Local Adaptation and Phenotypic Plasticity in Alpine Plants

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Ich widme diese Dissertation meiner Mutter. Ihr Beispiel inspiriert mich allen Widrigkeiten zum Trotz weiterzumachen.

Contents

Chapter 1

General Introduction

General Introduction

Scientific background

„In considering the Origin of Species, it is quite conceivable that a naturalist, reflecting on the mutual affinities of organic beings, on their embryological relations, their geographical distribution, geological succession, and other such facts, might come to the conclusion that each species had not been independently created, but had descended, like varieties, from other species. Nevertheless, such a conclusion, even if well founded, would be unsatisfactory, until it could be shown how the innumerable species inhabiting this world have been modified so as to acquire that perfection of structure and co-adaptation, which most justly excites our admiration. ... It is, therefore, of the highest importance to gain a clear insight into the means of modification and coadaptation."

Charles Darwin, 1859

A beautiful feature of Charles Darwin's theory of natural selection is that it not only explains the diversity of species, it additionally explains the staggering match of the structure of organism to their environments that so much evoke a sense of purposeful design in us – their adaptations. In this thesis we studied aspects of phenotypic divergence among populations of alpine plants and asked whether they are due to adaptation to local environmental conditions.

Many widespread species are variable across their distribution range or present us with a number of sub-species or varieties. For the study of local adaptation these species are often the most important as they allow us to compare closely related groups of individuals that have recently diverged in only some specific characters (e.g. Gauthier et al. 1998; Kalisz et al. 2012). Alpine plant populations are frequently small and isolated, yet they often show striking degrees of phenotypic divergence (e.g. Fabbro & Körner 2003; Äegisdottir et al. 2009, Albert et al. 2010). This divergence might result from diversifying natural selection as well as other processes such as random changes in gene frequencies or purely environmental influences. The nature and significance of intraspecific phenotypic variation in alpine plants has attracted considerable attention over the past decade (e.g.: Weppler & Stöcklin 2005; Alvarez et al. 2009; Gonzalo-Turpin & Hazard 2009; Grassein et al. 2010; Hahn et al. 2012; Scheepens et al. 2013; Frei et al 2014), but some fundamental hypotheses about the ecological relevance of this variation remain untested.

In recent years it has become evident that the glacial history of the European Alps has had a major influence on the distribution of genetic diversity of alpine plants (Hewitt 2004; Schönswetter et al. 2005; Parisod 2008; Thiel-Egenter et al. 2011). The repeated oscillations of alpine glaciers during the Quaternary have lead to range shifts in alpine plants with recurring isolation and admixture of populations and communities (Hewitt 2000). These range shifts most likely have led both to population divergence unrelated to adaptation, but have also imprinted a signature of past selection in the genome of alpine plants (Paun et al. 2008; Alvarez et al. 2009; Scheepens et al. 2013). Past adaptations might not necessarily be of adaptive value today, possibly confounding signals of recent adaptive evolution.

Due to their sessile habit and their inability to evade adverse conditions, plants are expected to have evolved the ability to vary their characters according to the prevailing external conditions (Sultan 2000). We call this responsiveness of individuals to external conditions phenotypic plasticity (Bradshaw 1965). It is likely that many phenotypic responses

to changes in the environment are a by-product of the evolutionary process rather than the result of direct natural selection on phenotypic plasticity. However, it has been shown compellingly that the responsiveness of individuals to their environment can be the result of natural selection and therefore adaptive (Dudley & Schmitt 1996; Herrera & Bazaga 2013). Phenotypic plasticity is almost omnipresent and has long been considered a nuisance in evolutionary and ecological studies because the dependence of phenotypes on the environment significantly complicates the their study (Sultan 2000). Plasticity and genetically fixed adaptations are intricately entangled phenomena that can interfere with each other. Strong directional selection for example can eliminate genetic variability in plasticity by selecting (positively) only those genotypes with either a specific responsiveness to environmental change or with no responsiveness at all. On the other hand, genetic diversity in plasticity within a population (*GxE*) can interfere with selection, because in the different environmental states, different genotypes are fittest, and therefore different genotypes are favored by selection in the different environments (Via & Lande 1985). Yet again, such *GxE* is ultimately a major cause for the preservation of genetic diversity (McLeod et al. 2012), which is the basis upon which natural selection acts. The role of phenotypic plasticity for alpine plant survival, the significance for the preservation of genetic diversity in alpine plants, and the circumstances under which it is most likely to evolve remain incompletely understood despite considerable recent efforts (Hülber et al.2006; Ceriani et al. 2008; Richter et al. 2011; Cornelius et al. 2012; Frei et al. 2014).

Plasticity is adaptive when a more plastic genotype has higher fitness averaged across environments than a less plastic genotype (Relyea 2002). In this regard, the rate at which different environments are encountered by a genotype or lineage is critical for the evolution of phenotypic plasticity (Winn 1996). This rate is determined by the grain of environmental heterogeneity, the generation time, and dispersal capacity of the organism under study (Alpert & Simms 2002). Under fine-grained environmental heterogeneity, individuals within one generation may encounter various states of the external conditions and should theoretically evolve the capacity to respond appropriately, that is, they should evolve plasticity (Sultan & Spencer 2002). If the grain of environmental variation is very coarse and the same habitat type is prevalent over many generations, organisms should theoretically specialize in this habitat type and evolve fixed geneticadaptation (Kawecki & Ebert 2004). The link between grain of environmental variability and the evolution of phenotypic plasticity is well established theoretically. However, only few experimental investigations exist that demonstrate this link practically (Baythavong 2011).

In the Alpine landscape steep gradients in elevation, soil properties, exposure, and other factors occur commonly on short distances (Körner 2003; Scherrer & Körner 2010). These are superposed by larger-scale heterogeneity in the same and other environmental variables, for example precipitation. The Alps receive considerable amounts of precipitation at their margins, but there are particularly dry zones in the central Alpine regions such as the canton of Wallis in Switzerland (Ozenda 1995). In contrast to general edaphic properties that are spatially distinct and consistent, soil nutrients are likely to vary at a considerably finer grain over space and time. Seasonal variability due to litter decomposition for example is common (Chapin 1980). The relative importance of the two complementary strategies of phenotypic plasticity and genetically fixed local adaptation is therefore not straigthforward to predict in Alpine plants.

Thesis outline and aims

This thesis is part of the SNF-funded project „How local adaptation and phenotypic plasticity allow plants to survive in a changing Alpine landscape: effects of fine-grainded vs. coarse-grained environmental variability“ involving two doctoral students – Elena Hamann and myself - under the supervision of Prof. Dr. Jürg Stöcklin.

The main goal of this thesis is to elucidate the importance of local adaptation and phenotypic plasticity in the highly structured Alpine landscape. Using reciprocal transplantation experiments and common garden studies, as well as molecular genotyping at neutral microsatellite loci, this thesis aims to provide proof of the importance of local adaptation and phenotypic plasticity for Alpine plant survival. The main study organism is the polymorphic *Anthyllis vulneraria* L., which has a wide latitudinal and elevational distribution throughout Europe up to around 3000 m above sea level. The grain of environmental variability is implicitly considered in the sampling design of populations, and the geographic distance of reciprocal transplantations. Populations were sampled in two regions, the Eastern and the Western Swiss Alps, and transplanted within and between regions, representing fine- and coarse-grained environmental change, respectively.
Below, a short outline of each chapter is given.

Chapter 1
General Introduction

This chapter.

Chapter 2
New microsatellite markers for *Anthyllis vulneraria* (Fabaceae), analyzed with Spreadex® gel electrophoresis
Kesselring H, Hamann E, Stöcklin J, Armbruster GFJ

The first chapter presents the development of novel microsatellite markers for *Anthyllis vulneraria*. Neutral molecular markers have become a widely used and valuable tool in evolutionary ecology. They allow the measurement evolutionary processes at genomic loci presumably unaffected by natural selection. Comparing patterns of phenotypic variation against this neutral background often allows a better understanding of adaptive evolutionary processes.

Chapter 3
Signature of local adaptation increases with geographic distance in an alpine plant.
Kesselring H, Scheepens JF, Hamann E, Armbruster GFJ, Stöcklin J

This chapter addresses the fundamental question whether local adaptation is present in Alpine plants using the outcrossed *Anthyllis vulneraria* and the inbred *Arabis alpina*. We perfomed what is considered among evolutionists the definitive test of local adaptation, a reciprocal transplantation experiment among six populations of each of the two species. Populations were sampled in the Eastern and Western Swiss Alps and transplantations were performed at two spatial scales – within the Eastern and Western Swiss Alps and across both regions - to learn more about the geographic scale of local adaptation in the two species.

Chapter 4

Past selection explains differentiation in flowering phenology of nearby populations of a common alpine plant.

Kesselring H, Armbruster GFJ, Hamann E, Stöcklin J

Chapter 4 presents a common garden study that assessed variation in reproductive allocation and flowering phenology of *Anthyllis vulneraria* under two soil moisture regimes. Local adaptation is particularly likely in reproductive characters because they have a direct effect on fitness. Furthermore, the tight link between flowering phenology and snomelt in Alpine plants makes adaptive differentiation in this trait especially likely. We used the comparison of quantitative trait differentation with neutral molecular differentiation (Q_{ST}-F_{ST} comparison) to investigate whether variation in flowering phenology is the result of natural selection. Phenotypic plasticity in response to soil moisture is discussed for its potential evolutionary significance.

Chapter 5

Protandry in Alpine populations of *Anthyllis vulneraria* s.l. (Fabaceae): Genetic and environmental variability and significance for the mating system.

Kesselring H, Armbruster GFJ, Frich B, Stöcklin J

The mating system of an organism can crucially affect the adaptive potential of a species, because it determines the amount of genetic recombination in a population. Flowers optimally position female and male parts in the same locality allowing for the female receptive parts to pick up pollen from the pollinator exactly where they were deposited by the male parts of another flower. This allows for an effective pollination process and needs only little pollen. However, it also bears the risk of high rates of self-fertilization, which is considered an evolutionary dead-end track. A majority of flowering plants are hermaphroditic, but separate female and male parts either in space (herkogamy), or in time (dichogamy), which prevents excessive self-fertilization. In this chapter we study variation in the degree of dichogamy in Alpine populations of *Anthyllis vulneraria*, its plasticity in response to soil moisture, and its significance for the mating system.

Chapter 6

Alpine plants have reduced plasticity in flowering time in response to warming compared to lowland congeneric species.

Schmid SF, Stöcklin J, Hamann E, Kesselring H

Chapter 6 investigates whether alpine plants are generally more/less plastic than lowland plants in their reproductive phenology. We tested this hypothesis by comparing a considerable number of congeneric species pairs of lowland and highland plants. We measured plasticity in flowering phenology in response to elevational transplantation. Results are discussed in the context of the particularities of reproduction at high elevations with short growing seasons and cold temperature limitation.

Chapter 7

Lower plasticity exhibited by high- versus mid elevation species in their phenological responses to manipulated temperature and drought.

Gugger S, Kesselring H, Stöcklin J, Hamann E

In addition of the experiment described in chapter 6, this study uses a parallel design to explore phenotypic plasticity in flowering phenology, yet at higher elevations in the Alps. Furthermore, this study incorporated drought as an additional treatment factor crossed with

elevation. Precipitation and temperature are considered two rapidly changing environmental factors under global climate change, and both are key determinants of species distributions. This study aims to elucidate the respective plastic responses of alpine and lowland plants to increasing drought and warming, and their implications for future plant distributions.

Chapter 8

General Summary and Conclusions

This chapter summarizes the main results, discusses their implications and relevance as a whole, and gives an outlook on outstanding questions and future research directions.

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Chapter 2

New microsatellite markers for *Anthyllis vulneraria* (Fabaceae), analyzed with Spreadex® gel electrophoresis

H. Kesselring, E. Hamann, J. Stöcklin, G.F.J. Armbruster

Abstract

Premise of the study: New microsatellite primers were developed for the diploid herb *Anthyllis vulneraria* L. Primers will be used in a study focusing on random genetic variation, local adaptation, and phenotypic plasticity in alpine plants.
Methods and Results: The new primers were adjusted to separate PCR amplicons (70 bp to 170 bp) on precast Spreadex® gels using horizontal gel electrophoresis. No capillary sequencer was needed.
Conclusions: Our preliminary results showed that the three studied alpine populations are predominantly outcrossing, but including variable levels of self-fertilization.

Key words: Alpine plants, Ethidium bromide, Horizontal electrophoresis, Microsatellites

Introduction

Alpine environments are considered to be particularly heterogeneous. Two fundamental survival strategies for heterogeneous environments can be contrasted: local adaptation or specialization vs. phenotypic plasticity, a generalist strategy. A major hypothesis suggests that phenotypic plasticity is favored over local adaptation when the spatial scale of dispersal spans several environmental states (Sultan and Spencer, 2002). Reciprocal transplantation experiments (RTE) are suitable to study both, local adaptation, and the reaction norm of plant phenotypes at different transplantation sites (Kawecky and Ebert 2004). In the near future, we will apply RTE using populations from two spatial scales (representing fine vs. coarse grained environmental variation) of four alpine species including *Anthyllis vulneraria* L. The degree of neutral genetic differentiation will be estimated using microsatellites and will be compared to phenotypic differentiation (e.g., $F_{st} - Q_{st}$ analysis).

Methods and Results

In our lab, we used Spreadex® gels and the ORIGINS electrophoresis unit (ELCHROM SCIENTIFIC AG, Cham, Switzerland) for microsatellite analyses. Spreadex® gels resolve PCR amplicons with size differences of 2 bp in an electrophoresis time of 1 to 2 h. Amplicons should not be longer than ca. 170 bp. In nearly all cases, heterozygous character states show a 'third' top band in the gel (i.e. a heteroduplex) because the gels consist of a non-denaturing matrix (see Figure 1, and Armbruster et al., 2005). Homozygous individuals show a single prominent PCR band. For *Anthyllis vulneraria*, we checked the five microsatellite loci AV2, AV3, AV7, AV12, and AV23 and the respective primers described by van Glabeke et al. (2007). These loci promised to be suitable for Spreadex® electrophoresis because the amplicons are between 60 bp and 170 bp. Despite the infraspecific taxonomic uncertainties of *A. vulneraria* (Nanni et al., 2004) the above microsatellites finally proved to be useful for our populations from the Swiss Alps (data not shown). However, to study spatial genetic variation with greater power we needed additional polymorphic microsatellite sequences from the genome of *A. vulneraria*. The development of 10 additional microsatellite primer pairs was outsourced to ECOGENICS GmbH (Schlieren, Zurich; see Matter et al. 2012).

ECOGENICS started with leaf material of *A. vulneraria* from the alpine region of Davos, Switzerland. Size selected fragments from genomic DNA were enriched for simple sequence repeats (SSR) by using magnetic streptavidin beads and biotin-labelled CT and GT repeat oligonucleotides. The SSR enriched library was analyzed on a Roche 454 platform using the GS FLX titanium reagents (MICROSYNTH AG, Balgach, Switzerland). The total 23,720 reads had an average length of 188 bp. Of these, 574 contained a microsatellite insert with a tetra- or a trinucleotide of at least 6 repeat units or a dinucleotide of at least 10 repeat units. One prerequisite was that the newly developed amplicons should be in the size range from 70 to 170 bp (see above). Suitable primer design was possible in 120 reads. Subsequently, ten loci (Table 1) provided allelic polymorphisms in 15 individuals (using an 48 capillary ABI3730 sequencer; data not shown). ECOGENICS used M13-tailing at the 5'-end of each forward primer for PCR. Hence, PCR conditions of ECOGENICS were different from our protocol in the running phase (below). The 10 µL PCR mix of ECOGENICS consisted of 1 µL PCR stock buffer of QIAGEN (Hilden, Germany) with 15 mM $MgCl_2$, 200 µM dNTP's, 0.04 µM forward primer (with M13-tail), 0.16 µM reverse primer, 0.16 µM M13 primer (5'-TGTAAAACGACGGCCAGT-3', labeled with a fluorescent dye for multiplexing), 0.5 U Hotstar Taq polymerase (QIAGEN, Hilden, Germany), and 10 ng DNA. Cycling conditions

were: denaturation at 95 °C for 15 min, start PCR at 95 °C 30 sec, 56 °C 45 sec, and 72 °C 45 sec in 30 cycles, continued with 95 °C 30 sec, 53 °C 45 sec, and 72° 45 sec in eight cycles. Termination was set to 72 °C for 30 min.

In the running phase, we checked the ten loci with Spreadex® electrophoresis. Three distinct populations of *A. vulneraria* that were geographically close to Davos, Switzerland, were selected (each with N = 20): Schiahorn (46°48'59.64" N, 9°48'16.80" E), Monstein (46°41'16.92" N, 9°47'15.84" E), and Casanna (46°51'26.88" N, 9°49'37.74" E). Voucher specimens and seeds (sampled by H.K.) are stored in the collection of the University of Basel, section of Population Biology of Plants. DNA was extracted with the DNeasy Plant Mini Kit of QIAGEN (Hilden, Germany). We used self-dissolving illustra puReTaq Ready-To-Go PCR Beads (GE Healthcare, Buckinghamshire, UK). 25 pmol forward and reverse primer, ddH_20 and 5 ng of DNA were added to the beads (e.g., Steiner et al. 2012). PCR was run in a MASTERCYCLER GRADIENT (Eppendorf, Hamburg, Germany), with denaturation at 95 °C for 2 min, start PCR at 95 °C 30 sec, locus specific annealing temperature (Table 1) 45 sec, 72 °C 45 sec in 35 cycles. Termination was set to 72 °C for 8 min. Samples were loaded on EL 400 or EL 600 gels (Table 1, Figure 1). M3 ladder from ELCHROM was used as size marker. Finally, gels were stained with ethidium bromide. Nine loci provided PCR amplicons, and their alleles were identical in size (bp) to those reported by ECOGENICS (Table 1). We tested the observed allelic signals for repeatability. Repetition comprised DNA extraction of nine individuals (= 15 % of the 60 individuals; Table 2), PCR and electrophoresis. In the 81 microsatellite lanes on the gels (9 samples · 9 loci), two lanes gave unclear genotype re-assignment (i.e. an error rate of ca. 2.5 %).

Three to twelve alleles were found per locus depending on the population studied (Table 2). Observed and expected heterozygosity (Table 2), linkage equilibrium and Weir&Cockerham F_{is}-values were calculated with 'Genepop 4.2. On The Web' (http://genepop.curtin.edu.au/). *P*-values for each locus pair across all populations yielded no significant linkage (all p's > 0.07). The mean F_{is}-values over all loci were positive (Schiahorn = 0.12; Monstein = 0.33, and Casanna = 0.34). Micro-Checker (van Oosterhout et al. 2004) tested for null alleles, with maximum expected allele size set to 200 bp, and a confidence interval of 95 %. No unusual observations were found. Micro-Checker suggested null alleles for AV-021012, AV-021049, AV-021224, and some others (Table 2). However, in the 60 individuals tested just four blank lanes appeared, interestingly all at AV-021049. We believe that 'real' null alleles are therefore only likely for that particular locus. Hence, we suppose that the excess of homozygosity is mostly due to self-fertilization (e.g. three of the 60 specimens were homozygous in all nine loci). Inbreeding is also indicated by the positive F_{is}-values. Autogamy has been reported as the predominant mode of reproduction for French populations of *Anthyllis vulneraria* (see Couderc, 1971), whereas Navarro (2000) found that strong protandry constrained self-fertilization in an Iberian population. The molecular analysis of van Glabeke et al. (2007) of two Belgian populations indicated that they were predominantly outcrossing. As all flowers of an individual plant do not develop synchronously, it is very likely that insects transfer pollen from late flowers to stigmata of early flowers of the same plant (i.e. geitonogamy). Based on our results, we suppose that there is variation in the degree of outcrossing and inbreeding among our populations from the Swiss Alps.

Conclusions

The newly developed microsatellite markers are suitable for horizontal Spreadex® gel electrophoresis with simple ethidium bromide staining and a considerable short electrophoresis time. No sequencer is needed to resolve the allelic patterns. Multiplex of two loci can also be tested, e.g. if the locus-specific amplicons differ in their respective length (e.g, 80 bp to 100 bp *vs*. 110 bp to 130 bp). Central alpine populations seem to be predominantly outcrossing with variable levels of self-fertilization.

Table 1. Characteristics of the newly developed microsatellite markers in *Anthyllis vulneraria*.

Locus	Genbank accession	Primer sequence [1] (5'-3')	Repeat motif [2]	Amplicon length (bp) [3]	T_a (°C)	Spreadex® gel type [4]
AV-000290	KF379737	F: GCAGAGAAGTTATAGTAGCTGTGTG R: CAGCCTGAAAGTATTGGTGGG	$(GA)_{13}$	89 – 123	52	EL 400
AV-002128	KF379738	F: GCATCTAGCCTCGTTTGTTTTATG R: CACTCTTGCGATACGAGAGC	$(Funk\ et\ al)_{13}$	77 – 101	52	EL 400
AV-004868	KF379739	F: GTCTGTTTATATGCAATGCGTGC R: CAGCATAGCTGCTTCTGTGAG	$(Funk\ et\ alFunk\ et\ al)_{12}(AG)_{12}$	114 – 147	50	EL 600
AV-005692	KF379740	F: TGAAATCAACCCACTAGACAACG R: AACAATCTGGAAACCCTCGC	$(GTT)_7$	77 – 93	52	EL 400
AV-015354	KF379741	F: GACTATGGTGGGTGGGTGG R: TGCGCATACACGAAGAAACC	$(TC)_{11}$	89 – 117	50	EL 400
AV-020270	KF379742	F: ATGAAGGAGGTGGGGCATAG R: TGGGCCATTTGCTTCTATATATGTG	$(CA)_{12}$	136 – 155	52	EL 600
AV-021012	KF379743	F: ACCAGCACCCAAGACCATAG R: TGGAATCGGAGATTGATTCTGG	$(AGT)_8$	82 – 98	50	EL 400
AV-021049	KF379744	F: GGAGCTGCTTTTAGCGAGAG R: GGTCCTCTATGGCAATCCTCC	$(AG)_{17}$	88 – 120	52	EL 400
AV-021224	KF379745	F: TGCATTGTTAAATTGAAGCTAGGTG R: CAGTCGATTCTCCACCCCTC	$(AC)_{18}$	133 – 170	52	EL 600
AV-021803 [5]	KF379746	F: TCTTACTTTCTCACAAGAATGCTATC R: TTTGCTAGTGTTGGACCTGC	$(AC)_{12}$	74 – 104 [5]	---	-----

Table 1 (previous page).
T_a = annealing temperature in the running phase of our project (see text).

[1] Primers used for PCR and subsequent Spreadex® gel electrophoresis with the ORIGINS Elchrom™ electrophoresis chamber. Note that ECOGENICS used F-primers with an M13 tail at the 5'-end, and fluorescent labeled M13 primers in their developmental phase (see text).
[2] Protocol of ECOGENICS, based on genomic DNA sequences analyzed on a Roche 454 GS FLX platform.
[3] In the 60 individuals of Davos (see Table 2), except locus AV-021803 (see footnote 5).
[4] We recommend an electrophoresis time of 1.5 to 2.0 h, a temperature of 55 °C, and 10 V/cm (i.e., 120 V in the ORIGINS Elchrom™ electrophoresis chamber). Our preferred precast Spreadex® gels are the Mini S-2x25 with a loading capacity of 25 samples per gel (loading volume ca. 9 µL per slot). EL 400 and EL 600 differ in the density of the gel matrix. For longer amplicons we used EL 600, for shorter amplicons EL 400.
[5] This locus worked according to the protocol of ECOGENICS (with M13 tailing; see text) but could not be established in the running phase in our lab. Amplicon size is based on 15 individuals checked by ECOGENICS.

Tab. 2. Details on the three populations of *Anthyllis vulneraria*. A = number of alleles found, H_o = observed heterozygosity, H_e = expected heterozygosity.

	Schiahorn (n = 20)			Monstein (n = 20)			Casanna (n = 20)		
Locus	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e
AV-000290	5	0.600	0.601	6	0.600	0.715	8	0.650	0.750
AV-002128	7	0.750	0.695	8	0.650	0.820	8	0.600*	0.802
AV-004868	8	0.850	0.827	8	0.500*	0.729	12	0.600*	0.917
AV-005692	4	0.750	0.675	4	0.500	0.602	6	0.500	0.601
AV-015354	7	0.800	0.770	6	0.550	0.673	8	0.700	0.715
AV-020270	6	0.500*	0.764	5	0.550	0.689	7	0.700	0.764
AV-021012	4	0.700	0.714	4	0.300*	0.670	3	0.150*	0.678
AV-021049	4	0.300*	0.610	8	0.250*	0.720	10	0.200*	0.803
AV-021224	6	0.350*	0.667	6	0.300*	0.635	12	0.400*	0.769

* indicates excess of homozygotes / potential null alleles based on MICRO-CHECKER analysis (see text).

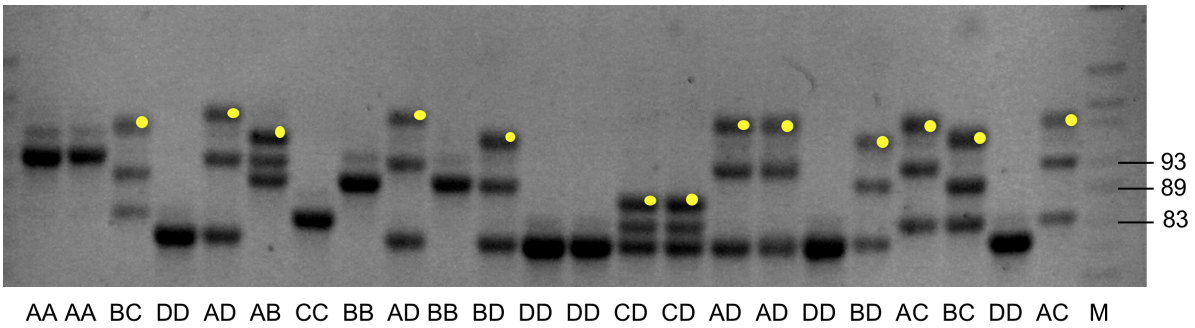

Figure 1 Spreadex® EL 400 gel with electrophoretic resolution of 8 µl to 9 µl of microsatellite amplicons at locus AV-005692. Fingerprints of 23 diploid individuals of *Anthyllis vulneraria* are shown. M = 7 µl of M3 marker from Elchrom Scientific (see bp at right margin). Genotypes are labeled in capitals. Alleles (A,B,C,D) are coded by size (A = 79 bp, B = 83 bp, C = 89 bp, D = 93 bp). Note that heterozygous individuals show a prominent heteroduplex signal (yellow dots).

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Chapter 3

Signature of local adaptation increases with geographic distance in an alpine plant.

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Abstract

Spatially variable selection is considered to result in local adaptation. Yet the generality of local adaptation of populations remains debated, and we know little about the spatial patterns of local adaptation.

We conducted reciprocal transplantations among six populations each of two common and well-studied herbaceous plants, *Anthyllis vulneraria* and *Arabis alpina*. We measured aboveground biomass, reproductive allocation and flowering propensity to test for local adaptation at two spatial scales: within and between the Eastern and Western Swiss Alps. Additionally, populations were genotyped using microsatellite markers to assess neutral differentiation and historic inbreeding.

Microsatellite analyses indicated neutral population differentiation according to spatial scale in both species, as well as mixed mating in *Anthyllis vulneraria* and strong inbreeding in *Arabis alpina*. The spatial scale was also mirrored in fitness variation of transplanted *Anthyllis vulneraria*: fitness decreased with geographic distance between population origin and transplant site. In *Arabis alpina*, reproductive biomass was lowered only in near away transplantations, but not in far away transplantations.

The findings suggest that habitat heterogeneity across the alpine landscape can drive local adaptation although results on *Arabis alpina* remain inconclusive. Selection-driven differentiation appears to increase with geographic distance in the outcrossed *Anthyllis vulneraria*.

Keywords: alpine plants; *Anthyllis vulneraria*; *Arabis alpina*; local adaptation; reciprocal transplantation; sympatric vs. allopatric contrast

Introduction

Despite the long history of research on local adaptation of plant populations (Clausen et al 1940), there is still debate over its pervasiveness (Leimu and Fischer 2008; Hereford 2009) and best definition (Kawecki and Ebert 2004; Blanquart et al 2013). Ideally, proof of local adaptation should demonstrate an increase in individual fitness through evolution from the ancestral state towards the descendant state (Lande and Arnold 1983; Travisano et al 1995). The ancestral state, however, is usually not conserved through time (but see Franks et al 2008), making this comparison difficult. The reciprocal-transplant design has therefore become the gold standard for testing local adaptation of populations (Kawecki and Ebert 2004; but see Peterson et al 2016 New Phytologist).

Two criteria have traditionally been applied to test for local adaptation in reciprocal transplant studies: the *local vs. foreign criterion* and the *home vs. away criterion* (Kawecki and Ebert 2004). The *local vs. foreign criterion* compares the fitness of the local population to foreign populations at each transplantation site, while the *home vs. away criterion* compares the fitness of each population at their home sites to their fitness at away sites. Both criteria are not free of confounding effects: the former is confounded by the intrinsic vigour of the populations and the latter by the fertility of the sites. One frequently finds differences among populations in their intrinsic vigour that need not be related to local adaptation (Hirst et al 2016), and likewise, the fertility of sites can vary greatly across the landscape. Therefore, these confounding effects are serious problems in the analysis of reciprocal transplant experiments.

Recently a novel criterion has been introduced, called the *sympatric vs. allopatric criterion* (Blanquart et al 2013). It compares the average fitness in naturally occurring population by site combinations (*sympatric*) to the average fitness in experimentally created population by site combinations (*allopatric*) across a preferably large number of populations. Before the criterion is assessed, effects of population vigour and site fertility are statistically removed, and therefore, effects unrelated to local adaptation do not confound the *sympatric vs. allopatric criterion*. It is noteworthy that the terms sympatric and allopatric have different meanings in the context of local adaptation sensu Blanquart et al (2013) than in classical ecology.

It is of particular interest to test local adaptation across multiple ecological scales (e.g. habitat types or geographical scales) to resolve some of the disagreement over the pervasiveness and conditions under which local adaptation evolves (Peterson et al 2016). The *sympatric vs. allopatric criterion* is ideal to incorporate multiple ecological scales because it can be divided into multiple levels with little loss of statistical power (Blanquart et al 2013), for example according to the geographic distance between a transplantation site and the site of origin of a population (e.g. *sympatric*, *near allopatric*, *far allopatric*).

The evolution of local adaptation among plant populations is expected when populations of a species experience consistent divergent selection, are sufficiently genetically isolated, and have high genetic variation. Accordingly, the signature of local adaptation should be strong when comparing plant populations at great geographic or environmental distance, and weak at small distances. Few studies have set out to explicitly test such hypotheses at multiple ecological scales simultaneously. Evidence is correspondingly rare, but mostly supportive (Sambatti and Rice 2006; Hereford and Winn 2008; Anderson et al 2015; Peterson et al 2016). Galloway and Fenster (2000) have found evidence for local adaptation of an annual legume at distances greater than 1'000 km, but not at closer distances. Likewise, Toräng et al (2014) have found strong local adaptation in the arctic-alpine *Arabis alpina* at distances of 3'000 km, but not at distances smaller than 600 km. The only study to date that has made use of the *sympatric vs. allopatric criterion* for testing local adaptation at multiple spatial scales is by Hamann et al (2016), who found that a common alpine fodder grass was locally adapted at the regional scale (>200 km), along with some evidence for adaptive differentiation at the within-region scale (>20 km).

Mountain plants are particularly interesting for the study of local adaptation because they frequently face diverse habitats across their range even at similar elevation (Körner 2003). While studies in mountain systems usually find considerable phenotypic differentiation among plant populations (e.g. Frei et al 2011), there is mixed evidence for the prevalence of local adaptation at high elevations (Galen et al 1991; Angert and Schemske 2005; Geber and Eckhart 2005; Byars et al 2007; Gonzalo-Turpin et al 2009; Sedlacek et al 2015; Hirst et al 2016). Furthermore, we lack studies that focus on local adaptation of mountain plants unrelated to elevational gradients, i.e. local adaptation of plant populations at similar elevation (Hirst et al 2016).

In the current study, we performed reciprocal transplantations across two spatial scales, within and between the Eastern and Western Swiss Alps using two alpine species, *Anthyllis vulneraria* L. and *Arabis alpina* L. These species were chosen because they are widely distributed and well-studied herbaceous plants of the European Alps. Each of six populations per species was transplanted to its site of origin, to another site in the same region, and to a site in the other region. We measured mortality, aboveground biomass, and flowering propensity as fitness proxies, and tested for local adaptation using the *sympatric vs. allopatric criterion*. We also investigated genetic population structure, genetic diversity, and inbreeding levels using microsatellite markers. The following questions were addressed: (1) Is there evidence for local adaptation in alpine *Anthyllis vulneraria* and *Arabis alpina*? (2) Does the geographic distance between transplant sites explain fitness variation, i.e. is the occurrence and strength of local adaptation related to the spatial scale? (3) Do the experimental populations show neutral genetic differentiation, and is this differentiation in line with their geographic distribution?

Materials and Methods

Study species

Anthyllis vulneraria L. sensu lato (s.l.; Fabaceae) is a clade of self-compatible short-lived perennial rosette plants common throughout Europe. It grows preferably on calcareous grassland and scree up to around 3'000 m above sea level (a.s.l.; Hegi 1975). Plants grow to a height of ca. 15-45 cm. Each plant comprises a variable number of shoots, of which each bears 2-6 inflorescences. Each inflorescence comprises a number of 7-19mm long white to yellow, sometimes claret to red flowers arranged in a capitulum (Hegi 1975; Navarro 1999a). Selfed and geitonogamous offspring may be produced due to the spatial co-location of self-pollen and stigma and the asynchronous flower ripening across capitulae and shoots. Populations of *Anthyllis vulneraria* may be exclusively selfing (Couderc 1971) or may be protandrous to a degree where selfing is effectively prevented (Navarro 1999b). *Anthyllis vulneraria* s. l. is a particularly polymorphic taxon with unclear infraspecific classification (Nanni et al 2004; Köster et al 2008). We have assigned the alpine populations studied here to *Anthyllis vulneraria* ssp. *alpestris* (Schult.) Asch. and Graben, and to *Anthyllis vulneraria* ssp. *valesiaca* (Beck) Guyot (Konrad Lauber 2012). Two populations in the western region (Findelgletscher and Findelwald) belong to *Anthyllis vulneraria* ssp. *valesiaca* while all other populations belong to *Anthyllis vulneraria* ssp. *alpestris*.

