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

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Abstract

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

Introduction

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

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

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

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

Here we tested the efficacy of an ecological connectivity network implemented in southern Germany since 1989 to reconnect abandoned calcareous grassland fragments through 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.

Methods

Data Collection

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

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

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

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

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

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

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

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

Genetic Analysis of Functional Connectivity

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

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

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

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

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

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

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

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

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

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

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

Genetic Diversity

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

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

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

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

Fitness

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

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

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

Results

Genetic Markers

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

Functional Connectivity

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

Effects of Shepherding Connectivity on Genetic Diversity

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

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

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

Effect of Genetic Diversity on Fitness

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

Mean *seed set* per population showed strong positive correlations with both A_r ($r=0.82$, $p=0.003$), 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 ($r=0.32, p=0.37$).

Discussion

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

Functional Connectivity

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

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

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

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

Effect of Shepherding Connectivity on Genetic Diversity

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

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

Effect of Genetic Diversity on Fitness

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

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

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

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

Conclusions

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

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

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

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

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

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_{IBS}	-0.45	(-0.663,-0.294)

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

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

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Figure 1: Location of calcareous grassland patches and grazing treatment of patches in the study area in the Franconian Alb, Germany. Grazing routes connecting consistently grazed populations are indicated by black lines and labelled with the associated route. Dashed circles indicate populations where fitness-related traits were measured. Semi-natural open habitat is shown in light grey and core calcareous grassland patches ('core areas', see Methods for details) are 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 (Si_{IBD}), among-population least cost path through open habitat
588 (Si_{LCP}), and among-population shepherding distance (Si_{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.

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

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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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