The effects of inbreeding and stress on plant performance

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"The result of some experiments which I have amused myself in making on plants, appearing to me interesting to the naturalist, […] I have taken the liberty to communicate them to you."

Thomas Knight (1799) on his pollination experiments

Content

Summary

The aim of this thesis was to enhance our understanding of the combined effects of inbreeding and environmental stress on plant performance. Inbreeding, the mating among close relatives, reduces the fitness offspring in most organisms. However, the magnitude of the resulting fitness reduction (inbreeding depression, ID) often differs among environments. From an evolutionary perspective it is important to understand the effect of an environment on ID, as the magnitude of ID is an important driver e.g. of the evolution of mating systems. In addition, ID can be a major factor in the extinction of species. Since early studies reported higher ID in plants grown in field than in greenhouse populations and in wild mammal populations than in zoo populations, it has often been assumed that ID is generally higher in more stressful environments. Today, the destruction and fragmentation of their habitats has restricted many rare species to small and isolated populations where inbreeding is common. Higher ID under stressful conditions would have important consequences for the conservation of rare species in the face of changing environmental conditions like climate change and land-use intensification. However, the evidence for a general increase of ID under stress is controversial, and a number of studies have found no differences among environments or even higher ID in the more favorable environment. As an alternative hypothesis it has been proposed that ID does not change with stress intensity, but is higher in environments that increase phenotypic variation (phenotypic variation hypothesis). Most of the previous studies on environment-dependent inbreeding depression have compared inbreeding in a species only under one type of stress, which makes comparisons among studies difficult. I conducted a series of experiments and compared the effects of various types of stress and inbreeding on plants to answer the following questions: (1) Does inbreeding depression (ID) differ among environmental conditions? (2) Does ID generally increase or decrease with the intensity of stress? (3) Does ID increase in environments which increase phenotypic variation?

Chapter II and III investigate the interacting effects of inbreeding and stress on performance and plasticity of the perennial herb *Silene vulgaris.* Seedlings derived from self- and cross-pollinations were clonally propagated, and replicates of each of the genotypes were grown under eight stress treatments in a greenhouse. These included a control, drought, copper addition, simulated herbivory and two levels of nutrient deficiency and of shade.

Inbreeding depression differed among stress treatments and decreased with stress intensity (Chapter II). This decrease of ID with stress was particularly strong for stress types to which the species may have become adapted in its population of origin (drought, nutrient deficiency), whereas ID did not change with increasing shade. To test if the results from the experiment can be transferred to the situation in natural populations, I planted selfed and cross-pollinated *S. vulgaris* into a common garden and into a more stressful field site. In contrast to the greenhouse experiment, ID was higher in the field site than in the common garden. However, the phenotypic variation hypothesis explained both the higher ID in the field compared to the common garden, and the decrease of ID with stress intensity in the greenhouse.

Inbreeding also affected the response of various traits of *S. vulgaris* other than biomass to stress (Chapter III). Offspring from self-pollination were less plastic in some important functional traits, like stem length, leaf area, specific leaf area and chlorophyll content. Plants changed their allocation patterns in response to specific stresses like shading and nutrient deficiency as predicted by optimal partitioning theory, but these allocation responses were not affected by inbreeding. Two traits that are often part of a general stress response, leaf anthocyanins and senescence, were higher under nutrient deficiency and copper stress, but lower under herbivory and shade than in the control. Inbreeding reduced anthocyanins, but increased senescence. Fluctuating asymmetry of leaves was not increased by inbreeding and was not consistently higher under stress than in the control, suggesting that fluctuating asymmetry of leaf traits is not a generally suitable indicator for environmental and genetic stresses.

A second study system was the hemiparasite *Rhinanthus alectorolophus* (Chapter IV), which was grown with a number of plant species representing a range in host quality for the parasite and thus in stress intensity. Selfed and open-pollinated offspring from two populations were grown with single individuals of 13 host species known to differ in their quality for the parasite. In a second experiment parasite seedlings were grown with four-species mixtures of the same 13 hosts differing in the number of legumes and of functional groups (grasses, legumes and non-leguminous forbs). Inbreeding reduced the performance of the hemiparasite *R. alectorolophus*. Inbreeding depression was strongest for parasites grown with good hosts and decreased with stress intensity, i.e. with declining host quality. When grown with mixtures of four host species, ID decreased with the number of host functional groups, suggesting a buffering of the effects of deleterious

alleles by host functional diversity. Grasses were on average the best hosts for *R. alectorolophus*, but host quality varied strongly within functional groups. In mixtures of species, parasite biomass increased with the number of host functional groups. In addition, more legumes in a mixture strongly benefited the parasites, as even non-host legumes increased mixture productivity by symbiotic nitrogen fertilization. The contribution of individual species to the quality of a mixture for *R. alectorolophus* could not be predicted from experiments with single hosts. The growth of good hosts was suppressed most strongly by the parasite, but some suitable host species were very tolerant to parasitization. Inbreeding did not influence the negative effects of the presence of the parasite on host growth.

In conclusion, the results of both studies suggest that in contrast to the predominant expectation, ID does not generally increase with stress intensity. In both studies, the magnitude of ID depended on the stress treatment. However, when ID changed with stress intensity, it was lower under more stressful conditions, which supports the alternative hypothesis that cross-pollinated plants are more capable of using favorable conditions than selfed plants. Differences in phenotypic variation explained some of the differences in ID among treatments in *S. vulgaris*, but not in *R. alectorolophus*. The phenotypic variation hypothesis thus does not provide a general explanation for environment-dependent inbreeding depression, but may be useful for understanding the mechanisms contributing to differences in ID. We further conclude from the experiments that stressful conditions will not generally exacerbate the negative effects of inbreeding for small and fragmented populations. However, inbred plants may be less able to cope with changing conditions because of reduced phenotypic plasticity. Inbreeding depression may increase under unpredictable, fluctuating conditions including multiple environmental stresses which are characteristic of many natural environments.

Zusammenfassung

Das Ziel dieser Dissertation war es, unser Verständnis der Auswirkungen von Inzucht und Stress auf Wachstum und Reproduktion von Pflanzen zu verbessern. Inzucht, d.h. die Paarung nah verwandter Individuen, wirkt sich in der Regel negativ auf das Wachstum und die Fortpflanzungsfähigkeit der Nachkommen aus. Das Ausmaß dieser negativen Auswirkungen, die als Inzuchtdepression (ID) bezeichnet werden, variiert jedoch in Abhängigkeit von den Umweltbedingungen. Aus evolutionärer Sicht ist es wichtig, den Einfluss der Umweltbedingungen auf die Stärke der Inzuchtdepression zu verstehen, da diese deutliche Auswirkungen z.B. auf die Evolution der Paarungssysteme von Pflanzen haben kann. Darüber hinaus kann ID eine entscheidende Rolle beim Aussterben von Arten spielen. Seit Studien gezeigt haben, dass die ID bei Pflanzen in natürlichen Populationen meist stärker als unter Gewächshausbedingungen und bei Säugetieren in Natur stärker als unter Zoobedingungen ist, wird oft angenommen, dass ID grundsätzlich unter stressreicheren Bedingungen (also Bedingungen, die im Mittel das Wachstum und Überleben reduzieren) stärker ist, als unter günstigeren Bedingungen. Viele seltene Arten kommen heutzutage aufgrund der anhaltenden Zerstörung und Fragmentierung ihrer Habitate nur noch in kleinen, isolierten Populationen vor, in denen Inzucht häufig ist. Eine Zunahme der Stärke der ID unter stressigen Bedingungen könnte sich im Zusammenhang mit dem Klimawandel und der Intensivierung der Landnutzung negativ auf den Erhalt seltener Arten auswirken. Die Ergebnisse von Studien zum Einfluss von Stress auf ID sind jedoch widersprüchlich, denn einige Studien fanden keinen Einfluss der Umwelt auf die ID oder sogar stärkere ID in einer weniger stressreichen Umwelt. Eine andere Hypothese postuliert, dass ID nicht mit der Stressintensität ansteigt, sondern unter Umweltbedingungen, welche die phänotypische Variation erhöhen (Hypothese der phänotypischen Variation). Die meisten der vorliegenden Studien zum Umwelteinfluss auf ID sind allerdings schwer zu vergleichen, da sie bei sehr unterschiedlichen Arten jeweils nur den Einfluss eines Typs von Stress auf die Stärke der ID untersucht haben. Ich habe deshalb eine Reihe von Experimenten durchgeführt die anhand jeweils einer Pflanzenart den Einfluss diverser Umweltbedingungen auf ID untersuchen. Meine Hauptfragen waren dabei: (1) Unterscheidet sich die Stärke der Inzuchtdepression (ID) unter unterschiedlichen Umweltbedingungen? (2) Nimmt die Stärke der ID generell mit

der Stressintensität zu oder ab? (3) Steigt ID unter Bedingungen, welche die phänotypische Variation erhöhen?

Kapitel II und III untersuchen den Einfluss von Inzucht und Stress auf Wachstum und phänotypische Plastizität von *Silene vulgaris*. Durch Selbst- und Fremdbestäubung gewonnene Keimlinge wurden klonal vermehrt, und Replikate jedes Genotyps wurden unter acht verschiedenen Bedingungen in einem Gewächshaus angezogen. Diese umfassten eine Kontrollbehandlung, Trockenheit, Kupferstress, simulierte Herbivorie, sowie Nährstoffmangel und Schatten in jeweils zwei Stärken.

Die Inzuchtdepression unterschied sich je nach Art der Behandlung und nahm mit der Stressintensität ab (Kapitel II). Diese Abnahme der ID war besonders stark unter Stressbedingungen, an die sich die Pflanzen in ihrer Herkunftspopulation möglicherweise angepasst hatten (Trockenheit und Nährstoffmangel), wohingegen sich die ID mit zunehmendem Schatten nicht änderte. Um zu testen, ob die Ergebnisse des Gewächshausversuchs auf die Bedingungen in natürlichen Populationen übertragbar sind, wurden in einem zweiten Experiment Keimlinge aus Selbst- und Fremdbestäubung in einen Versuchsgarten und eine Wiese gepflanzt, in der die Pflanzen schlechter wuchsen als im Versuchsgarten. Im Gegensatz zum Gewächshausexperiment war die ID in der Wiese im Vergleich zum Versuchsgarten höher. Die Hypothese der phänotypischen Variation erklärte sowohl den Anstieg der ID mit Stressintensität in der Wiese, als auch die Abnahme der ID mit Stressintensität im Gewächshaus.

