

Physiological and anatomical differentiation of two sympatric weed populations

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ABSTRACT

In the vineyards of Rhineland-Palatinate (Germany), two different types of Shepherd's Purse (*Capsella bursa-pastoris*) coexist: (1) the common type called 'wild type', and (2) the decandric type called *Capsella apetala* or 'Spe' with four stamens in place of the four petals. In this study, we compare the anatomical and physiological characters of rosette leaves of the respective types. Progeny of individual plants was cultivated in growth chambers under low- and high-light conditions. Under low-light conditions, the stomata densities of the adaxial and abaxial epidermis did not differ between the two types. When grown under high-light conditions, wild type and *Spe*, both exhibited increased stomata densities compared to low-light conditions, but *Spe* to a lesser extent than the wild type. The maximal photosynthetic capacity of *Spe* was lower in both, low-light and high-light conditions compared to wild-type plants. Under all CO₂ concentrations, *Spe* seemed to be less productive. The less effective CO₂ assimilation of the *Spe* mutant *C. apetala* was accompanied by later flowering. This fact prolonged the vegetative phase of *Spe* by about two weeks and was sufficient for the maintenance of both populations stably over years.

Subjects Ecology, Evolutionary Studies, Plant Science, Population Biology

Keywords Shepherd's Purse, *Capsella*, Photosynthetic capacity, Sympatric evolution, Anatomy, Flowering time, *Spe* mutant

INTRODUCTION

In the vineyards of southwestern Germany (Rhineland-Palatinate) two morphologically distinct types of the common annual weed Shepherd's Purse (*Capsella bursa-pastoris* (L.) Medik., Brassicaceae) occur side by side (Reichert, 1998, Fig. 1). The two populations can readily be differentiated by their characteristic flower structure. Wild type (wt) *C. bursa-pastoris* flowers exhibit the conserved body plan of Brassicaceae: it comprises four sepals in the first, four petals in the second, six stamens in the third, and two fused carpels in the fourth, the innermost whorl (Fig. 1).

In contrast, the flowers of the floral variant, also referred to as *Spe* (Hameister & Neuffer, 2017; Nutt et al., 2006), can easily be identified by four additional stamens replacing the wild-type petals thus giving it its unique decandric phenotype (Fig. 1). This floral variant of

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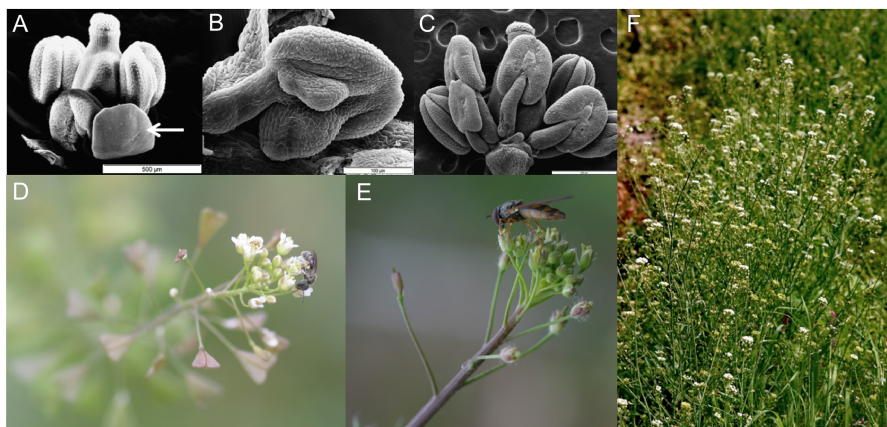


Figure 1 Flower morphology. Typical wild-type flower (A: arrow points to the petal) and its typical pollinator, a wild bee (D). Typical decandric flower (C) and its typical pollinator, a hoverfly (E). Proof for a rare hybrid between both flower types with a developing petal and pollen sacs beneath (B). In the field, both types are growing in mixed populations (F). Fotos: Gitta Schüttler, Birgit von Höveling.

