

1 **An ecological connectivity network maintains genetic diversity of a flagship wildflower,**
2 ***Pulsatilla vulgaris***

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14 Keywords: actual functional connectivity, calcareous grassland, ecological networks, fitness,
15 fragmentation, genetic diversity, landscape genetics, microsatellites, *Pulsatilla vulgaris*

16 **Abstract**

17 Ecological connectivity networks have been proposed as an efficient way to reconnect
18 communities in fragmented landscapes. Yet few studies have evaluated if they are successful at
19 enhancing actual functional connectivity (i.e. realized dispersal or gene flow) of focal species, or
20 if this enhanced connectivity is enough to maintain genetic diversity and fitness of plant
21 populations. Here we test the efficacy of an ecological connectivity network implemented in
22 southern Germany since 1989 to reconnect calcareous grassland fragments through rotational
23 shepherding. We genotyped 1,449 individuals from 57 populations and measured fitness-related
24 traits in 10 populations of *Pulsatilla vulgaris*, a flagship species of calcareous grasslands in
25 Europe. We tested if the shepherding network explained functional connectivity in *P. vulgaris*
26 and if higher connectivity translated to higher genetic diversity and fitness of populations. We
27 found that population-specific F_{st} was lowest in populations that had high connectivity within the
28 shepherding network, and that well-connected populations within the network had significantly
29 higher genetic diversity than ungrazed and more isolated grazed populations. Moreover, genetic
30 diversity was significantly positively correlated with both seed set and seed mass. Together our
31 results suggest that the implementation of an ecological shepherding network is an effective
32 management measure to maintain functional connectivity and genetic diversity at the landscape
33 scale for a calcareous grassland specialist. Populations with reduced genetic diversity would
34 likely benefit from inclusion, or better integration into the ecological connectivity network. Our
35 study demonstrates the often postulated but rarely tested sequence of positive associations
36 between connectivity, genetic diversity, and fitness at the landscape scale, and provides a
37 framework for testing the efficacy of ecological connectivity networks for focal species using
38 molecular genetic tools.

39 **Introduction**

40 Habitat loss and fragmentation are major threats to the persistence of populations across nearly
41 all taxonomic groups (Fischer and Lindenmayer 2007). Together, these processes can lead to
42 reductions in effective population size and gene flow among previously contiguous patches,
43 enhancing the effects of genetic drift in small populations and accelerating the loss of genetic
44 diversity (Frankham 2005; Reed and Frankham 2003). Small and isolated populations are more
45 prone to inbreeding depression and suffer a reduced potential for adaptation (Frankham 2005).
46 Preserving genetic diversity is not only important for the population or species at-hand, but also
47 plays a critical role in the functioning of communities and ecosystems, with positive influences
48 on species diversity, disease dynamics, food-web dynamics, and ecosystem cycling (Hughes et
49 al. 2008; Johnson et al. 2006; Lamy et al. 2013).

50 Ecological connectivity networks (i.e. sets of connected suitable habitats that allow
51 persistence of viable metapopulations for multiple species in a community), are increasingly
52 being implemented to restore dispersal linkages between remnant patches in fragmented
53 landscapes (Baguette et al. 2013; Boitani et al. 2007; Gilbert-Norton et al. 2010; Maiorano et al.
54 2015; Whitelaw and Eagles 2007). Dispersal linkages are often structural features of the
55 landscape, such as habitat corridors or stepping-stones that are created, protected, or maintained
56 under the assumption that they directly support the movement of organisms and their genes,
57 between fragments (known as functional connectivity). For plants, an alternative strategy to
58 reconnect populations exists where vectors of seed or pollen dispersal, instead of structural
59 aspects of the landscape, are restored for focal species. For example, rotational shepherding has
60 the potential to disperse seeds over very long distances (e.g. >100 km; Fischer et al. 1996;

61 Manzano and Malo 2006) and has been used as a strategy to reconnect grassland plant
62 populations in central Europe (Auffret et al. 2012; Butaye et al. 2005).

63 The spatial extent of ecological connectivity networks and their large number of corridors
64 often preclude the collection of base-line connectivity data to help inform decisions about the
65 suitability of linkages for protection. Most often, ecological connectivity networks are designed
66 solely based on the structural connectivity of the landscape (Cushman et al. 2009). More
67 recently, conservation managers have considered *potential* functional connectivity when
68 designing networks, using dispersal thresholds of focal species to select linkages for protection
69 (e.g. Bruinderink et al. 2003; Carroll et al. 2012; Cushman et al. 2009). However, it is rarely
70 tested if ecological connectivity networks are successful at maintaining *actual* functional
71 connectivity (i.e. realized dispersal or gene flow, Calabrese and Fagan 2004, but see Melles et al.
72 2012), or test if the enhanced connectivity provided by the network is enough to maintain genetic
73 diversity of populations. Consequently, there is a great need to evaluate the utility of ecological
74 connectivity networks for supporting functional connectivity for focal species and communities
75 (Boitani et al. 2007). Neutral genetic diversity on its own may not be a suitable conservation
76 target as the argument to conserve diversity is made on the basis of its presumed association with
77 fitness and potential for adaptation; for selectively neutral markers this evidence is equivocal
78 across the plant world (Leimu et al. 2006). Thus a better evaluation of the outcomes of
79 conservation planning involves applied targets such as fitness components or a demonstrated link
80 between diversity and fitness.

81 Here we tested the efficacy of an ecological connectivity network implemented in
82 southern Germany since 1989 to reconnect abandoned calcareous grassland fragments through
83 rotational shepherding. We measured genetic differentiation, genetic diversity and fitness-related

84 traits in the perennial wildflower *Pulsatilla vulgaris* Mill. (Ranunculaceae)— a flagship and
85 specialist species of calcareous grasslands in Europe and one of high conservation concern
86 (IUCN 2015). We tested the hypothesis that the shepherding network maintains seed dispersal
87 and thus gene flow of populations, and that this enhanced gene flow translates to higher genetic
88 diversity and fitness. Previous ecological research in this system showed that shepherding
89 connectivity is associated with increased species richness (Wagner et al. 2013), patch
90 colonization rates (Rico et al. 2012) and patch occupancy (Rico et al. 2014a) of characteristic
91 calcareous grassland plants. At the molecular level, research on a single species, *Dianthus*
92 *carthusianorum*, indicated that shepherding decreased genetic divergence among connected
93 populations (Rico et al. 2014b), and increased within-population neutral genetic diversity (Rico
94 et al. 2014a). The question that remains is whether such an increase in genetic diversity is
95 enough to make an impact on the fitness of populations, specifically for species of concern such
96 as *P. vulgaris*. We ask: (1) Does the shepherding network explain gene flow among *P. vulgaris*
97 populations, as quantified by population-specific genetic differentiation? (2) Does the potential
98 enhanced gene flow provided by the shepherding network translate to higher genetic diversity in
99 connected populations? (3) Does higher genetic diversity translate to higher fitness in this
100 system, beyond the effect of population size? This system is ideal as it allows us to compare
101 genetic diversity of patches in the network to ungrazed controls, but also allows a comparison
102 across patches with varying degrees of connectivity within the network to control for possible
103 direct effects of grazing on growth and reproduction.

104 **Methods**

105 *Data Collection*

106 This study was conducted in the Franconian Alb, Germany in a 10 km x 15 km study area,
107 containing 96 calcareous grassland fragments embedded in a matrix of agriculture, forest, and
108 settlements. Abandonment of traditional grazing practices over the past century has led to a
109 significant loss of grasslands, and previously contiguous patches have been fragmented by forest
110 succession and urban development (Dolek and Geyer 2002). In 1989, an ecological connectivity
111 network was implemented to reconnect fragmented grassland via rotational shepherding. The
112 network consists of three non-overlapping shepherding routes (Fig. 1). Prior to the
113 implementation of the network, a baseline survey was conducted to record all vascular plants in
114 previously abandoned grassland patches (Boehmer et al. 1990) and in 2009 this survey was
115 repeated (Wagner et al. 2013).