Inzucht beeinflusste nicht nur die Reaktion der Biomasse, sondern auch die anderer Merkmale von *S. vulgaris* auf verschiedene Umweltbedingungen (Kapitel III). Nachkommen aus Selbstbestäubungen waren weniger plastisch in einigen wichtigen funktionellen Merkmalen, wie der Sprosslänge, Blattfläche, spezifischen Blattfläche und im Chlorophyllgehalt. Pflanzen passten die Muster der Biomasseallokation in verschiedene Organe an bestimmte Umweltbedingungen, wie Schatten und Nährstoffmangel, so an, wie es von der Theorie der optimalen Partitionierung vorhergesagt wird, aber diese Allokationsplastizität wurde nicht durch Inzucht beeinflusst. Zwei Merkmale, die oft an einer generellen Stressantwort beteiligt sind, der Anthocyangehalt der Blätter und die Seneszenz, waren unter Nährstoffmangel und Kupferstress stärker ausgeprägt als in der Kontrolle, unter dem Einfluss von Herbivorie und Schatten dagegen weniger stark. Inzucht hatte eine geringere Anthocyanbildung, aber ein erhöhte Seneszenz zur Folge.

Die fluktuierende Asymmetrie der Blätter wurde nicht durch Inzucht beeinflusst, und war auch unter Stress nicht einheitlich höher als in der Kontrolle. Diese Größe ist deshalb nicht als Indikator für den genetischen oder umweltbedingten Stress geeignet.

Ein zweites Untersuchungssystem bildete der pflanzliche Hemiparasit *Rhinanthus alectorolophus* (Kapitel IV), der mit einer Reihe unterschiedlich geeigneter Wirte angezogen wurde, die einen Stressgradienten für den Parasiten darstellen. Selbst- und offenbestäubte Nachkommen aus zwei Populationen wurden mit einzelnen Individuen 13 verschiedener Wirtsarten angezogen, die sich in ihrer Qualität als Wirt für den Parasiten unterschieden. In einem zweiten Experiment wurden die Parasitenkeimlinge mit Mischungen aus jeweils vier der 13 Wirtsarten angezogen, die sich in der Anzahl Leguminosen und in der Anzahl funktioneller Gruppen (Gräser, Leguminosen, Kräuter) unterschieden. Inzucht wirkte sich negativ auf die Größe der Parasiten aus. Die Inzuchtdepression war am stärksten bei Parasiten, die mit guten Wirten wuchsen und nahm mit zunehmender Stressintensität, also mit abnehmender Wirtsqualität, ab. Bei den Parasiten, die mit Wirtsmischungen wuchsen, nahm die Stärke der ID mit der Anzahl funktioneller Gruppen in einer Mischung ab, was darauf hinweist, dass die Effekte negativer Allele durch die funktionelle Diversität einer Mischung abgepuffert wurden. Gräser waren im Mittel die besten Wirte für *R. alectorolophus*, aber die Wirtsqualität variierte innerhalb der funktionellen Gruppen stark. In Mischungen von Wirten nahm die Größe der Parasiten mit der Anzahl funktioneller Gruppen zu. Darüber hinaus profitierten Parasiten von mehr Leguminosen in einer Wirtsmischung, denn selbst Leguminosen, die nicht als Wirte geeignet waren, erhöhten die Produktivität einer Mischung durch symbiotische Stickstoffdüngung. Der Beitrag einzelner Arten zur Qualität einer Mischung für *R. alectorolophus* konnte nicht durch die Eignung der einzelnen Wirte vorhergesagt werden. Das Wachstum gut geeigneter Wirte wurde am stärksten durch die Parasiten reduziert, aber einzelne gut geeignete Wirte waren sehr tolerant gegenüber der Parasitierung. Inzucht hatte keinen Einfluss auf den negativen Effekt der Parasiten auf das Wirtswachstum.

Die Ergebnisse der Experimente mit beiden Arten verdeutlichen, dass im Gegensatz zur vorherrschenden Meinung die Stärke der Inzuchtdepression nicht grundsätzlich mit der Stressintensität der Umwelt zunimmt. In allen Experimenten variierte die Stärke der ID je nach Behandlung. In den Fällen, in denen sich die ID mit der Stressintensität änderte, war sie aber unter stressreicheren Bedingungen geringer. Dies unterstützt die Hypothese, dass

fremdbestäubte Pflanzen besser in der Lage sind, gute Bedingungen auszunutzen als selbstbestäubte Pflanzen. Unterschiede in der phänotypischen Variation erklärten einige der Unterschiede in der ID zwischen Behandlungen in den Experimenten mit *S. vulgaris*, aber nicht in jenen mit *R. alectorolophus*. Die Hypothese der phänotypischen Variation liefert deshalb keine grundsätzliche Erklärung für umweltabhängige Inzuchtdepression, aber sie kann helfen, die Mechanismen zu verstehen, die zu Unterschieden in der Stärke der ID führen. Eine Schlussfolgerung aus den Ergebnissen der vorliegenden Arbeit ist, dass stressreichere Umweltbedingungen nicht grundsätzlich die negativen Auswirkungen von Inzucht auf kleine, fragmentierte Populationen verstärken. Ingezüchtete Pflanzen sind jedoch aufgrund reduzierter phänotypischer Plastizität schlechter in der Lage, auf sich ändernde Umweltbedingungen plastisch zu reagieren. Unter nicht vorhersagbaren, wechselnden Bedingungen, wie sie für viele Habitate charakteristisch sind, kann Inzuchtdepression stärker sein als unter konstanten Versuchsbedingungen.

Chapter I

General introduction

General introduction

The performance of plants depends to a large degree on the environmental conditions they are exposed to, which can be benign or stressful (Levitt 1972, Grime 1977, Hoffmann and Parsons 1991, Graham et al. 2013). Another process which strongly affects plant performance is inbreeding, the mating between close relatives. An understanding of the effects of inbreeding is particularly important for conservation biology. Since the early days of quantitative conservation biology, inbreeding has been identified as an important threat to the survival of populations and species (Gilpin and Soulé 1986, Hedrick and Kalinowski 2000, Keller and Waller 2002, Frankham 2005), because inbreeding often reduces the fitness of offspring, a phenomenon called inbreeding depression. Small and fragmented populations are expected to be particularly prone to inbreeding. As a consequence of the lower fitness of inbred offspring in small populations, the size of such populations may further decrease, leading in turn to even higher levels of inbreeding, a process called an extinction vortex (Gilpin and Soulé 1986). Inbreeding depression and environmental stress often do not act separately, but have joint and sometimes interactive effects on plant fitness (Dudash 1990, Armbruster and Reed 2005, Cheptou and Donohue 2011). In this general introduction I will present the concepts underlying the effects of inbreeding and environmental stress on plants and briefly review what is known about the interaction of these two factors. I then present the aims of the studies that form my thesis, present the different study systems I used to answer my questions, and give a short outline of the chapters that make up this thesis.

Inbreeding and inbreeding depression

Inbreeding is the mating of related individuals. The degree of inbreeding is described by the inbreeding coefficient f as the probability that two alleles are identical by descent (Waser and Williams 2001). As all plants originate from a common ancestor, the comparison of inbreeding coefficients is always relative to that of a reference generation or population (Falconer 1981, Waser and Williams 2001). Inbreeding can be very strong in plants. In contrast to most animals, the majority of angiosperms are hermaphrodites (Renner 2014), which can self-fertilize (f = 0.5). Self-pollination is common in plants, either by direct pollen transfer within a flower, or among neighbouring flowers of the same plant (geitonogamy; De Jong et al. 1993).

Although the negative consequences of inbreeding were known from humans for centuries and are reflected in very old marriage rules (Waser and Williams 2001), this was not transferred to plants. Until the 19th century it was generally assumed that pollen from the anthers fertilizes the stamens of the same flowers (Baker 1979). After pioneering pollination studies by Christian Konrad Sprengel, Thomas Knight, Friedrich von Gärtner, William Herbert and Friederich Hildebrand published in 1793 – 1867 (Baker 1979), it was Charles Darwin who used his new theory of natural selection to conclude from the observed morphological barriers to self-pollination that inbreeding should have negative consequences for plants (Whitehouse 1959). Darwin (1878) initiated a series of experiments to test this hypothesis which resulted in the self and cross pollination and subsequent growth of more than 60 species of plants. He found that self-pollination nearly always resulted in reduced fitness of the offspring compared to cross-pollination (Darwin 1878). The reduction of fitness in inbred offspring became known as inbreeding depression (ID; Charlesworth and Charlesworth 1987).

Many plant species have evolved mechanisms to avoid self-pollination. A range of different sexual systems exists in which male and female flowers are separated on the same individuals (monoecy) or on different individuals (dioecy). Even in plants with hermaphrodite flowers, the male and female functions are often separated to reduce self-pollination, and many species possess physiological self-incompatibility mechanisms (Barrett 2002). A few plant species even produce different morphs of flowers, which can only reciprocally be pollinated because their stamens and pistils differ in length (heterostyly; Barrett 2002). However, most plants have a mixed mating system which allows inbreeding (Vogler and Kalisz 2001). Even if self-fertilization is impossible, pollination between related neighboring plants in a population often leads to biparental inbreeding ($f < 0.5$), and in small populations, all matings can be regarded as inbreeding (Falconer 1981).

Self-pollination also has some advantages for plants. Self-pollination can assure reproduction when either mates or pollinators are rare. Selfing is thus especially frequent in short-lived species and in populations which are small or close to the range margin of a species (Barrett 2010). In addition, selfing can facilitate local adaptation, as locally adapted genotypes are not diluted by gene flow through pollen from more distant habitats (Charlesworth and Charlesworth 2010). Finally, but most importantly, selfing increases the transmission of genes of the mother plant to the next generation. A completely selfing

mutant in an outcrossing population would transmit both alleles of a locus to the next generation via its seeds and in addition one of the alleles via its pollen, and thus pass 1.5 times as many genes to the next generation as outcrossing individuals (Fisher 1941). This transmission advantage is only neutralized by a strong fitness disadvantage of selfing. Models show that selfing is advantageous if ID is smaller than 0.5, whereas outbreeding will be favoured if ID > 0.5 (Lande and Schemske 1985, De Jong and Klinkhamer 2005).