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C. bursa-pastoris has initially been described as *C. apetala* Opiz (Opiz, 1821), a terminology which we follow here to differentiate between both types. Notably, the decandric flower shape does not affect floral symmetry, but the lack of petals might have an impact on pollination success (Hameister & Neuffer, 2017). Recent field surveys in vineyards revealed that the wild type is the predominant taxon with tens of thousands of individuals, and the *Spe* occurs with a stable frequency of about 10% (Hameister, Neuffer & Bleeker, 2009). Intriguingly, potential pollinators, especially wild bees, and hoverflies, tend to prefer one over the other type, probably due to differing volatile emissions of the two flower types (Ziermann et al., 2009; Hameister & Neuffer, 2017, Fig. 1). Recent investigations by Hameister & Neuffer (2017) demonstrated that flower induction is delayed in *Spe* when compared with wild type in both, field and controlled greenhouse conditions. Notably, the authors could show that in an overlapping period both types are fertile. However, both taxa exhibit a high “selfing” rate and a low crossing rate between the two flower types has been found (Hameister & Neuffer, 2017), Fig. 1). Furthermore, pollen tubes of *C. apetala* self-pollen grew faster compared with *C. apetala* crossed with wild-type pollen (*spe* × *wt*, Neuffer & Paetsch, 2013). Field experiments in the Botanical Garden of the University of Osnabrueck revealed that the two types differ in the physiological investments into their respective offspring. Wild-type individuals produced more siliques per plant, whereas the *Spe* generated a higher number of seeds in the siliques (Hameister & Neuffer, 2017). The total seed output per plant was balanced among the two flower types (Hameister & Neuffer, 2017) indicating that the fitness-related factor seed production remains unaffected.

Taken together, the previous investigations lead to the assumption that *C. bursa-pastoris* and *C. apetala* are separated by several strong isolation barriers. Thus, they form two morphologically distinct types that grow sympatrically in the vineyards of southwestern Germany (Reichert, 1998). Further support for this hypothesis can be extracted from genetic work by Hameister, Neuffer & Bleeker (2009). Here, the authors showed that wild type and

Spe differed in their isozyme genotype. Furthermore, the genetic difference was confirmed by creating a mapping population between a wild-type individual and a *Spe* individual followed by comparative linkage analysis (Hameister et al., 2013).

In this study, we focus on the properties of the leaves of the two types in the vegetative stage when the plant accumulates assimilates which then are used for building up the inflorescence and production of seeds. Rosette leaves provide the basis for an individual to withstand harsh environmental conditions, e.g., cold temperatures, high irradiation, and drought stress. When the apical meristem switches from the vegetative to the reproductive state, no further rosette leaves are generated. The question was: what is the contribution of leaf differentiation to the ecotypic variation between sympatrically growing individuals with normal (wild-type) flowers in contrast to individuals with decandric (*Spe*) flowers? Here, we studied the ecotypic differentiation of the rosette leaf after growth under stress (high-light) and non-stress (low-light) conditions using anatomical and physiological indicators.

MATERIAL AND METHODS

Plant material and cultivation of plants

Seeds of individual plants of *Capsella bursa-pastoris* (wild type) and *Capsella apetala* Opiz (*Spe*) have been collected in the vineyards located near to Gau-Odernheim (Rhineland-Palatinate, 25 km southwest of Mainz, 49.7847N, 8.1942E, elevation 150 m, Germany) and subsequently stored at minus 20 °C in the seed gene bank of the Botanical Garden of Osnabrueck. The material refers to the gene bank numbers 1951/19 and 1960/25 for the wild type and 1956/5 and 1961/4 for the decandric type. Note that plant material subjected to photophysiological and anatomic characterization in this publication is material derived from seeds of two independent population subgroups of each flower morphological type. Plants were initially cultivated under controlled conditions programmed with a 12-h photoperiod per day, resulting in four subgroups with six individuals each. The temperature was adjusted to 15 °C during day time and 5 °C during the night phase (initial growth conditions for seedlings). After six weeks, the plants were divided into two experimental groups. Three individuals of each subgroup were transferred into two different light conditions in an 8-h light period at 20 °C: (i) high-light setting ($600 \mu\text{mol quanta m}^{-2}\text{s}^{-1}$); (ii) low-light setting ($100 \mu\text{mol quanta m}^{-2}\text{s}^{-1}$) (Fig. 2). Later on sister individuals have been planted in the experimental field of the Botanical Garden.

