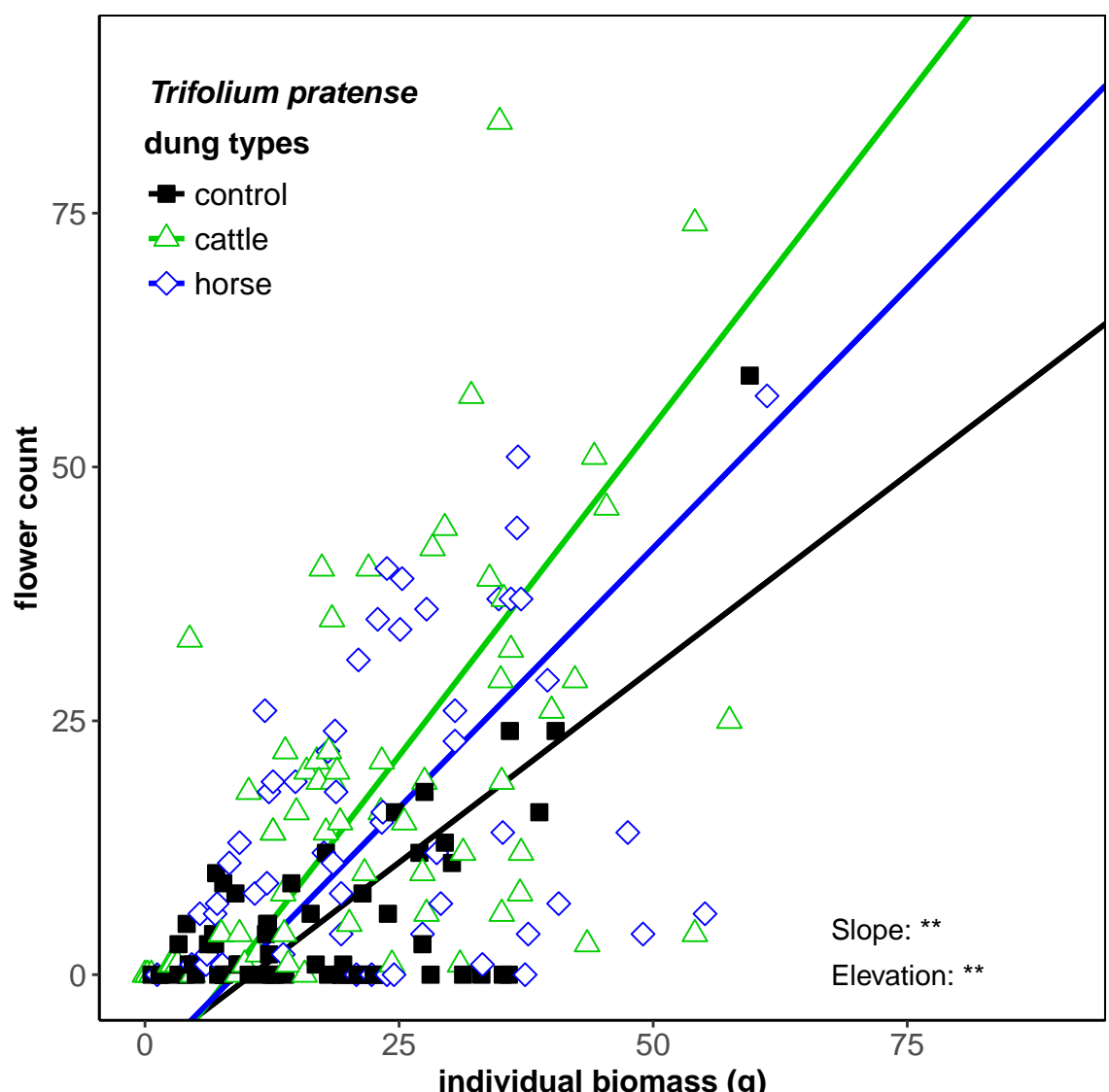