Two genetic mechanisms, which both result from the increased homozygosity of inbred offspring, can be responsible for inbreeding depression. Heterozygotes may have a higher fitness than both homozygotes (overdominance hypothesis). This hypothesis was favoured for a long time because of the common observation in plant breeding that crossing of inbred lines leads to an increased performance in the hybrid offspring, termed heterosis (Whitehouse 1959). Alternatively, the expression of recessive deleterious alleles which are masked in the heterozygous state may be responsible for the reduced fitness of homozygotes (partial dominance hypothesis). Today, partial dominance is regarded as the more important of the two mechanisms (Crow 1999, Charlesworth and Willis 2009). The hypothesis of partial dominance implies that the genetic load of recessive deleterious alleles can be removed by inbreeding and selection, a process called purging. Purging has been detected in controlled experiments (Crnokrak and Barrett 2002), but appears to be of minor importance in wild populations (Byers and Waller 1999, Keller and Waller 2002). Mathematical models show that purging can be effective for strongly deleterious alleles, whereas the purging of mildly deleterious alleles is effective only at intermediate or large population sizes, depending on the intensity of inbreeding and the recessiveness and selective disadvantage of the involved alleles (Glémin 2003). Some support for the importance of purging comes from reviews which found that forced inbreeding has less negative effects in short-lived and regularly selfing than in outcrossing species (Husband and Schemske 1996), and in small than in large populations (Angeloni et al. 2011). However, low levels of ID can also be due to a higher genetic load in inbred populations, if deleterious alleles have become fixed and not been purged (Keller and Waller 2002, Angeloni et al. 2011).

Inbreeding often reduces plant fitness, but crossing unrelated parents is also not always positive. Like inbreeding it can have negative effects on fitness, a phenomenon called outbreeding depression. Outbreeding depression can be caused by reduced local adaptation of offspring after crosses between plants adapted to different conditions. In

addition, co-adapted complexes of positively interacting genes can be broken up during meiosis and recombination (Waser and Williams 2001). The negative effects of outbreeding are often only visible in the second and later outbred generations, whereas in the first generation the positive effects of heterosis may prevail (Edmands 2007). Outbreeding depression is usually observed after crosses between distant populations, but it has also been observed at small spatial scales, and sometimes even within populations (Waser and Price 1994, Quilichini et al. 2001). In other cases, negative values of inbreeding depression are observed within populations which can hardly be regarded as outbreeding depression (e.g. Paland and Schmid 2003, Sandner 2009).

Environment dependent inbreeding depression

During his series of studies on the effects of inbreeding on plant performance, Darwin (1878) already noted that inbreeding depression was stronger when plants were grown under stress. He observed that "in several cases (but not so invariably as might have been expected)" ID was stronger when plants were grown in competition with other plants than when grown alone (Darwin 1878, p. 288). Similarly, he reported that in some cases crossed offspring were more resistant to unfavorable conditions, like cold weather or freezing. Related observations were made in plant breeding, where the heterosis after crossing inbred lines was often higher in less favorable environments (Lloyd 1980). However, the first planned studies on the effects of environmental stress on ID in plants were performed during the late 1980s. In a very influential study, Dudash (1990) reported that ID in *Sabatia angularis* was highest in a field site and lowest in a greenhouse.

Many studies on the magnitude of ID are performed in controlled environments, and as even these find substantial ID, an increase of ID under stressful conditions would have important consequences for the conservation of rare species. Many of the plants and animals in ex situ cultivation or captive breeding programmes are kept in very small populations where inbreeding is frequent. However, many of the negative effects of inbreeding may not be noted under the benign ex situ conditions, but would increase when the organisms are again released in the wild, which would undermine conservation success (Ralls et al. 1988). This new awareness initiated a series of studies on inbreeding depression in wild populations (reviewed by Crnokrak and Roff 1999, Keller and Waller 2002). In animals, estimates of ID were indeed found to be higher in wild populations than in captive zoo populations (Crnokrak and Roff 1999). This led to the generalization

that ID was always higher in the field than under benign conditions (e.g. Ralls et al. 2007, Frankham et al. 2010, Reed et al. 2012, Prill et al. 2014). In a first review, Armbruster and Reed (2005) found that ID was higher in more stressful environments in the majority of studies. However, the difference in ID was often not significant, and some studies even found the opposite pattern of decreasing ID under stress (e.g. Norman et al. 1995, Waller et al. 2008, Walisch et al. 2012). A recent review found no consistent effect of the environment (field, garden or greenhouse) on the magnitude of ID (Angeloni et al. 2011).

As the number of studies on environment-dependent inbreeding depression has grown, there are today at least three different interpretations of this heterogeneity of results. First, some authors argue that ID increases with the intensity of stress, and that the lack of an increase of ID under stress in a study can be explained by the low stress intensity applied (Fox and Reed 2011, Reed et al. 2012). The meta-analysis of Fox and Reed (2011) was based on 27 plant and animal species as different as *Drosophila* (Diptera) and *Costus* (Zingiberaceae). Most of the species were subjected to only one type of stress, e.g. temperature, viral infections or intraspecific competition. Only 10 plant studies were included, which was even less than in the older review of Armbruster and Reed (2005). More recent studies using only *Drosophila melanogaster* either confirmed (Reed et al. 2012, Enders and Nunney 2012) or questioned the increase of ID with stress intensity (Yun and Agrawal 2014).

As a second interpretation it has been pointed out that a pattern of decreasing ID with stress has also a convincing explanation (Cheptou and Donohue 2011): While it is usually expected that self-pollinated plants are more sensitive to environmental stress, which I will call the sensitive selfed hypothesis (Fig. 1a), it is also possible that cross-pollinated plants are more capable of exploiting favorable conditions, which would lead to decreasing ID with increasing stress. This I will call the capable crossed hypothesis (Fig. 1b).

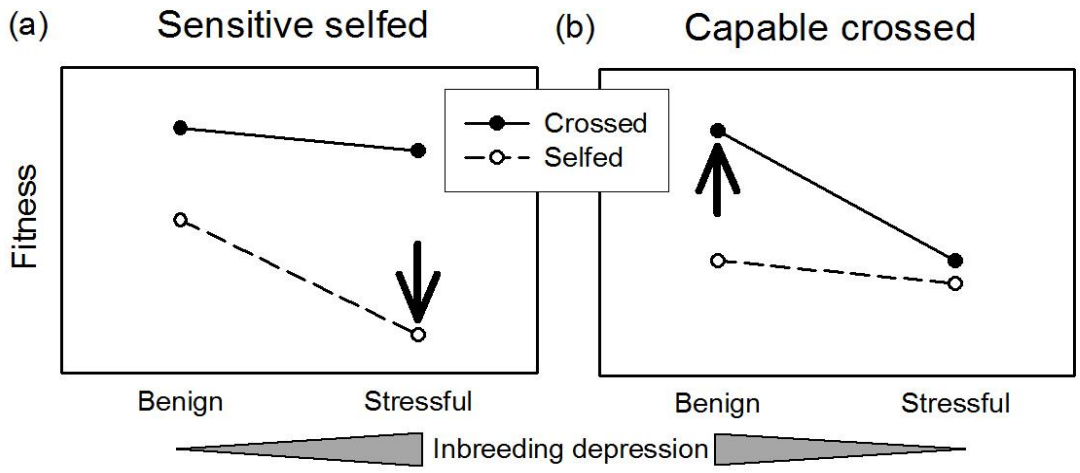

Figure 1: Opposing hypotheses on the effect of stress intensity on inbreeding depression; (a) stress reduces fitness in offspring from self-pollination ("sensitive selfed"), leading to increased ID under stress; (b) crossed offspring is better able to exploit benign conditions ("capable crossed"), leading to reduced ID under stress. Modified after Cheptou and Donohue (2011).

In a third approach, the environment-dependency of ID is not explained by the stress intensity of an environment, but by its effect on phenotypic variation. In their phenotypic variation hypothesis, Waller et al. (2008) argue that inbreeding depression represents the selection against selfed offspring. As selection is limited by the amount of variation exposed to selection, the opportunity for selection (measured as the squared coefficient of variation, CV^2), is expected to set an upper limit to selection (Crow 1958) and ID is thus expected to increase in environments which increase phenotypic variation (CV^2). Although stress may often increase CV^2, it may also reduce CV^2, in which case ID is also expected to be reduced (Waller et al. 2008). The phenotypic variation hypothesis was intended as a simple null-model to be preferred over other explanations due to its parsimony. However, the phenotypic variation hypothesis has been rarely tested (Reed et al. 2012, Long et al. 2013, Yun and Agrawal 2014).

What is stress?

The term stress is widely used but is one of the most controversial concepts in biology, as it is used with very different meanings in different contexts (see Harper 1982, Bijlsma and Loeschcke 2005). The biological stress concept was first introduced for humans by Selye in 1936 and later applied to plants (Levitt 1972, Lichtenthaler 1998). It divides the stress response of an organism into four phases: The beginning of stress is followed by an

(1) alarm phase, in which plants activate their physiological stress-coping mechanisms. When a stress continues, a (2) restitution phase follows, in which the hardened plants reach their resistance maximum and can grow with reduced growth rates, unless a stress is too strong and leads to exhaustion in the (3) end phase. When a stress ends, surviving plants recover in a (4) regeneration phase (Lichtenthaler 1998). In contrast to this physiological definition, Grime (1977) described different plant strategies and defined stress as a factor that reduces the size of plants, like nutrient limitation, water shortage or shade, whereas he defined a factor which destructs plant biomass as disturbance, like herbivory, frost or desiccation. In addition to the physiological and the ecological definition, stress can be defined as an energy drain on an organism (Graham et al. 2013). Although the different concepts are linked, only the effects on fitness matter from an evolutionary perspective (Hoffmann and Parsons 1991, Graham et al. 2013). However, it should be noted that especially in plants the concepts may strongly diverge. Due to the undetermined modular growth of most plants, small differences in relative growth rates can over time translate into large size and fitness differences, even at very low physiological stress levels, e.g. after hardening in the restitution phase of the biological stress concept (Lichtenthaler 1998). In nature most organisms exist under conditions below their optimum and thus under stress most of the time (Hoffmann and Parsons 1991).

For studies on the effects of stress intensity on inbreeding depression, stress has been clearly defined as the reduction of fitness in a certain environment compared to that in a control environment (Armbruster and Reed 2005, Bijlsma and Loeschcke 2005, Fox and Reed 2011). Stress intensity is then calculated as 1 minus the relative fitness of (outcrossed) individuals in an environment (Fox and Reed 2011), which allows the comparison of very different environments in their effects on fitness. I will use this fitness-related stress intensity concept throughout this thesis.

However, even with this clear definition, the stress intensity concept has some problems. Especially the trade-off between the two fitness components growth and survival may lead to different strategies, which is why Sibly and Calow (1989) differentiate among mortality stress and growth stress, related to the distinction among stress and disturbance by Grime (1977). A growth vs. survival trade-off may lead to very different interpretations of the stress intensity of an environment depending on the trait used for the definition of stress. As an illustrative example, the famous study showing increased ID

under stress in *Sabatia angularis* by Dudash (1990) is included in the review by Armbruster and Reed (2005) as an example of the opposing result of reduced ID under stress, because in contrast to the author herself, who regarded the field site as the harshest environment in terms of mortality (Dudash 1990), the greenhouse was interpreted as the more stressful environment in terms of a composite fitness measure.