Morphology and anatomy

Leaf characterization was performed on three-months-old leaves. Epidermal leaf tissue was prepared for microscopic examination (Fig. 3) by a customized technique applying nail polish. After solidification of the nail polish, the epidermal cell layer was removed by transparent adhesive tape (Tesafilm). The nail polish imprint, including adhesive tape, was observed with a microscope. Subsequently, stomata were quantified in a representative area of $400 \times 400 \mu\text{m}^2$ on one rosette leaf of every individual plant (Fig. 4).

Photosynthetic characterization

All measurements were performed using one fully expanded rosette leaf from plants two months after sowing when they did not yet flower. The CO₂ gas exchange was measured using the LI-6400/XT Portable Photosynthesis System (LI-COR Environmental, Lincoln, NE, USA). All measurements were conducted under saturating light intensities of 800 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ at 20 °C. The relative humidity was adjusted to approximately 50%. The rate of CO₂ assimilation (A) was measured as a function of sub-stomatal CO₂ concentration (C_i). C_i values were applied in the following order: 400 ppm; 200 ppm; 100 ppm; 50 ppm; 0 ppm; 400 ppm; 600 ppm; 1,000 ppm; 1,750 ppm. Here, the initial decrease in C_i concentration was chosen to ensure a sufficient leaf conductance. The leaf conductance is widely considered as an indicator of effective gas exchange of the leaf with the surrounding environment and is predominantly determined by the extent to which the stomata are opened. By applying decreasing CO₂ concentrations in the first half of a photosynthesis measurement, a conductance of at least 0.2 was maintained throughout the entire measurement (Fig. S1). In this way, a possible limitation of CO₂ assimilation due to inefficient gas exchange was avoided. Data points were logged within 2 min after the start of each C_i concentration when infrared gas analyzer (IRGA) parameters reached a steady-state value (raw data for 22 individuals see Table S2).

Determination of flowering time under field conditions

Seventy days after sowing, sister plants were planted into the experimental field of the Botanical Garden Osnabrueck and flowering time was determined by observation of the emerging inflorescence bud.

RESULTS

Stomata density on both sides of rosette leaves

After growth for three months in the climate chambers, epidermal peel from upper and lower leaf surfaces was prepared and inspected by light microscopy (Fig. 3). The number of stomata was determined for wild type and *Spe* after growth in low light and high light. As expected, the stomata density of the lower epidermis was found to be higher compared to the upper surface for both types in all light conditions (Fig. 4). When grown under low-light conditions, no obvious differences could be detected between the two flower types. In contrast, after growth under high-light conditions, both types exhibited a significantly increased stomata density on both leaf surfaces (Figs. 3 and 4, Table S1). Interestingly, the number of stomata on the adaxial (upper) surface of the leaves of *Spe* was significantly less increased when compared with wild-type individuals after growth in high-light conditions (Fig. 4). Notably, this trend could not be observed for the abaxial (lower) epidermis under the same conditions.

Photosynthetic characteristics of rosette leaves

To further elucidate the impact of the differentially increased stomata density observed in plants grown under high-light conditions, the photosynthetic efficiency was determined under saturating light (800 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$) using gas-exchange measurements. The



Figure 2 Habitus of individual plants cultivated under different light conditions. Habitus of individual plants of the same age (8th to 9th week after sowing) that had been cultivated under different light conditions. (A) Wild-type individuals under low-light conditions, (B) *Spe* individuals under low-light conditions, (C) wild-type individuals under high-light conditions, (D) *Spe* under high-light conditions. In C, the inflorescence shoot was distorted since the light source was mounted 20 cm above the rosettes.