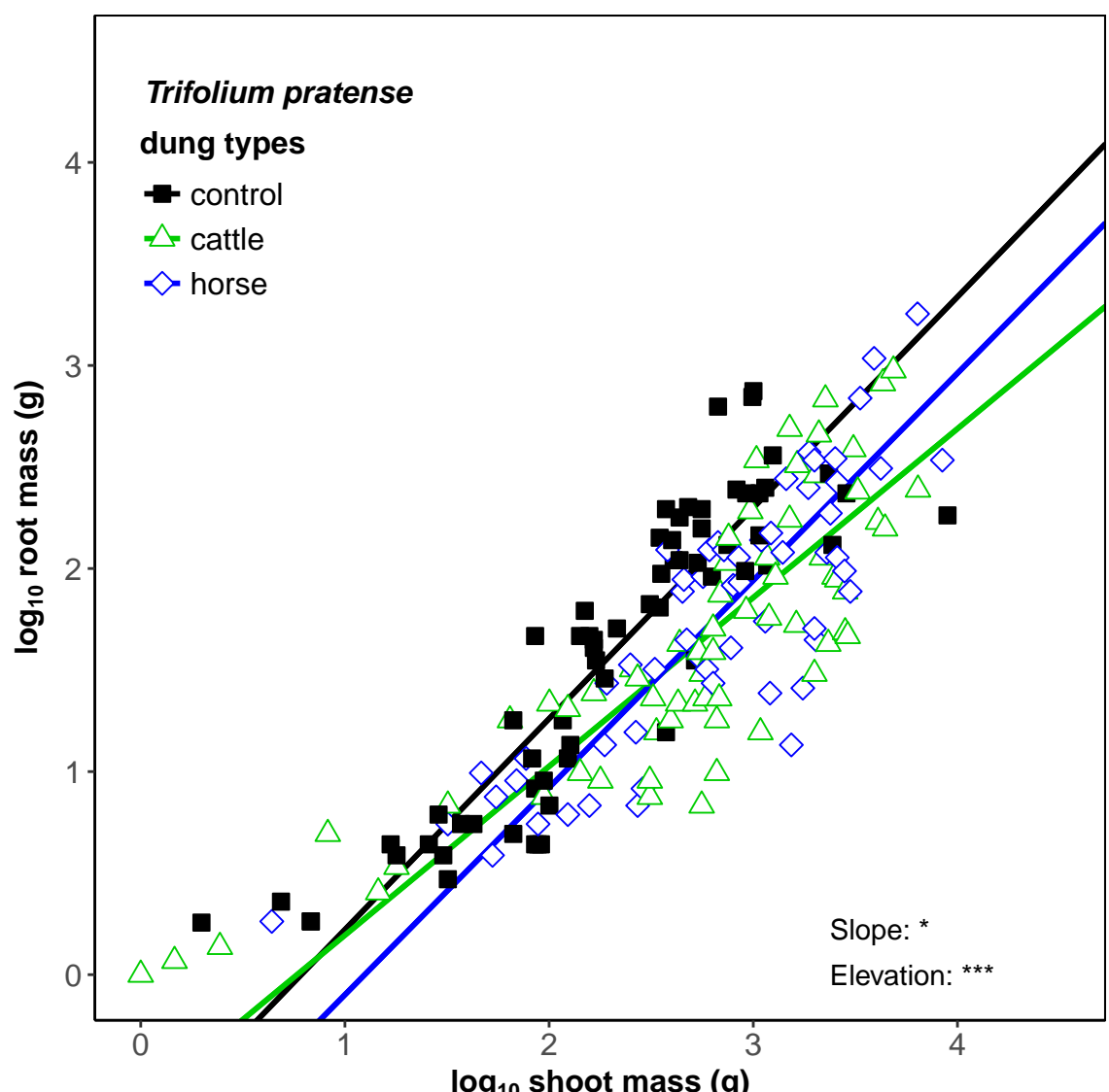
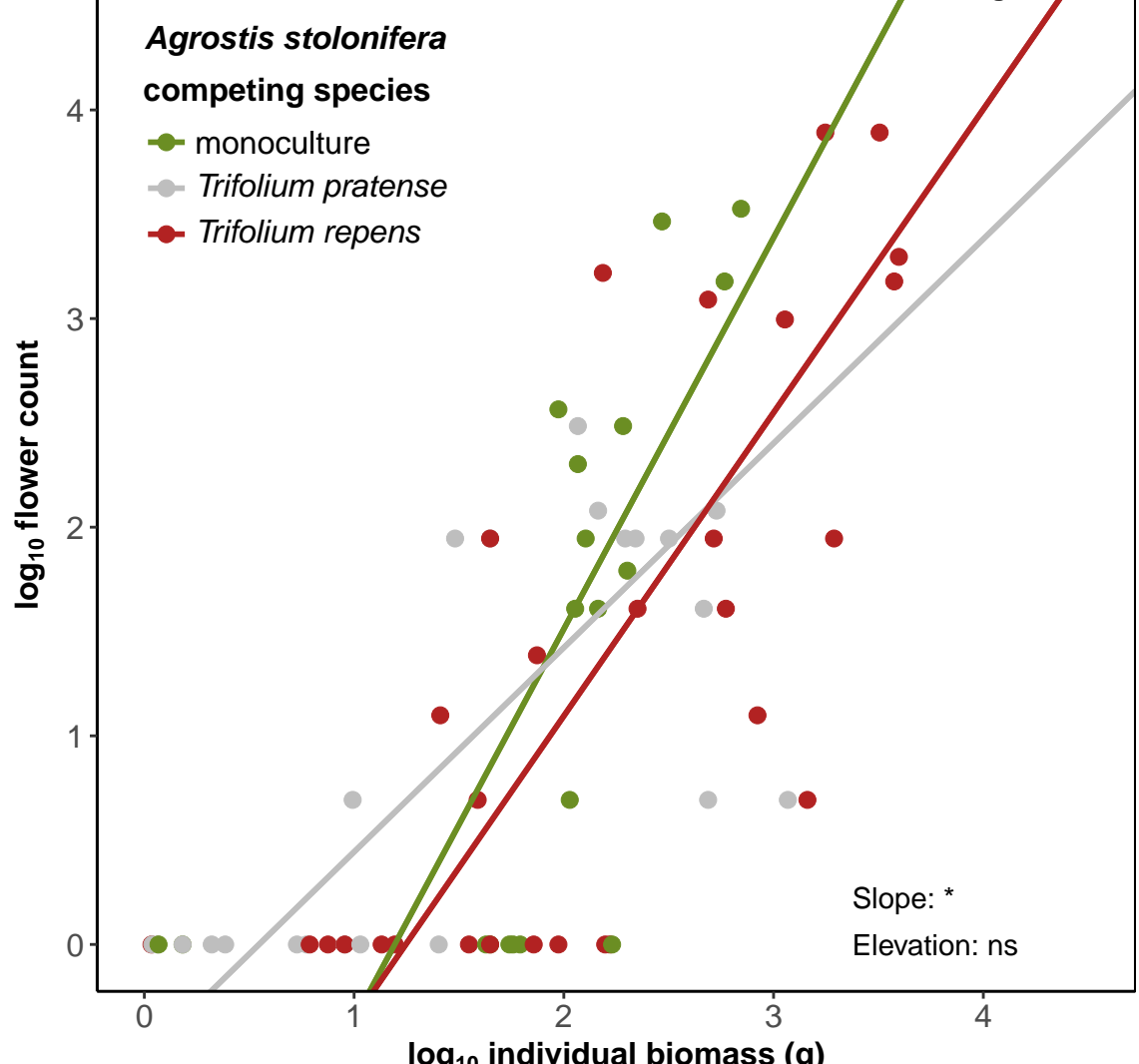