Arabis alpina L. (Brassicaceae) is a perennial rosette plant and is the type species of the Arabideae, the largest crucifer tribe (Hegi 1975; Karl and Koch 2013). *Arabis alpina* has a wide distribution range from the high mountains of northern Africa over the Pyrenees and the European Alps to the Near East, and across the whole arctic region. *Arabis alpina* is a pioneer plant and grows near glacier snouts and on screes up to around 3'200 m a.s.l., but can also be found at lower elevations down to 400 m a.s.l. It occurs commonly on disturbed and mildly moist sites with calcareous and alkaline bedrock (Hegi 1975; Koch et al 2006). *Arabis alpina* grows 6-40 cm tall. Vegetative shoots are short and horizontally crawling with leaf rosettes at the tip of the shoots, while reproductive shoots are upright. Flowers produce nectar and are arranged as raceme. *Arabis alpina* populations from the central and western Alps are highly inbred due to a non-functional self-incompatibility system (Tedder et al 2011; Buehler et al 2012a) resulting in frequent selfing along with bi-parental inbreeding.

Molecular genotyping

20 individuals per study population of *Anthyllis vulneraria* were scored for amplified fragments at 9 microsatellite loci, and 15 individuals per study population of *Arabis alpina* at 10 loci. Six study populations per species were used (Table 1; Fig. 1). Leaf samples for DNA extraction were taken from experimental plants. Each sample was taken from one randomly chosen offspring of a different maternal plant each, and stored in paper bags in silica gel. We used Spreadex® gels and the ORIGINS electrophoresis unit (Elchrom Scientific AG, Cham, Switzerland) to separate PCR amplicons with size differences as small as 2bp. Gels were stained with ethidium-bromide and scored by hand comparing against ELCHROM's M3 ladder. A detailed description of the microsatellite analysis and *Anthyllis vulneraria* loci can be found elsewhere (Kesselring et al 2013). New primers were designed for *Arabis alpina* based on published GenBank sequences of 10 loci described by Buehler et al (2011) to achieve an amplicon length of 90-150 bp, which is suitable for Spreadex electrophoresis (Kesselring et al 2013). PCR details and primer sequences are reported in the supplementary materials (S1). Error rate in electrophoretic genotyping of *Arabis alpina* was estimated with a repetition analysis, starting from DNA extraction of 11 of the 105 individuals (9.5% of the entire sample size). 216 signals of amplified DNA were found in the first run for these 11 individuals across all 10 loci. In the repetition analysis, 212 of the 216 alleles were identically re-scored, equalling an error rate of 1.8%. Error of *Anthyllis vulneraria* was estimated at 2.5% (Kesselring et al 2013). Null alleles were suggested for all loci in most populations of *Anthyllis vulneraria* by the software FreeNA (Chapuis and Estoup 2007). However, F_{ST}

values adjusted for null alleles were nearly identical for all but one locus and only this locus showed homozygote null alleles (blank lanes on the gel). Visual inspection of electrophoresis gels and the biology of the species furthermore suggest that the observed heterozygote deficiencies at the other loci are not due to artefacts. Therefore, we removed only the questionable locus from analyses and assumed the rest of the data to be free of artefacts. *Arabis alpina* showed only two blank lanes out of 900 making it highly unlikely that null-alleles exist in the studied samples, especially given the fact that *Arabis alpina* is highly inbred.

	Anthyllis vulneraria			*Arabis alpina*		
	population	coordinates	elevation	population	coordinates	elevation
Davos (Eastern Swiss Alps)	Schiahorn	780513.385 / 187874.756	2650	Schiahorn	780513.385 / 187874.756	2650
	Casanna	782301.543/ 192247.969	2320	Casanna	782301.543/ 192247.969	2320
	Monstein	779685.630 / 173389.160	2010	Weissfluhjoch	780324.165 / 189706.004	2700
Zermatt (Western Swiss Alps)	Findelwald	626828.986 / 95475.764	2170	Blauherd	627165.547 / 96339.072	2580
	Findelgletscher	629173.611 / 95175.270	2490	Findelgletscher	629173.611 / 95175.270	2490
	Stafelalp	619094.320 / 94427.436	2280	Trockener Steg	621622.757 / 90793.274	2880

Tab. 1 Elevations and coordinates (Swiss grid CH1903) of the 12 populations from two regions of *Anthyllis vulneraria* and *Arabis alpina* used in the reciprocal transplantation experiments.

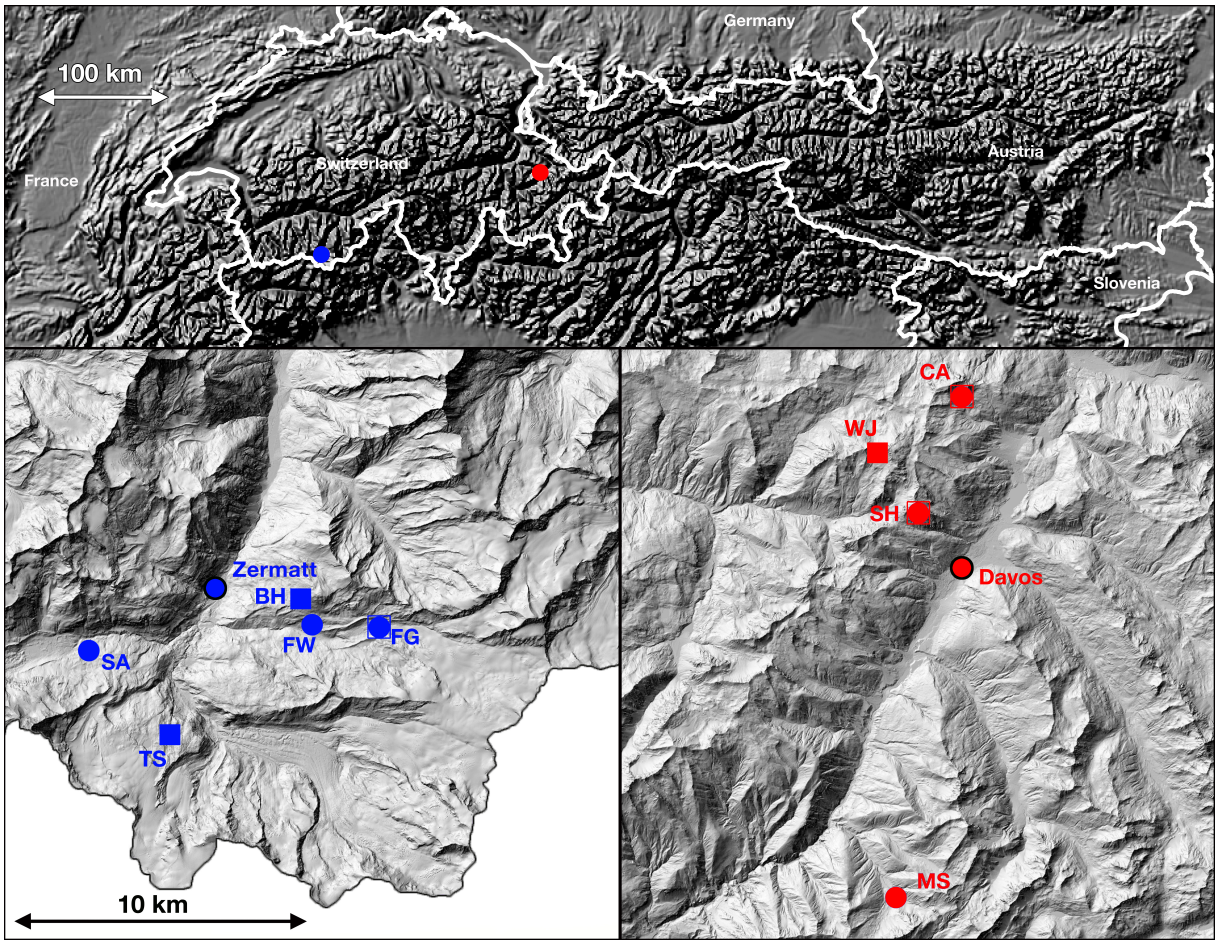

Fig. 1 The location of the populations and transplantation sites used in the experiments. Circles stand for populations of *Anthyllis vulneraria*, squares for those of *Arabis alpina*, and circles inside open squares for sampling sites of both species. Populations are located in the vicinity of Davos and Zermatt (circles with black outline) in Switzerland. Abbreviations are SA for Stafelalp, TS for Trockener Steg, BH for Blauherd, FW for Findelwald, FG Findelgletscher (both species) in the Zermatt region, and MS for Monstein, SH for Schiahorn (both species), WJ for Weissfluhjoch, and CA for Casanna (both species) in the Davos region.

Reciprocal transplantations

For both species, three populations from each of two regions near Davos and Zermatt, respectively, were used for transplantations (Table 1). The distance between regions roughly equals 180 km, and distances between populations within regions range from 2 to 18 km (Fig. 1). Juvenile offspring of each population were transplanted to their *home* site, to an *away* site in the same region, and to a *far away* site in the other region. It follows that each site received offspring from its *local* population, from a foreign population of the same region (*near foreign*), and from a foreign population of the other region (*far foreign*). Thus, a total of 18 transplantations were performed per species. Sensu Blanquart et al (2013), a transplantation where a site is combined with its local population is referred to as *sympatric*, and one of a site with a foreign population as *allopatric* (note that *sympatric* and *allopatric* therefore do not have the same meaning as in classical systematic biology). Here, we further specify the combination of a site with a population from the same region as *near allopatric*, and a combination of a site with a foreign population from the other region as *far allopatric*. Populations and sites for *near allopatric* and *far allopatric* transplantations were randomly matched. We preferred an unbalanced reciprocal design instead of transplanting all populations to all sites, as a fully factorial design has been shown to not make optimal use of resources in terms of statistical power (Blanquart et al 2013).

Open-pollinated seeds of *Anthyllis vulneraria* were collected in the second week of August 2012. Offspring of individual maternal plants were collected in separate paper bags, and family membership was traced subsequently. One week later, between 15-Aug-2012 and 17-Aug-2012, seeds of *Anthyllis vulneraria* were scarified and placed on wet filter paper in Petri dishes for germination. On 21-Aug-2012, three seedlings per maternal family were potted to 54-pot trays filled with 100ml low nutrient soil (Anzuchterde, Ökohum GmbH, Herrenhof, Switzerland) per pot. A total of 45 maternal families were used per population. Final transplantation to field sites was performed on 17 and 18-Sep-2012 in Davos and on 25 and 26-Sep-2012 in Zermatt. Of the three individuals per maternal family, one was transplanted to its *home* site, one to the *away* site, and one to the *far away* site. However, family membership was ultimately ignored, because uneven mortality soon after transplantation led to an unequal genetic make-up of transplanted populations. Plants were transplanted into the native soil at the field sites by digging a hole of the same volume and shape and placing the root ball inside the hole. Air spaces were filled with loosened soil from the field sites, and each plant was watered with 200 ml of water to facilitate root contact with the local soil. Plants were

transplanted in rows of 10 individuals, alternating between *local*, *near foreign*, and *far foreign* individuals, and spacing individuals at 20 cm distance from each other. Each site received 135 transplants that were arranged in 14 rows (leaving the last 5 slots empty). There were 810 transplanted individuals of *Anthyllis vulneraria* in total.

In the first week of July 2013, at the beginning of the growing season, we assessed survival at one site in Davos (Monstein) and one site in Zermatt (Findelgletscher). Towards the end of the first growing season in the field (2013), in the first week of September in Zermatt and in the fourth week of September in Davos, we assessed survival at all transplantation sites. Thus by comparing September survival against July survival, we could assess mortality of established individuals during the first summer at these two sites (n = 165). To assess mortality during the second winter, we compared data from September 2013 against survival at the time of harvest (all sites; n = 432). Initial mortality due to transplantation was very high (nearly 46% of individuals).

After two full growing seasons in the field, aboveground biomass of all *Anthyllis vulneraria* plants was harvested during the first week of August 2014. The Monstein site was excluded from final analyses because plants at this site never flowered and remained very small, most likely due to grazing by cattle. Individuals in the first five rows at the Findelwald site were already harvested in the first week of September 2013, because plants at this site flowered already in the first year. This was done to spread the risk of high mortality in the field. Time of harvest is implicitly accounted for in statistical analyses by the inclusion of site effects. Harvested biomass was divided into reproductive and vegetative biomass and dried in the oven at 80 °C for 48 h, starting no later than the day following harvest. For final analysis of biomass in *Anthyllis* vulneraria, 304 plants remained. At the time of harvest, it was also noted whether an individual had flowered or not (n=304).

Open-pollinated seeds of *Arabis alpina* were collected during September and October 2012. Seeds were kept at room temperature for one week and then stored at 4 °C. Seeds of *Arabis alpina* were sown on 27-Mar-2013 onto small trays filled with low nutrient soil and topped with a thin layer of soil (Anzuchterde, Ökohum GmbH, Herrenhof, Switzerland). They were subsequently cold-stratified at 4 °C for 4 days to improve germination rate (D. Buehler, pers. comm.). Between 18-Apr-2013 and 29-May-2013 seedlings were transferred to 54-pot multitrays containing the same soil type. Plants were transplanted to field sites between 03-

Jul-2013 and 26-Jul-2013 (this timing was not ideal and has caused severe mortality; we advise future studies to transplant during autumn). Transplantations were performed in the same fashion as with *Anthyllis vulneraria*. A total of 810 individuals were transplanted. Final harvest of aboveground biomass was conducted between 31-Jul-2014 and 05-Aug-2014. Reproductive and vegetative biomass was separated and dried in the oven at 80 °C for 48 h, starting on the day following harvest. Maternal families were traced throughout the experiment, but ultimately one average value per maternal family and transplant site was used. On average 8 maternal families (between 3 and 13) and 2 replicates per family (between 1 and 7) were used per population and site. Bad timing of transplantations has cause severe mortality and two sites were completely lost, resulting in a total of 200 individuals resp. 121 family averages for final analyses. Mortality of *Arabis alpina* was not analysed, because plants were in the field for only one growing season and mortality therefore could not be assessed independent of direct transplantation effects.

Statistical analysis

Given the clear sampling structure of populations, we used analysis of molecular variance (AMOVA) as implemented in GenAlEx to partition genetic variation at microsatellite loci into components according to the sources region, population within region, individual within population, and within individuals. AMOVA also estimates Wright's fixation indices (*F*) corresponding to structuring at the same hierarchical levels. We further report genetic diversity at microsatellite loci in terms of expected heterozygosity H_e and allelic richness.

We used linear mixed effects models for the analysis of biomass traits based on the *sympatric vs. allopatric* definition of local adaptation (Blanquart et al 2013). *Sympatric* refers to naturally occurring site by population combinations and *allopatric* (*near-* resp. *far allopatric*) to experimentally created site by population combinations. We specified models in the lmerTest package (Kuznetsova 2013) for R (R Development Core Team 2008), which included a factor for site, population and a factor describing whether a combination of site and population was *sympatric*, *near allopatric*, or *far allopatric* (the factor is referred to as *sympatric vs. allopatric*).

The factors site, population and *sympatric vs. allopatric* were tested for their effects on total aboveground biomass and reproductive allocation. Separate models were specified for each response variable and species. Site and population, were specified as random effects, while

sympatric vs. allopatric was a fixed effect. lmerTest is a package of convenience functions for lmer objects of the lme4 package (Bates 2014) that allow F-tests for fixed effects and likelihood-ratio tests for random effects using stepwise model reduction and comparison. We used Type 3 sums of squares and Satterthwaite approximations for degrees of freedom. We report *P*-values, mean squares, and χ^2 values that correspond to those from the model comparison (i.e. likelihood-ratio tests) using the step function in lmerTest.

Significant site or population terms indicate differences in the fertility of sites or intrinsic population quality, respectively. A significant *sympatric vs. allopatric* factor indicates that populations performed on average better when transplanted to either one of the *home*-, *near away*- or *far away sites*. If the *sympatric vs. allopatric* factor was significant, post-hoc pairwise comparisons were used to check if patterns of fitness variation conformed to local adaptation. A significantly positive difference between *sympatric* combinations of sites and populations and *near allopatric* combinations of sites and populations indicates local adaptation at the scale of populations within regions. A significantly positive difference between *sympatric* combinations of sites and populations and *far allopatric* combinations of sites and populations indicates local adaptation at the regional scale. We used differences of least squares means (dlsm) as output by the step function of lmerTest for post-hoc comparisons.

Flowering propensity and survival of *Anthyllis vulneraria* were analysed using generalized linear mixed-effects models of the lme4 package (Bates 2014) for R with a binomial distribution and the logit link function. Identical model specifications were used as for the mixed effects models above. Likelihood ratio tests were performed to assess significance levels of all factors. The glht function of the multcomp package (Hothorn et al 2008) in R was used for post-hoc pairwise comparisons of the levels of the *sympatric vs. allopatric* factor.

Results

Molecular Analyses

In *Anthyllis vulneraria*, average numbers of alleles per locus and population ranged from (mean±SD) 5.80±0.56 to 8.00±1.05 and expected heterozygosities (H_e) ranged from 0.68±0.02 to 0.76±0.02 across populations. The microsatellite analyses revealed a positive mean inbreeding coefficient (F_{IS}) of 0.254 across populations of *Anthyllis vulneraria* used in this study, ranging from 0 to 0.414 across populations. AMOVA showed that small but

significant amounts of molecular variation are explained by region (5%) and population structure within regions (4%; Table 2). Pairwise F_{ST}'s ranged from 0.014 to 0.084 within the Davos region, from 0.000 to 0.052 within the Zermatt region, and from 0.038 to 0.127 across regions. Only the populations Findelgletscher and Stafelalp were not significantly differentiated from each other (S2).

In *Arabis alpina*, average numbers of alleles per locus and population ranged from 2.60±0.34 to 3.80±0.44. Expected heterozygosities (H_e) ranged from 0.34±0.07 to 0.54±0.04 across populations. Our analyses confirmed the highly inbred nature of *Arabis alpina* with an average F_{IS} of 0.758 for the populations studied here (ranging from 0.459 – 0.975). Accordingly, AMOVA revealed that most of the molecular variability is between (55%) and not within individuals (17%; Table 2). Furthermore, regions and populations within regions explained relatively large amounts of variation with 11 % and 17 %, respectively. Pairwise F_{ST}'s ranged from 0.048 to 0.127 within the Davos region, from 0.101 to 0.257 within the Zermatt region, and from 0.155 to 0.282 across regions (S3).

	Anthyllis vulneraria					*Arabis alpina*				
	df	SS	est.var.	%	fixation index[a]	df	SS	est.var.	%	fixation index[a]
region	1	25.7	0.145	5	0.045 **	1	53.9	0.355	11	0.105 **
population	4	33.8	0.114	4	0.037 **	4	87.8	0.589	17	0.195 **
individual	114	422	0.751	23	0.081 **	84	359.7	1.847	55	0.279 **
within individual	120	264	2.2	69	0.254 **	90	53	0.589	17	0.758 **
total	239	744.7	3.21	100	0.315 **	179	554.4	3.38	100	0.826 **

Tab. 2 AMOVA (analysis of molecular variance) table of 12 populations from two regions of *Anthyllis vulneraria* and *Arabis alpina*.

[a]All fixation indices were significant at the P=0.001 level (**).

Transplant experiments

Anthyllis vulneraria

Mortality after establishment was assessed for *Anthyllis vulneraria* in the first growing season (summer 2013) at one site in each region and at all sites during the second winter (2013/2014). During summer 2013, only a single individual died at the Findelgletscher site in the Western region, and seven individuals died at the Monstein site in the Eastern region. Mortality during the second winter in the field (2013/2014) was high: out of 437 plants living at all six sites in September 2013, 122 (28%) died until the final harvest in September 2014. However, mortality during the 2013/2014 winter was neither dependent on site (df = 2, $\chi^2 = 0$, P =1), nor on population (df = 2, $\chi^2 = 0$, P =1), nor on the *sympatric vs. allopatric factor* (df = 2, $\chi^2 = 2.28$, P =0.319).

Total aboveground biomass of *Anthyllis vulneraria* was different between populations and sites (Table 2; S4c). The *sympatric vs. allopatric* factor was also significant (Table 2): the average total aboveground biomass across the experiment was lower in *far allopatric* plants compared to *sympatric* plants and compared to *near allopatric* plants (Fig. 2; dlsm: sym-far.allo=0.5, df=296, P <0.001; near.allo-far.allo=0.5, P <0.001). *Near allopatric* combinations of populations and habitat within the same region had equal total aboveground biomass compared to *sympatric* transplants (Fig. 2; dlsm: sym-near.allo=0.0, df=96, P =0.9).

Reproductive biomass was different among sites and among populations (Table 2; S4b). Moreover, a highly significant *sympatric vs. allopatric* effect was found for this trait (P =<0.001). Reproductive allocation of *Anthyllis vulneraria* decreased with increasing distance between transplantation site and population origin (Fig. 2). *Sympatric* transplantations yielded the highest reproductive biomass, *near allopatric* intermediate, and *far allopatric* transplantations lowest biomass (dlsm: sym-near.allo=0.1, df=73, P =0.038; sym-far.allo=0.3, df=84, P <0.0001, near.allo-far.allo=0.2, df=297, P =0.007). Furthermore, in four out of five sites, reproductive biomass tended to decrease with increasing distance between the site and the origin of the transplanted population, and at every site the *local* population had the highest reproductive biomass (S4b). Removing non-flowering individuals (with zero reproductive biomass) from the analysis did not qualitatively affect the results.

At the end of the experiment, populations of *Anthyllis vulneraria* in sympatric combinations of populations and habitats had 76 % flowering propensity, allopatric combinations 66 %, and

far allopatric only 62% (Fig. 3; *sympatric vs. allopatric* factor: df = 2, $\chi^2 = 6.96$, $P = 0.031$). Flowering propensity was not significantly affected by site (df = 2, $\chi^2 = 0$, $P = 1$) nor population (df = 2, $\chi^2 = 0.155$, $P = 0.694$). The local population at each site generally had the highest flowering propensity, the only exception being the Stafelalp site. In all populations transplanted to the Stafelalp site, less than 50% of plants flowered due to the low overall growth of transplanted individuals (compare with biomass results). The Stafelalp population was also the only one to flower at a higher rate at both away sites compared to the home site.

Arabis alpina

Of a total of 200 surviving *Arabis alpina* plants (individuals as opposed to family means for other analyses) at four sites, all but 28 flowered at the time of harvest. Of these 28 non-flowering plants, 11 were in *sympatric* combination with their habitat, 5 were *allopatric* within-region, and 12 were in *allopatric* population by habitat combinations across regions. None of the factors site (df = 2, $\chi^2 = 0$, $P = 1$), population (df = 2, $\chi^2 = 0$, $P = 0.999$) or *sympatric vs. allopatric* explained significant variation (df = 2, $\chi^2 = 2.38$, $P = 0.497$).

Total aboveground biomass of *Arabis alpina* was not differentiated among populations or sites. The *sympatric vs. allopatric* factor was also not significant for total aboveground biomass (Table 2; Fig. 2).

There were no significant differences among sites or among populations in reproductive biomass (Table 2; S5b). However, the *sympatric vs. allopatric* factor was significant for reproductive biomass in the *Arabis alpina* experiment, with *near allopatric* combinations having lower biomass than *sympatric* and *far allopatric* population by habitat combinations (Table 2; dlsm: sym-near.allo= 0.1, df=118, p=0.02; near.allo-far.allo=-0.1, df=118, p=0.03). Cross-regional population by habitat combinations (*far allopatric*) were not significantly different from *sympatric* combinations (Fig. 2; dlsm: sym-far.allo=0.00, df=118, p=0.78).

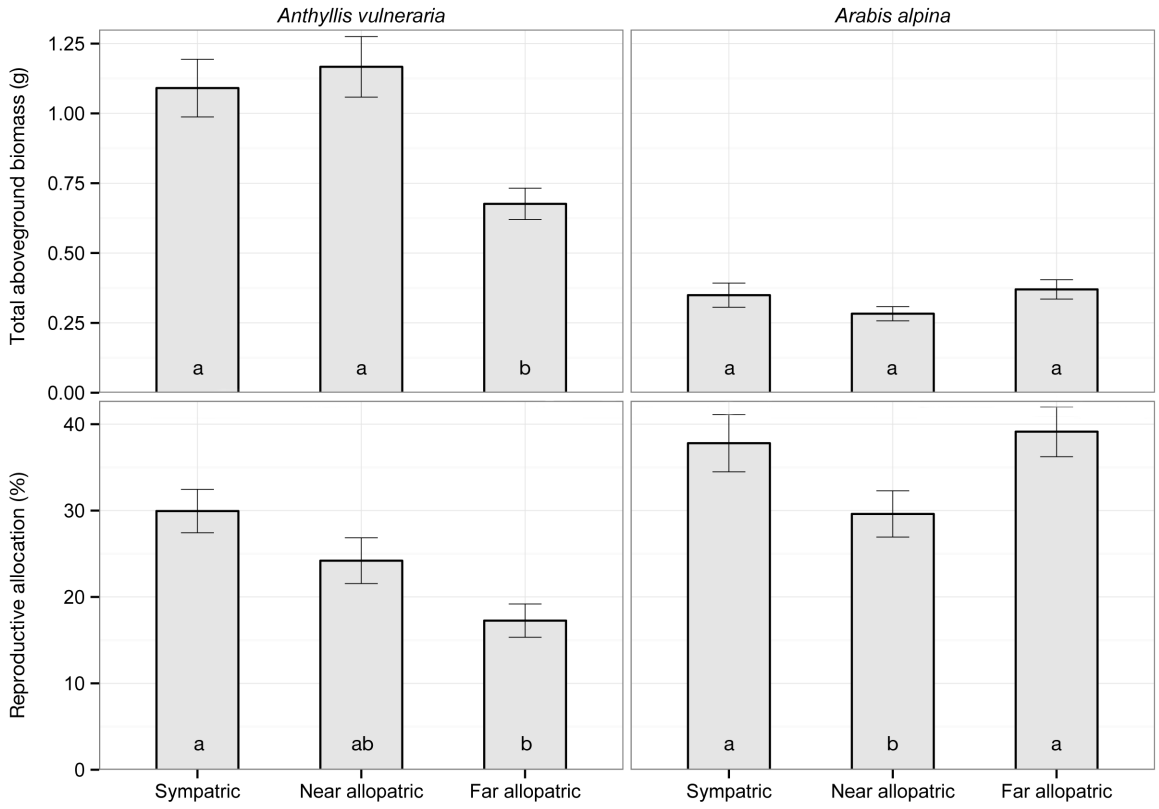

Fig. 2 Total aboveground biomass (top row) and aboveground biomass allocation to reproductive structures (bottom row) of *Anthyllis vulneraria* and *Arabis alpina* in *sympatric*, near *allopatric* and *far allopatric* transplantations. Error bars depict 1 standard error of the mean. Letters inside bars of each panel represent significantly different groups as per least squares means post-hoc tests. Non-flowering plants were disregarded in calculating mean values of reproductive biomass.

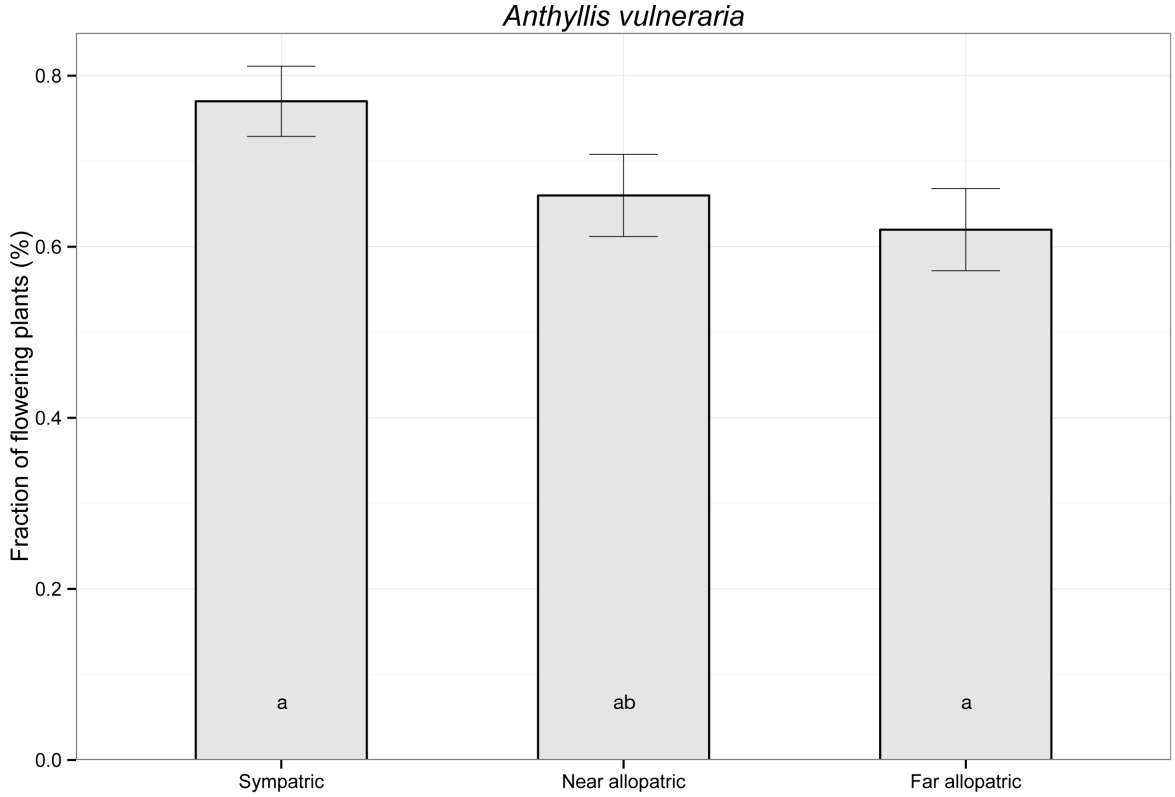

Fig. 3 The fraction of flowering plants across all transplantations of *Anthyllis vulneraria* in *sympatric*, *near allopatric* and *far allopatric* combinations of sites and populations. Error bars depict 1 standard error of the mean. Different small letters inside bars of each panel denote significantly different groups as per Tukey post-hoc tests.

Tab. 3 ANOVA table of mixed model analyses of total aboveground biomass and reproductive allocation for *Anthyllis vulneraria* and *Arabis alpina*.

		Anthyllis vulneraria						*Arabis alpina*					
		total biomass			reproductive allocation			total biomass			reproductive allocation		
	df	MS[b]	$F/\chi^{2,a}$	p	MS[b]	$F/\chi^{2,a}$	p	MS[b]	$F/\chi^{2,a}$	p	MS[b]	$F/\chi^{2,a}$	p
site	4	NA	106.36	<0.001	NA	25.37	<0.001	NA	106.36	<0.001	NA	0.00	1.000
population	5	NA	10.95	<0.001	NA	2.92	0.088	NA	10.95	<0.001	NA	0.87	0.350
sympatric vs. allopatric	2	7.96	15.52	<0.001	0.43	8.81	<0.001	7.96	15.52	<0.001	0.14	4.44	0.015
residual	299	0.51			0.05			0.51			0.03		

[a]F-tests were used for fixed effects and likelihood ratio tests for random effects. [b]lmerTest allows for variances of 0, resulting in NA's for mean square values.

Discussion

Determining the structure of fitness variation and the scale of adaptive differentiation in widespread plant species is important for understanding the ecology of these species and their evolutionary potential. In the current study, we transplanted six populations of each of two widespread alpine species to 5 respectively 4 different sites of origin across two spatial scales and tested for local adaptation. We found a signature of local adapation in *Anthyllis vulneraria*: reproductive allocation and flowering propensity decreased with increasing distance of transplants from population origin. In *Arabis alpina*, no conclusive evidence for local adaptation was detected.

Neutral genetic differentiation

Differentiation at microsatellite loci of both species accorded to the spatial distribution of populations with stronger differentiation across than within regions. The additional genetic isolation between regions should therefore favour regional phenotypic differentiation over local differentiation. Populations of *Anthyllis vulneraria* appear to be largely outcrossing. High levels of genetic diversity are maintained within populations and individuals of *Anthyllis vulneraria*, close to average values as reviewed for microsatellites in plants by Nybom (2004). In *Arabis alpina*, we found high F_{IS} values indicative of low rates of outcrossing (Tedder et al 2011; Buehler et al 2012a) and lower genetic diversity than in *Anthyllis vulneraria*. Of 13 alpine plant species studied by Manel et al (2012) across the entire European Alps, *Arabis alpina* was in fact the least genetically diverse. Genetic diversity is a key determinant of the potential for local adaptation (Geber and Eckhart 2005; Dlugosch and Parker 2008; Blanquart et al 2012) and a possible explanation for the lack of evidence for local adaptation in our study is the high level of inbreeding and low genetic diversity in this species.

Local adaptation in Anthyllis vulneraria

When individuals of *Antyllis vulneraria* were transplanted away from their home site, their fitness on average decreased, and more strongly so at larger geographic distance from the home site. Although one would intuitively assume greater divergence in environmental conditions with increasing geographic separation between sites, this is not necessarily given. Levin and Clay (1984) have shown that transplantations across range limits cause much stronger fitness reduction than those across habitat types within the native range, even if the geographical distance of the latter is much greater than that of across-range transplantations

(Geber and Eckhart 2005). This suggests that geographic distance alone is not necessarily a surrogate for ecological divergence. Our results, however, suggest that within the native range of *Anthylllis vulneraria*, geographic distance can substitute for ecological distance and that regional and local environmental variation sum up. As we have not specifically set out to test the effect of certain environmental variables, it is difficult to pinpoint selective agents responsible for local adaptation in *Anthyllis vulneraria*. However, when comparing the two regions used in this study, Zermatt has substantially warmer and dryer summers than Davos and less snow-fall during winter (MeteoSwiss). It is well accepted that climatic factors are largely responsible for species distibutions (Good 1931; Angert and Schemske 2005). If we presume that within-region small-scale variation, such as variation in exposition, elevation and inclination are similar in both regions, then these regional differences could cause additional differentiation between populations from the two regions. Therefore it is conceivable that climatic divergence between the regions in part explains the fitness variation observed for cross-regional transplants of *Anthyllis vulneraria*.

The signature of local adaptation in *Anthyllis vulneraria* was present in reproductive biomass and flowering propensity. Reproduction and flowering in alpine plants are commonly associated with vernalisation (Wang et al 2009) and photoperiod (Keller and Körner 2003). However, it is unlikely that the pattern of local adaptation found here is caused by adaptive differentiation in vernalisation requirements or photoperiodic control. All populations used here need only little vernalisation and were able to flower in the botanical gardens of our institute at 300 m a.s.l., where winter is much warmer and shorter compared to any of the transplantation sites. Moreover, all populations are situated at roughly equal latitude, and so differentiation according to photoperiod is unlikely. Due to the higher vegetative biomass in *near allopatric* population by habitat combinations (dlsm: *sym-near.allo* = 0.2, df=111, p=0.032), we can also rule out reduced growth as the sole cause for lower flowering propensity or the inability to reach reproductive maturity of allopatric transplantations (Angert and Schemske 2005).