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rate of carbon assimilation (A) in response to various externally applied CO_2 concentrations given as internal CO_2 (C_i) was monitored for wild type and *Spe*. The resulting A/C_i plots show the typical saturation behavior for all plants grown under both light conditions (Fig. 5).

Photosynthesis was limited by the availability of CO_2 until a calculated internal CO_2 concentration (C_i) of approximately 350 ppm was reached. The initial linear increase of CO_2 assimilation reflects the maximal carboxylation rate of RubisCO (Figs. 5C and 5D). Above 350 ppm, the curves enter saturation as net photosynthesis becomes limited by other factors such as the electron transport and, at higher CO_2 concentrations, the utilization of triose phosphate. At saturating CO_2 concentrations, maximum net-photosynthesis rates of *Spe* were always lower than of the wild type when cultivated under both, low- and high-light conditions (Figs. 5A, and 5B). However, the initial carboxylation rate was rather similar for the two types. At ambient CO_2 concentrations of approximately 400 ppm which more accurately reflect the photosynthetic efficiency of the plants, the rates of CO_2 assimilation are higher when the plants have been grown under high light, and significantly lower when grown under low-light conditions (Figs. 5C, and 5D). No premature stomatal closure had limited gas exchange as shown by plotting the stomatal conductance (g_s) against the C_i values for each measurement (Fig. S1).

Flowering time

In order to identify traits of *Spe*, which explain the observed stable co-existence in the field even with a lower photosynthetic performance, the flowering time was analysed. After 70 days sister plants (wt 72 individuals, *Spe* 63 individuals) were transferred from the

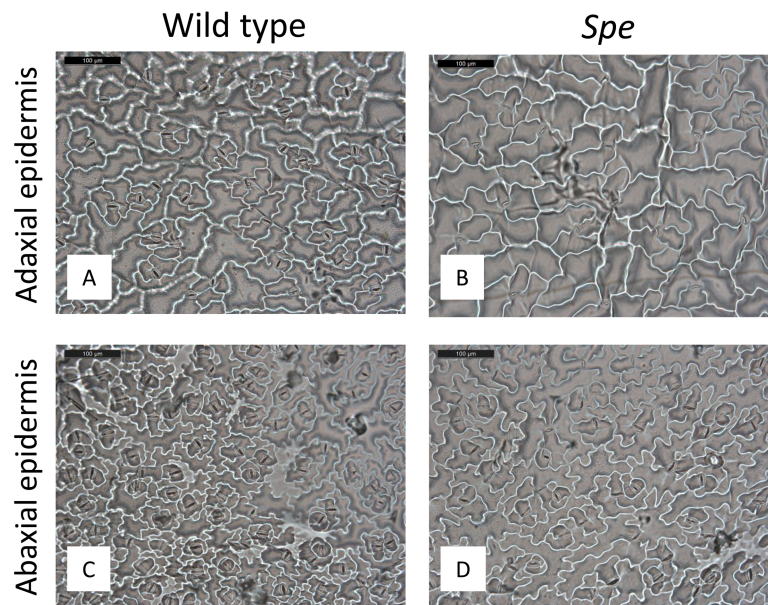


Figure 3 Stomata density of plants grown under high-light conditions. Microscopical views of stomata of wild type (A adaxial, C abaxial) and *Spe* (B adaxial, D abaxial) epidermis. Nail polish tracks of upper and lower leaf surfaces were taken. Black scale bar corresponds to 100 μm .