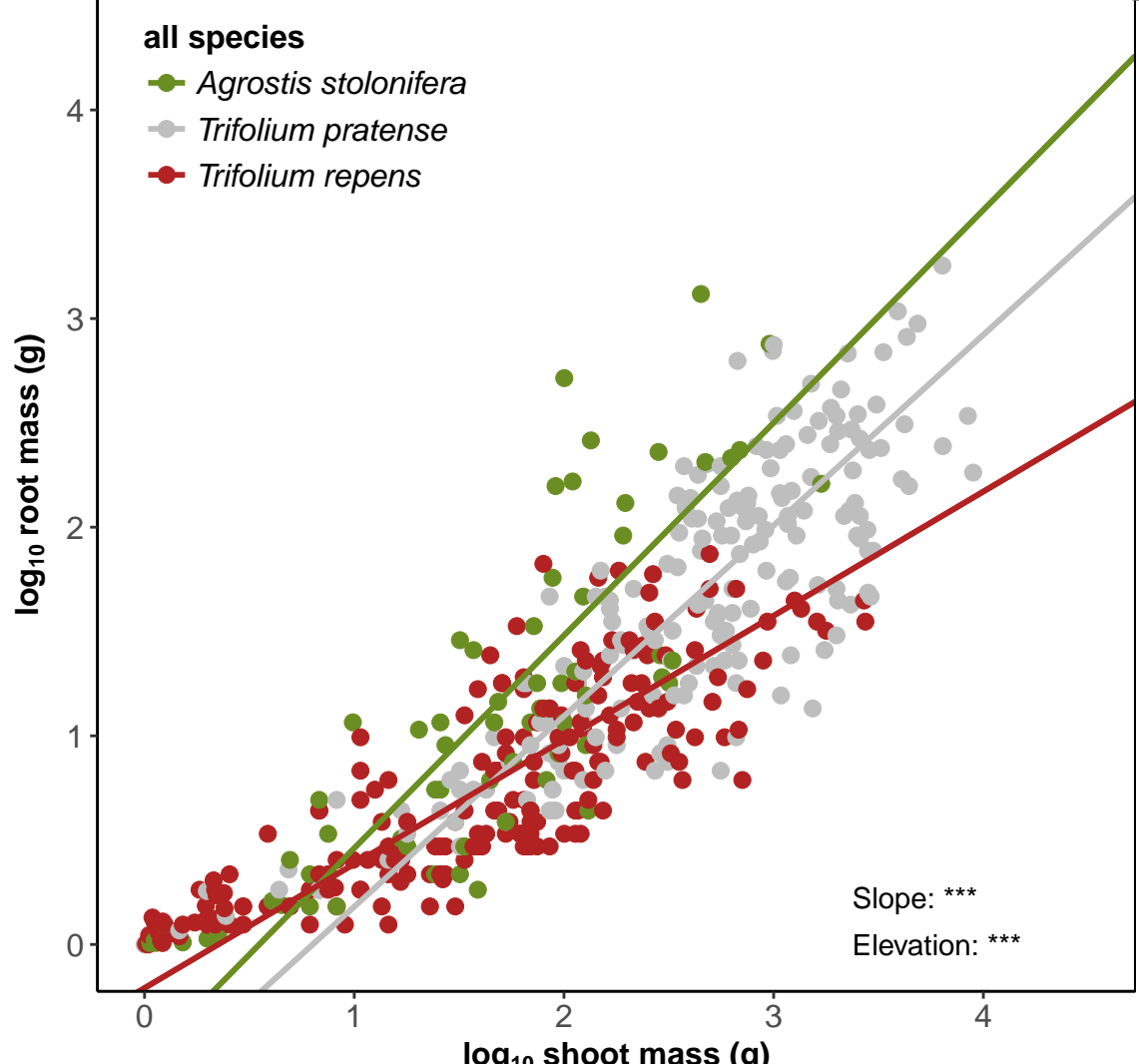