Radiation and light quality could be factors of interest explaining the pattern of local adaptation in reproductive traits of *Anthyllis vulneraria*. Radiation is likely to differ across sites, depending on the amount of snowfall during winter and temperatures during spring. Measurements of the snow-free period at one site in each region in the years 2015 and 2016 suggest that sites differ in the timing of the snow-free period by approximately 2 weeks. A

recent study using the same populations of *Anthyllis vulneraria* furthermore found that population differentiation in the onset of flowering is larger than expected by chance, suggesting that the date of snow-melt differs consistently across sites and that populations are locally adapted to the timing of snow-melt (Kesselring et al 2015). Snow does not transmit all wavelengths equally well, and so the light composition changes with increasing snow depth (Richardson and Salisbury 1977). As *Anthyllis vulneraria* retains green leafs over winter, this difference in the light quality and the change thereof in spring can certainly be sensed by the plants.

Alternatively to light, Alexander et al (2015) have recently shown that soils from different elevations of the same mountain slope have large effects on biomass production and flowering propensity of *Anthyllis vulneraria* ssp. *alpestris* grown in these soils. Soil-borne minerals or soil biota might therefore play a part in the signature of local adaptation observed here for *Anthyllis vulneraria*. In summary, the results suggest that different climatic conditions across regions, and small-scale variability, possibly in the timing of snow-melt and edaphic properties might have caused the signature of local adaptation observed in *Anthyllis vulneraria*.

Local adaptation in Arabis alpina

Even though we must be cautious when interpreting the current results for *Arabis alpina* due to the low final sample size and short observation period, our reciprocal transplant experiment represents an important direct test for local adaptation in this emerging alpine model organism of the European Alps. Evidence for local adaptation in *Arabis alpina* of the central European Alps until now was based on the detection of outlier loci (Buehler et al 2012b) or association studies of marker loci with environmental variables (Poncet et al 2010; Manel et al 2012), which rest on statistical assumptions or environmental and molecular data of coarse resolution. In the current experiment, we find only little evidence for the hypothesis that populations of *Arabis alpina* within the Swiss Alps are locally adapted: while three of four near away transplantations had reduced reproductive biomass production, all four populations that were transplanted to far away sites performed equally well as the respective local population. It is conceivable that the ecologically relevant factors for *Arabis alpin*a are different than those for *Anthyllis vulneraria*, and that the spatial distribution of variation in these factors is not structured along the geographic distances used in the current study. Toräng et al (2014) have conducted a reciprocal transplant experiment with *Arabis alpina* across wide latitudinal gradients (between Spain and Sweden; 3’000 km) and found local adaptation in

flowering phenology and other traits related to temperature, water availability, and growing season length. However, they did not find significant fitness differences between populations of the same region (within 690km circumference). In the present study we use a maximum transplantation distance of 180 km, and therefore both transplantation studies carried out to date with *Arabis alpina* come to the conclusion that local adaptation does not occur at distances smaller than a few hundred kilometers.

Conclusions

We performed two independent reciprocal transplantation experiments to test for local adaptation at two spatial scales using two unrelated but common alpine species. In *Anthyllis vulneraria*, flowering propensity and reproductive biomass decreased with increasing geographical distance between transplantation site and population origin, a clear signature of local adaptation. The results provide evidence for the role of natural selection in shaping phenotypes in populations of the European Alps and suggest that environmental differences between sites increase on average with geographic distance. In the highly selfing *Arabis alpina*, no evidence for local adaptation was found across regions, and only little evidence for local adaptation within regions. Whether inbreeding eliminates the potential for local adaptation in *Arabis alpina* at spatial scales smaller than a few hundred kilometres requires further tests.

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Author contributions

J.S. planned the research. J.S., H.K. and J.F.S. designed the experiments. H.K. performed the experiments, analysed the data and wrote the manuscript. G.F.J.A. and H.K. performed the molecular laboratory work. J.F.S. helped writing the manuscript. E.H., J.F.S. and J.S. helped with fieldwork and manuscript improvement.

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Appendix

S1 Nested primers developed from GenBank sequences of *Arabis alpina* published by Buehler et al (2011).

GenBank accession number; locus name	nested forward and reverse primers (5'-3')	annealing temp. (°C)	amplicon length (bp)	quality of Spreadex fingerprints
HQ599555; 4GHI	F: CGATAAGTATCCAGTCAAGC R: CAATGAGTTCTTAGATCCAC	52	135- 143	Good
HQ599551; 6U3A	F: GGAAGCGAAATGTTTGGAAACG R: CGTTTGTTTCATATAGTGCGTCC	56	151-165	Good
HQ599549; DEET	F: ATGTGTCGCCTCATAATTGG R: AGGAAACGACGAGGGAGTTAC	54	116-127	Good
HQ599548; 3XGR	F: GTTGATTGCTCAACACTAGTCG R: TACGATGCGTTCTCGTACAGG	56	101-107	Good
HQ599547; A1T8T	F: CAAGCGGTATCTGTTACTGC R: ACTATCAGATTCAGTTCCAC	52	120-127	Very good
HQ599546; 3JUY	F: CTTGTTTGAAACGCTACCC R: GTGGTCGTCTTTCTTAAGGG	53	93-117	Very good
HQ599545; A4JW7	F: GCATACCTTCCAAGCTTCC R: CTTGCCTGATCAAAACTTC	51	123-135	Very good
HQ599550; A25GM	F: AGATTCTGGGTTTCCTGTAATGG R: GTTCTTATATGTACCTAGCTTC	53	110-118	Very good
HQ599542; 9VSH	F: GTTGATTGCTTATGCTGGAACC R: CTAGGTACACAACAGCATC	54	131-149	Very good
HQ599553; BWF1	F: TAGTAAGGCATCAAGAAG R: CCATGATAATACGACTGACC	50	125-139	Moderate

Amplicon length is the range found in the entire sample of N = 105 individuals. Locus-specific PCR programs for *Arabis alpina* were: pre-denaturation at 95 °C for 2 min, followed by 35 cycles of 95 °C 30 sec, 30 sec at locus-specific annealing temperature, and 72 °C for 30 sec.

S2 Population pairwise F_{ST} values for *Anthyllis vulneraria* (below diagonal) with probabilities for significant F_{ST}'s (i.e. FST confidence interval does not include zero; above diagonal).

Pairwise Population Fst Values for ANTHYLLIS VULNERARIA

	Schiahorn	Monstein	Casanna	delgletscher	Findelwald	Stafelalp
Schiahorn	0.000	0.001	0.001	0.001	0.001	0.001
Monstein	0.084	0.000	0.037	0.001	0.001	0.001
Casanna	0.051	0.014	0.000	0.001	0.001	0.001
Findelgletscher	0.127	0.096	0.078	0.000	0.014	0.001
Findelwald	0.095	0.064	0.038	0.020	0.000	0.450
Stafelalp	0.098	0.085	0.039	0.052	0.000	0.000

Fst Values below diagonal. Probability, P(rand >= data) based on 999 permutations is shown above diagonal.

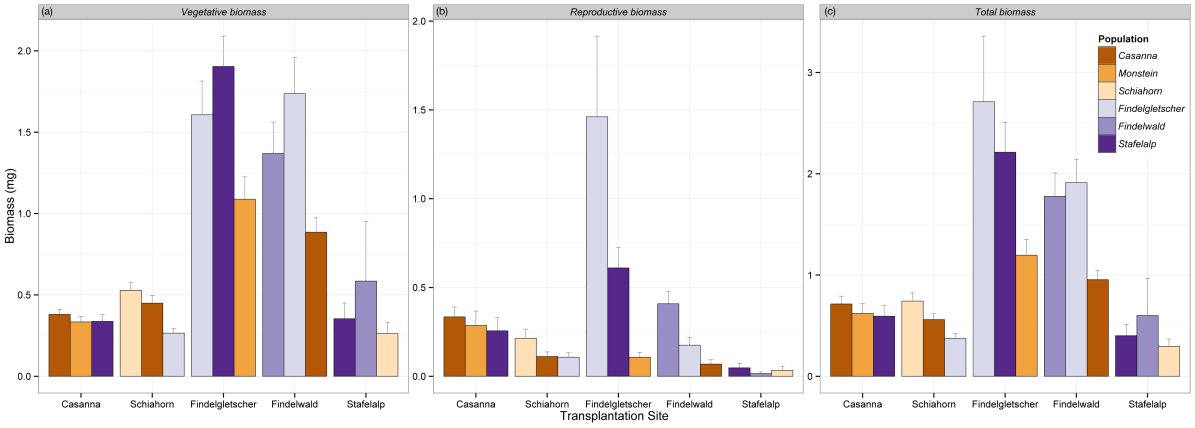
S3 Population pairwise F_{ST} values for *Arabis alpina* (below diagonal) with probabilities for significant F_{ST}'s (i.e. FST confidence interval does not include zero; above diagonal).

Pairwise Population Matrix of Fst Values for ARABIS ALPINA

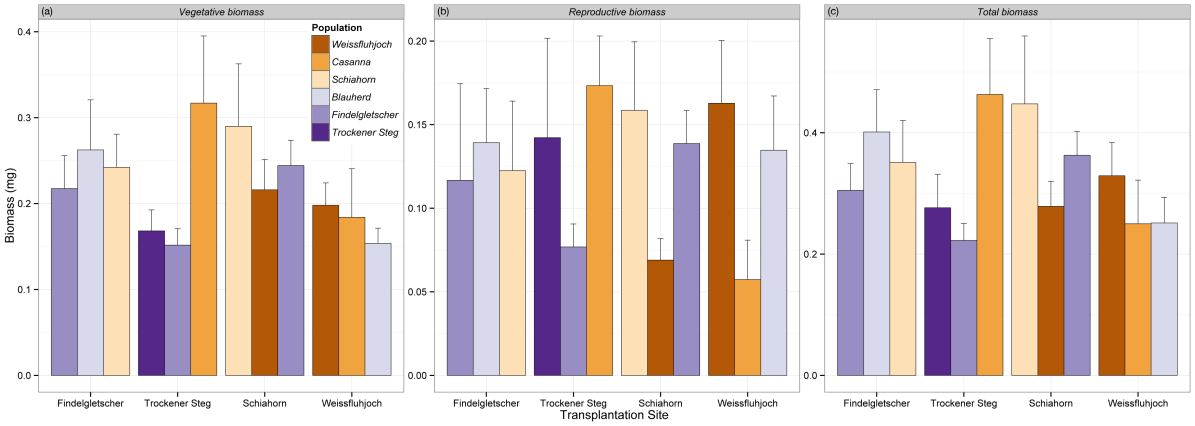
	Schiahorn	Casanna	Weissfluhjoc	Blauherd	Findelgletscl	Trockener Steg
Schiahorn	0.000	0.001	0.048	0.001	0.001	0.001
Casanna	0.125	0.000	0.001	0.001	0.001	0.001
Weissfluhjoc	0.048	0.127	0.000	0.001	0.001	0.001
Blauherd	0.165	0.163	0.155	0.000	0.003	0.001
Findelgletscl	0.282	0.210	0.266	0.101	0.000	0.001
Trockener St	0.181	0.171	0.155	0.177	0.257	0.000

Fst values below the diagonal. Probability, P(rand >= data) based on 999 permutations is shown above diagonal.

S4 The panels depict (a) vegetative, (b) reproductive, and (c) total aboveground biomass of *Anthyllis vulneraria* at five transplantation sites. Each site received plants from the *local* population and from two *foreign* populations. One of the *foreign* populations transplanted to each site originated from the same region and one from the other region. Bars are ordered within each site by increasing distance from the site (i.e. *local* first, *foreign* within region second, *foreign* across region last). Error bars depict 1 standard error of the mean. Note different scales of y-axis on all panels. Sites are written in normal face and populations in italic face. Brown shaded bars are used for populations from the Eastern Swiss region, and blue shades for the Western region. Mean values of reproductive biomass were calculated on the basis of only those plants that had flowered.



S5 Vegetative (a), reproductive (b), and total aboveground biomass (c) of *Arabis alpina* at four remaining transplantation sites. To each site, plants from the *local* population and plants from two *foreign* populations were transplanted. One of the *foreign* populations at each site originated from the same region and one from the other region. Bars are ordered within each site by increasing distance from the site (i.e. *local* first, *foreign* within region second, *foreign* across region last). Error bars show 1 standard error of the mean. Note different scales of y-axis on panels. Sites are written in normal face and populations in italic face. Brown shades refer to populations from the Eastern Swiss Alps, and blue shades to those from the Western region. Mean values of reproductive biomass were calculated on the basis of only those plants that had flowered.



Chapter 4

Past selection explains differentiation in flowering phenology of nearby populations of a common alpine plant.

Halil Kesselring, Georg Armbruster, Elena Hamann, Jürg Stöcklin

Abstract

The timing of and relative investment in reproductive events are crucial fitness determinants for alpine plants, which have limited opportunities for reproduction in the cold and short growing seasons at high elevations. We use the alpine *Anthyllis vulneraria* to study whether flowering phenology and reproductive allocation have been under diversifying selection, and to assess genetic diversity and plastic responses to drought in these traits.
Open-pollinated maternal families from three populations in each of two regions from the Swiss Alps with contrasting precipitation were grown in low and high soil moisture in a common garden. We measured onset, peak, and end of flowering, as well as vegetative and reproductive aboveground biomass. Population differentiation for each character (Q_{ST}) was compared to differentiation at neutral microsatellite loci (F_{ST}) to test for past selection.
We found population differentiation in onset and peak of flowering which results from natural selection according to Q_{ST}-F_{ST}. End of flowering and biomass were not significantly differentiated among populations. Reduced soil moisture had no consistent effect on mean onset of flowering, and advanced peak and end of flowering by less than one week. Reproductive biomass was strongly decreased by lowered soil moisture. No genetic variation within or among populations was found for plasticity in any trait measured.
The results suggest past heterogeneous selection on onset and peak of flowering in alpine *Anthyllis vulneraria* and potentially indicate local adaptation to differences in snowmelt date over distances < 5 km. Limited variation in plastic responses to reduced soil moisture suggests that soil moisture might not vary between populations.

Keywords: local adaptation; Q_{ST}-F_{ST} comparison; phenotypic plasticity; drought

Introduction

Evolution by means of divergent natural selection in spatially heterogeneous environments is considered the major cause of phenotypic variation (Linhart and Grant 1996; Schluter 2009). Apart from divergent natural selection, phenotypic variation among populations can also result from genetic drift – random variation in allele frequencies eventually resulting in the fixation of alleles in some populations and their extinction in other populations (Wright 1931). Furthermore, population differentiation may result from the expression of different phenotypes by the same genotypes in different environments, a phenomenon referred to as phenotypic plasticity (Bradshaw 1965). In alpine plants, both local adaptation and phenotypic plasticity have been hypothesized particularly important due to the steep environmental gradients across short geographical distances. On the other hand, populations of alpine plants are frequently small owing to the highly structured landscape, thereby intensifying genetic drift. The relative significance of genetic drift, local adaptation, and plasticity for population differentiation in alpine environments, however, is a question that remains insufficiently answered (Leimu and Fischer 2008; Frei et al. 2014).

The timing of reproductive events and the allocation of resources to reproduction are particularly crucial fitness determinants in the highly seasonal alpine environments (Rathcke and Lacey 1985; Ozenda 1995; Körner 2003). As temperature decreases along rising elevation with 0.55 °C/100 m, the snow free period and the time window for reproduction narrow down (Schroeter 1923). At high elevations, flowering phenology is therefore tightly linked to the date of snowmelt (Hülber et al. 2006). Among animal-pollinated plant species, the phenology and allocation of reproductive effort must also be coordinated with pollinator abundances and behaviour (Müller 1881; Kudo 1996). Temperature and the date of snowmelt at high elevations are subject to strong microhabitat effects, which may outweigh elevational effects of a few hundred meters (Scherrer and Körner 2010; Wheeler et al. 2014). Therefore the tight links of temperature and snowmelt with phenology and pollinator behaviour (Bergman et al. 1996) is expected to result in strong population differentiation in flowering phenology. The sensitivity of flowering phenology to external conditions can further facilitate reproductive isolation via asynchronous flowering time and therefore promote differentiation and local adaptation (Linhart and Grant 1996; Hall and Willis 2006; Hülber et al. 2010).

Contrarily, the evolution of phenotypic plasticity is expected when genotypes or lineages are likely to experience various external conditions due to high spatial or temporal environmental heterogeneity (Sultan and Spencer 2002). Flowering phenology is an inherently plastic trait that is strongly environmentally controlled through temperature, photoperiod, or both (Keller and Körner 2003). Phenotypic plasticity can slow the response to selection when genetic variation in plasticity exists in a population, i.e. when all genotypes in the population do not respond to environmental change in the same way. Natural selection then cannot operate on trait means as efficiently as when environments and phenotypes are stable, because the same genotype does not have highest fitness under all conditions (Via and Lande 1985). The question, whether mean flowering time is subject to divergent selection among populations in the highly variable alpine landscape remains rarely addressed (Scheepens et al. 2011; Scheepens and Stöcklin 2013; Frei et al. 2014).

Besides temperature and photoperiod, reproductive characters such as reproductive allocation are likely to respond to soil water availability (Caruso 2006, and references therein), because flowering incurs substantial water costs to the plant. The transpirational water loss of flowers can exceed that of leaves (Galen et al. 1999; Lambrecht 2013). Alpine plants often have big

flowers relative to the vegetative body (Körner 2003). Disproportionately large flowers therefore might further raise water costs. Under drought, plants produce smaller flowers and smaller reproductive structures in general (Mal and Lovett-Doust 2005; Caruso 2006), and were also found to advance flowering phenology as a plastic as well as an evolutionary response (Dunne et al. 2003; Franks 2011). Precipitation is very variable across the European Alps as a result of the interplay of climatic patterns with the obstructing effect of mountain ranges. This leads to regions of particularly low precipitation in the deep valleys inside the highest mountain ranges (Ozenda 1985).

Here we use comparisons of quantitative trait differentiation and genetic differentiation at neutral marker loci (Q_{ST}-F_{ST} comparisons; Spitze 1993) to test for the role of past selection in shaping patterns of population differentiation in reproductive allocation and phenology in a common alpine herb. Q_{ST}-F_{ST} comparisons allow to infer natural selection as opposed to random processes such as genetic drift as a cause of population differentiation when Q_{ST} is either significantly smaller or larger than F_{ST}. Q_{ST} values smaller than F_{ST} values indicate stabilizing selection across environments, whereas Q_{ST}'s larger than F_{ST} indicate population divergence as a result of heterogeneous selection across environments. If $Q_{ST} = F_{ST}$, we have no reason to infer a role of selection as drift alone can explain the observed population differentiation. We also assess plastic responses to drought in reproductive allocation and phenology by subjecting plants to two soil moisture treatments in a common garden. We asked (i) if population differentiation in reproductive allocation and flowering phenology is likely the result of past selection and therefore adaptive evolution, and (ii) if soil moisture availability has an effect on reproductive allocation and phenology (i.e. presence of phenotypic plasticity).

Methods

Study species

Anthyllis vulneraria L. sensu lato (s.l.) is a polymorphic fabacean taxon with unclear infraspecific classification (Nanni et al. 2004; Köster et al. 2008), and consists of a self-compatible clade of short-lived herbaceous plant species very common throughout Europe. It grows preferably on calcareous meadows and scree grounds from sea level to the alpine belt up to around 3000 m a.s.l. (Hegi 1975). Here we examined three alpine populations of *Anthyllis vulneraria* in each of two regions in the Swiss Alps. Plants grow to a height of around 15-45 cm. A variable number of shoots sprout from the basal leaf rosette, each bearing 2-6 inflorescences. Each inflorescence comprises a number of 7-19 mm long white to yellow, sometimes claret to red flowers arranged in a capitulum (Hegi 1975; Navarro 1999). Shoots are usually unbranched, but may have up to three side-branches originating from the axils of evenly pinnate compound leafs. Leafs of the basal rosette consist of the enlarged terminal leaflet of a compound leaf. *Anthyllis vulneraria* is representative of a type of fabacean flower characterized by a *pump mechanism* adapted to insect-mediated pollination (Müller 1881). Flower development of *Anthyllis vulneraria* takes approximately 4 weeks. Flowers ripen from bottom to top along a shoot and from top to bottom within a capitulum. Asynchronous flower ripening allows for geitonogamous selfing across capitulae, but suggests multiple paternity per maternal offspring. Microsatellite analyses found a variable degree of inbreeding in the studied populations (F_{IS} 0 - 0.42, unpublished results), suggesting regular outcrossing. A single flower is open and accessible to pollinators for about 6 to 7 days and produces a single seed.

Experimental Procedures

In August 2012, seeds from open-pollinated wild flowers in three populations from each of two regions (eastern and western Swiss Alps near Davos and Zermatt, respectively) were sampled (Tab. 1). The offspring of the same maternal plant are referred to throughout the article as *seed family*. Members of a seed family presumably are mostly half-sibs, as populations are outcrossed, and the asynchronous ripening of flowers within a capitulum makes it unlikely that they are sired by the same father (Pannell and Labouche 2013). Populations are situated between 2000 m a.s.l. and 2650 m a.s.l. Distances between populations within regions range from 2 km to 18 km, and regions are 180 km apart. Regions were specifically chosen for their difference in growing season precipitation, with the Davos region getting approximately 50% more precipitation in the months of June through September than the Zermatt region (Tab. 1; Zimmermann and Kienast 1999). We have identified populations as belonging to *Anthyllis vulneraria* ssp. *alpestris* after Hegi (1975). Seeds were stored in the refrigerator until they were scarified and sown in early August 2013 directly into their final high mineral potting soil mixture (210 l Ökohum Anzuchterde® with 14 l sand and 8 kg pumice). 5 individuals per seed family, and 6 seed families per population were used (180 individuals in total). Seedlings were kept in the greenhouse in 10 by 10 cm pots and watered *ad libitum*. Plants were randomized twice per week. Greenhouse heating and cooling systems were set so that temperatures would not fall below 16 °C and 8 °C at day and night, respectively, nor exceed 20 °C and 10 °C at day and night, respectively. Early leaf size was measured on every individual as length*width/2 of the first true leaf as soon as it was fully developed (2 leaf stage). After 4.5 weeks, on 11-Sep-2013, seedlings were potted into larger 2 l pots into the same soil mixture and watered to carrying capacity. At the same time, plants were moved to the outside garden under a UV-B transmissible rain shelter (folitec Agrarfolien-Vertriebs GmbH, Westerburg, Germany) and arranged in a regular array, alternating between individuals of all levels of hierarchy from seed family to region. Eleven days later, on the 22-Sep-2013, treatment began by watering plants designated for the wet treatment. Alternating between seed families, 3 or 2 of the 5 individuals per seed family were allocated to the dry treatment. Subsequently, volumetric soil moisture content was monitored with a moisture meter calibrated to the soil mixture used in the experiment and plants were watered accordingly (HH2 Moisture Meter with Theta Probe ML2x, Delta-T Devices Ltd. Cambridge, England). Wet plants were watered when mean soil moisture fell below 18 %, and dry plants when soil moisture fell below 5 %. Ten different plants of each treatment were randomly chosen each time at irregular intervals for soil moisture measurements (Supplemental Figure 1). Plants were sitting on a thick sand bed and marginally striked roots into the sand 2cm deep at maximum. Throughout the duration of the experiment, air temperature was logged hourly with a TidbiT® v2 Temperature Logger (Onset Computer Corporation, Bourne, Massachusetts, U.S.A.; Supplemental Figure 2). The logger was hung-up under a reversed 2 l plastic flowerpot painted in white and with perforation to allow air circulation. All plants were preventively treated with a ready-to-use fungicide powder (Maag Pirox®, Syngenta Agro AG, Dielsdorf, Switzerland) on a few occasions during growth phase, because alpine *Anthyllis vulneraria* is susceptible to mildew when grown at low elevations. *Anthyllis vulneraria* needs vernalisation to induce flowering (Halil Kesselring, personal observation), so we left plants outside over winter. During winter, from 19-Nov-2013 onwards, the rain shelter was temporarily removed and water treatment was suspended. Treatment was re-established on the 19-Mar-2014 by watering with 100ml and 60ml for wet and dry plants, respectively, and subsequently continued as described above.

region	population	coordinates (°E/°N)	elevation (m a.s.l.)	summer precipitation (mm)
Davos	Schiahorn	780513.385 / 187874.756	2650	1463
	Casanna	782301.543/ 192247.969	2320	1454
	Monstein	779685.630 / 173389.160	2010	1225
Zermatt	Findelwald	626828.986 / 95475.764	2170	809
	Findelgletscher	629173.611 / 95175.270	2490	939
	Stafelalp	619094.320 / 94427.436	2280	898

Table 1 Coordinates (Swiss coordinate system LV03), elevation, and mean monthly precipitation during the growing season (June – September) of the six populations of *Anthyllis vulneraria* studied in the common garden. Precipitation data is interpolated from monthly precipitation data using a digital elevation model (Zimmermann and Kienast 1999).

Once reproductive shoots became visible, plants were checked daily and the date of the following critical stages of flowering phenology were noted for each individual: i) *onset of flowering* defined as the date when the first flower opened on an individual; ii) *peak of flowering* defined as the date at which the maximum number of open flowers was observed; iii) *end of flowering* defined as the date when the last flower opened and no more flower buds were visible. Flower opening is very easily observed in *Anthyllis vulneraria* when the brightly coloured corolla appears from the calyx, a process that takes less than 24 hours. Onset of flowering was always a representative measure because the opening of the first flower was never an isolated event, but led to the onset of flowering of the whole plant.
Once a plant had reached the flower end, aboveground biomass was harvested and dried at 75 °C for 72 hours. Aboveground biomass was then separated into the vegetative leaf rosette and into reproductive parts, and weighed to the nearest mg. We also estimated *reproductive allocation* as the ratio of reproductive biomass over total aboveground biomass.

Statistical analyses

We performed separate linear mixed-effects models for each of our flowering phenology variables and for the biomass variables in R version 3.0.2 (R Development Core Team 2008). In these models, *water treatment* and *region* as well as their interaction were included as fixed effects, and *population* and *seed family* and their interactions with *water treatment* were included as random terms. Each seed family was given a unique identifier, which leads to the models implicitly nesting *seed family* in *population*. Likewise, *population* was nested in *region*. In these models, a significant *water treatment* effect indicates that soil moisture availability has an effect on either flowering phenology or aboveground biomass allocation, i.e. the focal trait is plastic in response to soil moisture. A significant interaction between *water treatment* and *region* indicates that populations from both regions differ in their plastic responses to soil water availability. Analogously, a significant *seed family* effect indicates that related individuals are more similar to each other in the focal trait expression than randomly grouped individuals, and an interaction of *seed family* with *water treatment* indicates genetic variation in phenotypic plasticity among seed families within populations. In order to control for maternal effects to the maximum possible extent, early leaf size (length*width/2) was included in all models as covariate. Statistical models were computed with the lmerTest package (Kuznetsova 2013). lmerTest applies F-tests to lmer objects of the lme4 package for fixed effects and likelihood-ratio tests for random effects using stepwise model reduction and

comparisons. We used type 3 errors and Satterthwaite approximations for denominator degrees of freedom. We report *P*-values, mean squares, and chi-square values that correspond to those from the model comparisons using the step function in lmerTest (i.e. likelihood-ratio tests). All random terms were specified as simple scalar terms. Phenological variables were analysed as date objects. Contrasts for fixed effects were tested using differences of least squares means as implemented in the step function of lmerTest.

Molecular analyses

20 individuals per population were scored for amplified fragments at 9 microsatellite loci. We used Spreadex® gels and the ORIGINS electrophoresis unit (Elchrom Scientific AG, Cham, Switzerland) to separate PCR amplicons with size differences as small as 2bp. Gels were stained with ethidium-bromide and scored by hand comparing against the M3 ladder from ELCHROM. Polymorphic microsatellites were developed to be suitable in length for analysis on Spreadex® gels (Kesselring et al. 2013). PCR programs were run in a Mastercycler Gradient (Eppendorf, Hamburg, Germany). 35 cycles with denaturation for 30 s at 95°C, start PCR for 30 s at 95°C, locus-specific annealing temperature (50 or 52°C) for 45 s, followed by 45 s at 72°C were repeated. Termination was set to 72°C for 8 min. A detailed description of the microsatellite development and loci description can be found in Kesselring et al. (2013). The free software FreeNA (Chapuis and Estoup 2007) was used to check for null alleles. Null alleles were suggested for several loci, but taking their frequencies into account resulted in nearly identical F_{ST} estimates for each locus except locus 8. Mean F_{ST} was slightly lower with null alleles taken into account, therefore inclusion of null alleles would render tests of $Q_{ST} > F_{ST}$ less conservative. Since a low degree of inbreeding is suggested by the data and the floral biology of *Anthyllis vulneraria*, and since blank lanes (homozygote null alleles) were only present at locus 8, we are confident that increased homozygosity at all but one locus is not due to null alleles, but results from bi-parental inbreeding and selfing. Estimation of null alleles rests on untested assumptions (e.g. a single null allele is present) and is not free of bias (Chapuis and Estoup 2007; David et al. 2007). Consequently, we preferred to remove the outlier locus with clear signals of null alleles instead of including null allele frequencies for final analyses. Genotyping error was estimated at 2.5 % (Kesselring et al. 2013). Population pairwise F_{ST}-values were calculated in GenAlEx (Peakall and Smouse 2006) based on allele frequencies. Probabilities of finding the observed F_{ST}-values are based on comparison of the observed value against 999 random permutations of the samples.

Q_{ST}-F_{ST} comparison

Q_{ST}-F_{ST} comparisons were performed for all traits to test whether population differentiation in quantitative traits is the result of natural selection. We followed the method described by Whitlock and Guillaume (2009), which provides a powerful significance test of the hypothesis that Q_{ST} is not equal to F_{ST}. For each trait, a null-distribution of Q_{ST}-F_{ST} is first constructed based on the observed within-population genetic variance for the focal trait and observed F_{ST}. Since it is based on the F_{ST}, this null-distribution is the expected distribution of Q_{ST}-F_{ST} under neutral evolution of the trait. The tail probability of the observed Q_{ST}-F_{ST} under the assumption of neutral evolution is then calculated from the null-distribution.
The software Nemo version 2.2.0 (Guillaume and Rougemont 2006) was used to calculate Weir and Cockerham's coefficients a,b, and c for each of the 8 microsatellite loci as a basis to estimate Wright's F_{ST} (Weir and Cockerham 1984). Whitlock and Guillaume (2009) provide an R script for a nonparametric bootstrap of F_{ST} values, which was used to generate 10^3 bootstrap replicates of F_{ST}, from which a probability distribution of F_{ST} was constructed. The script calculates F_{ST}'s by randomly sampling with replacement from the Weir and Cockerham coefficients calculated by Nemo a number of times equivalent to the number of loci used in the analyses. The Q_{ST} replicates were calculated by parametric bootstrapping using the

Lewontin-Krakauer distribution and the observed within-population variances and observed F_{ST} according to Whitlock and Guillaume (2009). For the calculation of Q_{ST}'s, we used Spitze's (1993) formula, estimating within-population variances as 4 times the seed family variance components, and among-population variance as the population variance components from the statistical models. Some degree of inbreeding is indicated in 5 of the studied populations by heterozygote deficiencies. Therefore the assumption of half-sibs may not always hold, and render tests of $Q_{ST}>F_{ST}$ too conservative and those of $Q_{ST}<F_{ST}$ too relaxed. We have therefore repeated the analyses under the assumption of full-sibs which is the opposite extreme. Results were identical, except that significant results were even more strongly significant under the assumption of full-sibs. We only report results assuming half-sibs as this is the more realistic and more conservative assumption. We used the minimal adequate models resulting from stepwise model reduction as implemented in the step function of lmerTest for estimation of all variance components used in the Q_{ST}-F_{ST} comparisons. The entire sample across both treatments was used for analyses to achieve reasonable sample sizes. If the minimal model did not include *population* or *seed family* those terms were re-included as they are necessary for calculations of Q_{ST}.

Results

In the experimental garden, global mean peak flowering was on the 25-April-2014. Peak flowering in the natural stands of these populations is roughly in the last week of June (Halil Kesselring, personal observation). Therefore, plants in this experiment flowered approximately 2 months earlier than the natural stands, equalling a 1h 45 min shorter photoperiod. All populations were significantly differentiated at microsatellite loci from one another (average F_{ST} = 0.079, 95% CI: [0.063, 0.098]) except populations Stafelalp and Findelwald (Tab. 2). Mean pairwise population differentiation within regions (F_{ST}=0.04) was lower than mean pairwise population differentiation across regions (F_{ST}=0.08, Tab. 2).

		Schiahorn	Casanna	Monstein	Findelgletscher	Findelwald	Stafelalp
Davos	**Schiahorn**	-----	0.001	0.001	0.001	0.001	0.001
	Casanna	0.051	-----	0.037	0.001	0.001	0.001
	Monstein	0.084	0.014	-----	0.001	0.001	0.001
Zermatt	**Findelgletscher**	0.127	0.078	0.096	-----	0.014	0.001
	Findelwald	0.095	0.038	0.064	0.020	-----	0.450
	Stafelalp	0.098	0.039	0.085	0.052	0.000	-----

Table 2 Population pairwise F_{ST} values (below diagonal) with tail probabilities based on 999 random permutations of samples as implemented in GenAlEx given (above diagonal).

Effects of origin on allocation of biomass and phenology

Differentiation at the regional level was only indicated for vegetative biomass and reproductive allocation (ratio of reproductive biomass over total aboveground biomass), but not for reproductive biomass by statistical analyses (Tab. 3). Populations nested within region were not significantly differentiated for biomass traits (Tab. 3), with observed Q_{ST}'s of 0.074 and 0.11 for vegetative and reproductive biomass, respectively. Accordingly, observed Q_{ST}-F_{ST} values fell within the 95%-confidence limit of the corresponding null-distributions for both vegetative (P=0.666) and reproductive biomass (P=0.429), giving no reason to infer selection as a driver of population evolution. Variance components analysis of the random terms revealed that large amounts of variability in vegetative and reproductive aboveground biomass were explained by *seed family* (31% and 22%, respectively; Tab. 3), indicating high within-population genetic diversity.