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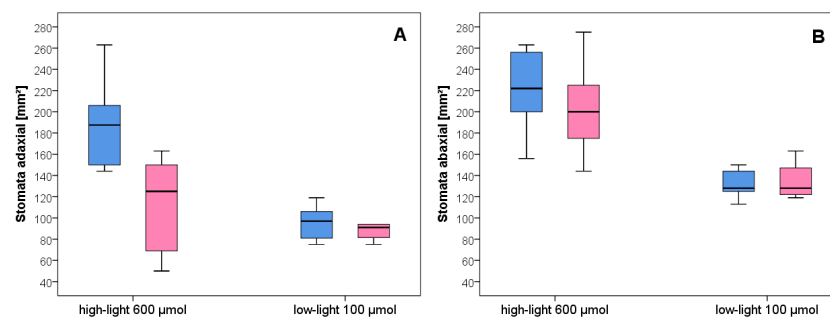


Figure 4 Stomata density of wild-type *C. bursa-pastoris* versus *C. apetala* (= *Spe*) grown under different light conditions. (A) Adaxial. (B) Abaxial leaf surface. Blue bars represent wild-type and pink bars *Spe* individuals. For significant differences between experimental groups see Table S1.

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initial growth conditions for seedlings to the experimental field in the Botanical Garden of Osnabrueck. Under these field conditions, wild-type individuals in average flowered about two weeks earlier ($x = 77.64 \pm 3.562$ days after sowing) than *Spe* individuals ($x = 91.75 \pm 12.618$ days after sowing (Fig. 6).

DISCUSSION

Finely scaled ecotypic differentiation for Shepherd's Purse is well known and quite substantial regarding germination, the onset of flowering, and growth forms (Linde, Diel & Neuffer, 2001; Neuffer & Linde, 1999, reviewed in Neuffer et al., 2011). Already at the

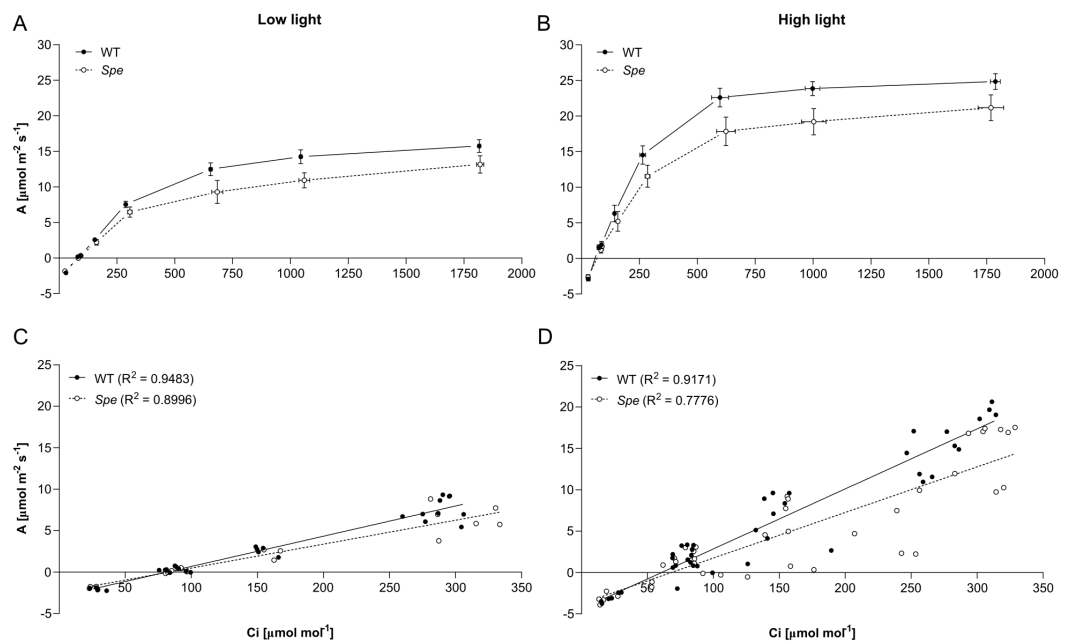


Figure 5 CO₂-concentration dependence of the rate of CO₂ assimilation. Wild type (WT, five individuals low light, seven individuals high light) and *C. apetala* (Spe, three individuals low-light, seven individuals high light) had been grown either under low-light (A and C) or under high-light conditions (B and D), respectively. Determination of the rates of CO₂ assimilation was performed under saturating light using one leaf for each individual as described in Material and Methods. A and B: After application of descending CO₂ concentrations, the internal CO₂ concentration (C_i) was calculated in each case, and similar C_i values were clustered (horizontal error bars). Stomata aperture was recorded for each measurement and is shown in Fig. S1. (C and D) Single measurements in the linear range up to 350 ppm were plotted against C_i.