Competing species

- Agrostis stolonifera*
- Trifolium pratense*
- Trifolium repens*





We conducted a competition experiment using three common perennial grassland species in different combinations and densities. By adding dung, we examined plant establishment, growth and flowering following endozoochory. High seed densities implied a cost due to a reduced establishment while the nutritive environment of dung acted as a compensation resulting in faster growth and an increased investment in reproductive tissues.



835x835mm (72 x 72 DPI)

The impact of dung on inter- and intraspecific competition of temperate grassland seeds

Tanja Milotić and Maurice Hoffmann

Supporting information

Contents

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Appendix S1 - Two-way ANOVA results with establishment success in monocultures as measured variable and dung type and seed density as nominal variables. Establishment success is expressed as the percentage of sown seeds that established to visible plants at the end of the experiment and was analysed for each size class. Significant results are written in bold.

Species	Factor	Df	Size class			
			All	Non-flowering	Flowering	Juvenile
<i>Agrostis stolonifera</i>	dung	2	F: 6.649; p=0.003		F: 2.726; p=0.079	F: 7.605; p=0.002
	seed density	2	F: 4.604; p=0.017		F: 1.515; p=0.233	F: 3.891; p=0.030
	dung: density	4	F: 1.872; p=0.137		F: 0.537; p=0.709	F: 1.905; p=0.131
<i>Trifolium pratense</i>	dung	2	F: 67.459; p<0.001	F: 5.300; p<0.001	F: 26.554; p<0.001	F: 37.908; p<0.001
	seed density	2	F: 4.714; p=0.015	F: 5.799; p=0.007	F: 34.252; p<0.001	F: 9.342; p<0.001
	dung: density	4	F: 1.542; p=0.211	F: 3.071; p=0.028	F: 14.701; p<0.001	F: 1.189; p=0.332
<i>Trifolium repens</i>	dung	2	F: 33.246; p<0.001		F: 0.006; p=0.994	F: 1.726; p=0.192
	seed density	2	F: 4.063; p=0.026		F: 1.484; p=0.240	F: 2.020; p=0.147
	dung: density	4	F: 0.990; p=0.425		F: 0.349; p=0.843	F: 0.584; p=0.677