116 The early-flowering perennial herb *Pulsatilla vulgaris* (Ranunculaceae) is a flagship
117 species of calcareous grasslands across central Europe and of considerable conservation concern,
118 designated as “near threatened” (IUCN 2015). It is hermaphroditic and mainly outcrossing, and
119 typically produces between one and three purple flowers in early spring (March-April) that each
120 yield 40-100 seeds (Wells and Barling 1971). Seeds have long, feathery styles, and although they
121 appear to be adapted for wind dispersal, those carried by wind rarely disperse further than 20 cm
122 from the plant (Wells and Barling 1971). Although it is possible that deer and small mammals
123 occasionally transport seeds of *P. vulgaris*, these effects are likely small compared to the
124 potential movement of seeds by the large flocks of sheep (400-800 ewes per herd) that move
125 among grassland patches within the ecological connectivity network. The main pollinators of *P.*
126 *vulgaris* are the small-bodied bees *Andrena bicolor*, *Lasioglossum lineare*, and *Osmia bicolor*

127 (Fay and Barlow 2014; Kratochwil 1988). They have limited foraging ranges (180-600 m
128 maximum foraging range for similar size bees; Gathmann and Tschardtke 2002; Zurbuchen et al.
129 2010) and thus likely do not contribute strongly to between-population gene flow.

130 From April to May 2009, we collected leaf material of flowering individuals ($n=1,449$)
131 from all patches ($n=57$) containing *P. vulgaris*. Seven patches have been colonized by *P.*
132 *vulgaris* since the survey conducted in 1989 (see Table A.1 for details), and analyses of patch
133 occupancy and colonization rates can be found elsewhere (Rico et al. 2012; Wagner et al. 2013).
134 All flowering individuals were sampled in populations with fewer than 40 individuals. For
135 populations that exceeded 40 individuals, we collected leaves from 30-40 flowering plants from
136 across the population. We made note of eight populations that had large numbers of non-
137 flowering individuals. Three of these were consistently grazed, four were intermittently grazed,
138 and only one was ungrazed. With the exception of two large, intermittently grazed populations,
139 we collected a mix of flowering and non-flowering individuals from these populations. Of the 57
140 patches containing *P. vulgaris*, 19 are ‘core areas’ that have been consistently grazed over the
141 last 200+ years. The remaining 38 are ‘previously abandoned’ patches that had been abandoned
142 since at least 1960, and since the implementation of the management program have been either
143 consistently grazed (every year, 3-5 times/season, $n=15$), intermittently grazed (grazed only
144 within the first few years after 1989, $n=7$), or have remained ungrazed or grazed only late in the
145 growing season ($n=16$). Note that we found no differences in allelic richness between core areas
146 and previously abandoned patches and so do not distinguish between them in further analyses.
147 Unlike Rico *et al.* (2012), we lumped patches that are grazed only late in the season (August and
148 onwards, once per year) together with ungrazed patches, since *P. vulgaris* is an early-flowering
149 species and we expect seed dispersal to occur shortly after seeds ripen in May (Wells and Barling

150 1971). This did not have an effect on our results (see Results). Populations with fewer than five
151 individuals were excluded to prevent bias of population-level measures of genetic diversity and
152 differentiation, giving a final sample size of 49 populations (31 consistently grazed, 6
153 intermittently grazed, 12 ungrazed). In the field, population size was estimated from the number
154 of flowering and non-flowering individuals and categorized as <40 individuals, 40-100
155 individuals, or > 100 individuals, following the scheme of Rico *et al.* (2014a). Census population
156 size (i.e. actual counts of individuals) was recorded only for patches with fewer than 40
157 individuals and in patches where we collected fitness data (see below). Note that because plants
158 can reproduce clonally by forming satellite rosettes, individuals within 30 cm of each other were
159 counted as a single individual.

160 We extracted genomic DNA from dried leaves using QIAGEN DNeasy Plant Mini Kit
161 (QIAGEN, Mississauga, ON, Canada) following the manufacturer's protocol. *P. vulgaris* is
162 allotetraploid ($2n=4x=32$; Wells and Barling 1971), and to simplify analysis we used
163 microsatellite markers developed for this species that could be analyzed as diploid (DiLeo *et al.*
164 2015; Wolfe 2001). For each individual, we amplified seven species-specific microsatellites in
165 two multiplex reactions (Multiplex A: PV2, PV27, PV65a PV65b; Multiplex B: PV7, PV33,
166 PV56), conducted fragment analysis and genotyping, following the protocols described in DiLeo
167 *et al.* (2015). Microsatellite loci were tested for departures from Hardy-Weinberg Equilibrium
168 (HWE) and linkage equilibrium using exact tests in GENEPOP 4.2 (Raymond and Rousset
169 1995).

170 In June 2013 we collected mature seed heads to measure fitness-related traits from 7-10
171 individuals in 10 populations (Fig. 1). We chose a combination of populations that differed in
172 size, isolation, and grazing intensity, with wide coverage across the study region. To control for

173 variation in seed production due to flowering phenology, flower buds were marked in
174 populations within the same two-day time period during peak flowering in April, and seeds were
175 collected from the marked flowers once matured. In the laboratory, developed and undeveloped
176 seeds were counted, and the developed seeds were weighed per seed head with feathery styles
177 removed. Developed seeds were easy to visually distinguish from undeveloped seeds based on
178 size of both the seed and style (Fig. A.1). Fitness traits were measured from only a single seed
179 head per individual plant.

180

181 *Genetic Analysis of Functional Connectivity*

182 We tested three hypotheses of functional connectivity in *P. vulgaris*:

183 (1) *Isolation by distance (IBD)*. This is a simple hypothesis of diffusion, where populations
184 that are geographically close will exchange more migrants, and thus experience more
185 gene flow, than populations that are more distant.

186 (2) *Least cost distance (LCP)*. This hypothesis posits that the intervening landscape matrix
187 shapes gene flow among populations. We specifically test the hypothesis that semi-
188 natural open habitat between populations facilitates movement of pollinators and thus
189 gene flow.

190 (3) *Isolation by shepherding distance (IBS)*. This hypothesis assumes that sheep are acting as
191 seed dispersal vectors, and thus populations that are closer together in the topology of the
192 shepherding network will exchange more gene flow.

193 For each of these hypotheses, we calculated an index of connectivity for each population
194 using Hanski's S_i index (Hanski 1994). The S_i index calculates population connectivity by
195 summing distances between focal population i and all source populations j using the equation:

196
$$S_i = \sum_{i \neq j} \exp(-\alpha d_{ij})$$

197 where d_{ij} is the distance between population i and population j , and α is a constant scaling
198 parameter accounting for dispersal capacity, which we fitted through optimization (see below).
199 For S_{iBD} , d_{ij} was calculated as the Euclidean geographic distance between patches, and for S_{iLCP} ,
200 d_{ij} was calculated as the least cost path through open habitat between patches. Open habitat was
201 delimited from digital land use maps, resampled to a resolution of 25 m (Tatsaechliche Nutzung,
202 ALKIS 2008-2009; Bayerische Vermessungsverwaltung, Munich, Germany; Fig. A.2), and
203 included permanent grasslands, groves, mires, barren land, orchards, and small roads/tracks,
204 which are known to be suitable habitat for wild bees (Steffan-Dewenter et al. 2002). Least cost
205 paths were calculated using the *gdistance* library (van Etten 2012) in *R*. For S_{iBS} , d_{ij} was
206 calculated as the number of patches traversed by sheep to get from j to focal patch i , and pairs of
207 patches from different grazing routes or that were intermittently grazed or ungrazed were given a
208 d_{ij} value of 100 assuming that gene flow outside of the grazing routes is rare (Rico et al. 2012).
209 Intermittently grazed populations and those grazed only late in the season were not included as
210 steps in the network because they likely do not contribute as strongly to functional connectivity
211 compared to consistently grazed populations, which have been in the network since 1989 and are
212 grazed up to five times per year. However, for comparison, we also calculated shepherding
213 connectivity including intermittently grazed and late grazed populations as steps in the network.
214 Grassland patches in the network that did not contain *P. vulgaris* populations (Fig. 1) were
215 counted as steps traversed by sheep in the calculation of S_{iBS} .