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beginning of the 20th century, *Almquist (1907)* determined several elementary species based on fruit and rosette-leaf morphology. This variable leaf morphology attracted the interest of the geneticist *Shull (1909)* and *Shull (1911)*. *Aksoy, Hale & Dixon (1999)* included the system of Shull in their attempt towards a simplified taxonomy of *C. bursa-pastoris* and *Ianetta et al. (2007)* widened the system of Shull.

Combining anatomical and physiological traits provides the first insight into the value of leaf differentiation other than the morphology of leaf types after Shull (*Neuffer et al., 2018*). The ecotypic differentiation of the leaf in various habitats and conditions at geographically and climatically different places of origin of many populations is an obvious character that somehow serves adaptation and persistence in new growth conditions. The occurrence of a specific flower morphology of *C. bursa-pastoris* individuals in the vineyards south of Mainz in southwestern Germany is known for quite some time (*Reichert, 1998*). At first sight, this natural mutant is predominantly important for studies of plant geneticists (*Nutt et al., 2006; Ziermann et al., 2009; Hintz et al., 2006; Hameister et al., 2013*). With a more detailed analysis, several traits became apparent, and population studies led to the insight that the wild type of *C. bursa-pastoris* and its variant *C. apetala* even form two different ecotypes, sympatrically occurring at one place over many years (*Hameister & Neuffer,*

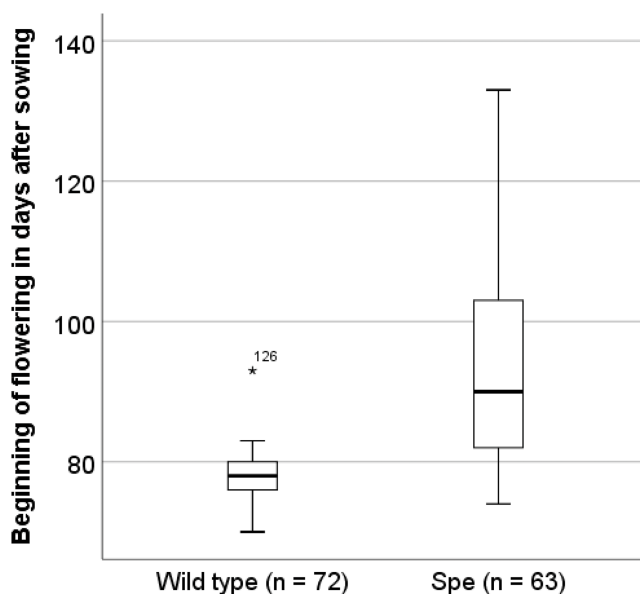


Figure 6 Onset of flowering in days after sowing. Sister individuals of the plants that had been used for the physiological and anatomical analyses have been planted into the experimental field of the Botanical Garden Osnabrück on day 70 after sowing.

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2017; Hameister, Neuffer & Bleeker, 2009). This differentiation is also evident from genetic differences (Isozyme Genotypes, AFLPs, see Hameister, Neuffer & Bleeker (2009).

As a typical leaf characteristic, stomata density was determined in the experimental populations (Fig. 4). In general, all leaves had developed more stomata under high-light growth conditions than in low-light as was already observed for *C. bursa-pastoris* provenances from Morocco and Norway, but not from Russia (Neuffer et al., 2018). Furthermore, stomatal density on the abaxial epidermis was about twice as high as in the adaxial leaf surface both in wild type and *Spe* after growth under high light. However, as an exception, *Spe* had not increased its stomata density on the upper leaf surface to the same extent as did the wild type. It had developed about 35% fewer stomata per area unit on the upper surface of its rosette leaves as compared to the wild type (Fig. 4A). A possible explanation for this phenomenon might lie in the fact that the incident light primarily impacts the adaxial surface of the rosette leaves and is exposed to adverse conditions more than the abaxial surface.