Plant responses to stress

Environment-dependent inbreeding depression may be caused by effects of inbreeding on plant responses to stress (Cheptou and Donohue 2011). As plants are sessile and cannot move away from unfavorable conditions, plants have evolved the ability to respond to stress in a variety of ways. Changes in the phenotype of a genotype in response to different environmental conditions are called phenotypic plasticity (Scheiner 1993, Sultan 2000). Plasticity is often regarded as adaptive when it represents a functionally appropriate response to a certain environmental factor, although it is difficult to proof that plasticity is really adaptive (Sultan 2000). For example, an increased elongation of stems is regarded as a functional response to competitive shading. This shade-avoidance response is triggered by a change in the ratio of red to far-red light. By producing different phenotypes under different light spectra of the same intensity and transplanting them into competitive and non-competitive environments, the elongation response has been proven to be adaptive, because each of the two phenotypes was superior at one of the density levels (Dudley and Schmitt 1996). Other responses that can be regarded as adaptive include the allocation of biomass to organs invoked in the uptake of the limiting resource, as predicted by the optimal partitioning theory (Bloom et al. 1995, Poorter et al. 2012). For example, plants usually invest more resources into their roots when water or nutrients are limiting, whereas they produce proportionately more leaves under shade (Fig. 2). These allocation patterns can to some degree be explained by allometric growth of the different organs (Weiner 2004), but nevertheless they can be regarded as functional responses for the plants to increase resource uptake (Sultan 2003).

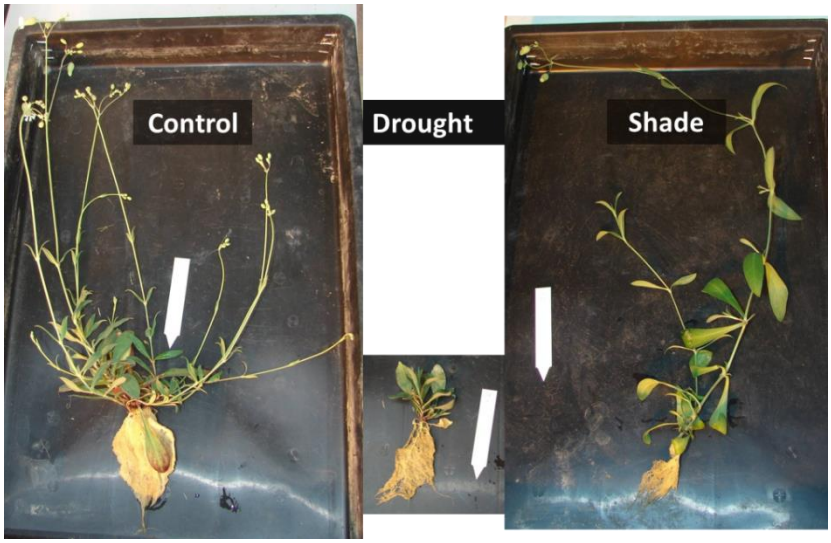

Figure 2: Patterns of biomass allocation in *Silene vulgaris* grown under control, drought and shade conditions. Note the large proportion of biomass allocated to roots in the drought treatment, and the large leaves of plants grown in the shade.

Plasticity in fitness traits is more difficult to interpret. Phenotypic plasticity is regarded as adaptive when it helps to maximize fitness under different environments. An increased phenotypic plasticity in non-reproductive traits should thus correspond to a reduced plasticity in fitness-related traits between two environments ("fitness homeostasis", Hoffmann and Parsons 1991, Richards et al. 2006). However, due to hidden costs and limits of plasticity, a very plastic genotype may have a reduced fitness in some environments compared to a less plastic genotype (van Kleunen and Fischer 2005, Auld et al. 2010). Thus, a genotype which is more plastic in a fitness-related trait like biomass can be regarded as adaptive if its mean fitness is higher in most environments, whereas a higher plasticity in fitness has to be regarded as maladaptive if mean fitness is lower than in a less plastic genotype.

Phenotypic plasticity can be studied by analyses of variance (ANOVA), by analyzing the effects of different environments, different genotypes and their interaction. A significant environment effect in an ANOVA indicates plasticity in the studied trait, and a significant lineage x environment interaction indicates that lineages differ in their plasticity in response to the environment (Whitman and Agrawal 2009). However, ANOVA does not differentiate between plastic responses in different directions, and thus cannot help to distinguish between adaptive and maladaptive plasticity. For example, when treatments as different as shade and drought are studied, a high plasticity of a genotype in specific leaf area can be due to an increased specific leaf area (SLA) in the shade and a reduced SLA

under drought, which would be regarded as adaptive, or the opposite response, which would be probably maladaptive. Traditionally, plasticity is analyzed by norms of reaction, by plotting the trait means of each genotype across two or more environments (Via et al. 1995, Sultan 2000). A steeper slope then represents a higher phenotypic plasticity. However, this concept becomes complicated in the case of more than two environments. Especially when the environments (like shade and drought) require different adaptations, linear reaction norms are not appropriate. To combine the advantages of both approaches (ANOVA and reaction norms), the mean trait values of all plants in an environment can be included as a linear contrast in the ANOVA. This orders the studied treatments by their mean trait value (Finlay and Wilkinson 1963, Via et al. 1995). Although this does not prove that a higher trait value is adaptive in a certain environment, it facilitates the interpretation of results. A lower slope of the individual trait value on the mean trait value indicates that the genotype has a reduced environmental sensitivity (Falconer 1981), i.e. it is less plastic than the population mean (Genotype 2 in Fig. 3). In contrast, a higher slope indicates that a genotype is more plastic than average (Genotypes 1 and 3 in Fig. 3).

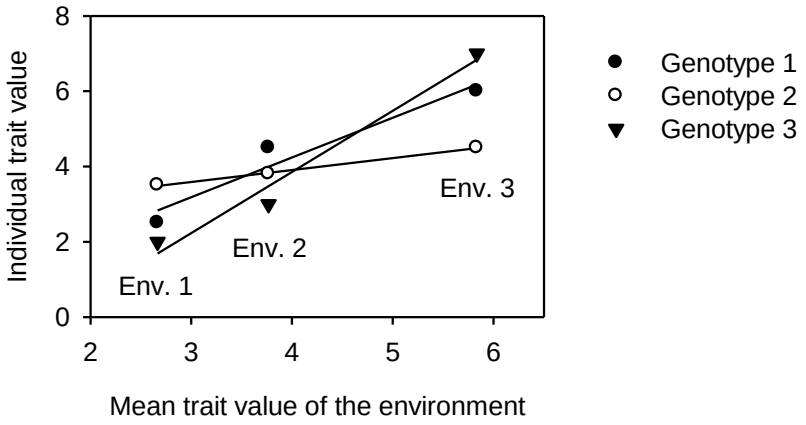

Figure 3: Hypothetical reaction norms of three genotypes in response to three environments with increasing mean trait values.

While it is often adaptive to change a phenotype in response to an environmental change, in other cases it is advantageous to keep the phenotype constant in spite of environmental variation. This capacity of a genotype to express a constant phenotype is called canalization (Debat and David 2001). Canalization does not necessarily imply that a phenotype does not change among environments, as reaction norms can be canalized as well (Scheiner 1993, Debat and David 2001). Within individuals, the capacity to buffer the development against random noise is called developmental stability (van Dongen

2006). Developmental instability is sometimes expected to increase with environmental and genetic stress, such as inbreeding (Møller and Shykoff 1999). A common way to estimate developmental instability is by analyzing fluctuating asymmetry, the deviation from bilateral symmetry in otherwise symmetric organs (Palmer and Strobeck 1986).

Main questions and study systems

System 1: *Silene vulgaris* grown under controlled environmental stresses

To answer the question whether ID increases with the intensity of stress, or whether differences among different types of stress are independent of their intensity, it is essential to compare ID in one species grown in multiple environments. To date, only a few studies have studied the effect of two or three stress types on inbreeding depression in the same species (Daehler 1999, Kéry et al. 2000, Waller et al. 2008, Walisch et al. 2012). In addition, I decided to use cloned lines of selfed and crossed offspring to separate genetic and environmental effects and to analyze the effects of inbreeding on phenotypic plasiticity in non-reproductive traits, which have rarely been studied.

The choice of *Silene vulgaris* as a model species was the result of a long selection process. Because I wanted to grow plants in the greenhouse, pollinate them in different ways and clonally propagate their offspring for a greenhouse experiment, I needed a species that was fast growing, early flowering, quickly germinating, easy to pollinate, self-compatible but not regularly selfing. Furthermore, the species should have many flowers per plant, keep its seeds when they are ripe, allow for clonal propagation and it should be frequent enough to sample a large population. The choice of the species was restricted by trade-offs, e.g. between short generation time and self-compatibility. Many short-lived, fast flowering species are regular selfers (Barrett et al. 1996) and show less inbreeding depression (Angeloni et al. 2011), and most species producing clonal offspring invest less in sexual reproduction (Silvertown and Charlesworth 2001). I thus decided to choose a non-clonal species and propagate the seedlings in-vitro by tissue-culture. Seeds were collected from different mother plants of 15 potential species, of which after germination tests four were chosen for further studies.

The four candidate species, *Anthyllis vulneraria*, *Centaurea scabiosa*, *Lotus corniculatus* and *Silene vulgaris*, were used for three preliminary studies. First, plants of all species were grown to test pollination treatments. Second, seeds of all species were sterilized and

germinated under sterile conditions to test the potential for in-vitro clonal propagation. And third, plants of all species were grown under three intensities of each of six different stress types to find out for each type of stress which is the maximum stress intensity the species can tolerate without mortality, as in the main study mortality should be avoided. Of the four species, only *Silene vulgaris* flowered in the first year, and as this species could also successfully be cloned it chosen for the main experiment (Chapters II and III). *A. vulneraria*, *C. scabiosa* and *L. corniculatus* flowered in the second year and were cross- and self pollinated and offspring of *A. vulneraria* were grown under different stress treatments by Finn Rehling during his BSc thesis (Rehling 2014).

The six stress types in the pilot study had different effects on biomass in the four species. Some of the stress effects were very strong for some of the species, but did not affect biomass in others (Fig. 4).