	Vegetative biomass			Reproductive biomass			Reproductive allocation			Onset of flowering			Peak of flowering			End of flowering		
factor	df	F/χ2	*P*	df	F/χ2	*P*	df	F/χ2	*P*	df	F/χ2	*P*	df	F/χ2	*P*	df	F/χ2	*P*
early leaf size	1	15.40	**		23.35	**	1	10.33	*	1	0.02	*ns*	1	1.29	*ns*	1	0.71	*ns*
Region	1	15.77	**	1	26.17	*ns*	1	12.30	*	1	8.40	**	1	8.54	*	1	7.79	***
Treatment	1	5.40	*	1	27.06	***	1	31.26	***	1	0.09	*ns*	1	4.36	*	1	12.20	***
Region x treatment	1	4.74	*	1	3.34	*	1	8.44	*	1	0.24	*ns*	1	0.03	*ns*	1	0.27	*ns*
Population (region)	na	1.94	*ns*	na	1.60	*ns*	na	0	*ns*	na	17.51	**	na	21.37	***	na	0.88	*ns*
Seed family (population)	na	32.91	***	na	24.98	***	na	14.54	***	na	8.58	**	na	12.35	**	na	23.18	***
Population x treatment	na	0	*ns*	na	0.03	*ns*	na	0	*ns*	na	0	*ns*	na	0.29	*ns*	na	0	*ns*
Seed Family x treatment	na	0	*ns*	na	1.14	*ns*	na	0	*ns*	na	0	*ns*	na	0	*ns*	na	0	*ns*

Table 3 The effects of population origin, soil moisture treatment, and family membership on aboveground biomass and reproductive phenology (linear mixed-effects analyses). Early leaf size (measured as length*width/2 of the first fully developed true leaf) was included as a covariate to control for maternal effects. *** $P<0.001$, ** $P<0.01$, * $P<0.05$, *ns* not significant. F-ratios are given for fixed effects and χ2-values for random effects. Reproductive allocation is the ratio of reproductive biomass over total aboveground biomass.

The two regions were significantly differentiated for all stages of reproductive phenology (Tab. 3). All populations from Zermatt flowered later than any of the populations from Davos, and the difference in peak flowering date between the first population from Davos and the last one from Zermatt was more than 5 weeks (Fig. 1). Significant population differentiation within regions was indicated by the statistical models for onset and peak of flowering, but not for end of flowering (Fig. 1; Tab. 3). Observed Q_{ST}'s ranged from 0.020 for end of flowering to 0.353 for peak flowering (Fig. 2). The observed Q_{ST}-F_{ST} value for end of flowering fell within the 95%-confidence limit of the corresponding null-distribution (P=0.984), and therefore differentiation in this trait can be explained by drift alone. Q_{ST}-F_{ST} values for onset and peak of flowering were greater than expected under the null-hypothesis and appeared in the tail of the corresponding null-distributions with an associated probability of finding the observed value or one that is greater of 0.002 and 0.001, respectively. Therefore, divergent selection is indicated for onset and peak of flowering. The observed F_{ST} as inferred from the microsatellite data was 0.079 (95% CI: [0.063, 0.098]) averaged over all loci. Genetic variation within populations was indicated for all phenological variables by significant *seed family* terms (Tab. 3). *Seed family* explained 10%, 11%, and 37%, of the variability in the random terms for onset, peak, and end of flowering, respectively.

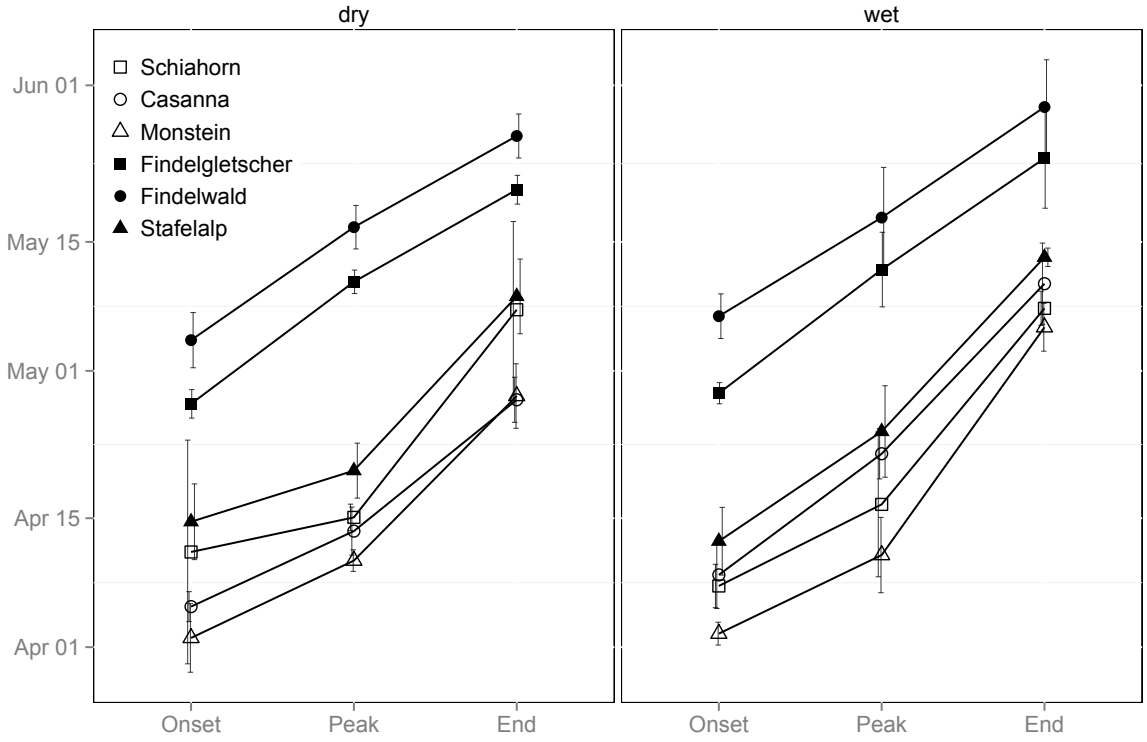


Fig. 1 Flowering phenology of six alpine populations of *Anthyllis vulneraria* in the common garden of the Botanical Institute in Basel in the two soil moisture treatments. Open symbols represent populations from Davos (Schiahorn, Casanna, Monstein), and closed symbols represent populations from Zermatt (Findelgletscher, Findelwald, Stafelalp). Error bars denote one standard error of the mean based on individual variation within population.

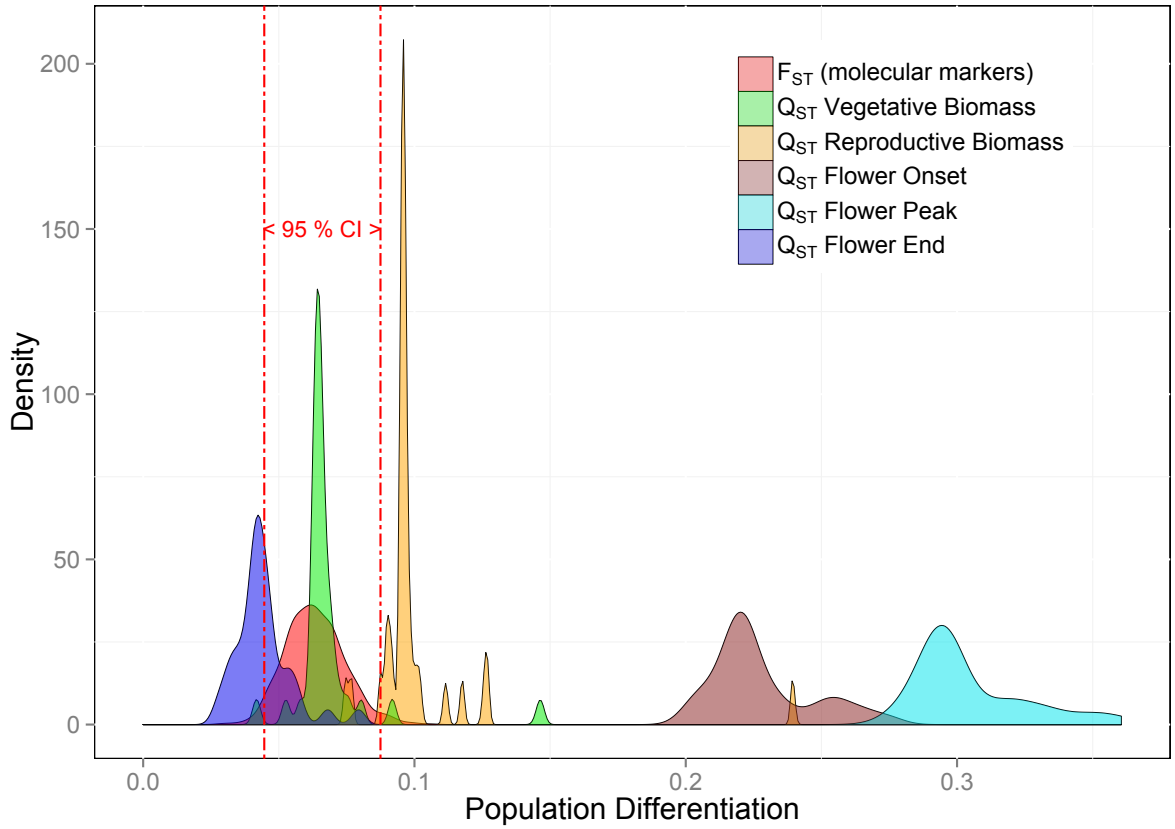


Fig. 2 Probability density distributions of F_{ST} and Q_{ST}'s for biomass and phenological traits. 10^3 replicate values of F_{ST} were generated by a non-parametric bootstrap. F_{ST} is based on microsatellites. Distributions of Q_{ST}'s for this plot consist of only 36 replicates per trait, which were generated by jackknifing over seed families. Vertical lines indicate the 95 % confidence interval of the estimate of F_{ST}. 95% confidence intervals of Q_{ST}'s of onset and peak of flowering do not overlap with the 95 % confidence interval of the estimate of F_{ST} and therefore reveal a signature of past selection.

Effects of treatment on biomass allocation and reproductive phenology

Regions responded differently in their aboveground biomass to the soil moisture treatment (significant *region x treatment* for vegetative and reproductive biomass, and reproductive allocation; Tab. 3). Contrasts of the treatments in each region revealed that vegetative biomass decreased in Zermatt under lowered soil moisture (p=0.003), while it was not significantly affected in Davos (p=0.93; Fig. 3). Concerning reproductive biomass, contrasts of the treatments in each region revealed that both the Davos and Zermatt regions were significantly affected by lowered soil moisture (p=0.04; resp. p<0.01), but the Zermatt region more strongly so. However, Figure 3 suggests the *region x treatment* for reproductive biomass was largely driven by the two geographically adjacent populations of Findelgletscher and Findelwald, which responded with a strong decrease in reproductive biomass to the drought treatment. The third population from Zermatt (Stafelalp) reacted very similarly to the Davos populations with a small decrease in reproductive biomass (Fig. 3). Overall, the population-level decrease in reproductive biomass in response to drought was proportional to the mean reproductive biomass across both treatments (Pearson's product moment, t=4.81, df=4, P<0.01). There was no correlation of reproductive allocation with vegetative biomass across all populations at the level of seed family (Pearson's product moment, t= 1.75, df=34, P>0.09), and therefore no indication of a trade-off between reproductive and vegetative biomass in the studied populations. No significant interactions of treatment with population or seed family were found.

Reduced soil moisture treatment had no consistent effect on the onset of flowering, but significantly advanced peak flowering and end of flowering of all populations by an average of 3 and 6 days, respectively. No significant interactions of treatment with population or seed family were found.

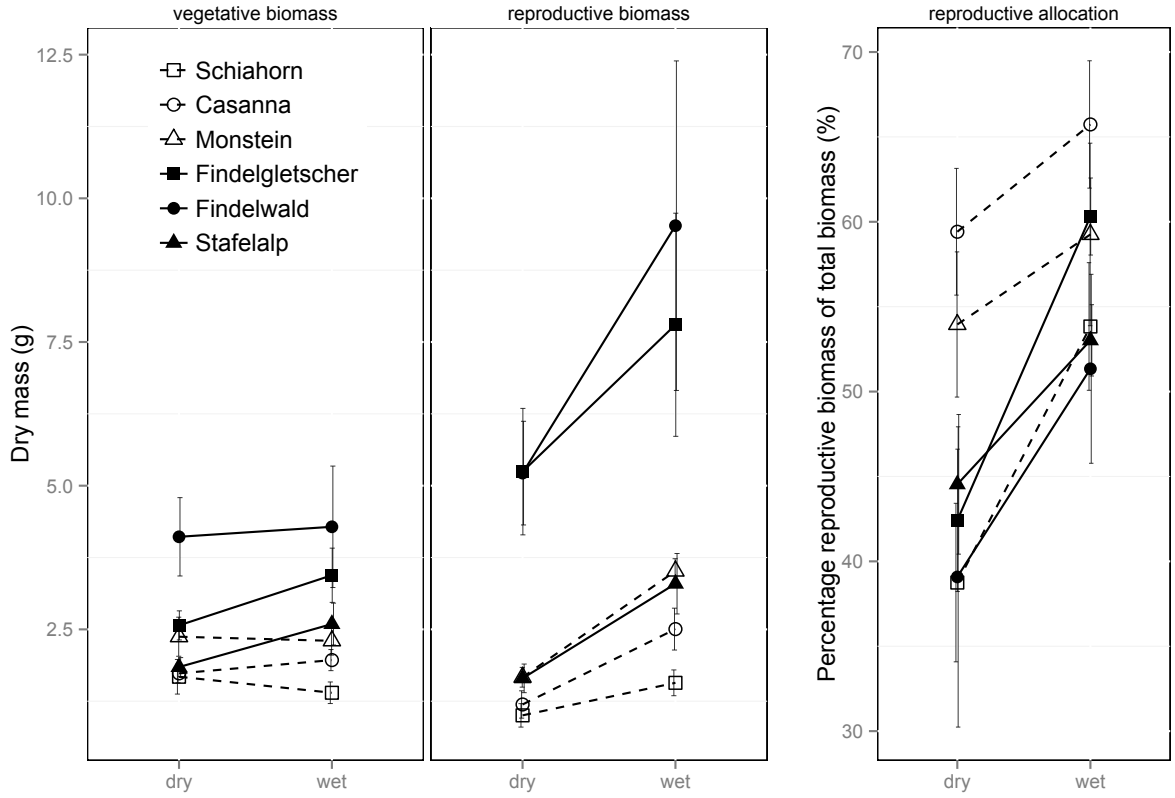


Fig. 3 Reaction norms of vegetative and reproductive aboveground biomass in response to two soil moisture treatments of six alpine populations of *Anthyllis vulneraria* in the common garden in Basel. Open symbols and dashed lines represent populations from Davos (Schiahorn, Casanna, Monstein), and closed symbols and solid lines represent populations from Zermatt (Findelgletscher, Findelwald, Stafelalp). Error bars denote one standard error of the mean based on individual variation within population.

Discussion

The current study demonstrates considerable variation in reproductive phenology and aboveground biomass among six alpine populations of *Anthyllis vulneraria* sampled in two contrasting regions. Q_{ST}-F_{ST} comparisons suggest that divergent selection likely caused population genetic differentiation in onset and peak of flowering but not in biomass. Substantial amounts of variation in all measured traits are explained by family membership, indicating within-population genetic variation and sustained potential for future evolution. Soil moisture treatment had a significant effect on biomass traits, and resulted in a small but statistically significant forward shift of peak and end of flowering. No genetic variation in plastic responses to soil moisture was found within populations, neither for flowering phenology nor for biomass traits. Moreover, populations within each region did also not differ in their plastic response to drought, but across regions populations responded differently in vegetative biomass.

Variation in biomass allocation

There is good evidence that reproductive allocation increases along elevation (Fabbro and Körner 2004; Zhu et al. 2010). Since our populations are not spread along elevation very far and since we study only three populations per region, it is not surprising that we do not find differentiation beyond the neutral expectation in reproductive allocation across such short geographic distances as indicated by Q_{ST}-F_{ST} comparison.

Both vegetative and reproductive biomass were plastic in response to soil moisture availability suggesting that summer precipitation plays a role for growth and reproduction of alpine *Anthyllis vulneraria*. Drought stress in the alpine life zone is a phenomenon mostly reserved to special microhabitats such as extremely shallow or exposed substrates (Neuner et al. 1999), and tolerance to desiccation is often high (Körner 2003). However, reduced soil moisture - even if it does not cause problems with maintaining turgor - frequently leads to nutrient limitation and therefore reduced growth (Körner 2003). Interestingly, reduced soil moisture did not have a negative effect on vegetative biomass for populations from Davos. Previous results also show no effect or a slightly positive effect of drought for alpine grassland sites receiving high annual precipitation (Gilgen and Buchmann 2009). Drought stress therefore seems to be avoided in the Davos populations through slow growth resulting in lower total leaf surface area and consequently in lower transpiration. Whether this pattern is a genetic adaptation driven by regional differences in precipitation should be further investigated with a larger number of populations and measurements of in situ water availability. Statistical models and graphical inspection also showed that there is no significant within-population genetic variation in phenotypic plasticity in response to soil moisture, meaning that all seed families within a population responded similarly to soil moisture change. This is in line with the absence of divergent selection across populations as found in the Q_{ST}-F_{ST} comparison, because environmental heterogeneity in water limitation and associated divergent selection are predicted to preserve genetic variation in plasticity (Via and Lande 1985). Although stabilizing selection across populations on trait means was not indicated by the Q_{ST}-F_{ST} comparison for biomass traits, stabilizing selection on reaction norms rather than on trait means might still be present and explain the absence of genetic variation in plasticity within populations.

Variation in reproductive phenology

Populations of *Anthyllis vulneraria* are differentiated in their reproductive phenology at all spatial scales from more than a hundred km to a few km. Snowmelt date was drastically advanced in our common garden compared to the natural sites because the garden is situated at much lower elevation. Genetic differences in photoperiodic sensitivity between populations, i.e. *G x E* in photoperiodic control, might therefore have become visible in our garden (Pigliucci 2003). Likewise *G x E* in vernalisation requirement might also contribute to the variation that was found in the common garden (Mendez-Vigo et al. 2013). Yet the strong forward shift in the phenology of all populations compared to the natural sites suggests strong insensitivity to photoperiod of all populations. Furthermore, we observed comparable differences in flowering time in an accompanying experiment with transplantations into the original field sites (Halil Kesselring, personal observation). Therefore it is more plausible, as the Q_{ST}-F_{ST} comparison suggests, that heterogeneous selection on onset and peak of flowering is the reason for the within-region population differentiation in these traits. Because a total of only 6 populations and a regional subdivision were used in our study, it is not feasible to correlate flowering dates with environmental variables at the sites of origin. Such correlations could strengthen the case for past and current adaptive evolution of flowering time and inform about selective agents. An emerging key environmental determinant of plant distributions in alpine habitats is spring frost (Bannister et al. 2005; Ladinig et al. 2013; Lenz et al. 2013; Briceño et al. 2014; Wheeler et al. 2014). The likelihood of spring frost at any elevation is largely determined by the date of snowmelt, which in turn is a function of winter precipitation, and topography. A thick snow cover in spring buffers temperature fluctuations and protects critical plant tissues from freezing damage due to very low temperatures. Reproductive structures of flowering plants are highly frost-susceptible and much less frost-tolerant than vegetative plant tissues (Neuner et al. 2013). Consequently, the timing of reproduction is expected to evolve so as to avoid periods with a high likelihood of frost. Sites with little snow accumulation during winter and relatively early snowmelt experience spring frost more commonly and should extend the pre-flowering duration. Similarly to spring frost, the emergence of pollinating insects of *Anthyllis vulneraria* can also potentially select for corresponding peak flowering times. As the activity of pollinating insects is strongly temperature-dependent, differences in elevation and exposition among populations could lead to divergent selection through pollinators (Kudo 1996). Flowering phenology is a trait particularly likely to be differentiated even over short geographical distances, because it is also a mechanism to reduce gene flow via pollen movement between individuals flowering at different times (Linhart and Grant 1996). There is a shortage of studies investigating whether populations of alpine plants at similar elevations experience divergent selection on flowering time by the local environments, and future studies should test the link between snowmelt date, pollinator abundances, and flowering time.

The observed advances of peak and end of flowering in response to decreased soil moisture availability - although mild as they were - are in keeping with a strategy of quick reproduction under stressful conditions. This is a previously observed reaction of short-lived plants on short as well as evolutionary time-scales (Dunne et al. 2003; Franks 2011). Peak flowering date per seed family was not a function of the reproductive biomass (ANCOVA, $F=1.25$, $p=0.27$). Hence, it is unlikely that the advanced dates of peak and end of flowering are merely the result of decreased biomass. Likewise, the time between re-establishment of the treatment in the second growing season to onset of flowering was not correlated to plasticity in flowering onset either, meaning that later-flowering populations were not more plastic. Therefore, the absence of a plastic response of flowering onset to soil moisture is unlikely the result of the suspension of the treatment during winter. Advanced peak and end of flowering therefore potentially reflect an adaptive drought escape strategy in *Anthyllis vulneraria*. No variation in

the response of the flowering phenology to soil moisture availability was indicated by statistical analyses, neither at the among-population nor at the within-population level (Tab. 3). As theory predicts that genetic variation in plasticity should be preserved under conditions of heterogeneous selection (Via and Lande 1985), one might conclude from these results that variability in soil moisture leading to heterogeneous selection on flowering phenology does not exist at the scale at which populations were sampled in this experiment (< 20 km within regions). Alternatively, evolutionary constraints or stabilizing selection on reaction norms may be present.

Accuracy of Q_{ST} and F_{ST} estimation

The method of comparing Q_{ST} to the neutral expectation using F_{ST} has been scrutinized, because both indices are not without problems (e.g. McKay and Latta 2002; O'Hara and Merila 2005). The accuracy of the estimation of Q_{ST} depends on how well we can separate additive genetic variance (V_A) within and between populations from environmental effects, maternal effects, and non-additive genetic effects such as dominance. Since we have largely reduced environmental variation by raising all plants in a common garden and controlling for soil moisture availability to our best ability, direct environmental effects should be minimal in our study (Leinonen et al. 2008). Indirect environmental variation can still occur through maternal effects in our design as we used maternal half-sibs to estimate V_A. However, maternal effects in plants have so far almost exclusively been found to affect only early life-history stages and to diminish over time (Bischoff and Müller-Schärer 2010 and references therein). As our plants were in the second growing season when traits were measured, maternal effects might not have had a considerable effect on the outcome. Furthermore, we have included early leaf size as co-variate in the analyses, a method commonly used to control for maternal effects (Scheepens and Stöcklin 2013). We used the phenotypic resemblance of open-pollinated half-sibs to assess V_A, a method that confounds additive with non-additive genetic effects such as dominance. However, non-additive effects always cause a downward bias in estimating Q_{ST} (Lynch and Walsh 1998), and therefore render tests of $Q_{ST}>F_{ST}$ conservative. As we found no Q_{ST} smaller than F_{ST}, this bias is unlikely to affect our conclusions. Finally, F_{ST} has been hotly debated as an accurate measure of neutral population differentiation and the molecular markers used to assess it are criticised (e.g. Hedrick 2005; Jost 2008). In this study we used microsatellites, which are notorious for having a high mutation rate resulting in lower estimates of F_{ST}. Indeed, Jost's estimate of differentiation was more than twice as big as F_{ST}. However, since this difference is still mild compared to many previous microsatellite studies, and since far less than 1 private allele per locus and population was found (results not shown), we suspect that our microsatellites do not have an exceedingly high mutation rate and are therefore suitable for comparisons of Q_{ST} with F_{ST} (Edelaar and Björklund 2011). Furthermore, Jost's estimate of differentiation was still smaller than both Q_{ST}'s concluded to be significantly larger than F_{ST}. Consequently, if our F_{ST} falsely underestimates population differentiation at neutral marker sites, then this would mostly affect our conclusions that none of the traits is under stabilizing selection across populations. In summary, we are confident in the accuracy of the results with the exception of underestimating stabilizing selection across the Alps in plant size. Nonetheless, we caution the reader against taking Q_{ST}-F_{ST} comparisons as definitive proof for the presence or absence of selection.

Conclusion

Our results suggest that the timing of onset and peak of flowering has been under divergent selection among populations of alpine *Anthylllis vulneraria*. Populations were differentiated in onset and peak of flowering up to 5 weeks across regions and more than 2 weeks within regions when grown in the common garden. Analyses suggest that differentiation resulting from selection occurs even at spatial scales < 20 km and we hypothesize it is the result of temperature differences at the population sites resulting in divergent snowmelt and pollinator conditions. We found ample genetic variation within populations for all traits, supporting the idea that future adaptations in flowering phenology and reproductive allocation to novel conditions are possible. However, genetic variation in phenotypic plasticity in response to soil moisture availability was absent for all traits studied. This indicates either the absence of significant heterogeneity in soil moisture across populations, or stabilizing selection on reaction norms across populations, or in the case of flowering phenology might be due to the low overall plasticity in flowering phenology in response to soil moisture.

Acknowledgements

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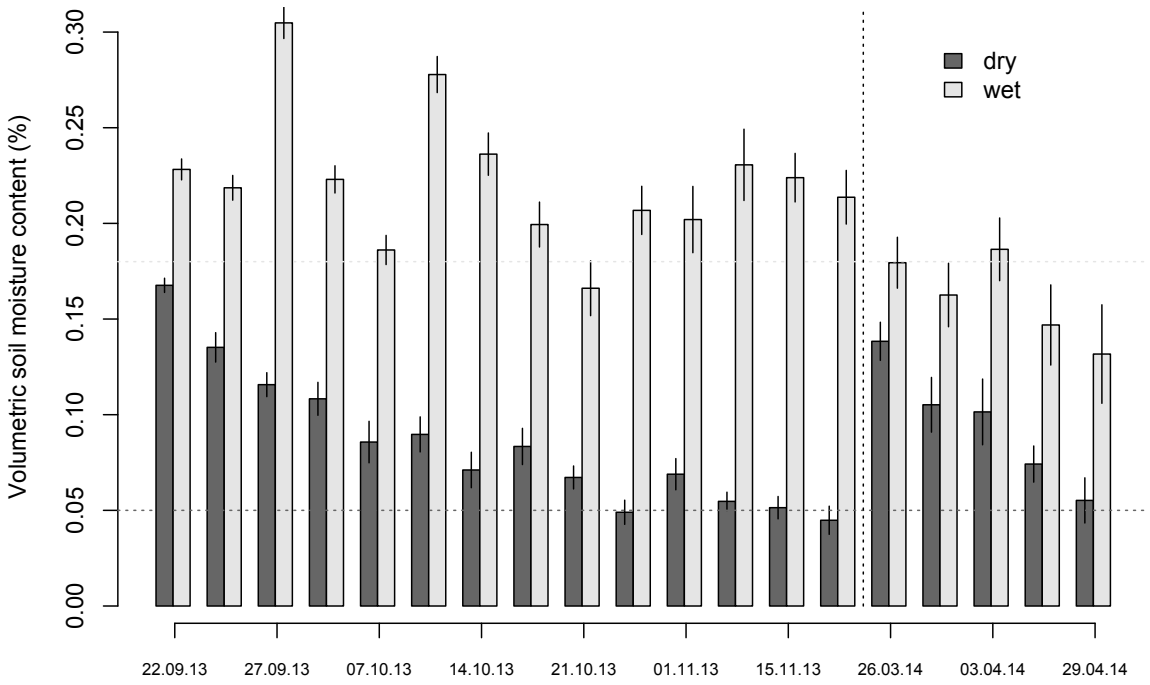
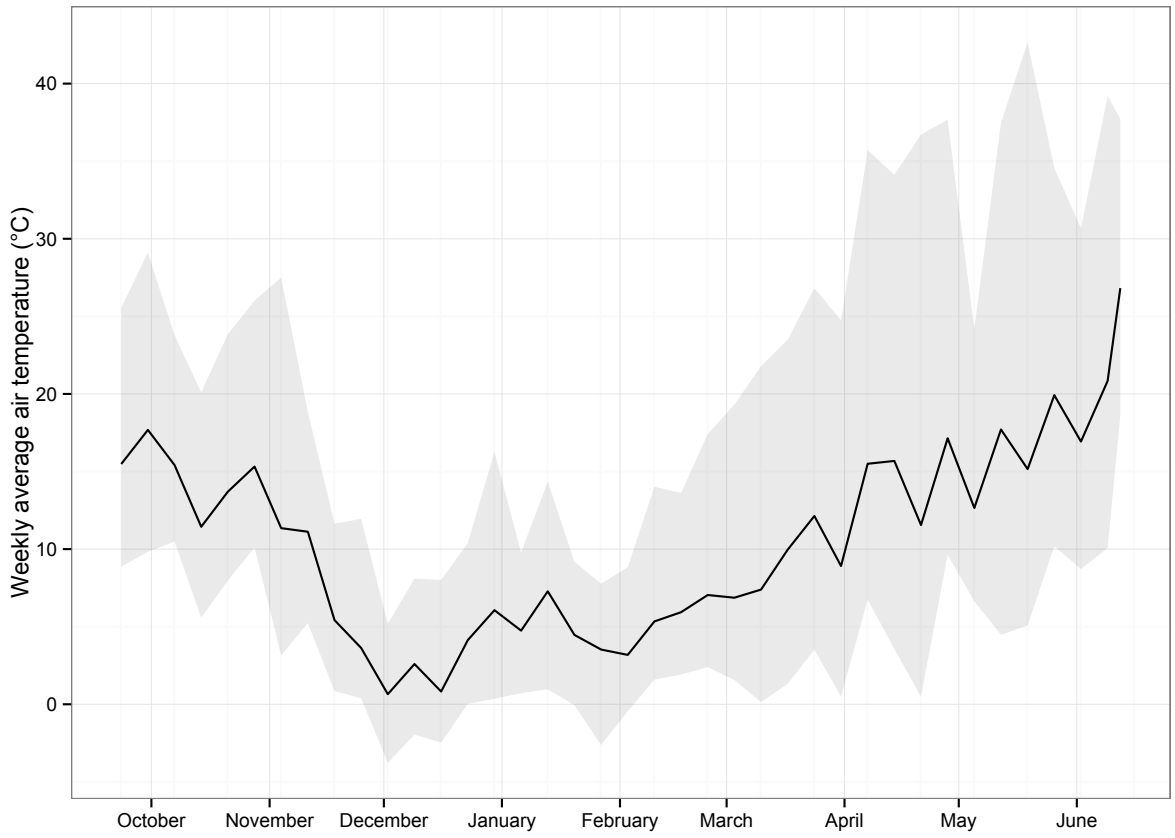
Appendix



Supplemental Figure 1 Volumetric soil moisture measurements were taken twice per week at random intervals and at different times after watering on 10 haphazardly selected individuals per treatment. Wet and dry plants were watered when the average moisture content undercut 18 % and 5 %, respectively as indicated by the horizontal lines. The vertical line indicates the suspension of the treatment during winter. Wet plants were given between 100 ml and 190 ml of water and on each watering, and dry plants between 60 ml and 100 ml, depending on water status.



Supplemental Figure 2 Weekly average (black solid line) and weekly maximum and minimum (grey shade) air temperatures for the duration of the experiment.

Chapter 5

Protandry in alpine populations of *Anthyllis vulneraria* s.l. (Fabaceae): Genetic and environmental variability and significance for the mating system.

Halil Kesselring, Georg Armbruster, Elena Hamann, Jürg Stöcklin

Abstract

Premise of the study

Despite the long history of research into angiosperm reproduction, there still remains large uncertainty about its evolution, particularly the maintenance of mixed mating. Here we use alpine populations of *Anthyllis vulneraria* s.l. to study genetic and environmental variation in protandry, presumably a key trait leading to mixed mating and determining the level of inbreeding in this clade.

Methods

We determined the timing of pistillate and staminate phases within flowers of six populations from two regions and calculated additive genetic variance for these characters in an open-pollinated maternal half-sib design under control and drought conditions. Neutral genetic differentiation, diversity, and inbreeding coefficients of the same populations were assessed using microsatellites.

Key results

We found a broad range of inbreeding coefficients that is matched by an equally broad range of degrees of dichogamy as a result of delayed stigmatic receptivity. A marginally significant relationship between protandry and inbreeding was found within but not across regions. Drought affected pollen quality negatively, but had no influence on stigmatic receptivity. Genetic variation in both the timing of stigmatic receptivity and pollen germinability indicates the potential for evolutionary change towards more of less selfing.

Conclusions

Our results suggest that dichogamy at least in part explains the degree of inbreeding in this species and that even a minimal delay in stigma receptivity can maintain a mixed mating strategy. The strong regional difference in the relationship between protandry and inbreeding stresses the importance of the pollinator environment for the realized outcrossing rate.

Keywords: alpine plants; mixed mating; phenotypic plasticity; soil water availability

Introduction

The ancestral flower is generally believed to be a hermaphroditic reproductive organ with male and female parts co-located in the same structure for effective pollen exchange between flowers (Darwin, 1876; Stebbins, 1957; Charnov et al., 1976; Theissen and Melzer, 2007). Early on it was pointed out that in many outcrossing plants the female sexual organs pick up pollen from the pollinator exactly where it was deposited by the male sexual organs of another flower, making this is a very effective way of achieving pollination that requires only little pollen (Sprengel, 1793). Most flowering plants also show either temporal (dichogamy) or spatial (herkogamy) separation of male and female function within flowers, or both (Lloyd and Webb, 1986; Bertin and Newman, 1993). Ever since Darwin (1876) showed that offspring resulting from self-fertilization usually suffer from reduced vigour compared to outcrossed offspring (Charlesworth and Charlesworth, 1987), dichogamy and herkogamy have been considered adaptations for outcrossing and their effect on the mating system has been demonstrated to be particularly strong in self-compatible species (Brunet and Eckert, 1998; Kalisz et al., 2012; Van Etten and Brunet, 2013). Furthermore, unlike self-incompatibility, which has no direct effects on male and female functionality, dichogamy also is an effective way of reducing interference of co-located male and female organs in hermaphroditic flowers (Nagylaki, 1976; Lloyd and Yates, 1982). In fact, since many if not most outcrossing species showing herkogamy or dichogamy are actually self-incompatible (Lloyd and Webb, 1986; Bertin and Newman, 1993), the long-held notion that these floral features evolved solely through selection against self-fertilization is largely abandoned, and selection against pollen-stigma interference offers itself as an alternative explanation for their evolution (Barrett, 2002b). Promotion of outcrossing and avoidance of male-female interference are not mutually exclusive explanations for the evolution of dichogamy (Barrett, 2002a). Further selective forces have been invoked to explain especially protandry, the most prevalent form of dichogamy (Bertin and Newman, 1993). Protandry might for example lead to intensified pollen competition and thus to selection against inferior pollen (Lankinen and Madjidian, 2011) or the potential for female choice (Galen et al., 1986).