216 The parameter α should be set to a value that is equal to 1/mean dispersal distance. Since
217 we do not know the mean dispersal of *P. vulgaris*, we optimized α for each connectivity
218 hypothesis separately by testing a range of values and choosing the value that gave the highest R^2

219 in univariate regression with allelic richness (A_r). Sheep have the ability to transport seeds of
220 grassland plants up to several hundred kilometers, and can retain seeds in their wool for several
221 months (Fischer et al. 1996), thus for Si_{IBS} we explored a wide range of α values from 0.08-1
222 which translates to mean dispersal distances between 1-13 steps (the maximum in our network)
223 through the grazing network. In contrast, gene flow via pollinators or wind-dispersed seeds
224 should occur at much smaller spatial scales for *P. vulgaris*, and so for both Si_{IBD} and Si_{LCP} we
225 explored α values between 0.2-2 which translates to mean dispersal distances between 0.5-5 km.
226 Based on this optimization procedure, we set $\alpha=0.2$ (mean dispersal of 5 km) for both Si_{IBD} and
227 Si_{LCP} and $\alpha=0.08$ (mean dispersal of ~12 steps through the network) for Si_{IBS} . Note that that our
228 goal was not to determine true α , but to compare the predictive power of each of the three
229 hypotheses of functional connectivity.

230 We tested the strength of evidence for each of these hypotheses of functional connectivity
231 by testing their associations with genetic differentiation using the program GESTE 2.0 (Foll
232 and Gaggiotti 2006). This program calculates population-specific F_{st} values, which represent
233 how differentiated each population is from others in the sample. GESTE uses a Bayesian
234 approach that includes non-genetic information as a prior, and models associations between these
235 non-genetic predictors and population-specific F_{st} with generalized linear models. This approach
236 has low Type I error rates compared to the commonly used Mantel test, and has performed well
237 in recovering landscape and ecological drivers of gene flow in simulations (Balkenhol et al.
238 2009; Foll and Gaggiotti 2006). We first ran GESTE on all populations ($n=49$), including four
239 predictors: Si_{IBD} , Si_{LCP} , *population size class* (<40, 40-100, >100 individuals included as an
240 ordinal variable: 1,2,3), and *grazing intensity* (ungrazed, intermittently grazed, or consistently
241 grazed, included as ordinal: 1,2,3). Note that Si_{IBS} could not be included since connectivity of all

242 ungrazed populations would be zero. To explicitly test the effects of shepherding on functional
243 connectivity, we ran GESTE separately for consistently grazed populations ($n=31$), including the
244 three measures of connectivity (Si_{IBD} , Si_{LCP} , Si_{IBS}), *population size class*, and *grazing route* (route
245 1, 2, or 3) as predictors. We ran GESTE for a total of 250,000 iterations with 50,000 iterations of
246 burn in.

247

248 *Genetic Diversity*

249 We evaluated the effects of grazing treatment (*ungrazed*, *intermittently grazed*, *consistently*
250 *grazed*), *population size class* (<40, 40-100, >100 individuals) and their interaction, on genetic
251 diversity and the inbreeding coefficient (F_{is}) with a two-way ANOVA followed by Tukey HSD.
252 F_{is} was calculated using GENEPOP 4.2 (Raymond and Rousset 1995).

253 Genetic diversity was measured as the mean number of alleles per population (A_r) using
254 the *gstudio* package (Dyer 2014) in R 3.02 (R Core Team 2016). We used rarefaction with a
255 sample size of five and 999 permutations to control for differences in sample size between
256 populations. Allelic richness was chosen over heterozygosity because it is more sensitive to
257 recent demographic change (Allendorf 1986) and is a better indicator of long-term adaptive
258 potential of populations, even when based on neutral markers (Caballero and Garcia-Dorado
259 2013; Vilas et al. 2015). However, for comparison, we also present results of analyses using two
260 alternative measures of genetic diversity: expected heterozygosity (H_e ; correlation with A_r :
261 Pearson $r=0.93$), and observed heterozygosity (H_o ; correlation with A_r : Pearson $r=0.50$).

262 Limiting analyses to consistently grazed patches, we tested the hypothesis that
263 populations that are well-connected *within* the grazing network (i.e. populations that are
264 separated by shorter shepherding distances from others) have higher genetic diversity than more

265 isolated populations using linear mixed effect models. We constructed a set of linear mixed
266 effect models to quantify the relationship between genetic diversity (A_r) and four predictors as
267 fixed effects: Si_{IBD} , Si_{LCP} , Si_{IBS} , and *population size class*, and grazing route (route 1, 2, or 3) as a
268 random effect. We found no significant interactions between connectivity indices and population
269 size class and so we included the latter only as a main effect. We tested sub-models of $A_r \sim Si_{IBD}$
270 + Si_{LCP} + Si_{IBS} + *population size class* and used AIC_c for model selection, however Si_{IBD} and
271 Si_{LCP} were highly correlated (Pearson $r = 0.85$), therefore we never included them in the same
272 sub-model. We assessed the relative importance of each predictor by summing the Akaike
273 weights of all models that included that predictor. We used likelihood ratio tests to test the
274 significance of individual fixed effects in the top selected models, and report parameter estimates
275 averaged over models with $\Delta AIC_c < 2$. We used the protocol of Nakagawa & Schielzeth (2013)
276 to calculate marginal R^2 , which represents variance explained by the fixed effects. We also ran
277 the model on a subset of consistently grazed populations where information on census population
278 size was available ($n=15$), this time treating population size as a continuous predictor variable.

279

280 *Fitness*

281 We evaluated the relationship between genetic diversity (A_r) and two fitness-related traits (*seed*
282 *set*, *seed mass*) in linear mixed effect models in the 10 populations where we collected seeds.
283 *Seed set* was measured as the proportion of developed seeds per seed head, and *seed mass* was
284 measured as the mean mass of developed seeds per seed head. The proportion of seed set and the
285 total number of filled seeds per seed head were highly correlated (Pearson $r=0.71$). A_r was
286 included as a fixed effect and was exponentially transformed to linearize the relationship with
287 *seed set* and *seed mass*. Note that fitness-related traits were measured at the level of individuals

288 within populations, and genetic diversity was measured per population. Population was included
289 as a random effect to control for the non-independence of seed data collected from multiple
290 mother plants within the same patch. Models were estimated in *R* using *lme4* (Bates et al. 2015)
291 using maximum likelihood. We used likelihood ratio tests to determine the significance of the
292 fixed effect, and report marginal R^2 (variance explained by fixed effects, Nakagawa and
293 Schielzeth 2013). Data on the number of flowering stems and height of the flowering stem of the
294 plants where we collected seeds were available for seven populations. We tested if these were
295 important predictors of seed set and seed mass in addition to allelic richness in mixed models.

296 Fitness-related traits can be influenced not only by genetic diversity, but also by
297 population size, either directly (e.g. ecological Allee effects; Lande 1988; Reed 2005), or
298 indirectly by increasing genetic diversity (Leimu et al. 2006; Luque et al. 2016). We used
299 Pearson's partial correlations to tease apart the effects of genetic diversity and population size on
300 seed set in *P. vulgaris* populations. Note that for this analysis, seed set was included as a mean
301 value per population. Ideally, a full path analysis would be conducted to quantify the interactions
302 between these variables, but our low sample size ($n=10$ populations) precluded such a test. We
303 calculated Pearson's product moment correlations between mean *seed set* per population and A_r ,
304 mean *seed set* and *population size*, and partial correlations between mean *seed set* and A_r
305 controlling for *population size*, and mean *seed set* and *population size* controlling for A_r using the
306 *ppcor* 1.0 (Kim 2012) in *R*. *Population size* was defined as the number of flowering individuals
307 per population. A_r was exponentially transformed and we took the logarithm of *population size* to
308 linearize relationships.

309 **Results**

310 *Genetic Markers*

311 Markers PV7, PV27, PV65a and PV56 showed departures from HWE expectations, but there
312 was no consistent pattern across populations (Table B.1) and all markers were retained. Marker
313 pairs were found to be unlinked (Table B.2).