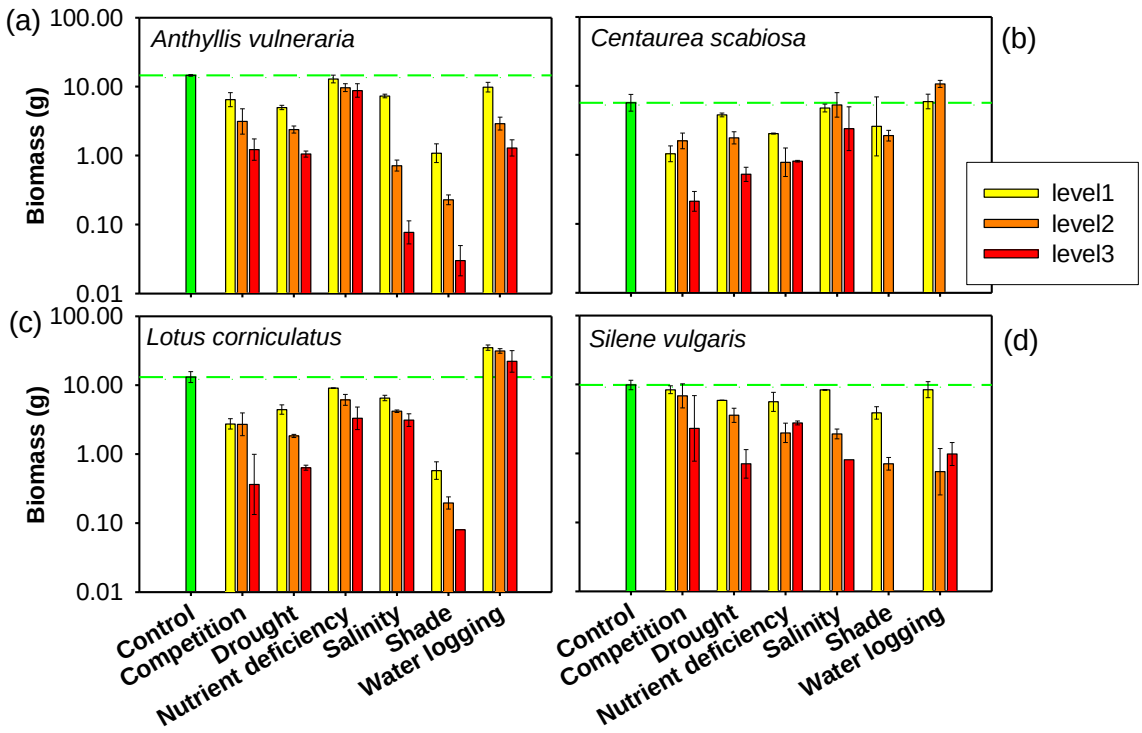

Figure 4: Aboveground biomass (dry) of four plant species grown in a pilot study under three levels of each of six types of stress. The dashed lines indicate the biomass of the control group. Error bars indicate ±1 SE.

For the main experiment with *S. vulgaris* I chose the strongest levels of each stress that did not cause mortality. Competition was not used as a stress type because it was not possible to determine below-ground biomass, and salinity and water logging were not

used because they were difficult to control and after a long period of no visible effects, plants suddenly died. Instead, I included copper contamination, because heavy metal tolerance is known from some *S. vulgaris* populations (Schat and Ten Bookum 1992), and simulated herbivory, because the species is frequently exposed to mowing and herbivory in its natural habitat.

System 2: *Rhinanthus alectorolophus* grown with hosts differing in quality

To test the generality of the effect of stress intensity on inbreeding depression, we studied the effects of a very special biotic stress, host quality for hemiparasitic plants. The biomass and reproductive success of hemiparasitic plants depend to a large degree on the quality of their host species (Fig. 5). Species reported to be good hosts for *Rhinanthus* spp. include some grasses and most legumes (Westbury 2004, Cameron et al. 2006, Hautier et al. 2010), whereas non-leguminous forbs are often reported to be poor hosts, and some of them have been shown to block the formation of haustoria by *Rhinanthus* (Cameron et al. 2006, Rümer et al. 2007). Species differing in host quality can be regarded to represent a gradient of stress intensity. Nothing is known about the effects of inbreeding on the performance of parasites along a gradient of stress by different host quality.

Figure 5: Gradient of host quality for *R. alectorolophus* grown autotrophically (left), with a poor host (*Leucanthemum vulgare*, center) and a good host (*Lolium perenne*, right).

Outline of the thesis

Chapters II and III use *Silene vulgaris* as a model species to understand the effects of inbreeding and environmental stress and their interactions on plant fitness. It is commonly expected that inbreeding depression increases under stress, but the results are controversial. We thus subjected cloned replicates of inbred and outbred offspring of *S. vulgaris* to different environmental conditions. **Chapter II** focuses on the effects on fitness and asks if ID differs among stress treatments, or more precisely, if ID increases with stress intensity or with phenotypic variation in an environment.

Chapter III represents a more in-depth analysis of the mechanisms underlying environment-dependent inbreeding depression by exploring the effects of inbreeding on the response of different plant traits to stress. Only few studies have addressed the effects of inbreeding on phenotypic plasticity in non-reproductive traits involved in stress responses. We could also test the hypothesis that developmental instability and fluctuating asymmetry increase with both stress and inbreeding.

Chapter IV uses *Rhinanthus alectorolophus* as a model system for a similar question: Does ID generally increase with stress intensity, in this case with decreasing host quality? To answer this question, we grew selfed and open-pollinated offspring of *R. alectorolophus* with 13 plant species differing in host quality, and with 15 different four-species mixtures of these 13 species, which better represent the situation in the field.

Chapter V is a short synthesis which returns to the main questions raised in this introduction, compares the results found with the different study systems, and presents a short outlook on future research.

Chapter II

The effects of stress intensity and stress type on inbreeding depression in *Silene vulgaris*

With D. Matthies, submitted to Evolution

Abstract

Inbreeding depression (ID) is generally assumed to increase under stressful conditions, but a number of studies have found the opposite pattern, i.e. that crossed offspring were more capable of exploiting benign conditions. Alternatively, the phenotypic variation hypothesis predicts that not stress intensity, but enhanced phenotypic variation in an environment leads to increased ID. We subjected inbred and crossed offspring of *Silene vulgaris* to drought, simulated herbivory, copper contamination, and two levels of nutrient deficiency and of shade. In contrast to the predominant expectation, most stress treatments decreased inbreeding depression. With increasing nutrient limitation, ID decreased strongly, whereas under increasing shade ID did not change. These differences may be due to purging at the site of origin that is nutrient-poor and dry, but not shaded. In contrast to the greenhouse experiment, ID was higher in a field site than in a more benign common garden. However, the predictions of the phenotypic variation hypothesis were met in both the greenhouse and the field vs. garden experiment. The results suggest that there may be no general relationship between ID and stress intensity, but specific effects of stress type and the novelty and variability of the environment.

Introduction

Inbreeding, the mating between closely related individuals, is common in plants. The majority of angiosperms is hermaphroditic (Renner 2014), which makes self-pollination within flowers or among neighboring flowers (geitonogamy) possible. Although plants employ many mechanisms to reduce self-pollination, including spatial (herkogamy) or temporal (dichogamy) separation of male and female organs in flowers, different floral morphs (heterostyly) or physiological self-incompatibility (Barrett 2002), most plants have a mixed mating system and selfing is common in plants (Vogler and Kalisz 2001). Because plants are sessile, and most pollen and seeds are not dispersed very far from the parents, many plant populations have a spatial genetic structure with higher relatedness among neighbors (Heywood 1991, Vekemans and Hardy 2004), which facilitates biparental inbreeding. Today, the frequency of inbreeding is further increased for many species due to the fragmentation of habitats (Ellstrand and Elam 1993, Young et al. 1996, Leimu et al. 2006).

Self-fertilization can be advantageous for plants, because it assures reproduction when mates are rare, facilitates local adaptation and increases the transmission of genes to the offspring (Barrett 2002, Charlesworth & Charlesworth 2010). However, selfing commonly has negative effects on fitness, which are called inbreeding depression (ID). Inbreeding increases homozygosity in the offspring leading to a reduction of fitness caused predominantly by the increased expression of recessive deleterious alleles in homozygotes (dominance hypothesis). ID may also be caused by an increased fitness of heterozygotes (overdominance hypothesis), but this is appears to be less frequent than previously thought (Crow 1999, Charlesworth and Willis 2009). Numerous studies have shown that inbreeding depression is very frequent in plants (Darwin 1878, reviews by Charlesworth and Charlesworth 1987, Husband and Schemske 1996, Keller and Waller 2002, Angeloni et al. 2011), but the magnitude of ID depends on the fitness trait studied (Angeloni et al. 2011). ID in traits which are expressed early in the life cycle is thought to be mostly due to strongly deleterious recessive alleles which are more likely to be purged by selection (Husband and Schemske 1996), in particular in small populations and regularly selfing species (Glémin 2003). In contrast, much of the ID in late traits is thought to be due to weakly deleterious mutations which may be difficult to purge (Husband and Schemske 1996, Byers and Waller 1999, Glémin 2003).

The magnitude of ID may strongly depend on environmental conditions (Armbruster and Reed 2005, Cheptou and Donohue 2011). In an influential study, Dudash (1990) found inbreeding depression in *Sabatia angularis* to be stronger in natural sites than in the greenhouse. Since then, it has often been assumed that ID is generally higher in stressful than in benign environments, because inbred offspring are more sensitive to stressful conditions than crossed offspring (Ralls et al. 2007, Frankham et al. 2010, Reed et al. 2012, Prill et al. 2014). However, the results of studies on the effect of stress on ID have been inconsistent (see review by Armbruster and Reed 2005). Most studies found that stress increased ID, but many found no effect of stress, and some even lower ID under stress (Armbruster and Reed 2005; Norman et al. 1995, Henry et al. 2003, Leimu et al. 2008, Waller et al. 2008). A literature survey found no consistent effects of competition on ID in plants (Willi et al. 2007) and a recent meta-analysis of the effect of different environments (field, greenhouse or garden) on the magnitude of ID found no general trend (Angeloni et al. 2011). A possible explanation for the inconsistent results is that the effect of stress on ID may depend on its intensity (Fox and Reed 2011). To make different

types of stress comparable in their intensity, stress intensity has been defined as the reduction in fitness compared to a no stress control (Hoffmann and Parsons 1991, Bijlsma and Loeschcke 2005, Fox and Reed 2011). It has been suggested that ID increases linearly with stress intensity, and that only studies using low stress intensities find no increase of ID with stress (Fox and Reed 2011). Alternatively, both crossed and selfed offspring may perform poorly under stress, while offspring from cross pollination may be more capable of exploiting benign conditions, which would cause ID to decline with stress (Cheptou and Donohue 2011).

Waller et al. (2008) proposed a phenotypic variation hypothesis, which states that an environment that increases phenotypic variation in a fitness-related trait increases the opportunity for selection (measured as the squared coefficient of variation, CV^2, Crow 1958). As inbreeding depression is the difference in relative fitness between selfed and crossed offspring, it represents the selection against selfed offspring and is expected to increase with the opportunity for selection. An environment that increases phenotypic variation may be in some cases the more stressful, in others the more benign environment (Waller et al. 2008). The phenotypic variation hypothesis can thus be regarded as a null-model: if the increase in CV^2 (measured within cross types to avoid autocorrelation with ID) between two environments is correlated with the increase in ID, more complex explanations for the effects of stress on the strength of inbreeding depression are not necessary. However, an environment might also increase phenotypic variation without increasing ID (e.g. because of random herbivory), or increase ID without changing CV^2, in which case more complex mechanisms must be sought.