Appendix S2 - ANOVA results with RNE based on establishment success and biomass as measured variable and dung type, seed density and competing species as nominal variables and a nested proportion: competing species term.

Focal species	Factor	RNE _{establishment}			RNE _{biomass}		
		df	F	p	df	F	p
<i>Agrostis stolonifera</i>	dung type	2	3.444	0.034	2	3.750	0.034
	seed density	2	115.2	<0.001	2	7.078	0.003
	competing species	1	3.251	0.073	1	4.420	0.043
	proportion	4	8.661	<0.001	3	3.586	0.024
	dung : density	4	110.9	<0.001	4	1.610	0.194
<i>Trifolium pratense</i>	dung type	2	8.727	<0.001	2	27.37	<0.001
	seed density	2	5.536	0.004	2	20.7	<0.001
	competing species	1	9.740	0.002	1	6.360	0.013
	proportion	4	4.689	0.001	4	1.423	0.230
	dung : density	4	2.845	0.025	4	15.3	<0.001
<i>Trifolium repens</i>	dung type	2	55.5	<0.001	2	0.240	0.787
	seed density	2	27.62	<0.001	2	3.309	0.039
	competing species	1	7.591	0.006	1	0.264	0.608
	proportion	4	1.258	0.287	4	2.801	0.028
	dung : density	4	11.836	<0.001	4	5.114	<0.001

Appendix S3 - Likelihood ratios and p-values for the SMA regressions between root and shoot biomass and between flower count and total biomass by species with dung types, seed densities and competing species as factors.

	species	factor	likelihood ratio (df)	p
root: shoot ratio	all	species	72.66 (2)	<0.001
	<i>Agrostis stolonifera</i>	dung types	0.16 (2)	0.923
		seed density	1.44 (2)	0.486
		competing species	4.10 (2)	0.129
	<i>Trifolium pratense</i>	dung types	6.86 (2)	0.032
		seed density	1.47 (2)	0.480
		competing species	100 (2)	<0.001
	<i>Trifolium repens</i>	dung types	11.82 (2)	0.003
		seed density	2.28 (2)	0.319
		competing species	7.74 (2)	0.021
flower count: biomass	<i>Agrostis stolonifera</i>	dung types	0.14 (2)	0.932
		seed density	3.05 (2)	0.217
		competing species	7.44 (2)	0.024
	<i>Trifolium pratense</i>	dung types	12.76 (2)	0.002
		seed density	8.88 (2)	0.012
		competing species	26.49 (2)	<0.001
	<i>Trifolium repens</i>	dung types	16.66 (2)	<0.001
		seed density	1.12 (2)	0.572
		competing species	1.79 (2)	0.409

Appendix S4 - Standardized major axis (SMA) regression coefficients between root and shoot biomass by species and factor. Only factors which differ significantly after a likelihood test are listed (see **Appendix S3**). Factor levels marked with different letters differ significantly ($p < 0.05$) after multiple comparisons with Šidák correction.