In our study, wild type individuals compared to *Spe* exhibited a higher maximal photosynthetic capacity both when grown under low light as well as high light. At saturating CO₂ and light, the rates of CO₂ assimilation were always higher for the wild type compared to *C. apetala* (Figs. 5A and 5B). This points to a limitation at the level of electron transport and would cause a disadvantage for *Spe*. However, as can be taken from the initial slopes of the A/C_i curves, under ambient conditions with limiting CO₂ concentrations up to 400 ppm, only slight differences were apparent when both types were compared (Figs. 5C and 5D).

One way to explain the coexistence of wild type and *Spe* in the same habitat is based on the fact that the flowering phenology of both types differs. Wild-type plants flower earlier by several days after sowing under greenhouse conditions (Hameister, Neuffer & Bleeker, 2009; Hameister et al., 2013) as well as under field conditions at the Botanical Garden of Osnabrueck (Hameister & Neuffer, 2017). The appearance of the inflorescences was monitored also for sister plants after transfer to field conditions in the Botanical Garden in Osnabrück (Fig. 6). On average, flowering started two weeks later in *Spe* individuals as compared to wild type. This might be explained by the disadvantage of *Spe* over the total growth period due to somewhat lower photosynthetic capacity and consequently a lower growth rate resulting in a delay of biomass production in the vegetative stage. Either due to the difference in the nutritional status or due to environmental signals, flowering induction occurs significantly later in *Spe* thus leading to a temporal niche for successful pollination and seed set. A small but stable population of *C. apetala* can apparently coexist in the presence of the large wild-type population. Both types might be adapted to slight differences in their growth properties and the environment by growing in the rosette stage for a longer or shorter time resulting in a difference in flowering time of about two weeks. Taken together, the selection pressure for both types is apparently similar leading to stable populations growing side by side.

CONCLUSIONS

In this study, we were able to analyse two independent sympatrically occurring populations of Shepherd's Purse. These two populations (wild type and *Spe*) occur intermingled with each other in stable frequencies of 9:1 over the years. This situation is rather specific and the question arose: What are the characters enabling these populations to coexist as they actually do? Both populations are isolated by strong (selfing) and smooth (flowering, reduced pollen growth activity between taxa) barriers. We have been able here to quantify anatomical and physiological traits which, with all probability, stay under selection pressure. Of course, many more characters are included in the ecotypic differentiation and already have been studied elsewhere (e.g., seed production). Here, in *Spe*, photosynthetic capacity appears to be lower, while growing for a longer time in the vegetative stage and later flowering induction. As both populations occur in stable frequencies, the selection pressure on single traits might be different, however, when combined, they seem to be neutralized. The specific combination of characters in each taxon is linked due to predominantly selfing.

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ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

Renate Scheibe is an Academic Editor for PeerJ.

Author Contributions

- Barbara Neuffer conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Michael Schorsch performed the experiments, analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.
- Steffen Hameister conceived and designed the experiments, authored or reviewed drafts of the paper, and approved the final draft.
- Johannes Knuesting conceived and designed the experiments, prepared figures and/or tables, and approved the final draft.
- Jennifer Selinski analyzed the data, prepared figures and/or tables, and approved the final draft.
- Renate Scheibe conceived and designed the experiments, authored or reviewed drafts of the paper, and approved the final draft.

Data Availability

The following information was supplied regarding data availability:

The raw data of CO₂-concentration dependence is available as [Table S2](#).

Supplemental Information

Supplemental information for this article can be found online at <http://dx.doi.org/10.7717/peerj.9226#supplemental-information>.

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