One potential source of increased phenotypic variation is the size-dependence of stress effects. In contrast to animals, plants show a huge plasticity in size, and as they grow their perceived stress intensity may change. When stress intensity is higher for smaller plants, existing size differences will be magnified by stress, and both ID and phenotypic variation (CV^2) will increase. Similarly, intraspecific competition has been shown to increase size hierarchies by dominance and suppression (Weiner 1985), and to increase ID (Schmitt and Ehrhardt 1990, Cheptou et al. 2001, Yun and Agrawal 2014). In contrast, in greenhouse experiments stress intensity may often increase with plant size as pot size and nutrients become limiting. A stress which is stronger for large than for small plants will decrease ID and phenotypic variation.

Studies on environment-dependent inbreeding depression in plants have usually either compared ID between greenhouse, common garden and field environments (e.g. Dudash 1990, Eckert and Barrett 1994, Koelewijn 1998) or have experimentally applied single types of stress like competition (e.g. Schmitt and Ehrhardt 1990, Van Treuren et al. 1993, Wolfe 1993, Eckert and Barrett 1994, Cheptou et al. 2000b), drought (e.g. Nason and Ellstrand 1995, Hauser and Loeschcke 1996, Cheptou et al. 2000a, Sedlacek et al. 2012) or herbivory (e.g. Carr and Eubanks 2002, Hayes et al. 2004, Ivey et al. 2004, Stephenson 2004, Kariyat et al. 2011, Campbell et al. 2013). In comparing the results of these studies, it is not possible to distinguish between the effects of different stress types, species or lineages within species (Armbruster and Reed 2005). Therefore, studies on the interaction of the effects of inbreeding with those of different environmental stresses in the same species are needed (Reed et al. 2012). However, to date only few studies have investigated ID in plant species under two or three types of stress (Daehler 1999, Waller et al. 2008, Walisch et al. 2012).

The aim of this study was to compare the effect of multiple types of stress on inbreeding depression in *Silene vulgaris*, a species known to show inbreeding depression (Glaettli and Goudet 2006, Emery and McCauley 2002). To distinguish between the effects of stress type and stress intensity, we subjected inbred and crossed plants of *S. vulgaris* to drought, simulated herbivory, heavy metal contamination, and two levels of nutrient deficiency and shade. To increase the precision of estimates of effects, cloned individuals were subjected to each stress type. In a second experiment, selfed and crossed individuals were grown both in a common garden and in the field. Specifically, we asked the following questions: (1) Does the studied population of *Silene vulgaris* show inbreeding depression in early and late components of fitness? (2) Does ID differ among treatments in the greenhouse? (3) If so, does ID increase or decrease with the intensity of stress? (4) Is ID higher in environments that increase phenotypic variation (phenotypic variation hypothesis)? And more specifically, (5) do environments that increase size differences among small and large plants also increase ID (size-dependent stress hypothesis)? Finally, (6) can the results from the controlled greenhouse environments explain the differences in ID between a field site and a more benign common garden?

Methods

Study species

Silene vulgaris (Moench) Garcke (Caryophyllaceae) is a perennial herb with white, protandrous flowers. The main pollinators are moths and long-tongued bees (Friedrich 1979, Clapham et al. 1987). Most plants have hermaphrodite flowers, but plants with only female flowers also occur, whose proportion in the population has been shown to increase after selfing (Emery and McCauley 2002). *S. vulgaris* is distributed throughout Eurasia and has been introduced to North America and Australia. In Central Europe, the species is shade intolerant (Ellenberg et al. 1992) and occurs in moderately dry, more or less nutrient-poor meadows, on roadsides and in quarries and gravel-pits (Oberdorfer 2001). Some populations have evolved tolerance to heavy metals, especially copper (Schat and Ten Bookum 1992) and occur on contaminated soils, but this does not apply for our study population. The species was chosen for the study because it is outcrossing, but self-compatible (Glättli and Goudet 2006), is fast growing, flowers after a few months, and in a pilot study proved to be suitable for in-vitro propagation.

Pollination treatments

In August 2011 seeds were collected from 15 plants that were at least 2 m apart in a nutrient-poor meadow near Bad Sooden-Allendorf in northern Hesse, central Germany (51°16’N, 9°55’ E). Seeds were germinated in Petri dishes, and five seedlings per plant were grown in a greenhouse until they flowered. One hermaphroditic descendant from each seed family was chosen as mother plant for the pollination experiment and all open flowers were removed. In the following weeks, flowers were emasculated once they had opened and two days later pollinated with a similar amount of pollen from either different flowers of the same plant (self treatment) or with pollen from the other plants (cross treatment). Similar to the situation in a natural population, we did not use single, specific fathers for the cross treatment, but a pollen mix from 3-6 of the other plants in the pollination experiment. Both crossing treatments were carried out on each plant. Non-pollinated flowers were removed to keep the number of flowers per mother plant similar and avoid resource allocation to non-target flowers. In March 2012 all seeds were collected, counted and weighed per capsule.

Germination and clonal propagation

Seeds from each pollination treatment were pooled per mother plant and from each of 12 of the pollinated plants 50 seeds were chosen randomly per treatment. The seeds were surface-sterilized in ethanol (1 minute) and chlorine disinfectant (10 minutes) and then rinsed in sterile water. Seeds were germinated in Petri dishes containing 25 ml of a MS basal medium (Murashige and Skoog 1962, pH = 5.8) under ambient light at 25 ± 1 °C. Every three days the number of germinated seeds was counted. After four weeks, when most seeds had germinated, the length of the cotyledons was measured and the number of malformed seedlings was counted. Malformed seedlings had either one or three cotyledons instead of two, or lacked chlorophyll. Nine healthy seedlings per combination of mother plant and treatment were selected for further propagation. They were transferred without roots into 440 ml screw-capped glasses filled with 100 ml shoot induction medium (MS + 2 mg/L BAP [6-benzylaminopurine]), to induce the formation of multiple shoots. The plants were kept at room temperature under natural light and the position of the glasses was frequently randomized. After three months, when a sufficient number of shoots had formed, shoot tips were cut and transferred into 440 ml screw-capped glasses filled with 100 ml of MS without hormones to induce the formation of roots. The cuttings were kept at room temperature under natural light and frequently randomized in their position. When roots started to form six weeks later, the cloned seedlings were planted into 0.5 L pots filled with 600 g of sterilized sand and covered with transparent bags to avoid desiccation. Two days later the bags were cut open and five days later completely removed. From six of the mother plants, at least one seedling from self- and one from cross-pollination was available, each of which had produced c. 16 surviving clonal replicates, resulting in a total of 447 clones from 29 seedling genotypes and six mothers (Fig. 1).

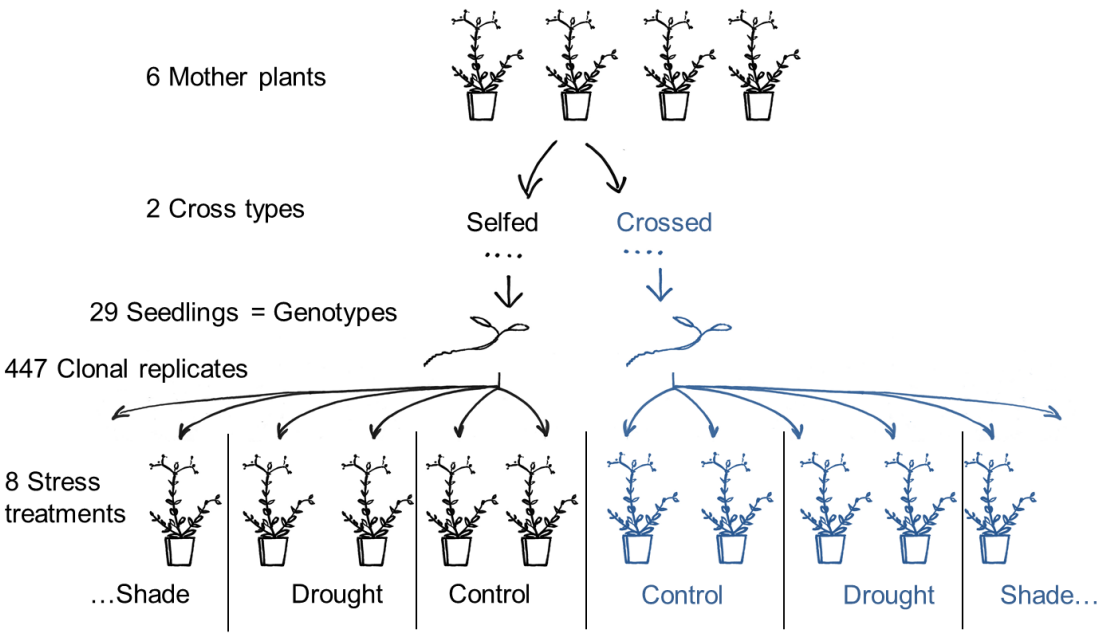

Figure 1: Schematic diagram showing the sequence of pollination treatments, clonal propagation and stress treatments in the experiment.

Greenhouse experiment

From September to November 2012, two clonal replicates of each genotype were grown under eight different treatments: (1) Plants in the control group received 16 h of full light by sodium high pressure lamps, were watered from above until saturation every 2 days and received once a week 125 mg of a commercial fertilizer (N:P:K = 14:7:14%; Hakaphos Gartenprofi, Compo, Wien). Plants in the seven stress treatments were grown for two weeks under control conditions and then treated in exactly the same way, except for the following modifications: (2) Drought plants were placed on a balance every second day and water was added until pots weighed 106% of their dry weight. (3) Plants in the simulated herbivory treatment were clipped 2 cm above ground after five weeks of growth. (4) Pots in the heavy metal treatment received after two and three weeks of growth 20 ml of a 20 mM $CuSO_4$ solution, corresponding to an overall concentration of 132.6 mg copper per kg soil. (5) Plants in the low-nutrient treatment received only 1/4, and (6) those in the very low nutrient treatment only 1/16 of the amount of nutrients of the control plants. (7) Plants under light shade grew under one layer of neutral shading cloth (37% of control irradiance), (8) and those under strong shade under two layers (14% of the control irradiance). The stress types were chosen to be of importance to the species in the wild. At the same time, we selected conditions which require a broad spectrum of

different plant responses. Based on the results of a pilot study, the intensity of each of five stress types was chosen to have a strong negative effect on plant growth without causing mortality. In addition, two of the stress types, nutrient deficiency and shade, were also applied at medium intensities to estimate the effects of stress intensity within stress type.