Species, factor	Factor level	n	α_{SMA} (95% CI)	β_{SMA} (95% CI)	r^2	p
all		453	-0.47 (-0.55; -0.38)	0.81 (0.77; 0.85)	0.74	<0.001
	<i>Agrostis stolonifera</i> ^a	70	-0.55 (-0.80; -0.32)	1.02 (0.89; 1.17)	0.69	<0.001
	<i>Trifolium pratense</i> ^a	187	-0.73 (-0.93; -0.54)	0.91 (0.84; 0.99)	0.7	<0.001
	<i>Trifolium repens</i> ^b	196	-0.21 (-0.29; -0.13)	0.59 (0.55; 0.64)	0.70	<0.001
<i>Trifolium pratense</i>						
dung types	control ^a	42	-0.82 (-1.10; -0.53)	1.04 (0.93; 1.16)	0.80	<0.001
	cattle ^b	16	-0.64 (-0.96; -0.32)	0.83 (0.73; 0.95)	0.71	<0.001
	horse ^a	12	-1.12 (-1.54; -0.70)	1.02 (0.88; 1.18)	0.72	<0.001
competing species	monoculture ^a	45	-0.05 (-0.24; 0.15)	0.80 (0.74; 0.86)	0.73	<0.001
	<i>Agrostis stolonifera</i> ^b	79	-0.62 (-0.79; -0.45)	0.80 (0.74; 0.86)	0.65	<0.001
	<i>Trifolium repens</i> ^c	63	-0.47 (-0.62; -0.32)	0.80 (0.74; 0.86)	0.83	<0.001
<i>Trifolium repens</i>						
dung types	control ^a	70	-0.14 (-0.23; -0.05)	0.61 (0.56; 0.66)	0.65	<0.001
	cattle ^b	59	-0.31 (-0.43; -0.20)	0.61 (0.56; 0.66)	0.78	<0.001
	horse ^b	67	-0.26 (-0.36; -0.15)	0.61 (0.56; 0.66)	0.69	<0.001
competing species	monoculture ^a	25	-0.32 (-0.61; -0.03)	0.74 (0.60; 0.91)	0.77	<0.001
	<i>Agrostis stolonifera</i> ^{ab}	88	-0.25 (-0.39; -0.11)	0.59 (0.53; 0.68)	0.66	<0.001
	<i>Trifolium pratense</i> ^b	83	-0.12 (-0.21; -0.02)	0.53 (0.48; 0.59)	0.76	<0.001

Appendix S5 - Standardized major axis (SMA) regression coefficients between flower counts and total biomass by species and factor. Only factors which differ significantly after a likelihood test are listed (see **Appendix S3**). Factor levels marked with different letters differ significantly ($p < 0.05$) after multiple comparisons with Šidák correction.

Species, factor	Factor level	n	α_{SMA} (95% CI)	β_{SMA} (95% CI)	r^2	p
all		453	-1.49 (-1.74; -1.24)	1.37 (1.27; 1.47)	0.37	<0.001
<i>Agrostis stolonifera</i>						
competing species	monoculture ^a	19	-2.24 (-3.69; -0.79)	1.88 (1.30; 2.72)	0.45	0.002
	<i>Trifolium pratense</i> ^b	22	-0.54 (-1.10; 0.02)	0.98 (0.72; 1.34)	0.54	<0.001
	<i>Trifolium repens</i> ^{ab}	29	-1.81 (-2.71; -0.91)	1.45 (1.12; 1.88)	0.56	<0.001
<i>Trifolium pratense</i>						
dung types	control ^a	42	-8.04 (-11.25; -4.82)	0.76 (0.63; 0.93)	0.38	<0.001
	cattle ^b	16	-10.93 (-18.71; -3.15)	1.30 (1.05; 1.61)	0.26	<0.001
	horse ^{ab}	12	-9.16 (-17.18; -1.13)	1.02 (0.79; 1.32)	0.09	0.026
seed density	low ^a	61	-9.68 (-15.98; -3.38)	1.12 (0.90; 1.39)	0.30	<0.001
	medium ^a	63	-15.82 (-24.13; -7.51)	1.40 (1.12; 1.74)	0.26	<0.001
	high ^b	63	-7.91 (-13.25; -2.56)	0.86 (0.69; 1.09)	0.18	<0.001
competing species	monoculture ^a	45	-12.92 (-19.04; -6.79)	0.58 (0.43; 0.78)	0.04	0.186
	<i>Agrostis stolonifera</i> ^b	79	-6.85 (-10.93; -2.78)	1.27 (1.11; 1.45)	0.64	<0.001
	<i>Trifolium repens</i> ^b	63	-10.65 (-15.30; -6.01)	1.42 (1.21; 1.66)	0.62	<0.001
<i>Trifolium repens</i>						
dung types	control ^a	70	-3.51 (-5.91; -1.11)	1.56 (1.25; 1.95)	0.15	0.001
	cattle ^b	59	-9.54 (-15.03; -4.04)	2.40 (2.02; 2.86)	0.56	<0.001
	horse ^b	67	-10.33 (-14.78; -5.89)	2.77 (2.35; 3.27)	0.55	<0.001