Despite the prevalence of so many outcrossing mechanisms and despite inbreeding depression as a chief barrier, the transition from outcrossing to selfing is considered one of the most frequent changes in plant evolution. Stebbins (1957) has argued that transitions to selfing occur frequently due to the short-term advantages of reproductive insurance and colonization success, but that it leads to a decreased potential for evolutionary novelty (Goldberg et al., 2010). This explains why only 10-15% of extant flowering plants are predominantly self-fertilizing(Goodwillie et al., 2005). Intermediary stages of mixed mating are a potential solution to overcoming inbreeding depression through the purging of deleterious mutations and therefore have traditionally been considered only a temporary state after loss of self-incompatibility, quickly leading to complete selfing (Lande and Schemske, 1985; Charlesworth and Charlesworth, 1987; Holsinger, 1991). Transitions in the reverse direction are supposedly very rare (Stebbins, 1957; Takebayashi and Morrell, 2001; Igic et al., 2008; Chantha et al., 2013). Recent data, however, suggests that mixed mating strategies are most common and possibly reflect an evolutionarily stable strategy (Goodwillie et al., 2005; Eckert et al., 2010; Winn et al., 2011). In mixed-mating taxa, it is easily conceivable that shifts from highly selfing to predominantly outcrossing and vice versa may occur frequently (Wright et al., 2013). The effect of dichogamy on the degree of selfing has been demonstrated not only for single species, but has also been shown to define the selfing syndrome across a whole self-compatible genus (Kalisz et al., 2012). There is also evidence for heritable variation in dichogamy (Van Kleunen and Ritland, 2004; Lankinen and Kiboi, 2007) so that this trait may respond to selection in either direction. Furthermore, some evidence suggests this trait is

labile to environmental conditions or there might even be adaptive phenotypic plasticity (e.g. the ability to increase selfing under unfavourable conditions and the reversion to outcrossing in benign environments; (Elle and Hare, 2002; Van Etten and Brunet, 2013).

The alpine environment is a particularly interesting arena for the study of mating systems. Due to the inhospitable conditions such as short growing seasons and reduced pollinator activity, the traditional view has been that selfing should be an exceedingly common phenomenon in high-elevation environments (Schroeter, 1923). However, recent empirical studies have failed to detect a clear relationship between elevation and selfing rate (Gugerli, 1998; Wirth et al., 2010). The key argument in favour of maintained or increased outcrossing rates at high elevations is that genetic variability is important for long-term survival under highly stochastic conditions frequently encountered at high elevations (Lande and Shannon, 1996), and most population genetic studies normally find moderate to high genetic diversity in high-elevation populations (Stöcklin et al., 2009). Apart from work on gene flow (e.g. (Scheepens et al., 2012) and reproductive allocation (Hautier et al., 2009; Guo et al., 2010), the study of traits associated with mating system and their evolution and functional consequences in the alpine landscape has largely been amiss (Bertin and Newman, 1993; Totland and Schulte-Herbrüggen, 2003; Van Etten and Brunet, 2013).

Here we study the degree of dichogamy and inbreeding in alpine populations of *Anthyllis vulneraria* s.l., a monophyletic group of short-lived herbaceous plants (Nanni et al., 2004). Large variation in the degree of outcrossing is found within and among closely related species in the genus *Anthyllis* (Couderc, 1980; Nanni et al., 2004), and the highly variable *Anthyllis vulneraria* clade. French populations of a number of subspecies of *Anthyllis vulneraria* were found to be exclusively selfing due to the receptivity of stigmata to auto-pollen before flower opening (Couderc, 1971). A Spanish population of *Anthyllis vulneraria* s. str. on the other hand was shown to have pollen fertility peaking 3 days earlier than stigma receptivity making self-fertilization unlikely (i.e. protandry; (Navarro, 1999). Finally, a microsatellite study of two Belgian populations of *Anthyllis vulneraria* s. str. found that they were largely outcrossing (Van Glabeke et al., 2007). All populations of *Anthyllis vulneraria* s.l. seem to be genetically self-compatible in general (above references). Thus, it seems that dichogamy defines the mating system in *Anthyllis vulneraria* s.l. However, no study to date has investigated the degree of dichogamy and inbreeding rates in *Anthyllis vulneraria* s.l. simultaneously to confirm their relationship.

In the present study we quantify variation in dichogamy in six alpine populations of *Anthyllis vulneraria* s.l. from two regions by studying the development of the pistillate and staminate activity over the lifespan of the open flower. Using a maternal half-sib design in a common garden under pollinator exclusion, we further detail genetic variation in dichogamy, a key prerequisite for its evolvability (Fisher, 1958). In order to assess the influence of environmental factors, we measure plasticity in dichogamy in response to soil water availability, an ecologically relevant factor for past, present and future plant survival (Manel et al., 2012). Finally, the study populations were scored with microsatellites to measure genetic diversity and inbreeding coefficients, and to relate these measures to the degree of dichogamy. Self-compatibility and autonomous selfing were assessed in a subset of individuals for all six populations. We expected to find considerable variation in inbreeding among regions and populations reflecting divergent selection in the highly variable alpine environment, and that this variation in inbreeding is caused by the degree of dichogamy in those populations. We also hypothesized that plants should increase the rate of selfing by earlier maturation of stigmata under drought conditions so as to ensure reproduction in a stressful environment.

Methods

Study species

Anthyllis vulneraria s.l. is a self-compatible clade of short-lived herbaceous plant species very common throughout Europe. It grows preferably on calcareous meadows and scree grounds from sea level to the alpine belt up to around 3000 m.a.s.l. (Hegi, 1975). *Anthyllis vulneraria* s. l. belongs to the tribe of Loteae within the Fabaceae. It is a particularly polymorphic taxon with unclear infraspecific classification (Allan and Porter, 2000; Nanni et al., 2004; Köster et al., 2008). Plants grow to a height of around 15-45cm. On a variable number of shoots per plant 2-6 inflorescences are found. Each inflorescence bears a larger and a smaller bract at its base, and comprises a number of 7-19mm long white to yellow, sometimes claret to red flowers arranged in a capitulum (Hegi, 1975; Navarro, 1999). Bracts are incompletely subdivided compound leafs. Shoots are usually unbranched, but may have up to three side-branches originating from the axils of evenly pinnate compound leafs. Leafs of the basal rosette consist of the enlarged terminal leaflet of a compound leaf. *A. vulneraria* is representative of a type of fabacean flower characterized by a 'pump mechanism' adapted to insect-mediated pollination. In short, the stigma and surrounding anthers act as a piston to pump the pollen mass through an opening at the tip of the keel. The pumping action is induced when a heavy insect pushes down the keel in an attempt to reach the nectar. The pollen mass is sticky and accumulates densely in the tip of the keel. The stigma is surrounded by auto-pollen inside the keel throughout anthesis, however, it generally becomes adhesive to the latter only later in development. Additionally, pollen is removed from a non-adhesive stigma by the elastically retracting edges of the keel opening upon departure of the pollinator. The adhesiveness of stigmata is enhanced by mechanical impacts such as pollinator visitations (Müller, 1873). Flower development of *Anthyllis vulneraria* takes approximately 4 weeks (Fig. 1). Flower ripening is not synchronous and geitonogamous offspring may be produced. The flower is open and accessible to pollinators for only about 5 to 7 days. The gynoecium always contains two ovules, of which usually only one develops into a seed (Couderc, 1971). There is no taxonomic description available that fits populations used in this study precisely, therefore no conclusive assignment to one the numerous described sub-species could be achieved (Müller, 1873).

Experimental procedures

In August 2012, seeds from open-pollinated wild flowers in three populations from each of two regions (Eastern and Western Swiss Alps near Davos and Zermatt, respectively) were sampled: Schiahorn (46°48'59.64''N,9°48'16.80''E), Casanna (46°51'26.88''N,9°49'37.74''E), Monstein (46°41'16.92''N,9°47'15.84''E), Findelgletscher (46° 0'25.84"N, 7°48'43.94"E), Findelwald (46° 0'37.08"N, 7°47'6.00"E), Stafelalp (46° 0'4.08"N, 7°41'6.37"E). Populations are situated between 2000m.a.s.l. and 2650m.a.s.l. Distances between populations within regions ranged from 2.2 km to 18 km, and regions are 180 km apart. Seeds were stored in the refrigerator until they were scarified and sown in early August 2013 directly into their final high mineral potting soil mixture (210l Ökohum Anzuchterde® with 14l sand and 8kg pumice). Seedlings were kept in the greenhouse in 10 by 10cm pots and watered ad libitum. Plants were randomized twice per week. Greenhouse heating and cooling systems were set so that temperatures would not fall below 16°C and 8°C or exceed 20°C and 10°C at day and night, respectively. After 4.5 weeks seedlings were potted into larger 2l pots into the same soil mixture and watered to carrying capacity. At the same time, plants were moved to the outside garden under a rain shelter and arranged in a checkerboard array. Eleven days later, on the 22.09.13, treatment began by watering those plants designated to fall into the wet treatment. Subsequently, volumetric soil moisture

content was monitored with a moisture meter calibrated to the soil mixture used in the experiment and plants were watered accordingly (HH2 Moisture Meter with Theta Probe ML2x, Delta-T Devices Ltd. Cambridge, England). Wet plants were watered when mean soil moisture fell below 0.18%, and dry plants when soil moisture fell below 0.05%. Throughout the duration of the experiment, air temperature was logged hourly with a TidbiT® v2 Temperature Logger (Onset Computer Corporation, Bourne, Massachusetts, U.S.A.). All plants were preventively treated with a ready-to-use fungicide powder (Maag Pirox®, Syngenta Agro AG, Dielsdorf, Switzerland) on a few occasions during growth phase, because plants from the same populations were heavily infested with mildew in a former experiment. *Anthyllis vulneraria* needs vernalisation to induce flowering (personal observation), so we left plants outside over winter. During this time the water treatment was suspended. Treatment was re-established on the 19.03.2014 by watering with 100ml and 60ml for wet and dry plants, respectively. From then on treatment was held up as described above.

Measuring dichogamy

In order to trace pistillate and staminate phases over time, we used 24-hour intervals to define 7 flower age classes as follows: the base line was flower opening when the corolla is just visible but not yet fully expanded (Fig. 1). The first age class is 24 hours after start of opening of the flower. Age classes 2 to 5 are the open flower 48 to 120 hours, respectively. Age classes 6 and 7 are the fading flower 144 to 168 hours, respectively. As soon as an individual plant had reached a flowering stage at which enough flowers could be expected to open over the next 7 days, labelling of flowers was started with marking flowers for age class 7 days. On the next day, flowers for age class 6 were marked, and so on the following days until flowers for every age class had been marked after one week. As our goal was to assess dichogamy in the absence of all other confounding factors, plants were kept under a large pollinator-exclusion net from the start of flower labelling until work was finished. Each flower age class was labelled with indelible ink of a unique colour. Two flowers per age class were chosen haphazardly on every individual. As we used 6 populations (3 from each of two regions), 5 maternal half-sib families per population, and 4 individuals per family, we ended up with a total sample size of 120 plants and 1680 flowers (14 flowers per plant). From the four individuals per maternal family, two were under the dry treatment and two under the wet treatment. Flowers from five to ten plants were finally measured on every working day between Monday 31.3.14 and Tuesday 15.5.14. As we started this experiment late in 2013, and finished it early in 2014, thereby avoiding the hot summer in Basel in both years, climate throughout the duration of the experiment resembled the climate in the Alps very closely. Especially, temperatures were quite stable and uniform during the time of measurement of the stigmatic and pistillate phases (weekly mean air temperature ranged from 11.6°C to 17.7°C).

Fig. 1. Flower development of *Anthyllis vulneraria*. The process of flower opening happens within 24 hours. The flower then remains open for approximately 6 to 7 days. Seed ripening takes another 3 weeks, approximately. The arrow indicates time zero from where we counted intervals of 24 hours to define 7 flower age classes.

Pollen germinability at each flower age was assessed by transferring pollen onto a Petri dish with a sterile solid 30% sucrose medium. The medium was prepared in the following fashion: 2% agar in distilled water, 100ppm boric acid (H3BO3, 61,83 g/mol), 100ppm potassium nitrate (KNO3, 101.103 g/mol), 200ppm magnesium sulphate (MgSO4 ,120.363 g/mol), 300ppm calcium nitrate (Ca(NO3)2, and 30% sucrose (Barshalom and Mattsson, 1977; Navarro, 1999). Pollen was incubated for four hours. Estimation of germination rate was done under the stereomicroscope. Grains with a pollen tube longer than the diameter of the grain were regarded as germinated. To investigate stigma receptivity at each flower age, the pistil was excised and put into a drop of 3% hydrogen peroxide on a microscope slide with a depression coated in Sigmacote® (SL2, Sigma-Aldrich). If the stigma is receptive it has peroxidase activity and breaks the hydrogen peroxide. Instantly oxygen bubbles are produced (Dafni et al., 2005). Coating makes slides water repellent and therefore gives rounder water drops. Stigmatic receptivity was judged in 25% steps from 0 to 100%. The two flowers per individual therefore gave nine possible mean values for stigmatic receptivity for every individual and flower age.

Test of self-compatibility

In order to confirm the self-compatibility of our plants we actively and passively self-fertilized 10 flowers on two plants from two distinct seed families per population under pollinator exclusion (n = 2 individuals x 6 populations x 10 flowers = 120). Five flowers per individual were self-pollinated by abundantly activating the pump mechanism of the flower thereby applying mechanical impact and self-pollen to the stigma. The other five flowers were left untreated. Ten days later, flowers were harvested and examined for seed set. As unfertilized flowers dehisce soon after wilting, flowers that could not be found anymore were regarded as unfertilized (this occurred in low frequency and evenly across groups). The fraction of flowers with seed set was noted. We cannot exclude the possibility of agamospermy in those flowers that had set seed (apomixis), since we did not perform pollen removal experiments. Agamospermy has never been documented in the genus of *Anthyllis*, however, and is unlikely due to the frequent personal observation of flowers that bear no seeds in natural populations.

Molecular analyses

20 individuals per population were scored for amplified fragments at 9 microsatellite loci. We used Spreadex® gels and the ORIGINS electrophoresis unit (Elchrom Scientific AG, Cham, Switzerland) to separate PCR amplicons with size differences as small as 2bp. Gels were stained with ethidium-bromide and scored by hand comparing against the M3 ladder from ELCHROM. A detailed description of the microsatellite analysis and loci description can be found elsewhere (Kesselring et al., 2013).

Statistical analyses

The main purpose of the present study was to analyse if there was added variation over and above individual variability in stigma receptivity and pollen germinability attributable to the factors population and maternal half-sib family, and to quantify the effects of soil-water availability on the same traits. We therefore specified linear mixed models with region, soil-water availability, and flower age as well as their two-way interactions with region as fixed effects, and with population, seed family, and individual as well as interactions of population with flower age and with treatment as random effects. Untransformed data could be analysed despite the fact that it was proportion data because model assumptions were fulfilled. Models were specified and analysed with the lmerTest package (Kuznetsova, 2013) for R (R Development Core Team, 2008) which applies tests to lmer objects of the lme4 package that allow F-tests for fixed effects and likelihood-ratio tests for random effects using stepwise model reduction and comparison. We used type 3 errors and Satterthwaite approximations for degrees of freedom. We report *P*-values, mean squares, and chi square-values that correspond to those from the model comparison (i.e. likelihood-ratio tests) using the step function in lmerTest. Qualitatively these results were unchanged when applying F-tests using the anova command in lmerTest. In order to test the functional significance of delayed stigmatic receptivity for the mating system we modelled F_{IS} as a function of mean stigmatic receptivity and region as a grouping factor in a linear least squares regression. We calculated a regression and not correlations for mean stigmatic receptivity and F_{IS} because a minimum of four observations per group is required in R to compute correlations and *P*-values.

Hardy-Weinberg exact tests were performed on the molecular fingerprints in GENEPOP version 4.2 (Raymond and Rousset, 1995). Population differentiation (Hedrick's G'_{ST} (2005), inbreeding coefficients (F_{IS} sensu Writght (1922), and genetic diversity indices (expected heterozygosity H_e, total number of alleles, number of private alleles) were calculated in GenAlEx version 6.5 (Peakall and Smouse, 2006; Peakall and Smouse, 2012). Null alleles were suggested for one locus in a former analyses using a subset of the populations studied here by MICRO-CHECKER (Van Oosterhout et al., 2004; Kesselring et al., 2013). Since there were no additional blank lanes for any other loci on the electrophoresis gels (homozygote null alleles) we did not test for the presence of null alleles again in this study, but removed the questionable locus from the analyses.

Results

Effectiveness of treatment

Our soil moisture treatment was effective and lead to an average decrease in bulk aboveground biomass across populations of 33% in the dry treatment compared to the wet treatment (ANOVA, $F_{1, 165} = 8.69$, $P<0.005$). Furthermore, the same treatment has lead to a comparable biomass change and a number of measurable physiological effects (e.g. stomatal conductance) in a former experiment with plants from the same populations. Overall, drought-stress in the dry treatment was probably only moderate as plants were at most times turgid,

and drought stress was probably stronger in the first year as the time between continuation of the treatment after winter and harvest was relatively short.

Male and female phase duration

We observed that in *Anthyllis vulneraria*, complete pollen dehiscence always occurs a few hours before completion of flower opening. Our pollen medium afforded high pollen germinability with average values for populations at flower age 1 ranging from 73% to 94% across treatments. Pollen germinability was highest directly after dehiscence and decreased afterwards (Fig. 2). The timing of pollen germinability differed among populations (significant 'population x flower age', Tab. 1). The Findelgletscher population for example had very high pollen germinability even seven days after flower opening while it decreased by about 40% in the adjacent population of Findelwald. Generally however, pollen germinability remained high throughout the flower lifespan with a mean of 62% across populations 7 days after flower opening, indicating that self-fertilization is possible throughout the flower lifespan if self-pollen is not removed. Soil water treatment negatively affected pollen germinability in the Davos populations, but less so in the Zermatt populations (significant 'region x treatment'). The seed family effect was significant and explained 41% of the variation in the random terms, indicating substantial heritable variation for this trait (Tab. 1).

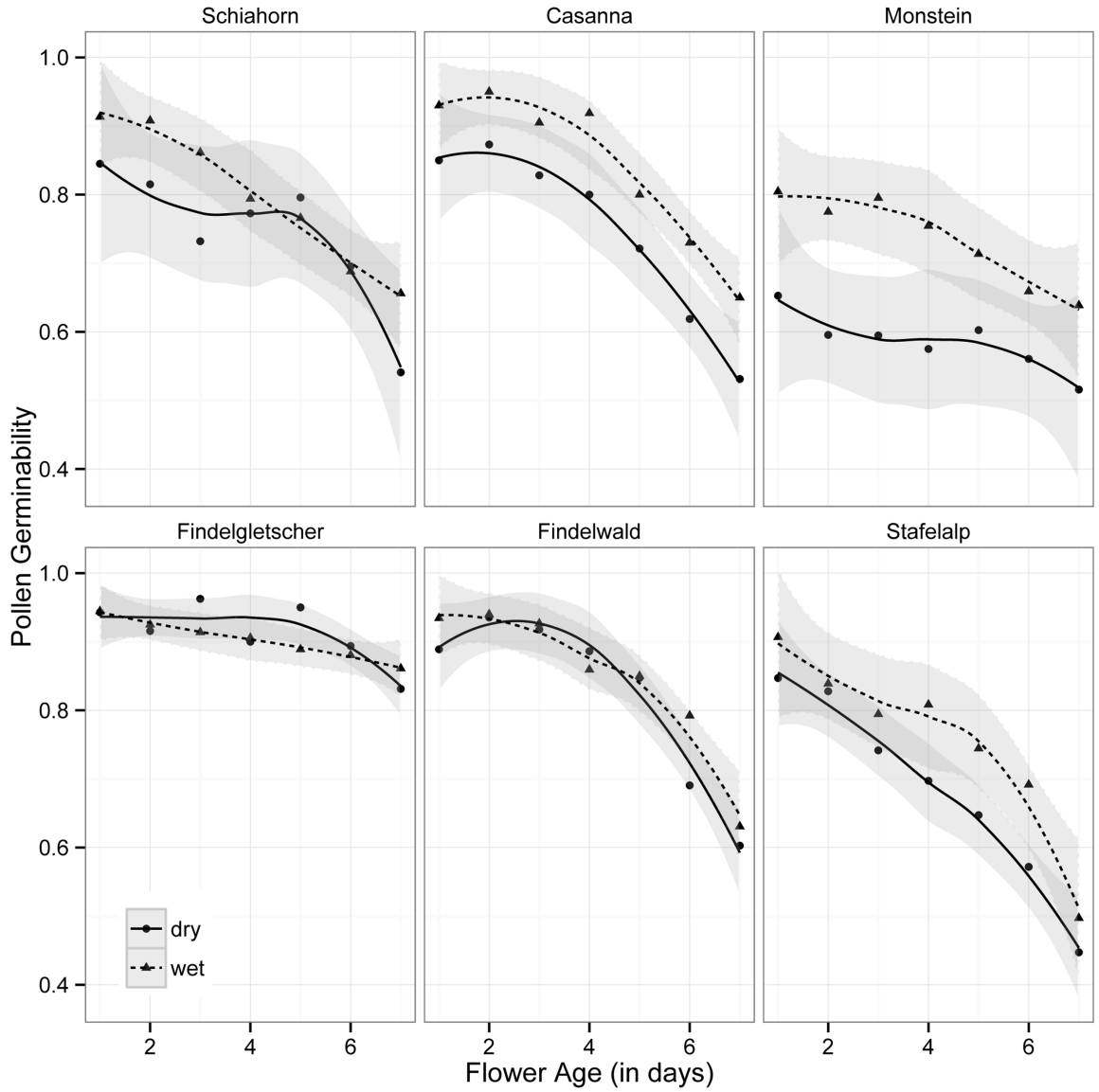


Fig. 2. Pollen germinability of 6 populations of *Anthyllis vulneraria* during 7 days when flowers were open. Top panels are the populations from Davos and bottom panels are the populations from Zermatt. Circles and solid lines refer to mean values and smoother lines in the low soil water availability treatment, respectively. Triangles and dotted lines refer to mean values and smoother lines in the high soil water availability treatment. Smoother and 95%-confidence intervals are calculated using the loess method based on a t-approximation. The fraction of pollen grains germinated was counted. A pollen grain was considered germinated when it had grown a pollen tube longer than the pollen grains diameter. Each estimate is the mean of 20 flowers from 10 individuals.

Stigmatic receptivity increased over time in all populations (Fig. 3). The change over time in stigmatic activity was significantly different in the two regions (significant 'region x flower age', Tab. 1). Populations from Zermatt had higher stigmatic activity upon flower opening and stronger increase in stigmatic activity over time compared to populations from Davos (Fig. 3). Soil water availability had no effect on stigmatic receptivity. Population within region explained no variance in the data. However, seed family within population explained 17% of the variance in the random terms, indicating that there is genetic variation within populations for stigmatic receptivity.

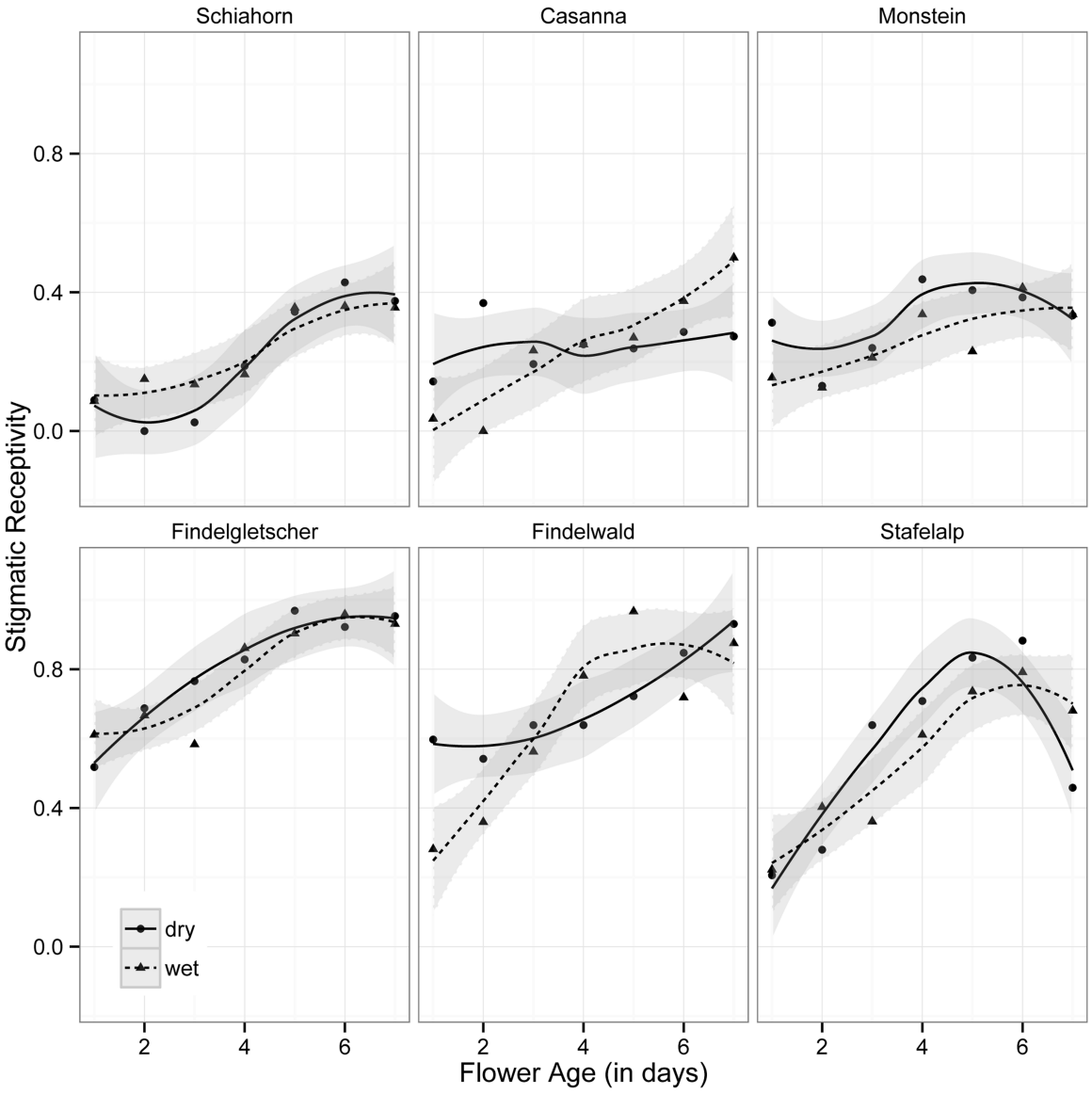


Fig. 3. Stigmatic receptivity of 6 populations of *Anthyllis vulneraria* during 7 days when flowers were open. Top panels are the populations from Davos and bottom panels are the populations from Zermatt. Circles and solid lines refer to mean values and smoother lines in the low soil water availability treatment, respectively. Triangles and dotted lines refer to mean values and smoother lines in the high soil water availability treatment. Smoother and 95%-confidence intervals are calculated using the loess method based on a t-approximation. Stigmatic activity was assessed using the hydrogen peroxide method. Activity was judged in 25% steps. Each estimate is the mean of 20 flowers from 10 individuals.

pollen				
effect	factor	df	MS/Chi.sq	*P*
fixed	Region	1	0.0229	0.145
	Flower Age	6	0.2363	**<0.001**
	Treatment	1	0.1515	**0.001**
	Region x Treatment	1	0.0689	**0.024**
	Region x Flower Age	6	0.0012	0.997
random	Seed Family	na	9.55	**0.002**
	Population	na	1.16	0.282
	Population x Flower Age	na	17.65	**<0.001**
	Population x Treatment	na	0.48	0.490

stigma				
effect	factor	df	MS/Chi.sq	*P*
fixed	Region	1	3.1525	**<0.001**
	Flower Age	6	2.0047	**<0.001**
	Treatment	1	0.0102	0.714
	Region x Treatment	1	0.0125	0.637
	Region x Flower Age	6	0.2077	**0.002**
random	Seed Family	na	18.74	**<0.001**
	Population	na	0.00	1.000
	Population x Flower Age	na	0.00	0.975
	Population x Treatment	na	0.00	1.000

Tab. 1. Results of the linear mixed model analyses of pollen germinability and stigma receptivity in *Anthyllis vulneraria*. Significant effects are in bold. Synthetic degrees of freedom were calculated using the Satterthwaite approximation. Mean squares were calculated for fixed effects using type 3 sums of squares. *P*-values were obtained by stepwise model reduction and comparison (i.e. likelihood-ratio tests). Chi-square values are reported for random effects.

Variation in inbreeding, genetic diversity, and population differentiation

The microsatellite analyses revealed that all populations and all loci deviated significantly from Hardy-Weinberg-Equilibrium showing significant heterozygote deficiency ($P<0.05$). The inbreeding coefficients (F_{IS}) were positive for all populations except Stafelalp, and varied strongly among populations and regions ranging from 0.00±0.05 to 0.41±0.04 (Fig. 4, Tab. 2). This indicates that all studied populations but one are mixed mating. Overall population differentiation was substantial with G'_{ST} =0.24±0.05 ($P<0.001$). All pairs of populations were significantly differentiated ($P<0.002$), except the geographically adjacent sites of Findelgletscher and Findelwald (G'_{ST} = -0.002; distance = 2.2 km). Pairwise differentiation within and between regions ranged from 0.00 to 0.26 and from 0.13 to 0.49, respectively. Expected heterozygosities (H_e) were high in all populations ranging from 0.68±0.02 to 0.76±0.02 (Tab. 2). All loci were polymorphic in all populations with allele numbers ranging from 5.80±0.56 to 8.00±1.05. Average numbers of private alleles ranged from 0.13±0.13 to 0.50±0.27.

Region	Population	Mean Stigmatic Activity	F_{IS}	H_o	H_e	Number of alleles (private alleles)
Davos	Schiahorn	0.234±0.028	0.050±0.078	0.665±0.058	0.701±0.024	5.875±0.515 (0.500±0.267)
Davos	Casanna	0.253±0.033	0.272±0.059	0.496±0.045	0.679±0.022	5.875±0.549 (0.125±0.125)
Davos	Monstein	0.277±0.025	0.265±0.090	0.538±0.066	0.731±0.032	8.000±1.052 (0.500±0.267)
Zermatt	Findelgletscher	0.800±0.026	0.414±0.036	0.446±0.035	0.755±0.015	5.750±0.559 (0.375±0.263)
Zermatt	Findelwald	0.681±0.032	0.366±0.062	0.456±0.047	0.717±0.026	5.875±0.953 (0.250±0.164)
Zermatt	Stafelalp	0.557±0.032	-0.001±0.048	0.694±0.030	0.695±0.030	5.875±0.743 (0.375±0.183)

Tab. 2. Summary table of mean stigmatic activity, inbreeding coefficient (F_{IS}), observed (H_o) and expected heterozygosity (H_e), mean number of alleles across all nine loci, and mean number of private alleles across all loci in parantheses of *Anthyllis vulneraria*.

Self-compatibility and autonomous selfing

Assisted self-fertilization always lead to considerable seed set demonstrating the self-compatibility of all populations of *Anthyllis vulneraria* used in this study. However, seed set under assisted self-fertilization was not 100% in all populations, indicating that there are variable degrees of partial self-incompatibility among populations in both regions (Fig. 4). Autonomous selfing did occur to various degrees in all populations but Schiahorn, which produced not a single seed out of 10 untreated flowers from two individuals under pollinator exclusion. In the Findelgletscher population all observed flowers in the passive selfing treatment did set seed (Fig. 4).

A linear least squares regression with F_{IS} modelled as a function of mean stigmatic receptivity and region as a grouping factor was only marginally significant for mean stigmatic activity

(b=1.81, t(2)=2.91, *P*=0.062). Given the low power of the test due to the small number of replicate populations and the small variation in F_{IS} among populations in Davos, this result suggests a relationship between mean stigmatic activity and population inbreeding within region, but not across regions.

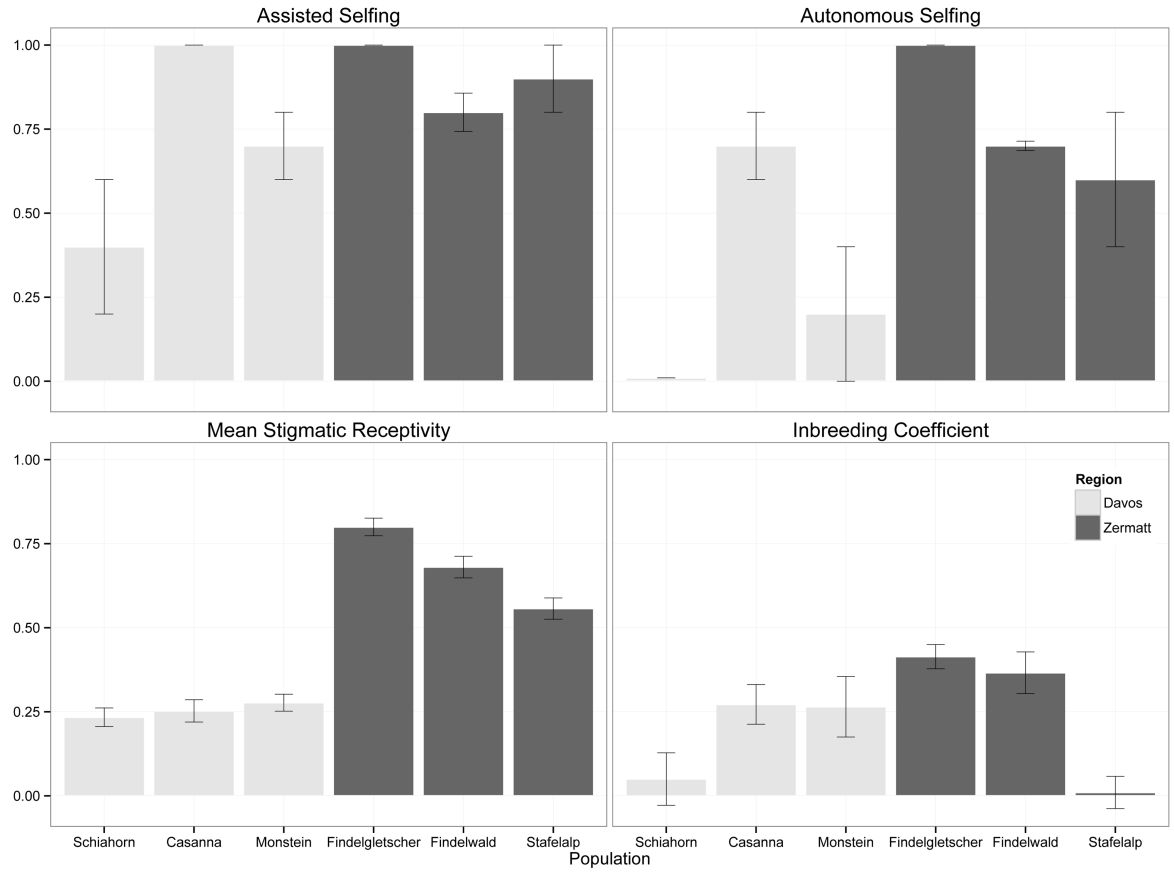


Fig. 4. Seed set of 6 populations of *Anthyllis vulneraria* under assisted and autonomous self-fertilization as well as mean stigmatic activity and inbreeding coefficients (F_{IS}) for the six studied populations (mean±s.e.). Standard errors for F_{IS} were calculated in GenAlEx by jack-knifing over loci. We use mean stigmatic activity in this study to compare against inbreeding coefficients and seed set under pollinator exclusion. Assisted selfing was achieved by abundantly activating the pump mechanism of the flower thereby applying mechanical impact and self-pollen to the stigma. Autonomous selfing refers to unassisted self-fertilization of flowers under pollinator exclusion. Light shaded bars refer to populations from the Eastern Alps (Davos), dark shaded bars refer to populations from the Western Alps (Zermatt).