314

315 *Functional Connectivity*

316 Connectivity indices Si_{IBD} and Si_{LCP} were highly correlated, but neither of these were
317 correlated with shepherding connectivity, Si_{IBS} (Table A.2). Analysis of genetic differentiation
318 across all 49 populations suggested that Si_{LCP} was a better predictor of overall structure than
319 Si_{IBD} , with the highest probability model including Si_{LCP} and *grazing intensity* as predictors
320 ($Pr=0.85$; Table 1, Fig. 2). When the analysis was restricted to consistently grazed populations
321 ($n=31$), population-specific F_{st} was best explained by a model including shepherding
322 connectivity (Si_{IBS}), with F_{st} showing a negative relationship with Si_{IBS} (Table 1 and 2, Fig. 3).
323 This model had a high posterior probability ($Pr=0.79$), and in comparison, the next-best model
324 had a very low probability ($Pr=0.045$), suggesting that Si_{IBS} alone fits the data well compared to
325 the other predictors for consistently grazed populations (Table 1). When Si_{IBS} was calculated
326 including intermittently grazed and late grazed populations as steps through the shepherding
327 network, GESTE failed to find an optimal model, with no model having a probability greater than
328 0.2 (Table A.3).

329

330 *Effects of Shepherding Connectivity on Genetic Diversity*

331 In a two-way ANOVA, both grazing treatment and *population size class*, but not their
332 interaction, had a significant effect on genetic diversity, A_r (ANOVA, grazing treatment:
333 $F_{(2,42)}=7.97$, $p=0.001$, population size class: $F_{(2,42)}=3.64$, $p=0.03$, interaction: $F_{(2,42)}=0.55$,
334 $p=0.58$). However, a posthoc Tukey test showed significant differences among grazing
335 treatments where ungrazed populations had significantly lower genetic diversity than
336 intermittently and consistently grazed populations, but no significant differences among
337 population size classes (Fig. 4). We found significant differences in expected heterozygosity H_e
338 among grazing treatments but not population size class in a two-way ANOVA (grazing
339 treatment: $F_{(2,42)}=6.56$, $p=0.003$, population size class: $F_{(2,42)}=2.66$, $p=0.08$, interaction:
340 $F_{(2,42)}=0.23$, $p=0.79$), and found no significant differences in observed heterozygosity H_o
341 (ANOVA, grazing treatment: $F_{(2,42)}=1.38$, $p=0.26$, population size class: $F_{(2,42)}=0.44$, $p=0.65$,
342 interaction: $F_{(2,42)}=1.26$, $p=0.29$) nor inbreeding coefficient F_{is} (ANOVA, grazing treatment:
343 $F_{(2,42)}=0.1$, $p=0.91$, population size class: $F_{(2,42)}=0.23$, $p=0.79$, interaction: $F_{(2,42)}=1.04$, $p=0.36$).
344 When late grazed populations were removed from the ungrazed category, we still found
345 significant differences in A_r between ungrazed and consistently grazed populations (ANOVA,
346 grazing treatment: $F_{(1,34)}=5.53$, $p=0.02$, population size class: $F_{(2,34)}=2.98$, $p=0.06$, interaction:
347 $F_{(1,34)}=1.09$, $p=0.3$).

348 Genetic diversity (A_r) of consistently grazed populations was best fit by a linear mixed
349 effect model including connectivity by shepherding (Si_{IBS}) and *population size class* as fixed
350 factors (Table 3, Fig. 3). This model explained significant variation beyond the grazing-route
351 random effect (likelihood ratio test, $\chi^2_{(1)}=18.4$, $p<0.0001$), and tests for the significance of
352 individual fixed factors showed that both Si_{IBS} (likelihood ratio test, $\chi^2_{(1)}=7.6$, $p=0.006$) and
353 *population size class* (likelihood ratio test, $\chi^2_{(1)}=4.9$, $p=0.03$) had a significant positive effect on

354 A_r (Table 3, Table 4, Fig. 3). In comparison, neither Si_{LCP} (likelihood ratio test, $\chi^2_{(1)}=2.1$, $p=0.14$)
355 nor Si_{IBD} (likelihood ratio test, $\chi^2_{(1)}=1.3$, $p=0.24$) had significant effects in the top models in
356 which they appeared (Table 3), and estimated effects on A_r were negative (A_r decreased with
357 Si_{IBD} and Si_{LCP} ; Table 4). The same model was chosen when we ran the model treating
358 population size as a continuous variable for the fifteen populations where census population size
359 was available (Table A.4, Fig. A.3), and when H_e was used as the response variable (Table A.5).
360 When H_o was used as the response variable, the top model included only the shepherding-route
361 random effects, and in general all models explained little variation as assessed by marginal R^2
362 (Table A.5).

363

364 *Effect of Genetic Diversity on Fitness*

365 *Seed set* and allelic richness (A_r) showed a significant positive linear association (Likelihood
366 ratio test: $\chi^2_{(1)}=9.2$, $p=0.002$; marginal $R^2=0.12$; Fig. 5a), and the same positive relationship was
367 found when seed set was measured as the total number of seeds per seed head, although the
368 strength of association was weaker (Fig. A.4). *Seed mass* also showed a significant positive
369 association with A_r (Likelihood ratio test: $\chi^2_{(1)}=11.76$ $p<0.0001$; marginal $R^2=0.23$; Fig. 5b).
370 *Seed set* and *seed mass* showed a significant positive linear association with expected
371 heterozygosity (H_e) but not observed heterozygosity (H_o ; Table A.6). In the seven populations
372 where data were available, we found that the height of the flowering stem had a significant
373 positive relationship with the total seed set in addition to significant effects of A_r (Table A.7, Fig.
374 A.5).

375 Mean *seed set* per population showed strong positive correlations with both A_r ($r=0.82$,
376 $p=0.003$), and *population size* ($r=0.71$, $p=0.02$). The partial correlation between mean *seed set*

377 and A_r remained significant after controlling for *population size* ($r=0.64$, $p=0.03$), whereas the
378 partial correlation between mean *seed set* and *population size* controlling for A_r was not
379 significant ($r=0.32$, $p=0.37$).

380 **Discussion**

381 Here we demonstrate the utility of an ecological connectivity network to maintain functional
382 connectivity and genetic diversity of populations of an imperiled wildflower, *Pulsatilla vulgaris*,
383 in calcareous grasslands in Germany. Genetic data of grazed populations were best fit by a model
384 of connectivity that incorporated the number of patches traversed by sheep in the shepherding
385 network, suggesting that connectivity by shepherding, and not the geographic distance separating
386 grassland patches or least-cost path distance through open habitat determines functional
387 connectivity. We further show that enhanced connectivity within the shepherding network
388 translated to enhanced genetic diversity, and that populations with higher genetic diversity
389 produced more and heavier seeds. Together these results suggest that shepherding is an effective
390 management measure to sustain functional connectivity among fragmented populations of *P.*
391 *vulgaris*, but populations that are small and unconnected, or small with low connectivity in the
392 shepherding network still suffer from reduced genetic diversity.

393

394 *Functional Connectivity*

395 Our results suggest that geographic distance is a poor predictor of genetic connectivity of *P.*
396 *vulgaris* populations. In contrast, connectivity through open habitat (S_{LCP}), and shepherding
397 connectivity (S_{IBS}) showed strong negative associations with population-specific F_{st} (Fig. 2, Fig.
398 3). When all populations were analysed together, S_{LCP} and grazing intensity explained patterns
399 of genetic differentiation better than either S_{IBD} or population size class. However, shepherding
400 connectivity was the single best predictor of genetic differentiation of consistently grazed
401 populations (Table 1). This suggests that shepherding contributes strongly to genetic connectivity
402 of consistently grazed populations, but that the movement of pollinators through open habitat

403 might be additionally important, particularly for populations outside of the shepherding network.
404 This highlights the importance of considering the potential vectors of pollen and seed dispersal
405 and their effect on functional connectivity when designing ecological connectivity networks, and
406 shows that considering patch proximity alone may not be enough to ensure dispersal and
407 subsequent gene flow between fragmented populations (Auffret et al. 2017; DiLeo et al. 2014;
408 Dyer et al. 2012; Kamm et al. 2010).