Each stress treatment was applied to 50 – 65 plants, which were kept in trays of 10 – 12 pots. The positions of the trays in the greenhouse were randomized every second week, and in between all pots were randomized among trays of the same type to avoid confounding effects. After nine weeks of growth, the inflorescences, leaves and stems of the plants were harvested separately, dried for 48 h at 80 °C and weighed. Because it was foreseen that cleaning the root systems would take a long time, the pots were frozen and kept at -12 °C to avoid decomposition of the roots. The roots were then washed free of soil, dried and weighed.

For the effects of inbreeding on early fitness components we analyzed fruit set (i.e. the probability of a flower to produce seeds), the number of seeds per capsule and the mean seed mass of flowers subjected to the different pollination treatments. We further analyzed the germination probability of the seeds, the cotyledon length of the seedlings and the proportion of malformed seedlings. As estimates of fitness influenced by the stress treatments we analyzed total biomass, inflorescence biomass and the probability of flowering of the offspring. To estimate lifetime inbreeding depression, a multiplicative fitness function was calculated per combination of family and stress treatment as fruit set x seed number x germination x biomass, which represents the total biomass produced per pollinated flower.

Total biomass was regarded as a the best estimate of fitness, because it is assumed to be more relevant for this perennial species than flowering traits and to be less influenced by allocation patterns or phenology. Biomass was square-root transformed for all analyses to achieve homoscedasticity and normally distributed residuals. Mean values were backtransformed before calculating inbreeding depression (ID) and stress intensity. ID was calculated for every combination of mother plant and treatment as one minus the relative fitness of the inbred (w_i) vs. that of the outbred (w_o) individuals: $\delta = 1 - (w_i/w_o)$. When inbred plants performed better than outbred plants, ID was calculated as $\delta = (w_o/w_i) - 1$ to keep all values between 1 and -1 (Ågren and Schemske 1993). This reversed formula was used to calculate inbreeding depression in biomass for seven out of

48 family by stress combinations in the greenhouse and for two out of ten families in the field site. However, the choice of formula did not qualitatively influence the results. Stress intensity was calculated as one minus the biomass of the crossed plants in each environment, relative to the biomass of crossed plants in the control (Fox and Reed 2011). The multiplicative fitness function was not used for this purpose, as the three early fitness components were not influenced by the stress treatments.

Field vs. common garden experiment

From the seedlings germinated in Petri dishes with MS medium, 100 seedlings from self and cross pollinations from 10 mother plants were planted into 0.9 L pots filled with a 1:1 mixture of sand and commercial potting soil (TKS1, Floragard Oldenburg) and transferred to flowerbeds in the Botanical Garden of the Philipps-University Marburg on 1 June 2012. They were watered regularly and received once a month 125 mg of a commercial fertilizer (N:P:K = 14:7:14%; Hakaphos Gartenprofi, Compo, Wien). Another 100 seedlings from the same mother plants were planted into soaked peat pellets (4 cm Jiffy pots) and kept in a greenhouse. After a week of growth they were transplanted into a field site near the Department of Biology, 500 m from the common garden. The seedlings were planted randomly in a 15 cm grid and their position recorded. The chosen site was located on a SE exposed slope and dominated by *Hieracium caespitosum* and *Leucanthemum vulgare*. *S. vulgaris* did not occur. Before the seedlings were planted, the site was mown to reduce competition by the established vegetation.

After 11 weeks of growth, plants in both the common garden and the field site were harvested 1 cm above ground. The field site was mown afterwards to reduce competition and allow resprouting and survival throughout the winter. Pots in the common garden were covered with fleece during the winter. In the second year they were watered regularly and received every three months 125 mg of the commercial fertilizer and their position was randomized. In July 2013, when most plants of both populations were flowering, plants in both the common garden and the field site were harvested a second time.

Statistical analyses

Hierarchical analyses of variance were used to test the effects of mother plant and pollination treatment on early traits, and of mother plant, pollination treatment, genotype, and the stress treatments on measures of plant performance. The corresponding error terms were chosen according to the rules for the analysis of mixed models (Zar 2010). To test for inbreeding depression (Question 1), the effect of cross type (fixed) was tested against the mother x cross interaction. To test for possibly confounding variation among lineages on early traits, the effects of mother plant (random) and the mother x cross interaction were tested against the residual variation. Possible lineage effects on late traits like biomass were tested against the variation among the plants resulting from the crossings (= genotypes, random). The effect of stress treatment (fixed) was tested against the stress x mother interaction, which, like the stress x mother x cross interaction, was tested against the genotype x stress interaction. To test for differences in ID among treatments (Q2), the stress x cross interaction was tested against the stress x mother x cross interaction. In a second step, we split the treatment effect into the linear contrast stress intensity (1 df) and the remaining treatment effect (rest, 6 df). This made it possible to analyze the interaction between the effects of stress intensity and cross type (Q3). Binary variables like germination, malformation of seeds and flowering probability were analyzed by generalized linear models with a logit link and a binomial error distribution with the same model structure as in the ANOVA models (analysis of deviance, Quinn and Keough 2002). The effects of mother and stress treatment on the multiplicative fitness function were analyzed by two-way analysis of variance without interaction.

As a measure of phenotypic variation, the opportunity for selection was calculated as the squared coefficient of variation (CV^2) separately for selfed and outcrossed individuals for every combination of mother and stress treatment. The separate CV^2 values for the selfed and crossed plants were then averaged to give one value per combination of mother and stress treatment which is mathematically independent of ID (Waller et al. 2008, Reed et al. 2012). To evaluate whether stress intensity or phenotypic variation is more important for explaining differences in inbreeding depression (Q3 and Q4), Reed et al. (2012) proposed to use a multiple regression approach with model averaging. Therefore, the AICc values of models including stress intensity and phenotypic variation in all possible combinations were compared using the package AICcmodavg version 2.0-3 with the

software R version 3.2.1 (R Core Team 2015). To illustrate the results of the best model, partial regression plots were constructed that show the relationship between inbreeding depression and individual predictors adjusted for the effects of all other predictors in the model (Moya-Laraño and Corcobado 2008).

To test the effects of the stress treatments on size differences independent of pollination effects (Q5), only the offspring from cross pollinations in the experiment were analyzed. Two different methods were used to define groups of small and large cross-pollinated plants. (1) Based on their initial size, all cross-pollinated plants were ranked by their leaf width at the start of the stress experiment. (2) Based on their size at harvest, all genotypes were ranked by their average biomass in the control treatment. The 33% largest plants identified with each method formed the group of large plants and the 33% smallest the group of small plants. Both groups together consisted of 156 plants in the classification based on start size and 115 plants in the classification based on genotypes. The effects of stress treatment, size class and stress x size class interaction on biomass at harvest were tested with analyses of variance. Coefficients of size depression were calculated for every environment based on mean total biomass of plants in the group of small (w_S) and large plants (w_L) at harvest as $1 - (w_S/w_L)$.

To compare the results of the greenhouse study with those of the field vs. garden experiment (Q6), inbreeding depression was calculated for offspring of each mother plant for biomass (square-root transformed), survival in the field or garden, and a multiplicative fitness function combining both (biomass of offspring per mother plant), but excluding early traits (fruitset and germination), because they were independent from the environment. The opportunity for selection (CV^2) was calculated, as in the greenhouse experiment, first for each combination of mother x cross x environment, and then averaged per combination of mother x environment. The effect of the environment (field or garden) on ID and CV^2 was tested in ANOVAs using the mother plants as replicates.

Results

Inbreeding effects on early traits

Of the selfed flowers, 22% were aborted and produced no seeds, compared to only 5% of crossed flowers (Table 1). Crossed flowers that were not aborted produced 36% more, but

not larger seeds (Table 1). Germination of seeds from most mother plants was reduced after selfing. After 28 days, 99% of the seeds from cross pollination had germinated, but only 90% of the seeds from self pollination, with no further increase in the next week. Inbreeding decreased germination of seeds from all mother plants, but to various degrees (Table 2). Selfed seedlings had 28% shorter cotyledons than crossed seedlings (6.6 vs 9.2 mm) and more than three times as many of them were malformed (12.4% vs. 3.8%).

Table 1: Analyses of deviance and variance of the effects of mother plant and cross type (self vs. outcross) on the reproduction of *S. vulgaris*. For seeds per fruit and seed mass, two mothers were excluded, because they formed no seeds after selfing. ***, $p < 0.001$, **, $p < 0.01$, *, $p < 0.05$.

		Fruit set				Seeds per fruit				Mean seed mass		
Source	df	MD	F		df	MS	F			MS	F	
Mother plant	14	300.2	1.29	**	12	1529.3	4.61	***		5.93	2.69	**
Cross type	1	1177.5	21.49	**	1	2048.0	7.88	*		0.76	0.63	
Mother x cross	14	54.8	0.24		12	259.9	0.78			1.20	0.55	
Capsule	81	232.9			64	331.9				2.20		

Table 2: Analyses of deviance and variance of the effects of mother plant and cross type (self vs. outcross) on germination, cotyledon length and the proportion of malformed seedlings in *S. vulgaris* after 28 days of germination. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

		Germination			Cotyledon length			Malformed seedlings		
Source	df	MD	F		MS	F		MD	F	
Mother plant	11	3.48	5.60	***	211.77	18.60	***	6.02	5.77	***
Cross type	1	41.78	20.47	***	1177.82	39.98	***	29.90	35.88	***
Mother x cross	11	2.04	3.29	**	29.46	2.59	*	0.83	0.80	
Petri dish	48	0.62			11.39			1.04		

Influence of stress type on inbreeding depression

At harvest, inbreeding depression was present in most fitness-related traits. Selfed offspring produced on average 37% less biomass (1.596 vs. 2.514 g) and 54% less inflorescence mass (0.109 vs. 0.236 g) than offspring from cross pollination, whereas the probability of flowering did not differ among cross types (Table 3). However, the effects

of cross type differed among the stress treatments (cross x stress interaction in Table 3). Inbreeding depression in total biomass was similar to the control (δ = 43.0%) in the two shade treatments (δ = 43.1% and 45.1%), while it was considerably lower in the other five stress treatments (16.5% - 36.1%, Fig. 2). ID in inflorescence mass was highest in the control treatment and reduced under all stress treatments. In the control treatment, all crossed offspring flowered, but only 81% of the selfed offspring. Flowering probability was reduced under all stress treatments, and in the strong shade treatment no plants flowered at all. However, when this treatment was excluded from the analysis, the cross x stress interaction effect on inflorescence mass did hardly change ($F_{6,30}$ = 6.46, $p < 0.001$).