Discussion

We found that alpine populations of self-compatible *Anthyllis vulneraria* are mostly mixed mating, and show a broad range of inbreeding that is matched by an equally broad range in the degree of dichogamy. We used the mean stigmatic activity as a proxy for the degree of dichogamy since we do not know what level of stigmatic activity allows for fertilization, and because pollen germinability, though significantly reduced over time, remained substantial throughout the flower lifespan. A marginally significant relationship between mean stigmatic activity and F_{IS} within regions but not across regions suggests that dichogamy indeed determines at least partly the degree of selfing, but that this relationship can be fundamentally different among regions. Whether this difference is an environmental one or an intrinsic genetic property of the populations in the two regions we do not know. Generally however,

extrinsic factors such as population density or pollinator abundance may have a large enough effect on the mating system to cause sufficient variation that masks the association between intrinsic floral characters and mating system (Becker et al., 1992). In order to fully trace the effect of dichogamy on the mating system one would have to relate within-population variation in dichogamy to the realized outcrossing rate of individuals or families (Takebayashi and Morrell, 2001). However, this approach is also limited in its capability to inform about the true outcrossing rate of a population since it is only a point-estimate, which is likely to change over time with changes in the environment such as pollinator activity (Barrett et al., 1993). F_{IS} is a good estimator in this respect as it integrates the long-term history of selfing in the studied plants.

In addition to external factors, intrinsic genetic differences of the populations in the two studied regions may also alter the relationship between dichogamy and inbreeding. The effects of dichogamy are likely to be somewhat different for example between strongly dichogamous and only slightly dichogamous populations. We expect the slope of the relationship between dichogamy and selfing in strongly dichogamous plants to be flatter because the chance that most seeds will be outcrossed is high in any case. Contrarily, we expect the slope to be quite steep in plants with little dichogamy and a short time-window of outcross opportunity, because in this situation, small changes in the length of the time-window are expected to have larger effects on the realized outcrossing rate (Takebayashi et al., 2006).

The regional difference in the relationship between stigmatic activity and F_{IS} is exemplified in the Stafelalp and Schiahorn populations. While they both have a similarly low F_{IS}, the Stafelalp population has more than twice as high a stigmatic activity. When pollinators very early visit flowers that have just opened or not even fully opened and deliver potent outcross pollen, cross-fertilization may take place and early autonomous selfers may achieve small inbreeding coefficients. In fact prepotency of outcross pollen and the pollinator environment can have such drastic effects on the outcrossing rate that early selfers resemble highly outcrossing species (Kalisz et al., 2012). In the Findelgletscher population, for example, where there is obviously no incompatibility barrier to self-fertilization and stigmas are often receptive directly upon flower opening the measured inbreeding coefficient of under 0.5 can only be explained by pollinators arriving very early and delivering outcross pollen within the first few hours of flower opening. Together, this points to prepotency of outcross pollen (Lloyd and Schoen, 1992) especially if we presume that pollinators often also exchange pollen among flowers of the same individual. Our data of the plants hand-pollinated with self-pollen furthermore point towards partial self-incompatibility as an additional modifier of the mating system in some of the studied populations. Lastly, differences in the amount of bi-parental inbreeding among the studied populations may also create variation in the mating systems (Griffin and Eckert, 2003). Bi-parental inbreeding is likely in populations with fine-scale genetic structure resulting from limited pollen and seed dispersal. Genetic drift and spatially heterogeneous selection can additionally amplify the effects of short-distance dispersal. We can only speculate on this, but as population size, density, and topography are likely to influence the relatedness of neighbouring plants as well as pollen dispersal, it is probable that the amount of bi-parental inbreeding differs among the studied populations.

Determining the heritability of key floral traits is an important step towards the solution of the puzzle of the evolution of mating systems. Here we could show that there exists genetic variation for dichogamy within the studied populations allowing the evolution of this trait within and among populations given either genetic drift or selection (Fisher, 1958). Environmental influence as measured in our drought treatment could only be detected in a

lowered pollen germinability, but not in stigmatic receptivity. A possible cause for the evolution of delayed stigma receptivity is its possible fitness advantage through increased pollen competition and female choice when outcross pollen accumulates before stigma receptivity (Galen et al., 1986; Lloyd and Webb, 1986; Lankinen and Kiboi, 2007). The results for the Findelgletscher population, the population with the lowest delay in stigma receptivity, are in agreement with this hypothesis. A high paternal expenditure in this population as exemplified by the high pollen germinability at the oldest flower age as well as robustness of pollen quality to drought points to either strong pollen competition or to bad pollinator services. The latter is unlikely given the F_{IS} is still smaller than 0.5 in this early autonomously selfing population. This population grows as a pioneer at a glacier fore-field site and is extremely large and dense making strong pollen competition likely. When pollen competition is naturally high, selection for delayed stigma receptivity should be low. Protandry through delayed stigma receptivity may concurrently also allow a species to function as an out-crosser when pollinators are available, and reassure seed set through delayed autonomous selfing when pollinators are absent (Fenster and Martén, RodrÍguez, 2007). Autonomous selfing is possible especially in the Findelgletscher population where it seems to function as 'competing autonomous selfing', however, rather than 'delayed autonomous selfing' (Lloyd and Schoen, 1992). In the Schiahorn population no autonomous selfing occurred in our experiment, which is in agreement with our frequent personal observation of wild plants setting little seed. Partial self-incompatibility as indicated by the low seed set under assisted selfing together with the low stigmatic activity probably rules out autonomous selfing in the Schiahorn population. It seems that in the Schiahorn population pollinators' assistance is needed not only to deliver potent outcross pollen but also to scratch the stigmatic surface and render it receptive. In all other populations, incomplete protandry seems to allow for some degree of delayed autonomous selfing due to the close proximity of pollen and stigma. Interestingly, the Schiahorn population is the one with the strongest delay in stigmatic activity and also the one with the strongest self-incompatibility, suggesting that both might have evolved due to strong inbreeding depression in this population.

One of the key arguments of Stebbins hypothesis explaining the relatively small percentage of predominantly selfing plant species in spite of the frequent transition to selfing in angiosperm evolution was the decreased potential of selfing lineages for adaptation due to reduced genetic variability (Stebbins, 1957; Hamrick and Godt, 1996; Charlesworth and Wright, 2001; Nybom, 2004). Mating system is the primary determinant of the distribution of genetic variation within and among populations of a species, and is key to the capacity of a species for local adaptation and evolutionary diversification (Glemin, 2007; Barrett and Schluter, 2008). In the studied populations there was no sign of reduced genetic diversity with increasing self-fertilization as both expected heterozygosities and number of alleles and private alleles were not only quite high in general, but even higher than in highly outcrossing populations of another study of *Anthyllis vulneraria* (Van Glabeke et al., 2007). Mixed mating potentially preserves high genetic diversity in our study populations.

Conclusion

Despite the overriding effects that environmental factors such as pollinator abundance can have on the realized mating system, we conclude that the timing of stigma receptivity is key to the degree of inbreeding in alpine populations of *Anthyllis vulneraria*. We find that at least one population has such a low stigmatic receptivity throughout the flower lifespan that together with some apparent degree of self-incompatibility autonomous selfing is prevented. Other populations with very high stigmatic receptivity upon flower opening still show high degrees of outcrossing which must be due to early pollinator visitation and possibly also the result of outcross pollen preference. Together these findings stress the interaction of timing of

stigmatic receptivity and pollinator environment: in one extreme, pollinators are obviously needed to scratch the stigmatic surface and make it receptive, and in the other extreme, early visiting pollinators serve as interrupters to programmed self-fertilization. The presently found degrees of mixed mating apparently suffise to preserve genetic diversity and therefore should not hamper responses to selection in more strongly selfing populations of *Anthyllis vulneraria*.

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Chapter 6

Alpine plants have reduced plasticity in flowering time in response to warming compared to lowland congeneric species.

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Abstract

Global warming has caused shifts in the flowering time of many plant species. In alpine regions the temperature rise has been especially pronounced and together with decreasing winter precipitation has led to earlier snowmelt. The close association between snowfall and plant growth at high elevations makes climate change for alpine plants particularly threatening.

Here we transplanted 11 congeneric pairs of alpine and lowland herbaceous species to common gardens differing c. 800 m in elevation, and c. 4 °C in growing season temperature to test whether reproductive phenologies of alpine and lowland plants differ in their respective responses to temperature.

We found that alpine plants were 7 days or 44 % less plastic in response to elevational transplantation than lowland congeners concerning phenophase onsets. Plasticity of phenophase durations was overall less strong than that of phenophase onsets, and slightly stronger in alpine plants compared to lowland congeners.

Our results suggest that spring frost constitutes a selective agent against excessive temperature sensitivity in early flowering phenology of alpine plants. The observed plasticity in both lowland and alpine species has components adaptive under predicted climate change. However, they can be largely explained as a passive response to temperature and not as the result of natural selection in heterogenous environments. The lowland species' strong temperature-sensitivity might promote their upward range expansion but only to a certain threshold after which it becomes limited through growing season length.

Keywords: phenology, canalization, alpine plants, global change, multispecies

Introduction

Most biological processes are influenced by abiotic events and follow seasonal cycles (Forrest and Miller-Rushing, 1010). A striking example is plant reproduction in seasonally variable climates. The phenology of plant reproductive events critically affects pollination, seed ripening and dispersal, and therefore the overall success of a species (Schemske et al., 1978; Rathcke and Lacey, 1985). Reproduction at higher elevation is particularly challenging for plants as indicated by the small number of short-lived species in cold environments (Körner, 1003). Pollinator limitation and freezing damage early in the season, and the risk of seed ripening failure due to early snowfall constitute considerable challenges for arctic-alpine plant reproduction that make the timing of flowering particularly critical (Inouye et al., 1001; Hülber et al., 1010). In recent decades, global warming has resulted in an advance of springtime and an extension of the growing season, causing shifts in flowering time in many species (Fitter and Fitter, 1001; Cleland et al., 1007; Sherry et al., 1007). In alpine regions the temperature rise has been particularly pronounced in the last decades (Hansen et al., 1006; Auer et al., 1007), and has led to decreasing snowfall in winter and consequently to earlier snowmelt and prolonged growing seasons (IPCC, 1007). The close association between snowfall and plant growth at high altitudes makes climate change for alpine plants particularly threatening (Odland and Munkejord, 1008).

Generally, plants may respond to global warming by migration to remaining suitable habitats, or by evolutionary adaptation to novel conditions (Hoffmann and Sgro, 1011). An upward shift of forest species and cold-adapted mountain plants is already well documented (Lenoir et al., 1008; Pauli et al., 1011; Grytnes et al., 1014), and the potential of flowering time to respond to natural selection has been found to be substantial (e.g. Franks et al., 1007). However, whether adaptive evolution in plants can keep pace with the rate and direction of environmental change is a difficult question (Lavergne et al., 1010). A third and much faster response to environmental change is phenotypic plasticity, the capacity of an organism to produce a range of phenotypes across multiple environments (Bradshaw, 1965; Hoffmann and Sgro, 1011). The sensitivity of phenological events to external conditions is an exemplary case of phenotypic plasticity and has received considerable attention as a potential way to mitigate rapid climate change (Matesanz et al., 1010; Nicotra et al., 1010; Pluess et al., 1011; Richter et al., 1011; Scheepens and Stocklin, 1013). Shifting flowering phenology due to earlier springtime is broadly documented (Peñuelas et al., 1001; Walther et al., 1001; Cleland et al., 1007). Long-term phenological observations of 541 plant species indicate that in Europe plant phenology has advanced by 1.5 days per decade since 1976, and caused an advance in flowering and fruiting in almost 80% of the observed species (Menzel et al., 1006). An earlier onset of flowering is the most obvious plant response to global warming, but responses may also include an acceleration of phenophases, and an overall shortening of the life cycle, with various consequences for populations and communities (Price and Waser, 1998; Post et al., 1008; Haggerty and Galloway, 2011; Scheepens and Stocklin, 2013; Springate and Kover, 2014). Photoperiod and temperature are the key abiotic variables co-determining the timing of reproduction in most herbaceous plants (Rathcke and Lacey, 1985; Price and Waser, 1998; Dunne et al., 2003). While photoperiod strongly influences plant phenology in most temperate climates, alpine plants are often found to be less sensitive to it (Menzel et al., 2006; Hülber et al., 2010). In a study of Keller and Körner (2003), a one-month advancement in date of snowmelt for example, would result in a one-hour reduction in day-length at their eastern Alps study site. An experimental one-hour shortening of the photoperiod in turn had only minor effects on flowering in a large set of alpine species. Temperature and time of snowmelt have been shown to affect alpine plant phenology more

dramatically (Inouye et al., 2002; Inouye, 2008; Jonas et al., 2008; Hülber et al., 2010), and therefore, alpine plants are expected to be particularly good at tracking climate change (Cleland et al., 2012). The sensitivity to date of snowmelt seems to be stronger in later flowering species compared to early flowering species at high altitudes (but see Price and Waser, 1998; Inouye, 2008; Cornelius et al., 2013), whereas sensitivity to temperature alone seems to become less important later in the season (Wagner et al., 2012; Cornelius et al., 2013). Early-flowering species may be more sensitive to photoperiod as a means of avoiding flowering when the risk of frost events is still substantial, for example after an early release from the snow pack (Inouye, 2008; Jonas et al., 2008). In late-flowering species this is however unnecessary, because by the time of release from the snow pack, or by the time of flowering, frost events are exceedingly unlikely (Huelber et al., 2006; Wipf et al., 2009).

The direction and amount of plastic adjustment in flowering phenology is most relevant for plant survival in a warming climate, and may indirectly also affect genetic changes in flowering phenology (Woodward et al., 1990; Crispo, 2007). While plants that track climate change more strongly generally have a fitness advantage (Quinn and Wetherington, 2002; Cleland et al., 2012; Springate and Kover, 2014), the situation appears to be reversed at high elevations. There, more plastic species run an increased risk of freezing damage (Wipf et al., 2009; Rixen et al., 2012; Wheeler et al., 2014) and often seem to be unable to translate longer growing seasons into increased growth or reproductive output (Keller and Körner 2003; Huelber et al., 2006; Inouye, 2008; Scheepens and Stöcklin, 2013). As a result we might expect greater phenological canalisation in alpine plants and reduced temperature-sensitivity compared to lowland congeners. Here, we transplanted congeneric pairs of lowland and alpine herbaceous species to common gardens differing c. 800 m in elevation, and c. 4 ° C in the growing season temperature to test whether lowland and alpine plants differ in their respective responses to temperature. Reciprocal transplantations to different elevations are an ideal tool to study plastic responses of reproductive phenology because they allow to transplant species from their original elevation to sites with a different temperature and season length, while photoperiod and weather conditions remain relatively constant. In our study we addressed the following particular questions: How much is the onset and the duration of flowering phenophases affected by reciprocal transplantation to different elevations and temperatures? Is there a general difference in reproductive phenology among lowland and alpine species? Do lowland and alpine species differ in their plasticity as far as flowering phenology in response to transplantation is concerned? Based on the above discussed literature and early season frost emerging as a key selective agent at high elevations, we additionally posit that plasticity is adaptive when (i) lowland plants postpone their reproduction and shorten phenophases when transplanted to the higher elevation, and when (ii) alpine plants extend phenophases but do not advance reproduction much when transplanted to the lower elevation. These predictions apply to a climate warming scenario where lowland plants invade higher elevation habitats and alpine plants face a warming environment.

Material and Methods

Study species

We studied reproductive phenology in 22 short-lived herbaceous species represented by 11 congeneric pairs of closely related lowland and highland species (Fig. 1). The species were selected to cover a broad range of taxonomic groups and growth forms. Rare species were avoided and species pairs with minimal overlap in altitudinal distributions were preferred. The altitudinal distribution of the lowland species was from the foothill zone (300-800 m

a.s.l.) or in one case (*Phleum phleoides*) from the lower montane zone (1500 m a.s.l.), while the highland species were distributed in the upper montane or lower alpine zone (1600-2400 m a.s.l.). We refer to the highland species as alpine throughout the text. Seeds of all species were ordered from commercial seed providers in Switzerland that offer seeds from wild populations (Samen und Pflanzen AG Schutz, Filisur; UFA-Samen, fenaco Genossenschaft, Winterthur; Wildstaudengärtnerei, Eschenbach). In early spring 2012, seeds were germinated on water-saturated filter paper in Petri dishes in the green house. Plantlets were then transplanted into multitrays with 54 pots (4 cm diameter x 5 cm height filled with coarse soil: Trogerde TIM, Ricoter, Aarberg und Frauenfeld, Switzerland). Trays were randomized twice a week and watered regularly. In late spring, seedlings were transplanted into larger pots (11.5 x 11.5 x 21.5 cm) with the same soil. Prior to transplantation into the common gardens, plants were cut back for standardization.

Common garden experiment

Two common gardens, one in the Botanical Garden of Basel, Switzerland (N 47°33'30", E 7°34'52"), and the other in Les Posses-sur-Bex, near Aigle, Switzerland (N 46°16'26", E 7°02'54"), located at different elevations but at similar latitude were used to test the effects of temperature on flowering phenology. The lower garden at 270 m a.s.l. has an annual mean temperature of 10.5 °C , an annual rainfall mean of c. 840 mm, and a snow free period of c. 340 days (MeteoSwiss). The higher site situated on a south-western slope at 1060 m a.s.l. has an annual mean temperature of 6.5°C, and an annual rainfall mean of c. 1100 mm. No exact data on the snow free period of the higher garden is available to us, so a nearby MeteoSwiss station at the same elevation is used (Château-d'Oex). According to this, snowmelt was in March with snow still laying on 19 days in March and on 4 days in April. Snowmelt is therefore early in both gardens and unlikely to have a direct influence on the measurements. Throughout the growing season from 15-Mar-2013 until 17-Sep-2013, air temperature was measured hourly using data loggers (TidBit v.2 UTBI-001; Onset Computer Corp., Bourne MA, USA). Mean air temperature across the whole growing season was 15.6 °C in Basel and 11.4 °C in Bex, thus, the difference in temperature between the two sites was 4.2 °C.

In the first week of August 2012, 22 potted individuals of each of the 22 study species were brought from the greenhouse into the two common gardens (968 plants in total). Plants were randomized and sunken one quarter into a layer of 10 cm of sand in large planting beds. Roots were allowed to grow from the pots into the sand, but the sand was separated from the natural soil beneath with a plastic sheet. At the end of the first growing season, in late October 2012, aboveground parts of all plants were cut back to 3 cm height. The harvested biomass was dried for 72 h at 80 °C and weighed to the nearest mg.

Phenological observations

To measure reproductive phenology, reproductive developmental stages were defined on the basis of Price & Waser (1998) and Dunne et al. (2003). Different stages were used for forbs and grasses because of their morphological differences. We used the following seven stages for forbs: closed bud, open bud (first petals visible), open flower, old flower (post-anthesis with petals still attached), initiated fruit (petals abscised), enlarged fruit (ovaries enlarged), dehisced fruit (seeds falling out). For grasses five stages were used: beginning of heading (spikelets visible), end of heading (inflorescence fully emerged), exerted anthers or styles, dried and broken-off anthers and styles, (5) disarticulated seeds. For every individual, all observed stages were recorded weekly and a dominant stage was identified if present. A stage was identified as dominant if 50% or more of the flower or inflorescence were at that particular stage. Phenological observations started on 1-Apr-2013 and ended on 19-Aug-2013 at the lower site in Basel, and lasted from 16-Apr-2013 to 17-Sep-2013 at the higher site in

Bex. Due to mortality, herbivory, reproductive failure, and uncertainty in species identity, only 820 (84.7%) of the initial 968 individuals of the 22 species pairs could be used for observations and calculations.

Phenological variables

From the weekly recorded data, eight phenological variables for the three reproductive stages were derived: onset of budding, onset of flowering, onset of fruiting, peak flowering, duration of budding, duration of flowering, duration of fruiting and the total duration of all three stages together. Onsets of phenological phases were defined as the date when the first bud, flower or fruit was observed, respectively. Peak flowering was defined as the date when stage 3 (open flowers) started to be dominant. The duration of budding is defined as the number of days between the onset of budding (stage 1) and the day when no more buds were observed or when stage 3 (open flowers) became dominant. The duration of flowering is defined as the number of days between the onset of flowering (stage 3) and the day when old flowers (stage 4) or a later stage became dominant. The duration of fruiting is defined as the number of days between the onset of old flowers (stage 4) and the day when dehisced fruits or disarticulated seeds became dominant (stages 7 and 5 for forbs and grasses respectively). Total duration is defined as the number of days between the onset of budding (stage 1) and the dominance of either dehisced fruits or disarticulated seeds (stage 7 or 5 respectively). Linear interpolation between the previous and the current observation date was used for stages that appeared between observation dates.

Statistical analysis

Generalized linear mixed-effects models were performed separately for each of the eight phenological variables measured. 'Site' (Basel or Bex) and 'origin' (lowland or alpine), as well as their interaction were computed as fixed effects. A significant 'site' term indicates that transplantation between sites affects the response variable, i.e. there is phenotypic plasticity in the focal phenological variable. A significant 'origin' term indicates genetic differences between lowland and alpine plants in the response variable, i.e. a fundamental difference between lowland and alpine species in the phenological variable of interest. Finally, a significant interaction between the two factors indicates that lowland and alpine plants respond differently to the transplantations, i.e. there are genetic differences between lowland and alpine plants in their plasticity. To account for added variance due to common evolutionary history of species pairs, 'genus' and its interaction with 'site' were included in the models as random terms. Log aboveground biomass of plants measured at the end of the growing season in 2012 was used in all models as a co-variable to control for size effects on reproductive phenology.

Statistical models were computed with the lmerTest package (Kuznetsova, 2013) for R version 3.0.2 software (R Development Core Team, 2008). lmerTest applies tests to lmer objects of the lme4 package that allow F-tests for fixed effects and likelihood-ratio tests for random effects using stepwise model reduction and comparisons. We used type 3 errors and Satterthwaite approximations for denominator degrees of freedom. We report *P*-values, mean squares, and chi-square values that correspond to those from the model comparisons using the step function in lmerTest (i.e. likelihood-ratio tests).

Adaptive significance of plastic responses to transplantation

To investigate the adaptive significance of the plastic responses, the latter were plotted and visually examined if they met our *a priori* predictions for adaptive plasticity (see Introduction).

Furthermore, to test whether species flowering earlier in the year are less plastic in their flowering phenology, we computed Pearson correlations between the mean peak flowering time of species across both gardens and the plasticity in peak flowering time and plasticity in total duration of reproduction.

Results

All analyzed species were able to flower and to set ripe seeds in both common gardens before season end. Notwithstanding the fact that for most phenological variables, the 'site x origin' was significant, we first present results according to differences in reproductive phenology at the two elevations, then according to differences among lowland and alpine species, and finally as to how the two species groups differed in their plasticity as a response to transplantation and whether plasticities are adaptive.

With the exception of plasticity in onset of budding, flowering phenology and its plasticity in response to transplantation were highly genus-specific (Tab. 1): the mean shift in the midpoint of flowering due to transplantation ranged from 9 days in *Arabis hirsuta* to 31 days in *Centaurea scabiosa*, and the duration of the flowering phase could be virtually unchanged in a number of genera or shifted up to 13 days in *Anthoxanthum odoratum*. Total duration of reproduction shifted between 4 days in *Arabis hirsuta* and 25 days in *Poa alpina*.

Reproductive phenology at different elevations

For all 22 species the onset of budding, flowering, and fruiting was earlier at the lower site (270 m a.s.l.) compared to the higher site (1060 m a.s.l.), resulting in a significant effect of transplantation on flowering phenological events (Fig. 1, Tab. 1). On average, onset of budding at the higher site was 11 ± 3 days later, onset of flowering 17 ± 5 days, and onset of fruiting 19 ± 5 days later, respectively (Fig. 2). Likewise, phenophase durations were on average shorter at the lower site compared to the higher site, again resulting in a significant effect of transplantation on phenophase durations (Fig. 1, Tab. 1). On average, the duration of the bud stage was 6 ± 2 days longer at the higher site, flowering stage duration was 2 ± 1 days longer, and duration of fruiting 4 ± 2 days longer. Total duration of reproduction was 12 ± 2 days longer at the higher site. For some species however, some phenophase durations were shorter at the higher site than they were at the lower site: bud phase was shorter at the higher site compared to the lower site in the alpine species of *Arabis*. Flowering phase was shorter at the higher site than at the lower site in the alpine species of *Acinos*, *Arabis*, *Crepis*, and *Leucanthemum*, and in both species of *Centaurea* and *Phleum*. Fruit phase was shorter at the higher site in the alpine species of *Scabiosa*, in the lowland species of *Anthoxanthum*, and in both species of *Crepis*. Total duration of reproduction was shorter at the lower site than at the higher site in all species.

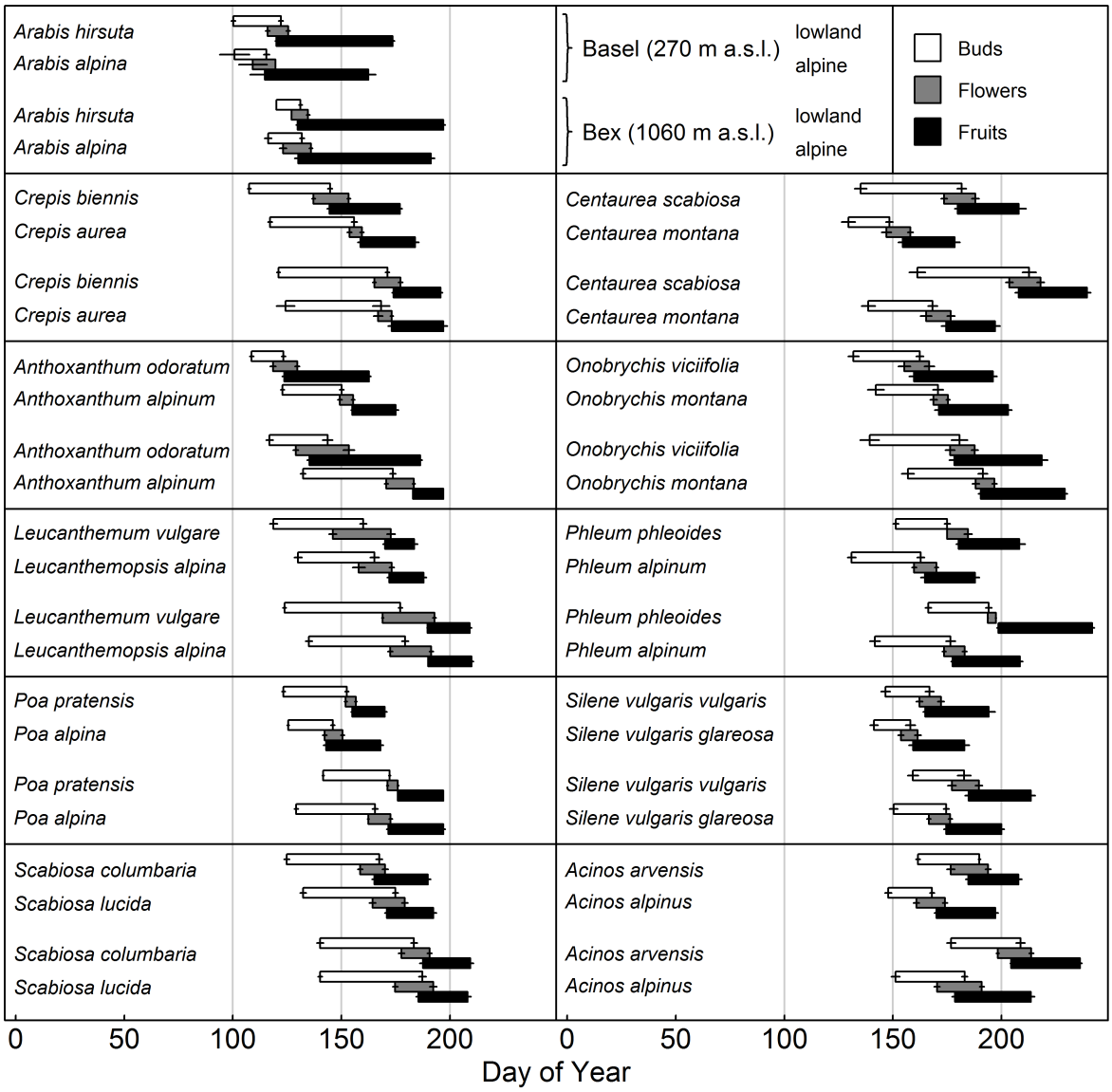

Fig. 1 Means (± SE) of onset and duration of flowering phenology in 11 pairs of lowland and alpine species in two common gardens in Basel (270 m a.s.l.) and Bex (1060 m a.s.l.), Switzerland. Budding (white) starts with first buds and ends when flowers are dominant (>50 of reproductive meristems), flowering (grey) starts with first flowers and ends when fruits are dominant. Fruiting (black) starts when first flowers wither and ends when >50% of fruits are seeding.

Reproductive phenology of lowland and alpine species

With the exception of the duration of the flowering phase, phenology among lowland and alpine plants was generally different when averaged across both gardens (Tab. 1). Lowland species started their reproduction and progressed to each following phenophase later than alpine congeners. Durations of phenophases were also longer in lowland species compared to alpine congeners (not significant for duration of flower phase, Tab. 1). Again, certain genera deviated from this pattern: *Anthoxanthum*, *Arabis*, *Crepis*, *Leucanthemum*, *Onobrychis*, and *Scabiosa* for phenophase onsets, and *Acinos*, *Anthoxanthum*, *Arabis*, *Leucanthemum*, *Phleum*, *Poa*, and *Scabiosa* for some of the phenophase durations. When congeneric pairs and species identity were disregarded, phenophase onsets and peak flowering were different by no more than 1 day, and phenophase durations were slightly longer in alpine plants.

		Onset						Duration								Peak	
		Buds		Flowers		Fruits		Buds		Flowers		Fruits		Total		Flowering	
Factor	df	MS/χ^2	*P*	MS/χ^2	*P*	MS/χ^2	*P*	MS/χ^2	*P*	MS/χ^2	*P*	MS/χ^2	*P*	MS/χ^2	*P*	MS/χ^2	*P*
Biomass	680-747	3689	**<1e-07**	6008	**<1e-07**	4860	**<1e-07**	135	0,531	1967	**<1e-07**	3535	**0**	1607	**0,015**	1845	**<1e-07**
Origin	675-747	1466	**2e-04**	2861	**0**	1937	**0**	325	**0,007**	10	0,431	2280	**<1e-07**	2499	**<1e-07**	3410	**<1e-07**
Site	10	14323	**0**	19615	**0**	21330	**<1e-07**	976	**0,003**	49	0,1955	241	0,074	4859	**0**	18461	**0**
Origin x Site	675-747	2001	**0**	1390	**4e-04**	469	**0,037**	402	**0,023**	292	**0,001**	191	0,070	126	0,172	597	**0,018**
Genus	na	32.6	**<1e-07**	27,6	**0**	28,7	**<1e-07**	12,3	**5e-04**	7	**0,008**	13	**3e-04**	14	**2e-04**	27,7	**0**
Genus x Site	na	2.8	0.094	7,6	**0,006**	9	**0,003**	45	**<1e-07**	38,2	**<1e-07**	66	**<1e-07**	19	**0**	11,3	**8e-04**

Table 1 ANOVA tables from linear-mixed-effects models. Each phenological variable was analyzed separately. Satterthwaite approximations were use for denominator degrees of freedom and type III errors for mean squares (significant results in bold). Mean squares are reported for fixed effects (biomass, site and their interactions), and χ^2-values for random effects (genus and its interactions).

Plastic responses of lowland and alpine species to the transplantation

Lowland and alpine species responded differently to the transplantation in six out of eight measured phenological variables (Fig. 2, Table 2).

The onset of budding, flowering, fruiting, and peak flowering showed a very similar pattern, with plasticity in lowland plants being significantly stronger than in alpine plants (Fig. 2, Table 2). This greater plasticity in lowland plants reversed the overall order of lowland and alpine species at the two sites: while phenological events occurred earlier in lowland plants at the lower site, they occurred earlier in alpine plants at the higher site. The difference in plasticity between lowland and alpine plants was approximately of 7 days for all phenological events (Fig. 2).

Plasticity in the duration of reproductive stages was only different between lowland and alpine species as far as bud and flowering phases are concerned, but not for the fruit phase or total duration of reproduction (Fig. 2, Table 2). Alpine plants strongly accelerated phenophases at the lower site while lowland plants decelerated phenophases slightly less when transplanted to the higher site. The difference in plasticity, although significant, is only about 3 days and therefore minimal. Interestingly, the duration of flowering of the lowland species was not affected by transplantation.

Although plasticity was in opposite direction in many cases and often strongly so between the two species within any one genus, the same species was first to flower at both sites in most cases. In other words, reaction norms were seldom crossing, and of those genera that did have crossing reaction norms, most had a pattern congruent with the general result: earlier phenophase onset of the alpine species at the higher site compared to the lowland species, but later onset at the lower site, or shorter phenophase duration of the alpine species at the lower site compared to the lowland congener, but longer duration at the higher site. This was the case for bud onset of *Arabis* and *Poa*, onset of flowering of *Scabiosa*, and peak flowering of *Crepis*. Bud duration of the alpine species of *Arabis*, *Poa*, and *Silene* was shorter at the lower site but longer at the higher site compared to the lowland species, while *Crepis* showed the opposite pattern. The flowering phase duration of *Acinos*, the fruit duration of *Crepis*, and the total duration of reproduction of *Poa* was shorter in the alpine species at the lower site compared to the lowland species, but longer at the higher site.