409

410 Previous work in this system showed that the rotational shepherding network predicted
411 demographic connectivity for *P. vulgaris* and other typical grassland species at the community
412 (Rico et al. 2012) and species level (Rico et al. 2014a), and at the genetic level for another
413 grassland plant, *Dianthus carthusianorum* (Rico et al. 2014b). The current study suggests that the
414 same trend holds true at the genetic level for *P. vulgaris*, where both genetic differentiation and
415 genetic diversity were best fit by a model including connectivity via shepherding (Tables 1 and
416 3). The effects of fragmentation can manifest at different spatial and temporal scales depending
417 on the population outcome measured (e.g. patch occupancy or abundance versus genetic
418 diversity; Jackson and Fahrig 2014; Takakis et al. 2013) and the importance of considering both
419 demographic and genetic factors for conservation planning is increasingly recognized (Landguth
420 et al. 2014; Luque et al. 2012). Demographic outcomes such as patch occupancy and abundance
421 are mediated by the processes of recruitment and colonization, which in plants occurs through
422 the dispersal of seed. Note that in *P. vulgaris* and other calcareous grassland plants, colonization
423 and establishment success depends on pre- and post-dispersal effects such as microsite
424 conditions (Piqueray et al. 2013), and this may explain why some patches remain unoccupied in
425 our study region (Rico et al. 2012; Wagner et al. 2013). In contrast, genetic differentiation and

426 genetic diversity of local populations is the product of gene flow over generations, and is thought
427 to mainly occur through pollination for most plant species (Ellstrand 1992; Ennos 1994).
428 However, in some systems the contribution of seed to overall genetic connectivity far exceeds
429 that of pollen, particularly when seeds are dispersed by animal vectors (Bacles et al. 2006;
430 Manzano and Malo 2006). This may explain why we find such a strong effect of shepherding
431 connectivity. The ecological network investigated in this study is different from many others as it
432 directly provides the functional vectors of seed dispersal (i.e. sheep) rather than protecting
433 physical aspects of the landscape that have the potential to support connectivity by either seed or
434 pollen. In comparison, our results suggest that pollen-mediated gene flow contributes less to
435 overall genetic connectivity; despite being highly connected by suitable pollinator habitat,
436 ungrazed populations had much higher genetic differentiation than grazed populations (Fig. 2).
437 These results are further supported by previous work conducted on a subset ($n=7$) of the *P.*
438 *vulgaris* populations included here, which showed that most pollination occurred within ten
439 meters of the mother plant, and only 16% of all pollination events could be attributed to among-
440 population pollen flow (DiLeo 2016).

441

442 *Effect of Shepherding Connectivity on Genetic Diversity*

443 We found that intermittently and consistently grazed populations had significantly higher allelic
444 richness than ungrazed populations (Fig. 4). This suggests that the populations of *P. vulgaris* that
445 are incorporated into the ecological connectivity network harbor more genetic variation and thus
446 may be better able to endure future environmental change. However, it is unclear if the observed
447 effect is due to the enhanced connectivity provided by the ecological network or a direct result of
448 the grazing process. For example, grazing can influence plant reproductive success by altering

449 vegetation height and habitat quality (de Vere et al. 2009; Jacquemyn et al. 2003), flowering
450 phenology (Lennartsson et al. 2012) or richness and abundance of pollinators (Kormann et al.
451 2015). When we restricted analyses to only those populations within the ecological connectivity
452 network to control for direct reproductive effects of grazing (i.e. ecological Allee effects; Lande
453 1988; Luque et al. 2016), we found that genetic diversity significantly increased with both
454 shepherding connectivity and populations size (S_{iBS} ; Table 4; Fig. 3). This suggests that the
455 functional connectivity provided by the ecological network maintains genetic diversity of
456 populations, but importantly, populations that are small and have low connectivity within the
457 network still experience low levels of allelic richness. These small, isolated populations may be
458 useful targets for connectivity network optimization and restoration (Mijangos et al. 2015). It is
459 also apparent from Fig. 3 that there are differences in the levels of diversity among the three
460 grazing routes, suggesting that they may have different levels of standing variation. Unsampled
461 *P. vulgaris* populations exist to the south of our study region, and it is possible that these
462 population exchange gene flow with populations in route 3, possibly explaining this route's
463 increased levels of genetic variation. The differences in shepherding route-level connectivity
464 likely also contribute to and compound this effect; for example, populations in route 1 tend to
465 suffer from the lowest connectivity and also have the lowest genetic diversity.

466

467 *Effect of Genetic Diversity on Fitness*

468 We found that higher genetic diversity translated to an increase in both mean seed set and seed
469 mass of populations (Fig. 5). Strong positive correlations between genetic diversity and fitness
470 have been observed in a number of plant species (reviewed in Leimu et al. 2006; Reed and
471 Frankham 2003), and populations with higher fitness have a higher probability of persistence

472 (Frankham 2005). However, our results should be interpreted with caution given our low sample
473 size ($n=10$ populations) and that we measured fitness-related traits in a single year for a long-
474 lived species. Given these caveats, it is surprising to see such a strong effect of genetic diversity
475 on seed set, with 64% of variation explained after controlling for population size. Further work is
476 required to determine how the magnitude of differences in seed set and seed mass between
477 populations of low and high diversity translates to germination success and seedling survival in
478 the field.

479

480 The lack of variation in F_{is} and observed heterozygosity (H_o) across populations was
481 surprising given that we found a strong correlation between shepherding connectivity and allelic
482 richness (A_r), and A_r and both measured fitness-related traits. Allelic richness degrades in
483 populations (or in this case, increases in populations) faster than heterozygosity (Maruyama and
484 Fuerst 1985), which suggests that the effects of shepherding connectivity are recent. This lack of
485 variation in F_{is} and H_o also suggests that inbreeding is not the reason for the low reproductive
486 success in populations with low diversity. One explanation for this result is that the increase in
487 fitness does not have a genetic basis but is rather the result of an ecological Allee effect, where
488 density-dependent mating success or facilitation in large populations leads to higher seed
489 production (Lande 1988). This has been demonstrated in a number of plant systems (reviewed in
490 Reed 2005), including *P. vulgaris* where Hensen *et al.* (2005) found strong positive correlations
491 between population size and both seed set and seed mass in central Germany. However, we
492 found that after controlling for the effects of genetic diversity, the partial correlation between
493 seed set and population size was no longer significant. This suggests that population size does
494 not directly influence fitness, but that large populations have higher genetic diversity, which in

495 turn have higher seed set (i.e. a genetic Allee effect; Luque et al. 2016). An alternative
496 explanation is that pollination plays an important role in maintaining both genetic diversity and
497 fitness of *P. vulgaris* populations. For example, Breed *et al.* (2012) found that the diversity of
498 pollen received by flowers was more important than inbreeding in determining progeny fitness in
499 *Eucalyptus socialis*. Sampling more diverse pollen sources opens up the opportunity for female
500 selection of more compatible pollen grains or ‘good genes’, resulting in increased fitness that is
501 independent of inbreeding (Armbruster and Rogers 2004; Yasui 1998). Populations with higher
502 diversity will act as more diverse pollen sources, and likewise populations with higher
503 connectivity will receive more immigrants which introduce new variation into populations by
504 providing outside pollen.

505

506

507 *Conclusions*

508 Here we show that an ecological connectivity network in southern Germany has been successful
509 at maintaining functional connectivity of a flagship wildflower, and that this enhanced
510 connectivity translated to enhanced genetic diversity of populations. We found a significant
511 relationship between genetic diversity and two fitness-related traits, emphasizing the importance
512 of preserving genetic diversity. Incorporating ungrazed populations into the existing network
513 may be a suitable conservation measure to boost genetic diversity and fitness, and small
514 populations that are more isolated within the network might benefit from further optimization of
515 the network topology. This study empirically demonstrates the pathway of predicted positive
516 associations between connectivity, genetic diversity, and fitness in the context of an ecological
517 network, and provides a framework for testing the efficacy of ecological networks for focal

518 species using genetic tools. Taken together with previous work in this system which showed that
519 shepherding maintains species richness (Wagner et al. 2013) and demographic connectivity
520 (Rico et al. 2012, 2014a) at the community level, these results suggest a positive role of
521 ecological shepherding networks for protecting two of the three levels of biodiversity
522 recommended for conservation by the International Union for Conservation of Nature (McNeely
523 et al. 1990) - species and genetic diversity (the third level is ecosystem diversity) – for
524 calcareous grassland plants.

525

526 *Acknowledgements*

527 Funding was provided by the Natural Sciences and Engineering Research Council of Canada
528 (NSERC CGS-D, CREATE-ADVENT and Michael Smith Foreign Study Supplement to M.F.D,
529 and Discovery Grant to H.H.W.) and the Government of Central Franconia, Bavaria, Germany.
530 We thank M-J. Fortin, M.T.J Johnson, R. Ness, and N. Keygohbadi for comments on an earlier
531 version of this manuscript, and R. Holderegger for helpful discussions. We thank Paul Beier and
532 two anonymous reviewers for comments that greatly improved this manuscript. We thank K.
533 Dadrich, D. Baumgartner, B. Raab, S. Haacke, and H. Lehnert for support in the field, A.
534 Lochab, and M. Liu for help in the lab, and the shepherds E. Beil, E. Neulinger, A. Grimm for
535 detailed information on management strategies and their implementation.

536 Table 1. The top five posterior probability models describing the effects of connectivity
 537 (geographic connectivity, Si_{IBD} ; least cost connectivity through open habitat, Si_{LCP} ; shepherding
 538 connectivity, Si_{IBS}) and population size class ($pop.size$) on population-specific F_{st} when all
 539 populations were analysed together (top) and when data were subset to consistently grazed
 540 populations (lower). Note that shepherding distance was not included as a predictor in the
 541 analysis of all populations (see Methods for details). Regression coefficients for the top
 542 supported models (bolded) are presented in Table 2.

543

Population subset	Model	Probability
All populations ($n=49$)	$F_{st} \sim \mathbf{grazing.intensity} + \mathbf{Si_{LCP}}$	0.85
	$F_{st} \sim grazing.intensity + Si_{LCP} + pop.size$	0.06
	$F_{st} \sim grazing.intensity + Si_{LCP} + Si_{IBD}$	0.04
	$F_{st} \sim grazing.intensity + Si_{IBD}$	0.02
	$F_{st} \sim Si_{LCP}$	0.01
Consistently grazed populations ($n=31$)	$F_{st} \sim \mathbf{Si_{IBS}}$	0.79
	$F_{st} \sim Si_{IBS} + Route$	0.05
	$F_{st} \sim Si_{IBS} + Si_{LCP}$	0.04
	$F_{st} \sim Si_{IBS} + Si_{IBD}$	0.04
	$F_{st} \sim Si_{IBS} + pop.size$	0.03

544

545 Table 2. Regression coefficients for the top probability model identified by GESTE explaining
 546 variation in population-specific F_{st} across all populations (top) and consistently grazed
 547 populations (bottom). The top model for the analysis conducted on all populations included
 548 grazing intensity and least cost connectivity through open habitat (Si_{LCP}) as predictors. The top
 549 model for consistently grazed populations included shepherding connectivity (Si_{BS}) as a
 550 predictor. The highest probability density interval (HDPI), which indicates the smallest interval
 551 that contains 95% of values, is shown.

552

Population Subset	Variable	Estimate	HDPI (95%)
All populations ($n=49$)	constant	-3.40	(-3.58,-3.22)
	grazing intensity	-0.33	(-0.522,-0.14)
	Si_{LCP}	-0.41	(-0.611,-0.232)
Consistently grazed populations ($n=31$)	constant	-3.40	(-3.62,-3.18)
	Si_{BS}	-0.45	(-0.663,-0.294)

553

554 Table 3: Results of model selection and model fit of linear mixed effect models testing the effect
555 of geographic connectivity (Si_{IBD}), least cost path connectivity (Si_{LCP}), shepherding connectivity
556 (Si_{IBS}), and population size class ($pop.size$) on genetic diversity (A_r) of *P. vulgaris* populations.
557 All models included grazing route (route 1, route 2, or route 3) as a random effect (1|Route).
558 Model weights (w_i) and marginal R^2 of fixed effects are shown. Model averaged estimates of
559 parameters for models with $\Delta AIC_c < 2$ are shown in Table 4.

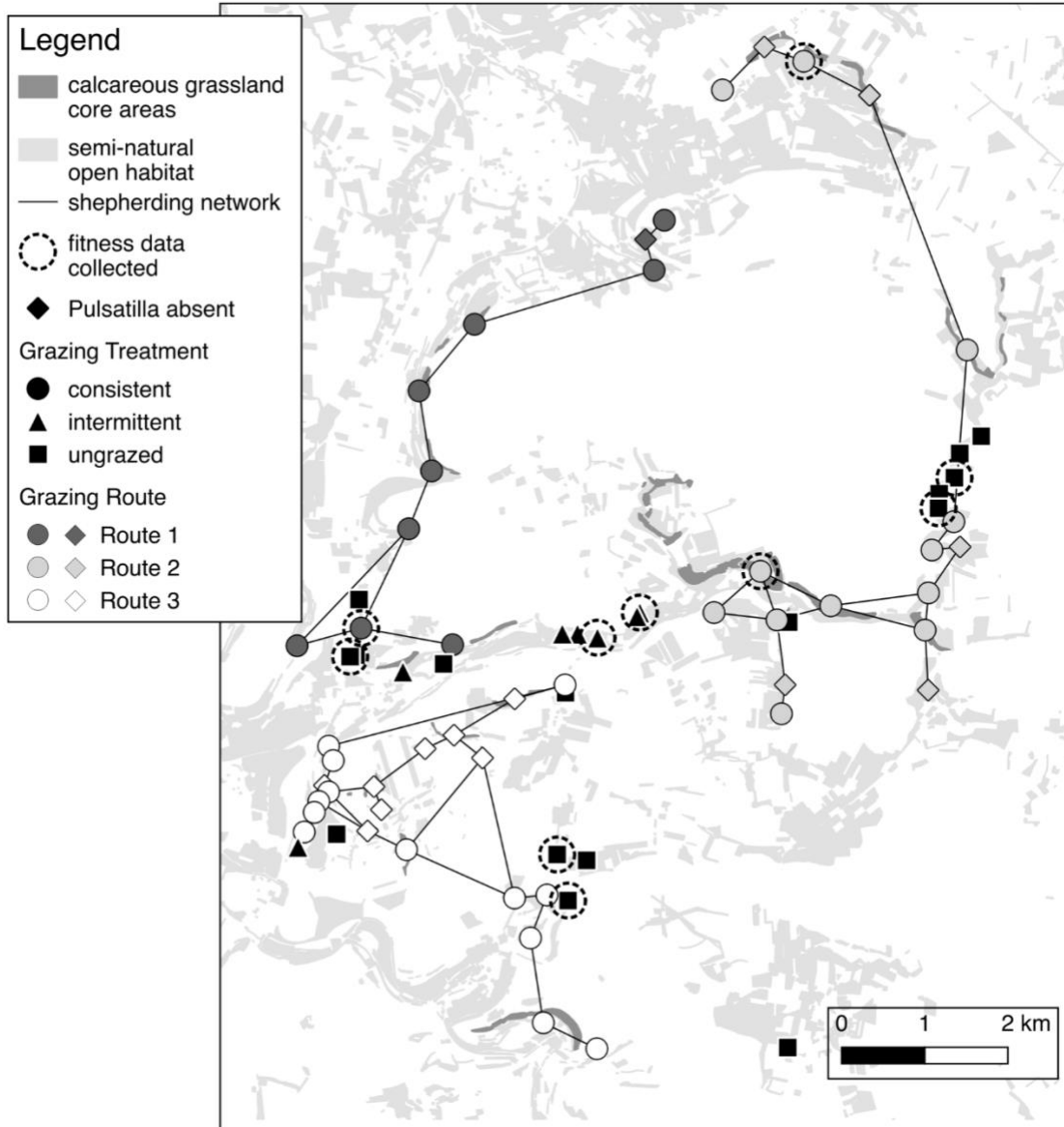
Model	AIC _c	ΔAIC _c	w_i	R^2
$A_r \sim Si_{IBS} + pop.size + 1 Route$	11.1	0	0.31	0.60
$A_r \sim Si_{IBS} + Si_{LCP} + pop.size + 1 Route$	12.0	1.0	0.19	0.63
$A_r \sim Si_{IBS} + Si_{IBD} + pop.size + 1 Route$	12.8	1.7	0.13	0.62
$A_r \sim Si_{IBS} + Si_{LCP} + 1 Route$	13.0	1.9	0.12	0.58
$A_r \sim Si_{IBS} + 1 Route$	13.2	2.1	0.11	0.53
$A_r \sim Si_{IBS} + Si_{IBD} + 1 Route$	13.9	2.8	0.08	0.56
$A_r \sim pop.size + 1 Route$	15.8	4.7	0.03	0.17
$A_r \sim Si_{IBD} + pop.size + 1 Route$	16.9	5.9	0.02	0.19
$A_r \sim Si_{LCP} + pop.size + 1 Route$	18.2	7.2	0.01	0.18
$A_r \sim 1 Route$	24.0	12.9	0	NA
$A_r \sim Si_{IBD} + 1 Route$	25.9	14.8	0	0.02
$A_r \sim Si_{LCP} + 1 Route$	26.6	15.6	0	0.001