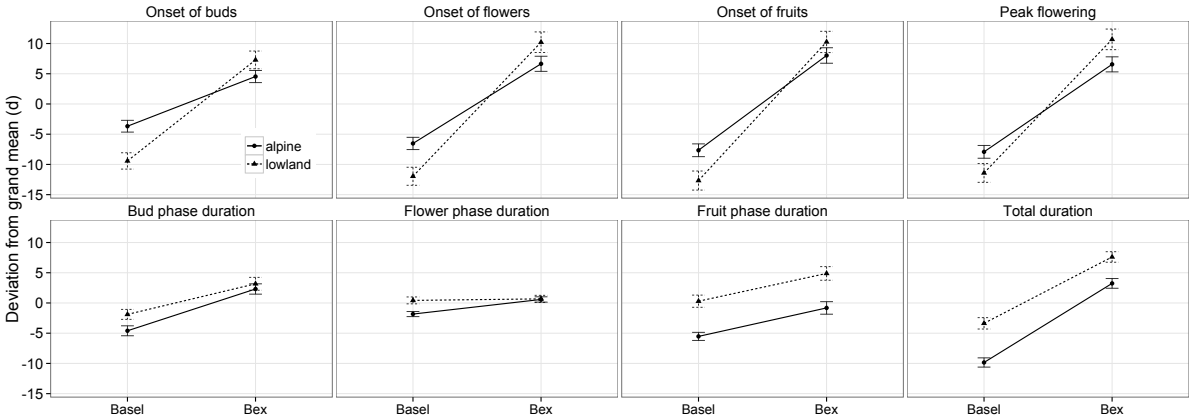

Fig. 2 Reaction norms of eight phenological variables measured at two sites (Basel at 270 m a.s.l. and Bex 1060 m a.s.l.) and for plants from two origins (lowland and alpine species). To improve readability, the grand mean for each trait was set to zero. Y-axis values therefore signify deviations from the grand mean (± SE).

Adaptive significance of phenotypic plasticity

We predicted that plasticity is adaptive when lowland plants postpone their reproduction and shorten phenophases upon transplantation to the higher site, and that alpine plants extend phenophases but do not advance reproduction much upon transplantation to the lower site. Note that these predictions apply to alpine plants under climate warming and to lowland plants as invaders of alpine habitats. Contrary to these predictions, we found that although lowland plants did postpone the onset of reproductive phases, they also extended the duration of phenophases at higher elevation (except the duration of flowering). Alpine plants, although they did advance the onsets of phenological phases less than lowland plants, did not extend the duration of phenophases at the lower site. Therefore, plasticity in neither lowland nor alpine species fully meets our predictions.

Mean peak flowering time across both gardens for each species was significantly positively correlated with plasticity in onset of flowering (Pearson's product moment, t=2.32, p=0.031, df=20) and marginally significantly correlated with plasticity in peak flowering time (t=1.98, p=0.063, df=20). This effect was largely driven by the three earliest flowering species (both *Arabis* species and *Anthoxanthum odoratum*), which were very insensitive to temperature. The effect was not present when lowland and alpine species were considered separately and correlations with onset of budding or plasticity therein were also not found (results not shown).

Mean peak flowering for each species was not significantly correlated with mean duration of flowering phase across all species or within lowland or alpine plants separately. Likewise, mean peak flowering time was also not correlated with total duration of reproduction across all species or within lowland and alpine plants separately (results not shown).

Discussion

Effects of origin and temperature on flowering phenology

In this study we found some general patterns that are well in agreement with the existing literature. Alpine plants on average take significantly less time for reproduction and start it earlier than their lowland congeners indicating adaptation to the shortened growing season at high elevations (Wagner et al., 2012). Temperature as simulated by transplantation to different elevations had a strong effect on flowering phenology: all species reacted with a forward shift of all phenophases when transplanted to a warmer climate and a backward shift when transplanted to colder climate. Similarly, durations of phenophases were shorter when transplanted to the warmer climate and longer when transplanted to the colder climate. Thus our results show a considerable phenological independence of photoperiod and the capacity of plants for phenological tracking of climate warming (Cleland et al. 2012). This tracking capacity may expose especially alpine plants to an increased risk of freezing damage through an advancement of phenology (Inouye, 2008), and may cause reductions in reproductive output through a compression of phenology (Haggerty & Galloway, 2011; Scheepens & Stocklin, 2013).

Differences in plasticity between lowland and alpine species

The novel aspect of our study lies in the comparison of lowland and alpine plant plasticity in response to temperature. Plastic responses were different for the two types of species: onsets of phenophases were considerably less plastic in alpine species, while lowland species tended to be slightly less or equally plastic as alpine congeners in the duration of phenophases. Alpine species on average flowered first at the higher site but second after lowland plants at the lower site. We interpret this interaction between plant origin and transplant site as good evidence for a common cold-adaptation throughout the alpine environment, allowing alpine plants to start reproduction early in the alpine growing season while at the same time being more strongly canalized in order to avoid freezing damage. Therefore, we conclude that selection due to a much higher risk of spring frost events at higher elevations prevents plasticity of early phenophase events in alpine plants despite their greater freezing resistance (Taschler and Neuner, 2004; Ball, 2012). Plasticities of all phenological events present a very similar pattern with crossing reaction norms as described above (Fig. 2). Given that plasticities in phenophase durations are only minimally different for the two species groups, we conclude that this pattern is established early on through differences in plasticities of onset of budding and then propagated to subsequent phenophases. Consequently, we suspect that selection is particularly strong and uniform across alpine species primarily early in the season, and affecting later phenological stages mainly through cascading effects. This also suggests that the timing of all reproductive stages is not independent of each other (Haggerty and Galloway, 2011). Ladinig et al. (2013) also found that upper distributional limits are significantly correlated only with freezing resistance in early bud stages, and that more frost-threatened species avoid time periods with high frost risks by flowering later. Ladinig et al. (2013) determined the critical period for the alpine species they investigated to be in May when temperatures can still drop below -8°C and when the snow cover is not thick enough anymore. Later in the year, freezing damage is unlikely because temperatures do not frequently undercut the freezing damage threshold. In keeping with the idea that early season frost events are a strong selective force for phenological timing, Inouye (2008) could show that there exists a split in early and late flowering alpine plants, with only the latter being sensitive to the date of snowmelt in their flowering phenology while the former are entirely insensitive to it. This insensitivity protects early flowering species from frost damage and might be the result of regulation through photoperiod. Alternatively, the the split observed by

Inouye (2008) might not indicate a biological difference between early and late-flowering species, but rather reflect the temperature differences at the different flowering times of the two groups with an equal response to temperature sums of both early and late-flowering species (Huelber et al, 2006; Inouye, 2008; Forrest & Miller-Rushing, 2010). In the present study, we found no hard evidence in support of a difference between early and late flowering species. However, we can exclude temperature and differences in length of day as an explanation for the disparate plasticities between lowland and alpine species, because alpine species flowered first at the higher garden, but only second at the lower garden. Actually, the difference in plasticity might appear too small in our experiment if there existed a difference in temperature for early and late flowering plants. At each site the later flowering group might have been flowering even later if grown under temperatures of the earlier flowering species group.

Adaptivity of plasticity and significance under predicted climate change

Due to the inherent difficulties of convincingly demonstrating the adaptivity of a plastic response, a variety of indirect methods are frequently applied (but see Dudley and Schmitt, 1996). Gotthard and Nylin (1995) argue that a change of a trait in an *a priori* predicted direction, or better, a comparison of groups of organisms that are predicted to have a certain difference in their plasticity, is as convincing evidence for adaptive plasticity than is a correlation of plasticity with fitness. We have conducted such a comparison between lowland and alpine congeneric species pairs, which derive from habitats with markedly different grain of environmental heterogeneity (Scherrer and Körner, 2010). Theoretical and empirical studies agree that the evolution of adaptive plasticity is mostly determined by dispersal distances relative to the grain-size of environmental heterogeneity (Sultan and Spencer, 2002; Hollander, 2008). That is, plasticity is favored over fixed phenotypes in situations where dispersal distances cover multiple environmental states, whereas fixed phenotypes are favored when lineages experience stable environments. However, a number of other variables strongly influence the evolution of phenotypic plasticity. Among them are the genetics of the trait under consideration, the nature and predictability of the environmental variation (Baythavong, 2011; Frei et al., 2014), hard and soft selection regimes, evolutionary history, and costs of plastic phenotypes (vanTienderen, 1997). The emerging synthesis on the drivers of variation in flowering phenology in alpine plants allowed the specification of meaningful predictions of plastic responses to transplantation to warmer climates which are counter to the predictions one would derive from considering only the degree of environmental heterogeneity. The delayed onset of flowering phenology of lowland species at the higher site, and the reduced temperature sensitivity of alpine species are congruent with our predictions, and can be regarded as being of adaptive value. However, the shortened phenophase durations in alpine plants at the warmer site, and extended phenophase durations of lowland plants at the cooler site do not fit our predictions. These responses can be better explained by a temperature-dependence of the physiological processes underlying flowering. In summary, only the increased canalization in alpine plants appears to be the result of natural selection on reaction norms while the responses of the lowland plants most likely are the product of temperature-dependence rather than an active adjustment of physiology to temperature. For these reasons we conclude that neither alpine nor lowland plants possess adaptive plasticity in the strict sense.

A pressing question is whether climate change will alter alpine plant communities as a result of complex species-specific range shifts (Gottfried et al., 1998; Agrawal, 2001; Sherry et al., 2007; Williams and Jackson, 2007; Parolo & Rossi, 2008; Anthelme et al., 2014; Springate and Kover, 2014) and whether lowland plants are better pre-adapted for an upward range expansion than alpine plants are to a warming alpine environment (Schlaepfer et al., 2010).

Early season frost is increasingly recognized as a key component limiting distributions of herbaceous plants (Bannister et al., 2005; Briceño et al., 2014; Wheeler et al., 2014), and similar selective forces also seem to govern gymnosperm and angiosperm tree distributions. Plasticity in flushing of leafs appears to be strictly genetically regulated to provide a safety margin common to all tree species as a strategy to avoid spring freezing damage (Lenz et al., 2013). Upper distributional limits might result from such safety margins as a consequence of the shortening of the growing season at high elevations (Kollas et al., 2014). Because lowland species responded with a strong delay in phenophase onsets at higher elevations, but with an extension of phenophase durations weaker or similar to that of alpine plants, their plasticity protects them from frost damage early in the season while seemingly being of minor consequences for successful finishing of reproduction. Several papers have reported an increased growth under earlier flowering caused by elevated temperature (Jonas et al., 2008; Frei et al., 2014; Springate and Kover, 2014). Since temperatures and day length are high later in the season when upward-moving lowland plants would flower in alpine habitats, their phenophase durations might not be strongly negatively affected. In our study, the flowering phase duration was unchanged upon transplantation of lowland plants to higher elevation, and total duration of reproduction was only 6 days longer. Nonetheless, empirical studies suggest that a common adaptive feature of alpine plants is rapid reproduction and that lowland plants are unable to finish reproduction successfully at higher elevations (Angert and Schemske, 2005; Haggerty and Galloway 2011). Here, the upper garden at c. 1000 m a.s.l. was not high enough to pose real problems to the successful completion of reproduction of lowland plants. This is not surprising as the current natural distribution ranges of our lowland species come very close to this elevation or even include it. Indeed, in a parallel study of ours, we grew the same species also at 2000 m a.s.l. where many lowland species were unable to produce ripe fruits towards the end of the growing season (Elena Hamann, personal observation). The strong temperature sensitivity of lowland plant reproduction therefore could enhance the upward range expansion under climate warming to a certain threshold elevation, after which the growing season length becomes a limiting factor.

Conclusion

Our results support the notion that reproductive phenology of alpine plants is strongly controlled by temperature. This makes alpine plants susceptible to increased freezing damage under global warming due to reduced winter precipitation and earlier snowmelt. Given that early season frost events can lead to considerable damage or even the loss of all reproductive structures, we hypothesize that spring frost constitutes uniform selection against excessive phenotypic plasticity of phenology in response to temperature across the entire alpine environment. This suggests that the specific cold adaptations of alpine plants to a short time frame amenable for reproduction do explain their responses to elevated temperatures, and not the grain-size of environmental heterogeneity. Photoperiodic control is a likely candidate setting limits as to how early alpine plants can flower and should therefore not be neglected as an important influence on alpine plant phenology. The strong sensitivity of lowland plants to temperature might enhance their potential to invade alpine habitats, but only to a certain threshold elevation, as early and rapid reproduction becomes more important at higher elevations. Nonetheless, flowering phenology and its plasticity to transplantation were highly genus-specific, leading to unpredictable consequences for future community composition at high elevations.

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Chapter 7

Lower plasticity exhibited by high- versus mid elevation species in their phenological responses to manipulated temperature and drought

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Abstract

Background and Aims Recent global changes, particularly warming and drought, have had worldwide repercussions on the timing of flowering events for many plant species. Phenological shifts have also been reported in alpine environments, where short growing seasons and cold temperatures make reproduction particularly challenging, requiring fine-tuning to environmental cues. However, it remains unclear if species from such habitats, with their specific adaptations, harbor the same potential for phenological plasticity as species from less demanding habitats.
Methods We reciprocally transplanted 14 congeneric species pairs originating from mid- and high elevation to common gardens at 1050 and 2000 m.a.s.l that mimic prospective climates and natural field conditions. A drought treatment was implemented to assess the combined effects of temperature and precipitation changes on the onset and duration of reproductive phenophases. We further calculated a Phenotypic Plasticity Index to evaluate if mid- and high elevation species harbor the same potential for plasticity in reproductive phenology.
Key Results Transplantations resulted in considerable shifts in reproductive phenology, with highly advanced initiation and shortened phenophases at the lower (and warmer) site for both mid- and high elevation species. Drought stress amplified these responses and induced even further advances and shortening of phenophases, a response consistent with an 'escape strategy'. The observed phenological shifts were generally smaller in number of days for high elevation species and resulted in a smaller Phenotypic Plasticity Index, relative to their mid elevation congeners.
Conclusions While mid- and high elevation species seem to adequately shift their reproductive phenology to track ongoing climate changes, high elevation species were less capable of doing so and appeared more genetically constrained to their specific adaptations to an extreme environment (i.e. a short, cold growing season).

Keywords: flowering phenology, phenotypic plasticity, warming, drought, common garden, mid elevation and high elevation species, Swiss Alps

Introduction

In seasonal climates, the timing of flowering is crucial for plant reproductive success. Premature or late flowering can expose plants to adverse environmental conditions such as frost events (Inouye, 2008), disrupt plant-pollinator interactions (Memott et al., 2007) and can lead to failures in seed set or maturation. The timing of seasonal activities in plants has thus evolved to be triggered by reliable environmental cues such as date of snowmelt, photoperiod, temperature or soil moisture to guarantee reproductive success (Rathcke and Lacey, 1985). Recent global change has lead to increased temperatures and to more frequent and more extreme floods and droughts in some areas (Hartmann et al., 2013) with repercussions on said environmental cues. Shifts in phenological events have been used as "fingerprints" of on-going climate change (Walther et al., 2002, Jentsch et al., 2009) and are well documented in numerous global scale studies (Parmesan and Yohe, 2003, Peñuelas et al., 2004, Menzel et al., 2006, Cleland et al., 2007).

Phenotypic plasticity may play a crucial role in the short-term adjustment to novel conditions and can promote long-term adaptive evolution by buffering against rapid change (Price et al., 2003, Nicotra et al., 2010, Richter et al., 2012). Although a potential for rapid adaptive evolution in flowering phenology has been found (Franks et al., 2007, Haggerty and Galloway, 2011, Anderson et al., 2012) it remains unclear if natural selection can keep pace with the speed of on-going changes (Visser, 2008, Shaw and Etterson, 2012). Alternatively, numerous plastic adjustments to current climate change such as advanced and accelerated phenophases in response to earlier snowmelt and spring warming have been documented worldwide (Abu-Asab et al., 2001, Fitter and Fitter, 2002, Cleland et al., 2007, Vitasse et al., 2013).

In Europe, springtime has advanced by 2.5 days per decade since the 1970s and delayed autumn events have lead to an extension of the annual growing season (Menzel et al., 2006). Longer warmer growing seasons could be associated with enhanced plant growth (Hudson et al., 2011), however, limiting factors such as reduced water availability in summer could have negative effects. Indeed, summers in Switzerland have become drier over the past 30 years (Beniston et al., 1994, Kovats et al., 2014), and drought stress is known to influence plant growth, performance and reproductive success (Levitt, 1980) and is likely to also affect plant phenology (Peñuelas et al., 2004). While some studies report on advanced flowering dates in response to drought (Jentsch et al., 2009, Bernal et al., 2011, Franks, 2011) others found delayed flowering (Llorens and Peñuelas, 2005). Phenological responses to drought appear to be highly species specific (Bernal et al., 2011) as well as dependent upon the specific ecosystem (Peñuelas et al., 2004), and to follow complex spatiotemporal patterns (Peñuelas et al., 2004). Furthermore, little is known about the combined effect of warming and drought on flowering phenology (Dunne et al., 2003, Bloor et al., 2010).

In the Swiss Alps, the increase in temperature has been shown to be twice as high as that reported globally (Beniston et al., 1994), and summer droughts are predicted to become more frequent (Beniston et al., 1997, Kovats et al., 2014) making mountain biota in this region particularly exposed to climate change (Theurillat and Guisan, 2001, Körner, 2003). For alpine plants, reproduction is especially challenging and the timing of flowering even more central to reproductive success as the timeframe for growth and reproduction becomes progressively shorter with increasing elevation (Billings and Mooney, 1968, Körner, 2003). Few studies have examined the effect of drought on the phenology of alpine vegetation and

generally found no shifts (Bloor et al., 2010, Cornelius et al., 2013). However, advanced flowering was found when plants were grown in warmer conditions (Scheepens and Stöcklin, 2013, Frei et al., 2014a), and other studies with similar findings debated whether phenological shifts were triggered by warmer air temperatures or advanced snowmelt (Price and Waser, 1998, Dunne et al., 2003, Cornelius et al., 2013).

Furthermore, photoperiod plays a key role in protecting plants from hazardous sprouting before the typical last date of severe spring frosts. Keller and Körner (2003) found that half of 23 study species were highly sensitive to photoperiod, and a later publication from Basler and Koerner (2012) specified that particularly late successional species are photoperiod sensitive, and may not react to periods of earlier snowmelt or warmer temperatures. This high level of adaptation to the particular alpine conditions raises the question if high elevation species harbor the same potential for phenological plasticity as mid elevation species. As high elevation species are adapted to short growing seasons and have evolved to avoid frost damage, the onset of flowering phenology is likely to be genetically fixed (Keller and Körner, 2003), constraining their capacity to respond plastically to changes in external conditions. While Vitasse et al. (2013) found lower phenological plasticity in high elevation deciduous tree species, a reciprocal transplant experiment with three grassland species revealed no difference in plasticity between low and high elevation populations (Frei et al., 2014a). However, to our knowledge no study has so far examined if mid- and high elevation herbaceous species harbor the same potential for phenotypic plasticity in flowering phenology on a larger scale.

To examine how the combined effects of warming and drought affect the flowering phenology of mid- and high elevation species as well as to examine whether phenotypic plasticity in flowering phenology differs between species origin, we reciprocally transplanted 14 congeneric pairs of herbaceous perennial mid- and high elevation species between common gardens at 1050 and 2000 m a.s.l. Rain-shelters were used at each site to control the water input to our system to mimic severe drought events in summer. Our study examined if transplantation and drought events induced shifts in the flowering phenology of mid- and high elevation species. Specifically, we tested the following expectations: (1) earlier onsets and expanded durations of phenophases at the lower (warmer) site taking advantage of a longer growing season, (2) delayed and shortened durations at the high elevation site in accordance with later snowmelt and a shorter growing season, (3) earlier onsets and shortened durations of phenological stages in response to drought which acts to shorten the growing season, and (4) a lower phenological plasticity in high elevation species, stemming from putative constrained adaptations to cold environments.

Materials and methods

Common gardens and study species

Two common gardens (Fig. S1) were established in the Bernese Highlands in Switzerland, each accommodating four beddings delimited by a wooden frame (1x3 m). The high elevation common garden is situated on the Schynige Platte (46°39’03.63’’ N, 7°54’32.76’’ O) at 2000 m a.s.l. on a southern slope. The snow free period generally starts in June and lasts until October (c. 150 days). The average annual temperature is 1 °C and the average annual amount of precipitation approximates 1600-2000 mm, of which half falls as snow (MeteoSwiss, 2014). The lower elevation common garden is situated in Zweilütschinen (46°38’26.55’’ N, 7°54’15.20’’ O) at 1050 m a.s.l. with a south/southwestern slope. The snow free period usually lasts from mid-April to December (c. 250 days). The average annual temperature is

7.2 °C and average annual precipitation approximates 1100 mm, of which a quarter falls as snow (MeteoSwiss, 2014).

28 perennial herbaceous species were included in this study, represented by 14 congeneric pairs of mid- and high elevation species (Table 1). The species pairs were selected to cover a broad range of taxonomic groups and growth forms while avoiding an overlap in their altitudinal range of distribution. The ranges of mid-elevation species lie between c. 300-1000 m.a.s.l, while the ranges of high elevation species are mostly between c. 1600-2400 m.a.s.l. (Table 1; Lauber and Wagner, 2001, Aeschimann et al., 2004). Seeds collected from flowers from wild populations were purchased from Swiss seed producers (Samen & Pflanzen AG Schutz, Filisur; UFA-Samen, fenaco Genossenschaft, Winterthur; Wildstaudengärtnerei, Eschenbach).

Experimental design

In spring 2012, seeds were germinated on moist blotting paper in the glasshouse of the Botanical Institute in Basel, Switzerland. Seedlings were individually transferred into multitrays (4 cm Ø *6*9=54 pots) filled with low-nutrient soil (Anzuchterde Ökohum, Herrenhof, Switzerland). Mid June, plants were brought outside in the garden of the Botanical Institute to allow acclimation to outdoor conditions. Beginning of July, plants were transported to the common gardens and transplanted into bigger pots (11.5*11.5*21.5 cm) filled with the same potting soil. At each site, 12 individuals of each species were randomized in the beddings previously enriched with potting soil and sunken to one-third into the soil. This design was systematically replicated in the beddings receiving rain-shelters, resulting in an experiment including a total of 1344 individuals across both sites and treatments (12 replicates x 2 sites x 2 treatments x 28 species = 1344 individuals; Fig. S1). The rain-shelters were installed after a week of acclimation and consisted of a triangular aluminium frame covered by an UV-B transmissible greenhouse film (Luminance AF Window, Folitec, Germany) with a base area of 2.4 x 3.0 m and a height of 1.2 m. The tunnel-shape with large openings allowed for constant wind flow preventing warming beneath the shelters. To minimise edge effects, the sheltered base was larger than the central 1*2.5 m area occupied by plants. To avoid lethal consequences of the drought treatment, a minimal water input was provided. 20 L of rainwater were distributed per bedding every two weeks (c. 0.12 L per individual). Accordingly, the difference in water availability between the beddings with and without rain-shelter equals the amount of precipitation. At the end of the first growing season, rain-shelters were removed and plants overwintered under snow.

In Spring 2013, rain-shelters were reinstalled right after snowmelt (early May at the low common garden and mid-June at the high common garden) initiating the start of phenological recordings (plants did not reproduce the first year). Air temperature was recorded hourly in each common garden and treatment at 0.5 m above the ground using sheltered data loggers (TidBit v.2 UTBI-001; Onset Computer Corporation, Bourne, MA, USA). Similarly, light intensity loggers (Hobo pendant light data logger 64K-UA-002-64, Onset Computer Corporation, Bourne, MA, USA) were installed in each common garden at 1 m above the ground in both treatments. The drought treatment consisted of a minimal water input as in the previous year. Once a month, the volumetric soil moisture content (VSCM m^3 m^{-3}) was measured randomly in 30 pots of each bedding with a HH2 Moisture Meter and a Theta Probe type ML2x (Delta-T Devices, Cambridge, England).

Lamiaceae	Acinos arvensis (Lam.) Dandy colline–montane	Acinos alpinus (L.) Moench subalpine
Poaceae	Anthoxanthum odoratum L. colline–alpine	Anthoxanthum alpinum Löve subalpine–alpine
Fabaceae	Anthyllis vulneraria ssp. vulneraria L. s.l. colline–montane	Anthyllis vulneraria ssp. alpéstris Schult subalpine–alpine
Brassicaceae	Arabis hirsuta L. colline–montane	Arabis alpina L. s.l. montane–alpine
Campanulaceae	Campanula rotundifolia L. Colline–subalpine	Campanula scheuchzeri Vill. subalpine–alpine
Asteraceae	Centaurea scabiosa L. s.l. colline–montane	Centaurea montana L. montane–subalpine
Caryophyllaceae	Dianthus deltoides L. colline–montane	Dianthus sylvestris Wulfen colline–subalpine
Rosaceae	Geum urbanum L. colline–montane	Geum montanum L. subalpine–alpine
Fabaceae	Lotus corniculatus L. colline–subalpine	Lotus alpinus Ramond alpine
Fabaceae	Onobrychis viccifolia Scop. colline–montane	Onobrychis montana DC. subalpine
Poaceae	Phleum phleoides (L.) Karsten colline–montane	Phleum alpinum L. subalpine–alpine
Plantaginaceae	Plantago lanceolata L. colline–subalpine	Plantago alpina L. subalpine–alpine
Caryophyllaceae	Silene vulgaris ssp. vulgaris (Moench) Garcke s.l. colline–subalpine	Silene vulgaris ssp. glareosa (Jord.) Marsd.-Jon & Turill alpine
Fabaceae	Trifolium pratense ssp. pratense L. colline–subalpine	Trifolium pratense ssp. nivale (Koch) alpine

Table 1 Overview of the congeneric pairs of mid- and high elevation species included in our study with their main range limits in the literature having only been given in terms of altitudinal zonations as defined for the European Alps by Lauber and Wagner (2001) and Aeschiman *et al*. (2004): "colline" = 300 m to 900 m; "montane" = 900 m to 1500 m; "subalpine" = 1600 m to 2300 m; "alpine" = 2300 m to 3000 m. "Mid-elevation" species mainly ranged from the colline to the lower montane zones, while "high elevation" species mainly ranged from the subalpine to the alpine zones.

Abiotic treatment effect

Averaged over the experimental period (May to October, Table 2), at the mid elevation common garden, the daily temperature was 15.5 °C in control beddings and 15.9 °C in beddings topped by rain-shelters. In the high elevation common garden, the average daily temperature equalled 11.2 °C in control beddings and 11.4 °C in beddings topped by rain-shelters. While there was a significant temperature difference between both common gardens, the rain-shelters increased the temperature at ground level only marginally by 0.25 °C.

The recorded light intensity (measured in lux at 1 PM) was higher at the high elevation common garden and was significantly reduced by rain-shelters (Table 2). At both common gardens, the rain-shelters intercepted approximately 30% of light but these values were not limiting for plant growth (see Fig.11.11 in Körner, 2003).

The volumetric soil moisture content (VSMC in m^3 m^{-3}, Table 2) differed significantly between the control and the drought treatment in both the common gardens ($W = 900$, $P = 10^{-4}$; $W = 844.5$, $P = 10^{-4}$, respectively). At the mid elevation site, the average VSMC of control pots equalled 0.40 ± 0.08 m^3 m^{-3}, while dry pots had a VSMC of 0.06 ± 0.02 m^3 m^{-3}. At the high elevation site, control pots had an average VSMC of 0.48 ± 0.1 m^3 m^{-3}, while dry pots had an average VSMC of 0.08 ± 0.02 m^3 m^{-3}.

	Temprature (°C)	Light Intensity (lux)	VSMC (m^3 m^{-3})
Low site / Control	15.5	115323.5	0.4
Low site / Dry	15.9	84554.8	0.06
High site / Control	11.2	139846.9	0.48
High site / Dry	11.4	101209.8	0.08

Table 2: Mean temperature, light intensity and volumetric soil moisture content (VSMC) for each treatment averaged over the experimental period (May-September).

Phenology monitoring

Phenological stages were defined after Price and Waser (1998) and Dunne et al. (2003). Different stages were used for forbs and grasses to account for their morphological differences. Seven stages were defined for forbs: unopened buds, opened buds, opened flowers, old flowers, initiated fruits, enlarged fruits and dehisced fruit. For grasses, five stages were defined: beginning of heading, end of heading, exerted anthers or styles, dried and broken-off anthers/styles and disarticulated seeds.

All observed stages were recorded weekly per individual and when 50% or more of the flowers or inflorescences were in a particular stage it was identified as dominant. Once all plants had completed their reproductive cycle and the growing season came to an end, all

plants were harvested. Aboveground biomass was cut at soil level and individuals were stored in parchment bags and transported to the laboratory within 24h, dried for 72h at 80 °C and weighed.

Phenological variables

Eight phenological variables were derived from the weekly recordings: onset of budding, onset of flowering, onset of fruiting, midpoint of flowering, duration of budding, duration of flowering, duration of fruiting and the total duration of all three phenophases combined. Onset of budding, flowering and fruiting were defined as the date (day of the year) when the first bud, flower or fruit was observed. Midpoint of flowering was defined as the average date when opened flowers or exerted anthers/styles (for forbs and grasses, respectively) were dominant. The duration of a phenophase was defined as the number of days between the onset of said phenophase and the dominance of the following phenophase.

Phenotypic plasticity in flowering phenology

The degree of phenotypic plasticity in response to warming and drought was calculated as a Phenotypic Plasticity Index (Pi_v) (Valladares et al., 2006). This index was calculated as the difference between the maximum and the minimum mean value of a given trait and species over all treatments divided by the maximum mean, which serves to standardize the index ranging from 0 (no plasticity) to 1 (maximum plasticity). Note that plasticity was considered at the species level rather than at the genotype level to compare the degree of plasticity between mid- and high elevation species.

Statistical analysis

To test treatment effects a linear mixed-effect model was performed for all eight phenological variables. 'Elevation' (mid or high elevation site), 'drought' (control or drought treatment), 'origin' of species (mid elevation or high elevation) and their respective interactions were computed as fixed effects. To account for variances between species, they were nested in their respective genus and computed as random effects. The effects of 'elevation' and/or 'drought' indicate trait variation due to different environmental conditions (i.e phenotypic plasticity), while the 'origin' of species effect indicates differences between mid- and high elevation species. The interaction between 'origin' of species and 'elevation' and/or 'drought' indicates a difference in the responses to treatment conditions between mid- and high elevation species. Aboveground dry mass was used as a covariate to correct for size effects on phenology, but was removed as it did not change the results or add value to the model. All linear mixed-effect models where performed with the 'lmerTest' package for R software (Kuznetsova et al., 2013), based on Type 3 errors and Satterthwaite approximation for denominator degrees of freedom. *Post-hoc* Tukey's HSD tests for multiple comparisons were performed using the 'multcomp' package (Hothorn et al., 2014) for R software.

To test for differences in the degree of phenotypic plasticity of flowering phenology between mid- and high elevation species, the Phenotypic Plasticity Index (Pi_v) calculated for each species was analysed with a paired Wilcoxon signed rank test. All the analyses were performed on R version 3.0.2 software (R Development Core Team, 2013).

Results

Many individuals died over winter, were subjected to herbivory or were not reproductive, leading to the total exclusion of 4 genera (*Centaurea*, *Geum*, *Onobrychis* and *Trifolium*) from the analysis. For the remaining species, an average of 8.3 replicates per treatment combination were included in the final analysis with a total of 667 individuals (i.e. 20 out of 28 initial species and c. 50% of the initial sample size). Mortality was however independent of species' origin and treatment combinations (Fisher's exact test for count data: P = 0.85). In 2013, the average temperature during the growing season differed by 4.4 K between common gardens and on average the drought treatment reduced the volumetric soil moisture content by 0.37 m^3 m^{-3}. These changes in abiotic conditions induced highly species-specific shifts in the onsets and durations of phenophases but important patterns emerged when groups of mid- and high elevation species were considered. To enhance clarity, we first report results from the control treatment, describing the shifts in reproductive phenology in response to temperature for mid- and high elevation species and second drought effects.

Transplantation effect

The reciprocal transplantation of species to a warmer or colder prospective climate induced major shifts in the time of initiation and the duration of reproductive phenology. The onsets of budding, flowering and fruiting were always initiated earlier at the low elevation site by at least a month, but mid- and high elevation species differed in their response to transplantations. While the differences between mid- and high elevation species in phenological onsets were not always revealed by *post-hoc* multiple comparisons (Fig. 1), they are highly significant overall, as indicated by the significant interaction between elevation and origin treatments (Table 3; budding F=7.64, P=0.006; flowering F=16.27, $P<10^{-4}$; fruiting F=15.48, $P<10^{-4}$, respectively).

Indeed, high elevation species consistently initiated the onset of budding, flowering and fruiting earlier than mid elevation species, and these differences were particularly pronounced at the high elevation site (Table 3; F=7.64, P=0.006; F=16.27, $P<10^{-4}$; F=15.48, $P<10^{-4}$, respectively). High elevation species started budding 8.4 ± 2.2 days earlier than mid elevation species when grown at the high elevation site and 5.5 ± 2.2 days earlier when grown at the mid elevation site (Fig. 1, Supplemental Table 1). High elevation species also started flowering and fruiting earlier than mid elevation species, with strongest responses at the high elevation site (Fig. 1). The same was found for the midpoint of flowering, which was always reached earlier by high elevation species relative to their lower elevation congeners, especially at the high elevation site (Table 3; F=29.47, $P<10^{-4}$). Midpoint of flowering was recorded 12.9 ± 2.3 days earlier for high elevation species grown at the high elevation site and 5.7 ± 2.6 days earlier when grown at the mid elevation site (Fig. 1, Supplemental Table 1).