560

561 Table 4: Model averaged parameter estimates showing the effect of each variable (geographic
562 connectivity, Si_{IBD} ; least cost connectivity through open habitat, Si_{LCP} ; shepherding connectivity,
563 Si_{IBS} ; population size class, $pop.size$) on allelic richness. Averaging was conducted over four
564 models with $\Delta AICc \leq 2$ after model selection (see Table 3). Adjusted standard errors and
565 confidence intervals of each parameter, and the relative importance of each predictor variable is
566 shown.

Variable	Estimate	Standard Error	Confidence Interval	Relative Importance
(Intercept)	3.04	0.38	(2.30, 3.78)	
Si_{IBS}	0.24	0.04	(0.16, 0.33)	1.00
$pop.size$	0.10	0.05	(0.01, 0.20)	0.84
Si_{LCP}	-0.02	0.01	(-0.04, 0.005)	0.41
Si_{IBD}	-0.01	0.01	(-0.04, 0.01)	0.17

567



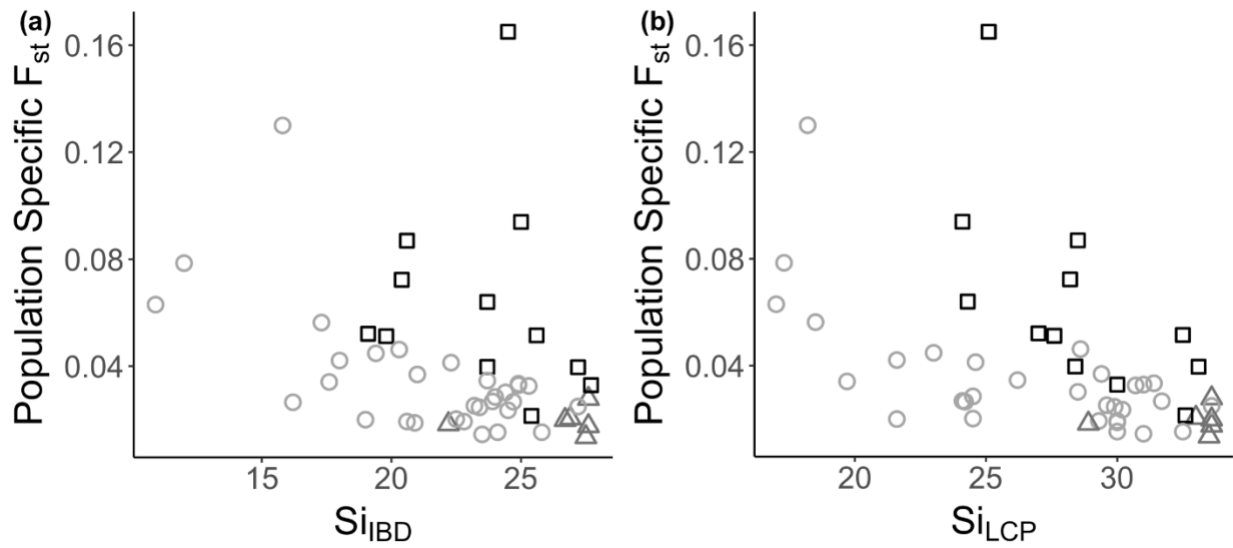
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569

570 Figure 1: Location of calcareous grassland patches and grazing treatment of patches in the study
 571 area in the Franconian Alb, Germany. Grazing routes connecting consistently grazed populations
 572 are indicated by black lines and labelled with the associated route. Dashed circles indicate
 573 populations where fitness-related traits were measured. Semi-natural open habitat is shown in
 574 light grey and core calcareous grassland patches ('core areas', see Methods for details) are
 575 indicated in dark grey.

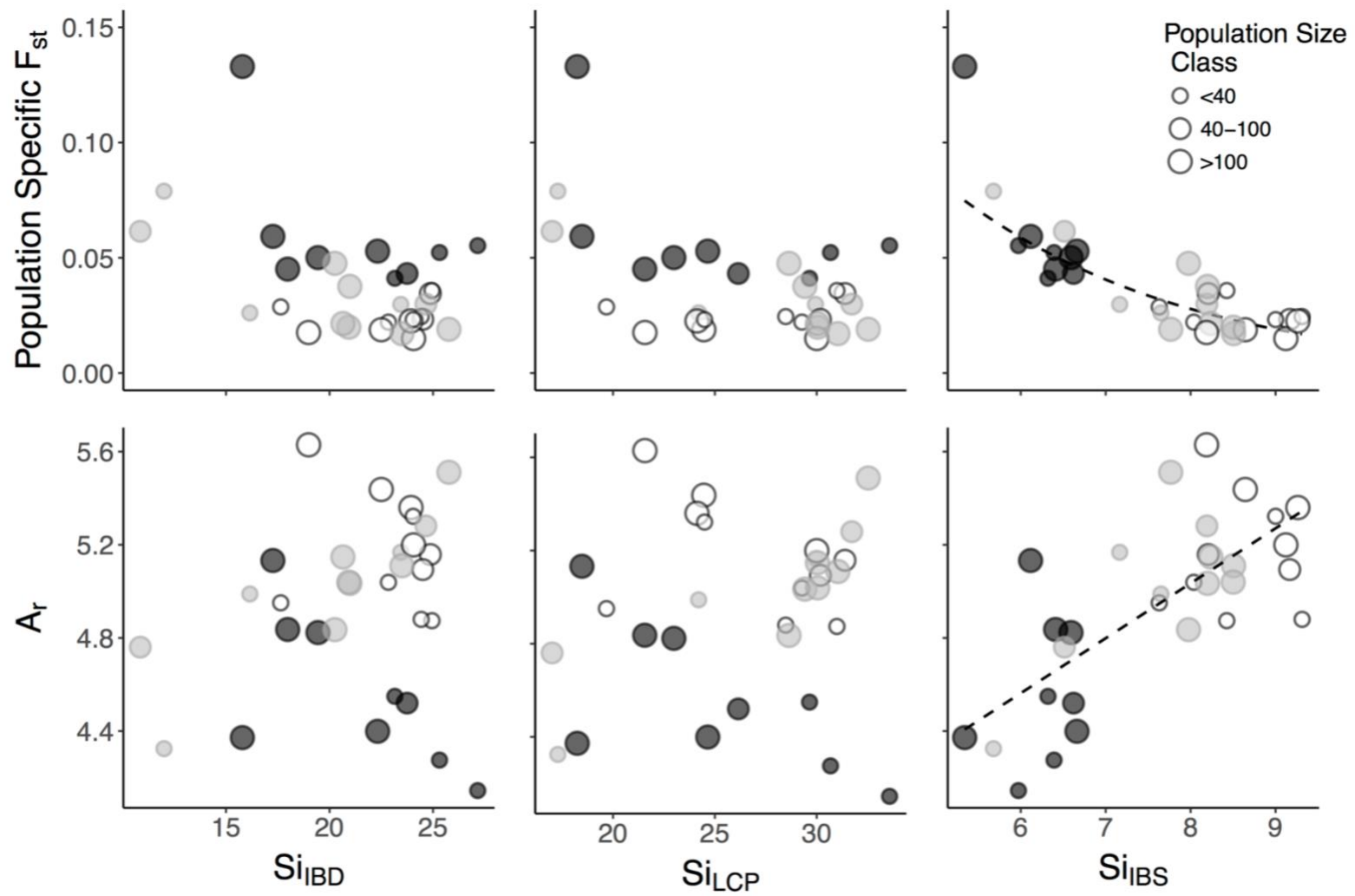
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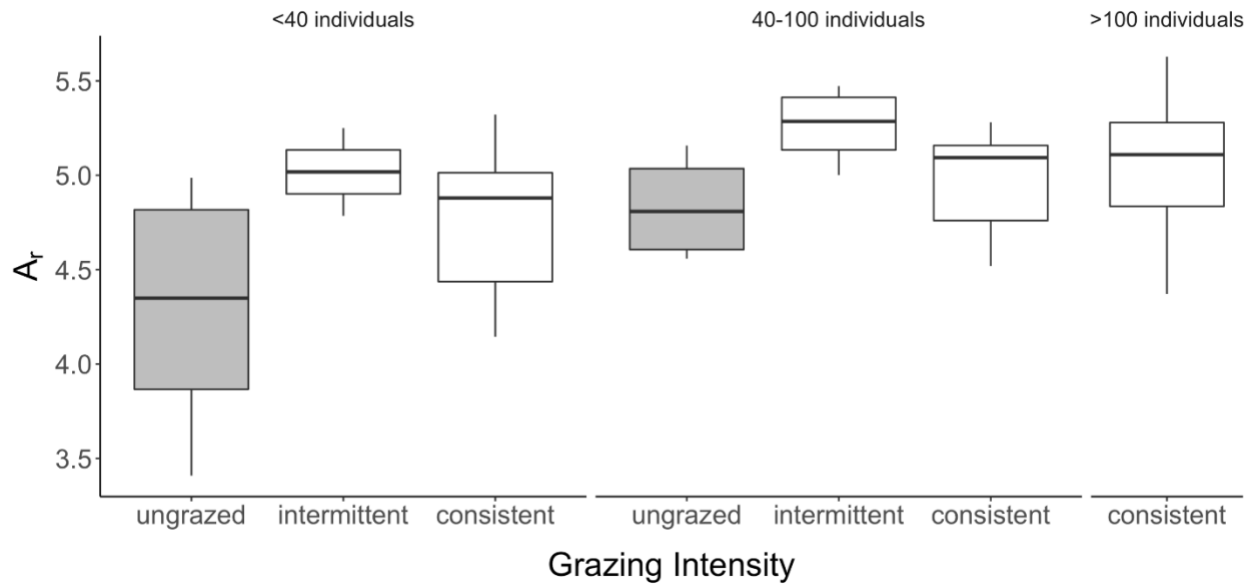


578

579 Figure 2: Scatterplots showing the relationship between genetic differentiation (population-
580 specific F_{st}) and geographic connectivity (Si_{IBD}), and least-cost path connectivity through open
581 habitat (Si_{LCP}). Each point represents a single population ($n=49$) and shape and color indicates
582 grazing treatment (circles, consistently grazed populations; triangles, intermittently grazed
583 populations; squares, ungrazed populations). See Table 1 for results of model selection on these
584 data.

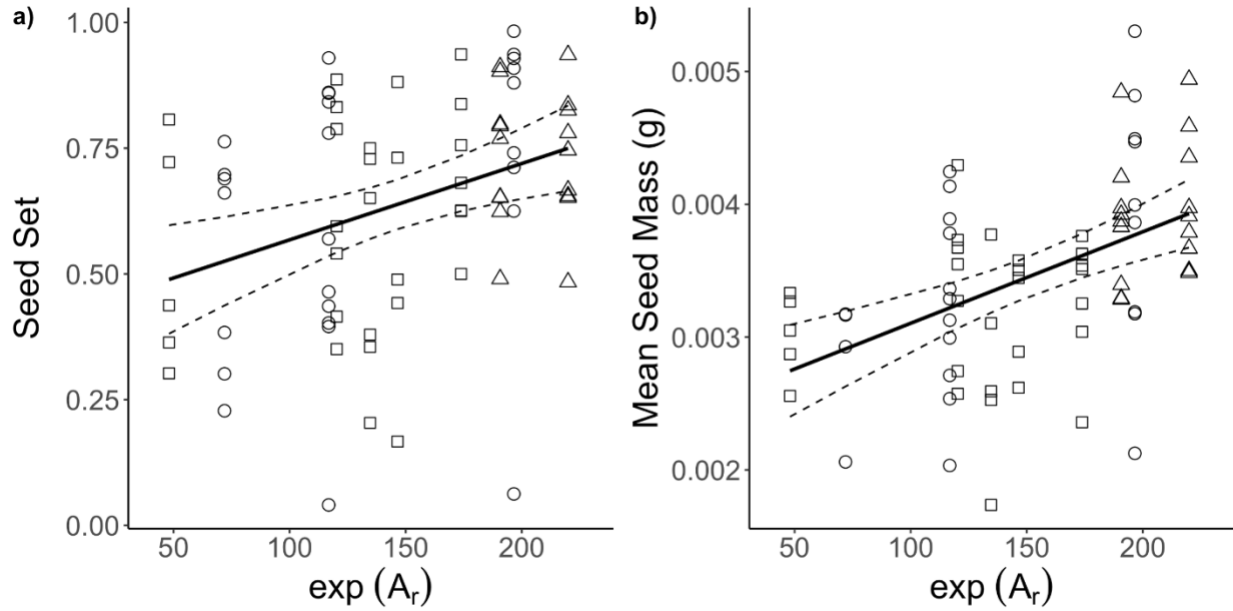


586 Figure 3: Scatterplots showing the relationships of population-specific F_{st} (top panels) and allelic richness (A_r ; bottom panel) with
587 connectivity indices based on among-population geographic distance (S_{iBD}), among-population least cost path through open habitat
588 (S_{iLCP}), and among-population shepherding distance (S_{iBS}) for consistently grazed populations ($n=31$). Each point represents a single
589 consistently grazed population and colour indicates the grazing route each population belongs to (dark grey, route 1; light grey route 2;
590 white, route 3). The size of points represents the population size class. The dashed lines in right panel shows the predicted values from
591 a generalized linear model produced from the program GESTE (top) and from a linear mixed model (bottom). See Table 3 results of
592 model selection on these data.



593

594 Figure 4: Boxplots showing differences in genetic diversity (A_r) among grazing treatment of *P.*
 595 *vulgaris* populations for each population size class (less than 40 individuals, 40-100 individuals,
 596 greater than 100 individuals). Boxes with different colours were found to be significantly
 597 different in post-hoc tests following a two-way ANOVA.



598

599 Figure 5: Scatterplots showing the relationships between a) seed set and genetic diversity (A_r),
 600 and b) mean seed mass and genetic diversity. Each point represents a single individual in one of
 601 ten populations, with point shape representing the population grazing treatment (squares =
 602 ungrazed; triangles = intermittently grazed; circles = consistently grazed). Solid lines show
 603 predicted values for the fixed effect (A_r) from linear mixed effect models controlling for
 604 population as a random effect, and dotted lines represent standard error of relationships. Genetic
 605 diversity was exponentially transformed to linearize relationships.

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