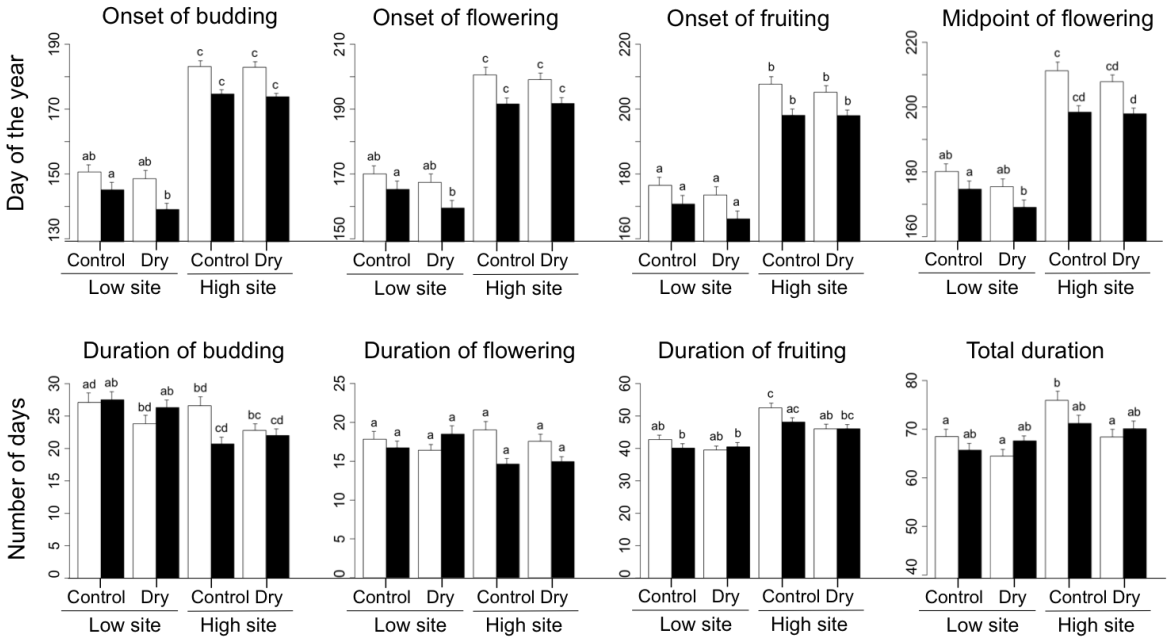

Fig. 1: Responses of mid elevation (white bars) and high elevation species (black bars) to transplantational elevation and drought treatment (mean ± s.e.) in onset, midpoint and duration of phenophases. The average onsets of budding, fruiting and flowering and the midpoint of flowering are shown in absolute days of the year, while the average durations of phenophases are shown in number of days. The letters above each bar represent the results of *post-hoc* Tukey tests for multiple comparisons. While they often provide detailed information about the differences between treatment combinations, some interactions between main effects are not revealed by the analysis, although they are significant on average in the more powerful ANOVA analysis.

Furthermore, differences in responses between mid- and high elevation species are also revealed in the fact that advancement of the onset of phenophases in response to transplantation between sites were consistently greater for mid elevation species relative to high elevation species (again, not revealed by *post-hoc* tests). For mid elevation species, the onset of budding at the mid- and the high elevation sites differed by 32.5 ± 2.0 days, whereas for high elevation species the difference was less (29.5 ± 2.1 days; Fig. 1). Similar trends were found for the onset of flowering and fruiting. The differences in responses between mid- and high elevation species were particularly pronounced for advancement in midpoint of flowering. Going from the 2000m site down to the 1050m site, mid elevation species advanced midpoint of flowering by 31.2 ± 2.4 days (Table 3; $F=29.47$, $P<10^{-4}$), whereas high elevation species advanced this stage by only 23.7 ± 2.2 days (Fig. 1, Supplemental Table 1).

The duration of phenophases responded to transplantations, with the exception of the duration of flowering (Table 3). A significant interaction between elevation and origin was found for the duration of budding (Table 3; $F=6.03$, $P=0.01$), indicating a difference in response between mid- and high elevation species. The duration of budding was generally shortened at the high elevation site compared to the mid elevation site, but this was significant only for high elevation species, for which a 6.8 ± 1.1 day contraction was recorded (Fig. 1, Supplemental Table 1). For mid elevation species on the other hand, this contraction was only of 0.5 ± 1.4 days. The duration of fruiting was significantly shorter at the mid elevation site for both mid- and high elevation species (Table 3; $F=44.87$, $P<10^{-4}$). The maturation of fruits took 9.8 ± 1.3 less days at the mid elevation site compared to the high elevation site for mid elevation species, and 8.1 ± 1.3 less days for high elevation species (Fig. 1, Supplemental Table 1).

One should note, that mid elevation species had a particularly long duration of fruiting when grown at high elevation (Fig. 1). The total duration of reproductive phenology was also shortened at the mid elevation site. However, this effect was only significant for mid elevation species, which had a 7.5 ± 1.6-day shorter duration of reproduction when grown at the lower site. The effects of transplantation on the total duration of reproductive phenology were similar to those on the duration of fruiting (Fig. 1), reflecting that this last stage was proportionally the longest.

Finally, it is important to note that at the mid elevation common garden, all reproductive individuals from mid- and high elevation species reached the final fruit maturation stage (defined as 50% or more flowers of one individual having reached stage 7: dehisced fruits for forbs and stage 5: disarticulated seeds for grasses). At the high elevation common garden, 99% of high elevation species finished fruit maturation but only 85% of mid elevation individuals reached the final fruit maturation stage before final harvest.

Table 3 (next page)**:** Linear-mixed effect model for the responses of onsets and durations of phenological stages to the elevation (mid elevation vs. high elevation site) and drought (control vs. dry) treatment, the origin of the species (mid elevation vs. high elevation species) and their respective interactions. Non-significant interactions were removed from the final model. The significant p-values are shown in bold.

	Onset of budding			Onset of flowering			Onset of fruiting			Midpoint of flowering		
	NumDf	F	P	*NumDf*	F	P	*NumDf*	F	P	*NumDf*	F	P
Elevation	1	1293.8	**$<10^{-4}$**	1	1206.9	**$<10^{-4}$**	1	1191.1	**$<10^{-4}$**	1	1099.4	**$<10^{-4}$**
Drought	1	3.53	0.06	1	3.20	0.07	1	3.53	0.06	1	8.80	**0.003**
Origin	1	2.62	0.14	1	2.97	0.12	1	3.57	0.10	1	4.30	0.07
Elevation : drought	1	7.55	**0.006**	1	7.15	**0.008**	1	3.92	**0.048**	1	6.51	**0.01**
Elevation : origin	1	7.64	**0.006**	1	16.27	**$<10^{-4}$**	1	15.48	**$<10^{-4}$**	1	29.47	**$<10^{-4}$**
Drought : origin	1	1.95	0.16	1	2.16	0.14	1	0.94	0.33	1	0.22	0.64
Elevation : drought : origin	1	0.15	0.70	1	0.83	0.36	1	0.49	0.49	1	0.22	0.64
	Duration of budding			Duration of flowering			Duration of fruiting			Total duration		
	NumDf	F	P	*NumDf*	F	P	*NumDf*	F	P	*NumDf*	F	P
Elevation	1	37.63	**$<10^{-4}$**	1	2.29	0.13	1	44.87	**$<10^{-4}$**	1	11.53	**0.0007**
Drought	1	3.96	**0.047**	1	0.04	0.83	1	15.94	**$<10^{-4}$**	1	11.39	**0.0008**
Origin	1	0.06	0.81	1	1.80	0.22	1	0.22	0.65	1	0.00	0.95
Elevation : drought	1	0.06	0.81	1	0.15	0.70	1	3.45	0.06	1	4.80	**0.03**
Elevation : origin	1	6.03	**0.01**	1	3.73	0.54	1	0.00	1.00	1	0.48	0.49
Drought : origin	1	2.63	0.11	1	3.09	0.08	1	6.27	**0.01**	1	6.43	**0.01**
Elevation : drought : origin	1	1.61	0.21	1	0.75	0.39	1	0.03	0.85	1	0.25	0.62

Drought effect

Drought had a tendency to advance phenophases, but had the greatest effect at the low elevation site. Drought consistently led to smaller advancement of phenophases than did transplantation to the warmer site (Fig. 1). Effects of drought on onset of budding, flowering, fruiting and midpoint of flowering varied depending on whether plants were grown at the mid- or high elevation sites, indicated by a significant interaction between elevation and drought (Table 3; ExD for budding P=0.006; flowering P=0.008; fruiting P=0.048; and flowering mid-point P=0.01). Drought initiated earlier phenophases at both sites but this effect was significant only at the mid elevation site for high elevation species (Fig. 1). At the mid elevation site, drought-stressed high elevation species initiated budding 6.1 ± 2.1 days earlier than individuals under control conditions, while drought-stressed mid elevation species started budding only 2.1 ± 2.3 days earlier. In contrast, at high elevation, the onset of budding was only marginally advanced in the drought treatment, namely by 0.2 ± 1.7 days for mid elevation species and by 0.9 ± 1.1 days for high elevation species (Fig. 1, Supplemental Table 1). The same results were found for the onset of flowering and fruiting, and for the midpoint of flowering (Table 3), although the difference between mid- and high elevation species was not revealed by *post-hoc* comparisons for the onset of flowering (Fig. 1).

The durations of phenophases were unequally affected by drought and only the duration of flowering did not change in response to drought (Table 3, Fig. 1). The duration of budding was significantly shorter on average under dry conditions (Table 3; F=3.96, P=0.047), however, this was not revealed by the *post-hoc* multiple comparisons (Fig. 1). For mid elevation species drought reduced the duration of budding by 3.3 ± 1.3 days at the mid elevation site, and by 3.8 ± 1.2 at the high elevation site. This effect was less pronounced and less consistent in high elevation species (Fig. 1, Supplemental Table 1).

While the duration of fruiting was also generally shortened by drought at the lower site, a significant interaction between drought and origin was found (Table 3; $F = 6.27$, $P = 0.01$) meaning that mid- and high elevation species responded differently to the drought treatment. At the lower site, drought affected the duration of fruiting only marginally for mid- and high elevation species (Fig. 1). In contrast, at the high elevation site, the duration of fruiting was significantly shortened by 6.4 ± 1.4 days for mid elevation species under drought stress but only marginally by 2.1 ± 1.3 for high elevation species (Fig. 1, Supplemental Table 1).

For the total duration, a significant interaction was found between drought and elevation, as well as between drought and origin (Table 3, $F = 4.8$, $P = 0.03$; $F = 6.3$, $P = 0.01$, respectively). Drought induced shifts in the total duration of reproductive phenology were more pronounced at the high elevation site than at the mid elevation site and in mid elevation species compared to high elevation species. Drought significantly shortened total duration of reproduction for mid elevation species when growing at the high elevation site, namely by 7.5 ± 1.7 days, however this effect was only marginal for mid elevation species when grown at the lower elevation site and for high elevation species at both sites (Fig. 1).

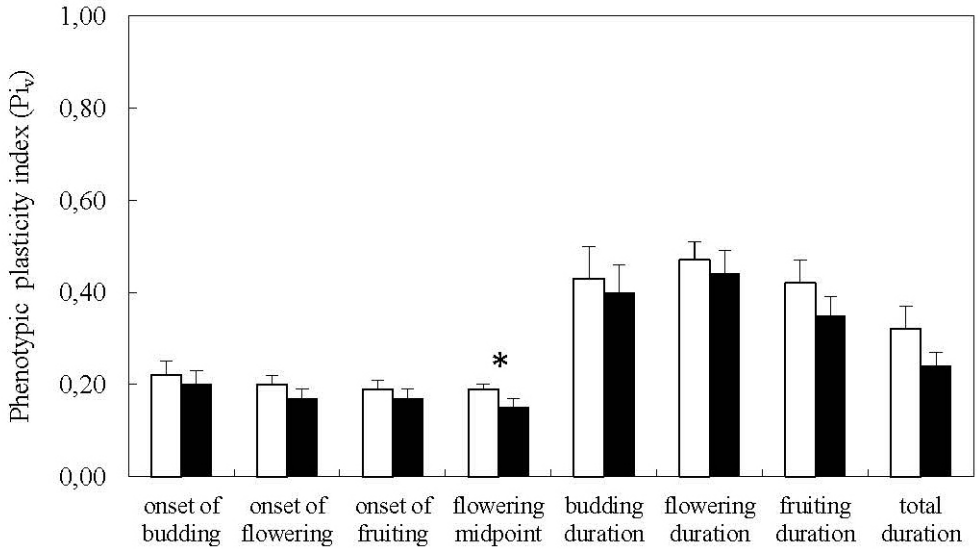

Fig. 2: Phenotypic Plasticity Index (Pi_v) of mid elevation (white bars) and high elevation species (black bars) calculated across all treatments for onsets and durations of phenophases. The error bars denote s.e. *, $P<0.05$.

Phenotypic Plasticity Index (Pi_v) of mid- and high elevation species

A significant difference in the Phenotypic Plasticity Index Pi_v was found for the midpoint of flowering (Fig. 2; $V = 40$, $P = 0.03$). Mid elevation species had a greater Pi_v than high elevation species, indicating that the shift in midpoint of flowering in response to elevation and drought was greater for mid elevation species than for high elevation species (0.19 ± 0.04 and 0.15 ± 0.05, respectively). These results are consistent with the previously reported shifts in number of days. Furthermore, as the Pi_v of mid elevation species was systematically greater (Fig. 2) we also compared the mean Pi_v across all traits between mid- and high elevation species and found a significantly higher mean value for mid elevation species ($Pi_{vMid}=0.31 \pm 0.1$, $Pi_{vHigh}=0.26 \pm 0.1$; $V=36$, $P= 0.01$). This overall result indicates that mid elevation species tended to have a greater degree of phenotypic plasticity in their reproductive phenology than high elevation species and hence a greater capacity to adjust these traits to environmental changes in temperature and water availability.

Discussion

Responses to transplantation and drought

Transplantation of high elevation species to a site with earlier springtime resulted in advanced onset of reproductive phenology, an overall pattern in agreement with existing literature (Price and Waser, 1998, Dunne et al., 2003, Scheepens and Stöcklin, 2013). Mid- and high elevation species initiated all reproductive phenophases approximately a month earlier at the lower elevation site, indicating the important role of temperature for phenophases. Interestingly, high elevation species initiated budding prior to mid elevation species at both sites, on average by 10 days at the high site and by 5 days at the low site. In contrast, other studies found that reproduction was always initiated first by low elevation populations at the low elevation sites (Haggerty and Galloway, 2011, Frei et al., 2014a, Frei et al., 2014b). However, in those studies, experimental gardens were situated at lower elevations relative to our study sites (at 514m and 600m compared to ours at 1050m). This resulted in high

elevation populations in prior studies being exposed earlier on in the year to days with warm temperatures, yet relatively shorter photoperiods than in our study, and may have driven the observed differences in results among our studies. As photoperiod is also a fundamental cue for a frost risk-free initiation of growth and reproduction for some alpine plants (Keller and Körner, 2003, Körner, 2003, Basler and Koerner, 2012), it is likely that high elevation populations in the prior studies waited for days with a sufficiently long photoperiod and did not rely solely on temperature to initiate reproduction. However, in our study, photoperiod was similar between both common gardens at the time of reproductive onset. Hence, advanced initiation of reproductive phenology in high elevation species at both of our study sites probably reflects other adaptations to cold climates and short growing seasons: *e.g.* low growing degree day (GDD) requirements (Haggerty and Galloway, 2011) and preformation of buds (Sørensen, 1941, Billings and Mooney, 1968, Bliss, 1971).

At the mid elevation site, most phenophases were shortened which is in agreement with previous studies (Sherry et al., 2007, Post et al., 2008, Steltzer and Post, 2009). However, the duration of budding was longer at the lower warmer site relative to the high elevation site, which highlights the contrasting effects of warming on individual phenophases (Post et al., 2008, Haggerty and Galloway, 2011, Cornelius et al., 2013). Contracted phenophases have generally been explained as resulting from increased developmental rates in warm temperatures (Sherry et al., 2007, Haggerty and Galloway, 2011). Alternatively, extended reproductive durations are often linked with an expanded growing season (Dunne et al., 2003). In our study, the average daily temperature during the budding phase was higher at the mid- than at the high elevation site (14.1°C and 12.3°C, respectively). Thus, temperature alone cannot explain expanded budding duration, which is in contradiction with fast developmental rates expected under warm temperatures. This result might be related mainly to the fact that high elevation species significantly contracted this phenophase at high elevation sites (Fig. 1) to guarantee sufficient time for flowering and fruit maturation, but it is also possible that plants tried to take advantage of a longer growing season at the lower site with advanced spring. The duration of fruiting was however highly accelerated for both groups of species by fast maturation rates under warmer temperatures. As this last stage was proportionally the longest it resulted in a shorter total reproductive duration at the low elevation site, which suggests that plants were not able to consistently prolong their reproductive cycle to take advantage of a longer growing season.

Limited water availability had considerable effects on plant reproductive phenology but drought induced shifts were less extensive than those in response to temperature changes (shifts in the order of magnitude of a few days against a month, respectively). However, when drought stress was combined with warmer temperatures, it generally emphasized the responses of species and consistently led to further advancements and shortenings of phenophases for mid elevation species in responses to drought. This result is in line with a 4-day advancement in mid-flowering date recorded after a simulated drought in Central Europe (Jentsch et al., 2009) and with a study which revealed that an ‘escape strategy’ inducing earlier flowering was selected for in *Brassica rapa* following a natural drought (Franks et al., 2007, Franks, 2011). In our study, species responded to drought by plastic shifts congruent with such an ‘escape strategy’. When the growing season is shortened by drought, plants with late reproductive initiations might be unable to mature seeds before conditions become lethal. Hence, when water availability is limited, a shift towards rapid development and maturation of flowers is advantageous and allows the maintenance of reproductive success (Vasek and Sauer, 1971, Franks, 2011).

Mid- and high elevation species generally advanced phenophases in response to drought but changes in the duration of phenophases were less pronounced in high elevation species. While the total duration of reproductive phenology was mainly shortened for high elevation species, a slight extension of budding and of fruiting was recorded at high- and mid elevation sites, respectively. This result highlights the divergent effects of drought on certain phenophases (Peñuelas et al., 2004, Llorens and Peñuelas, 2005). In our case, high elevation species are normally less exposed to drought periods than their congeners from lower elevations (Vasek and Sauer, 1971). Precipitation tends to increase with elevation and evapo-transpiration tends to decrease with elevation. Accordingly soil moisture availability generally increases with elevation (2003). Consequently, the inconsistent responses of high elevation species to drought at both sites suggest that although high elevation species also tended towards an 'escape strategy' when facing drought, they might be less efficient in doing so then their mid elevation congeners.

Constrained degree of phenotypic plasticity in high elevation species

In line with our hypothesis, the differences between herbaceous mid- and high elevation species affected their potential for phenological plasticity as previously found for low- and high elevation populations of deciduous tree species (Vitasse et al., 2013). Our results revealed that herbaceous high elevation species tended to have a smaller Pi_v than mid elevation species for flowering phenology even though this difference was only significant for the midpoint of flowering and when averaged over all phenological variables. Nevertheless, mid- and high elevation species both showed a notable capacity of tracking environmental changes through phenological shifts while maintaining a high performance. It is particularly interesting that high elevation species were found to have a lower Pi_v specifically for the midpoint of flowering. The exact timing of flowering might be the most crucial phenophase for successful reproduction. The timing of flowering is even more crucial in cold environments, where short growing seasons (Billings and Mooney, 1968, Körner, 2003) and adverse conditions such as frost events (Inouye, 2008) pose additional challenges to reproductive success. Consequently, strong directional selection decreasing temperature sensitivity and increasing photoperiodic control (Basler and Koerner, 2012, Vitasse et al., 2013) may have shaped the evolution of reproductive phenology of high elevation species to coincide with favourable environmental conditions, presumably contributing to local adaptation in heterogeneous landscapes (Hall and Willis, 2006, Verhoeven et al., 2008, Anderson et al., 2011).

The selective pressures controlling timing of reproduction become increasingly strong with elevation and thus we hypothesize that the difference in phenological plasticity would have been more pronounced if more strictly alpine species, from above treeline-elevation had been chosen. This would have provided a more extreme contrast with congeneric mid elevation species. Here, our results indicate that adaptation to short growing seasons in the alpine environment limits the potential for phenotypic plasticity in the reproductive phenology of high elevation species in response to environmental changes, leading to a higher genetic canalization of the timing of peak flowering (Price et al., 2003, Pigliucci et al., 2006, Ghalambor et al., 2007).

Consequences of phenological shifts

For high elevation species, transplantation to a lower elevation resulted in advanced phenophases, suggesting adaptive tracking of an advanced growing season (Cleland et al., 2012). However, warmer temperatures also accelerated developmental rates and led to shortened phenophases, indicating that high elevation plants were unable to take advantage of a longer growing season. Furthermore, in advanced growing seasons, the time frame for resource acquisition is abbreviated before environmental cues initiate reproduction. Consequently, advanced flowering could potentially lead to decreased fitness (Post et al., 2008, Scheepens and Stöcklin, 2013).

Alternatively, for mid elevation species, the upward transplantation resulted in delayed initiation and prolonged phenophases. While the later initiation of reproduction at the higher site might be adaptive, the prolonged phenophases suggest an entirely passive response to slower developmental rates in cold temperatures (Sherry et al., 2007). At the final harvest in late autumn 15% of mid elevation plants had not yet started to disperse their seeds and we estimate that in total c. 30% of flowers from mid elevation species would not have completed fruit maturation (Elena Hamann, pers. obs.). A prolonged reproductive period of upward migrated mid elevation species could thus have fitness costs if associated with uncompleted seed maturation before winter fall.

Limited water availability advanced and shortened phenophases, a result congruent with aforementioned 'escape strategy' limiting the negative impact of drought stress on plant fitness (Franks, 2011). However, drought induced phenological shifts were greater for mid elevation species suggesting that they were more capable of adopting an efficient 'escape strategy' than their high elevation congeners. Phenotypic plasticity has been suggested to be adaptive only when the environmental fluctuations experienced by populations do not fall outside of their native range (Ghalambor et al., 2007). While mid elevation species are frequently exposed to dry summer periods, high elevation species have rarely experienced such environmental conditions in the past (Körner, 2003), which could explain why they were unable to produce an 'escape strategy' as efficient as their mid elevation congeners.

We conclude that while the direction of plastic responses in reproductive phenology tended to track environmental changes, adaptation of species to their native range seem to constrain adaptive plasticity in novel conditions and could potentially lead to maladaptive responses (Ghalambor et al., 2007).

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Appendix

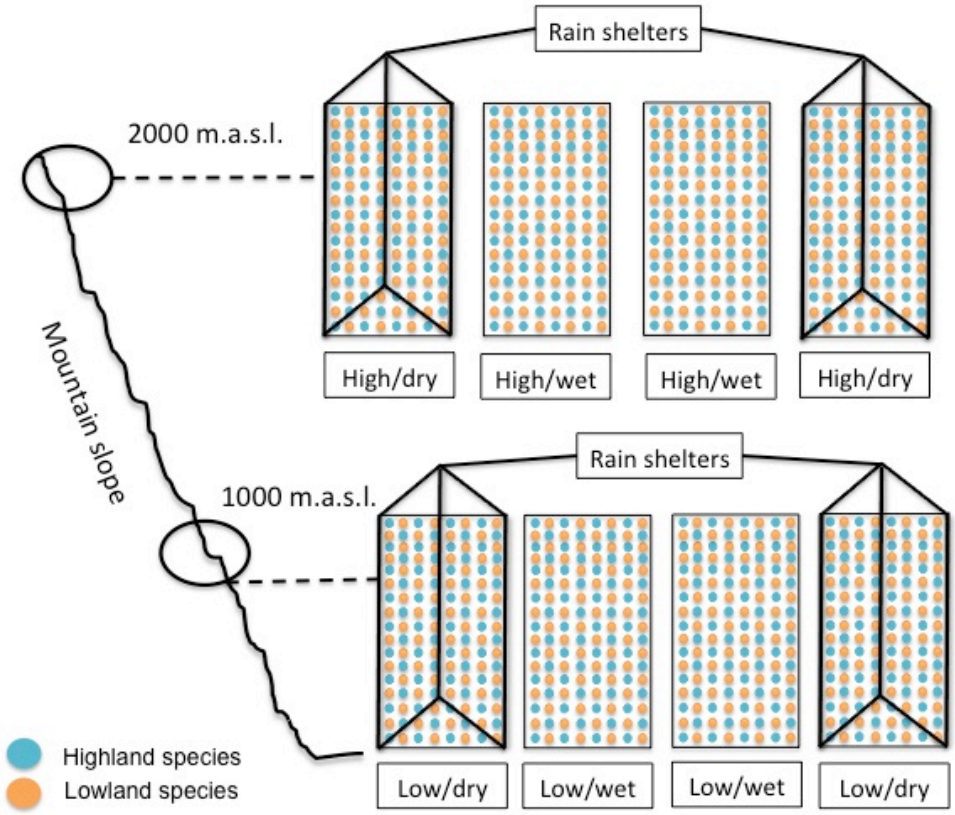

Fig. S1: Schematic overview of the experimental design.

Chapter 8

General discussion.

Summary

The pronounced seasonality and environmental heterogeneity in the Alpine environment (Körner 2003; Scherrer and Körner 2010) allow to hypothesize that both phenotypic plasticity and local adaptation should be particularly important for Alpine plant survival (Stöcklin et al 2009). It was the aim of this thesis to provide evidence for the respective importances of these two fundamental evolutionary strategies, which can be complementary, mutually exclusive, or otherwise intertwined in complex ways (Pigliucci, 2003).

Using a reciprocal transplantation experiment we have first established conclusively that populations of Alpine *Anthyllis vulneraria* and - with less compelling evidence – populations of Alpine *Arabis alpina* perform better at their site of origin compared to other sites. We found higher biomass, higher reproductive output and higher flowering propensity in these plants when they were transplanted to their home-sites compared to away sites. This suggests that the heterogenous external conditions, biotic and abiotic, at the population sites drive divergence in functional characters of these populations. Fitness did not decrease with geographic distance of transplantations in *Arabis alpina*, leaving us with the question whether species differ generally in the spatial pattern of local adaptation, or whether unknown meachinsm such as the release of genetic diversity upon stronger environmental change caused the observed results (Grether 2005; Ghalambor et al. 2007).

In the common garden, we were struck by the vast divergence in flowering time of the same populations of *Anthyllis vulneraria* as used in the reciprocal transplantation under common conditions. We found differences in the onset of flowering as large as five weeks, and two populations that were not genetically differentiated at neutral microsatellite loci and that were only 2 km apart from each other on the same mountain slope, showed a difference in the onset of flowering of two weeks. Despite high within-population genetic diversity for this character, a Q_{ST}-F_{ST} comparison indicated that this divergence was due to past natural selection. Given that the elevational difference between populations is only a few hundred meters at maximum, these results suggest that different snowmelt dates due to topographic heterogeneity among population sites have caused the divergence in flowering time. Flowering time responded only marginally to soil water reduction, indicating that this trait might be strongly canalized (Van Buskirk & Steiner 2009).

Our molecular analyses of *Anthyllis vulneraria* revealed variation in the degree of inbreeding among populations of this species. This was indicated by different degrees of heterozygote deficiencies in the studied populations. Tests for null-alleles and the floral biology of *Anthyllis vulneraria* clearly suggested, that these deficiencies are not due to technical artifacts. Therefore, Alpine populations of *Anthyllis vulneraria* are often mixed mating either through varying degrees of self-fertilization or through bi-parental inbreeding. Earlier studies of the floral biology and the mating system of this species in France and Spain have already revealed that the degree of the overlap of the male and female functions inside a flower (dichogamy) determine the degree of inbreeding (Couderc 1971; Navarro 1999). In our study of the degree of dichogamy in Alpine *Anthyllis vulneraria*, under drought and control conditions, we found large variation that matched the variation in the inbreeding coefficients, but did not fully explain. We could show that minimal degrees in delayed stigma receptivity could maintain large rates of outcrossing despite the close proximity of stigma and self-pollen. Drought did not affect the maturation of stigmas, but significantly reduced the longevity of pollen. We hypothesize that the degree of dichogamy may have evolved as an

instrument that allows plants to achieve optimal outcrossing rates under the give pollinator conditions at each site.

The study of phenotypic plasticity has a very long history that dates back to the late 19th century with the studies of Bonnier and Clements, who both found dramatic changes in the phenotypes of lowland plants when transplanted to higher elevations (Briggs & Walter 1984). The reciprocal transplantations of lowland and highland plants we performed during this thesis to study phenotypic plasticity in flowering phenology also revealed great advances of flowering time at lower elevations. Our studies further show that these advances are less strong in alpine species compared to their lowland congeners, suggesting that Alpine plants are more strongly canalized in their flowering phenology than lowland plants. This indicates that selection on trait means might be generally stronger at high elevations, possibly due to cold and short growing seasons which do not allow for much divergence from optimal phenotypes. This reasoning is in line with the fact that we could not show plastic responses to be of adaptive value, but rather they seem to be a passive by-product of physiological adjustments to changed external conditions as they can be expected under global climate change.

Conclusions

Alpine plants face a rapidly changing environment due to changes in land use (Fischer et al. 2008) and climate change (IPCC 2007). They can respond to these changes in three ways: one by migrating to remaining suitable habitats, two by responding plastically, and three by adapting to novel conditions in an evolutionary sense of the word. Migration is restricted in high-mountain plants by the ever decreasing recource of space towards mountain summits. Therefore, the adaptive potential and phenotypic plasticity gain in importance.

The results of this thesis clearly show that a wide-spread species, *Anthyllis vulneraria*, has adapted to heterogenous environmental conditions in the past. This process has not lead to a depletion of genetic diversity in this species. On the contrary, we have found ample genetic diversity in all traits that we have measured, suggesting that future adaptations should not be hampered by a lack of standing genetic variation. *Anthyllis vulneraria* is a very widespread species, and Darwin (1859) has already noted that it is the common and widespread species that will proliferate long into the future and give rise to new species. Therefore, it remains questionable whether our findings apply to Alpine species in general, which are often restricted in their distribution, and have small and isolated populations.

An adaptive value of phenotypic plasticity could not be shown in the presently studied examples, as they could all be explained by a passive physiological response. In fact few stringent tests of adaptive plasticity can be found in the literature (e.g. Dudley & Schmitt 1996). It is therefore conceivable that plasticity is not generally of much adaptive value apart from a few physiological characters such as the opening and closing of stomata according to humidity. This could be explained by the fact that several plant organs need to be integrated into a cooperative system to allow adaptive responses, and the synchronization of several organs or tissues into an integrated plastic apparatus by natural selection is statistically unlikely. Therefore, phenotypic plasticity remains largely a complicating factor for the biologist, but may still provide valuable phenotypic variation to allow certain evolutionary pathways otherwise locked (Ghalambor et al. 2007).

In conclusion it is therefore most likely that evolutionary adaptation from standing genetic variation will be crucial for the survival of Alpine plants in the coming decades and centuries.

Outlook

While a reciprocal transplantation experiment can arguably provide definitive proof of local adaptation (Blanquart et al. 2013), they usually and ideally attempt to measure life-time reproductive success, and therefore do not have as their primary goal the identification of targets of selection or selective agents. Targets of selection are functional traits and selective agents are the components of the environment of a focal individual, which exert selective pressure. The inclusive nature of reciprocal transplantation experiments therefore is not usually the weapon of choice for the identification of functional relationships among organisms and with their inanimate surroundings, and often will preclude the latter due to immense logistical requirements they bring about (Pigliucci 2003).

Once the fact has been established that local environmental conditions drive the evolution of adaptations, one naturally wishes to gain further insights and to uncover specifically which traits have adapted and to what circumstances. Methods such as Q_{ST}-F_{ST} comparisons are a very starting point to identify candidate traits that have likely been under selection. In this thesis we were able to apply this method successfully to the identification of the timing of the onset and peak of flowering as being most likely under divergent selection due to differences in the date of snowmelt across the alpine landscape. Unfortunately, the link to the reciprocal transplantation experiment is not quite given, because the timing of the flowering phenology will mostly affect seed set and this trait is difficult to measure in the field. Therefore, divergence in flowering phenology is not the cause of the local adapation documented in the reciprocal transplantation experiment. As one can hardly ever be certain that no viable seeds or failed embryos got lost, and as the regular visitation of multiple sites throughout the Alps and at the time of flowering is logistically nearly impossible, we did not attempt to measure seed set in the transplantation experiment. However, on the basis of our results – strong differentiation in flowering phenology suggested to be due to adaptive evolution – one could pick a limited number or only two strongly differentiated populations, and reciprocally transplant these to study seed set at the home and away sites and then extrapolate results to other populations. Likewise, the functional significance of the great phenotypic variation and differentiation in the degree of protandry in *Anthyllis vulneraria* (Chapter 5) could be assessed in such a study, to test thoroughly whether variation in protandry and flowering phenology in mixed-mating populations of *Anthyllis vulneraria* results from fine-tuning to local conditions such as the timing and abundance of pollinator availability. Moreover, protandry might have evolved to adjust flowers to the optimum degree of outcrossing, which is determined by the amount of inbreeding depression in a population, pollinator service, and the need for reproductive insurance. Such a study requires among others the measurement of the realized outcrossing rates at the levels of population and genotypes, which can be achieved using genotyping of multiple offspring of a number of maternal plants with co-dominant markers such as microsatellites.

Furthermore, *Anthyllis vulneraria* sensu lato (s.l.) is a particularly polymorphic taxon. Depending on the author, as many as 18 sub-species have been described in Central Europe alone (Hegi 1975). The polymorphic nature of this taxon is well documented throughout its distribution range (Jones & Turrill 1933; Köster 2008). Flower color in Swiss taxa for example can vary from pale white, over bright yellow, to red over distances as small as a few

kilometers. In the Zermatt region, the red-flowered *Anthyllis vulneraria* ssp. *guyotii* (Grenon 2006) occurs in sympatry with yellow-flowered *Anthyllis vulneraria* ssp. *alpestris*. Since no intermediate flower colors are known there, the two sub-species seem to be reproductively isolated. This seems not the case just a few kilometers to the East, where yellow- and white-flowered populations of an unidentified subspecies of *Anthyllis vulneraria* occur in sympatry and all intermediate colors can also be observed (Halil Kesselring, personal observation). Extensive genome-wide genotyping across Europe could elucidate the historical biogeography of this taxon (Holderegger et al. 2010). Combined with experimental approaches testing factors determining reproductive isolation and correlations of trait values with environmental factors, such studies could provide rare insights into the link between micro-evolution and speciation (Schluter 2009).

Lastly, *Arabis alpina* has recently been chosen as an alpine model species (e.g. Wang et al. 2009). Its close relatedness to *Arabidopsis thaliana* has facilitated and will continue to facilitate the development of molecular tools for the study of the molecular genetic bases of evolutionary processes. Of particular interest could be the elucidation of the molecular mechanisms of transcriptional regulation governing phenotypic plasticity in alpine plants. Since only few examples of adaptive phenotypic plasticity exist it would be interesting to study what circumstances allow natural selection to exploit gene regulatory systems for the control of adaptive phenotypic plasticity in alpine plants (Ackerly and Sultan 2006).

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44th annual meeting of the Ecological Society of Germany, Austria and Switzerland (GfÖ), Hildesheim 2014, Contribution: Poster

28th Conference of the Population Biology Section of the Ecological Society of Germany, Austria and Switzerland (GfÖ), Tuebingen 2015, Contribution: oral talk

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Publications

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Alpine Botany (Springer)