Table 3: Analyses of variance and deviance of the effects of mother plant, cross type, genotype, and stress treatment on inflorescence mass, the probability of flowering and total biomass of *S. vulgaris*. To allow an analysis of the cross x stress intensity interaction, the stress treatment was split into the linear contrast "stress intensity" and the remaining effect of stress treatment. However, the main effect of stress intensity and the interactions of stress intensity with mother or genotype were not of interest and are not listed in the table. ***, $p < 0.001$, **, $p < 0.01$, *, $p < 0.05$, +, $p < 0.10$.

		Total biomass			Inflorescence mass			Probability of flowering		
	df	MS	F		MS	F		MD	F	
Mother	5	1527	7.2	***	642	5.1	**	4.89	3.4	*
Cross type	1	9083	15.8	*	2537	8.1	*	3.30	1.1	
Mother x cross	5	573	2.7	+	311	2.5	+	3.04	2.1	
Genotype	17	212	1.5	+	125	2.4	**	1.42	1.9	*
Stress treatment	7	11860	44.3	***	4435	39.8	***	31.94	21.6	***
Mother x stress	35	267	1.6	*	111	1.6	*	1.48	1.9	**
Cross x stress	7	263	2.6	*	280	7.2	***	1.42	3.8	**
Cross x intensity	*1*	*1175*	*11.5*	**	*1703*	*43.5*	***	*5.54*	*15.0*	***
Cross x rest	*6*	*111*	*1.1*		*43*	*1.1*		*0.73*	*2.0*	+
Mother x cross x stress	35	102	0.6		39	0.6		0.37	0.5	
Genotype x stress	116	168	1.2		68	1.3	+	0.77	1.0	
Error	217	138			53			0.75		

ID in the multiplicative fitness function (offspring biomass per pollinated flower) differed among mother plants ($F_{5,35}$ = 12.193, $p < 0.001$), but not among stress treatments ($F_{7,35}$ = 0.791, $p = 0.60$). ID was higher than 0.5 in all stress treatments for two of the mothers,

while it was consistently lower than 0.5 in all stress treatments for one of the mothers (Fig. 3). Lineage effects were observed throughout the experiment. Mothers differed in fruit set, seed number and mean seed mass (Table 1), and they influenced the germination and seedling traits of their offspring (Table 2) as well as their biomass and flowering probability (Table 3). In addition, the resistance of plants to stress was influenced by the identity of their mothers (mother x stress interaction in Table 3). However, mother plants did not influence the interactive effects of inbreeding and stress on traits of their offspring at harvest, as the mother x cross x stress interaction was far from significant (Table 3). Offspring from individual seeds (= genotypes) differed in inflorescence mass and flowering probability, but little in total biomass (Table 3).

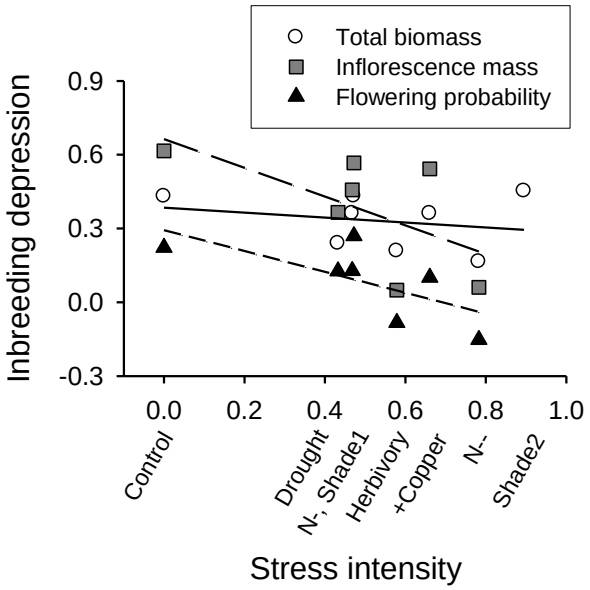

Figure 2: Effects of stress intensity and stress type on inbreeding depression in total biomass (continuous line), inflorescence biomass (dashed line) and probability of flowering (short dashed line) of *S. vulgaris*. Plants grown under strong shade did not produce any flowers and inbreeding depression for flowering traits could thus not be determined.

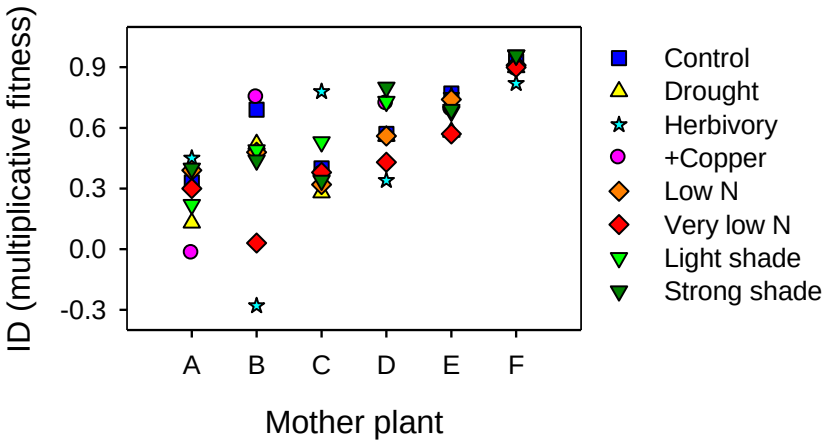

Figure 3: Mean inbreeding depression in multiplicative fitness (total biomass per pollinated flower) of offspring from six seed families of *S. vulgaris* under eight stress treatments. Mother plants are ordered in ascending order of mean inbreeding depression. Several symbols overlap in some mothers.

Effects of stress intensity on inbreeding depression

Most of the effects of the cross x stress interaction on biomass could be attributed to effects of stress intensity (see linear contrast in Table 3), as the effects of cross type on biomass decreased with stress intensity (Fig. 4). Maximum stress intensity in the experiment was high. Although only one plant died during the experiment (in the herbivory treatment), the biomass of crossed offspring was under stress reduced on average by 61% in comparison to the control. Stress intensity was highest in the strong shade treatment (89% less biomass than in the control treatment).

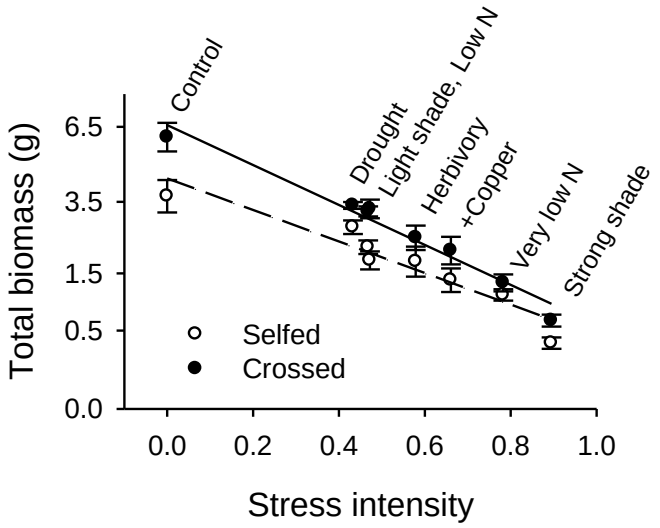

Figure 4: Effects of stress intensity (i.e. 1 - relative fitness of crossed plants per treatment) and stress treatment on total biomass of selfed (open) and crossed (filled symbols) offspring of *S. vulgaris*. Lines show the linear effect of stress intensity for crossed (continuous line) and selfed (dashed line) offspring. Error bars indicate standard errors of the predicted values. Note square-root scale for biomass.

The decrease of ID with increasing stress intensity was even stronger for inflorescence mass and flowering probability than for ID in biomass (linear contrast in Table 3, Fig. 2). For nutrient deficiency and shade it was possible to analyze the effects of stress intensity within stress type, as two different intensities had been applied. Family means of inbreeding depression in biomass decreased with increasing nutrient deficiency, but did not change with increasing shade (Table 4, Fig. 5).

Table 4: Analyses of covariance of the effects of mother plant and stress intensity on family means of inbreeding depression of biomass in *S. vulgaris* under two types of stress. **, $p < 0.01$, *, $p < 0.05$.

		Inbreeding depression (Stress = N-deficiency)			Inbreeding depression (Stress = shade)		
Source of variation	df	MS	F		MS	F	
Mother	5	0.087	4.09	*	0.141	7.48	**
Stress intensity	1	0.196	9.27	*	0.001	0.07	
Error	11	0.021			0.019		

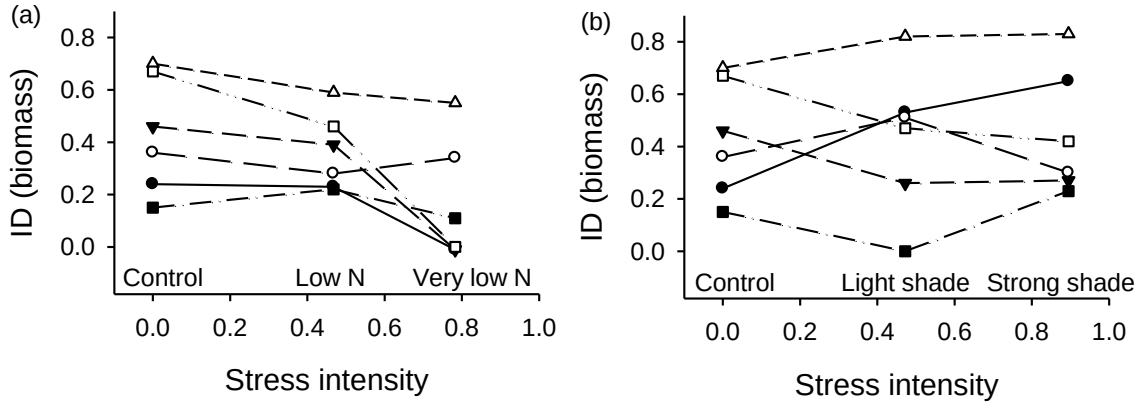

Figure 5: Effects of stress intensity on inbreeding depression in total biomass for plants from six seed families of *S. vulgaris* in a greenhouse. (a) Effects of increasing nutrient deficiency and (b) effects of increasing shade. Each family is represented by a different symbol.

Influence of phenotypic variation and size differences on inbreeding depression

Phenotypic variation of total biomass was not generally higher under stress, but differed among treatments. The opportunity for selection (CV^2) was highest under strong shade (0.62), intermediate in the copper (0.35), herbivory (0.32), control (0.31), light shade (0.29) and very low N (0.27) treatments, and lowest in the low nutrient (0.09) and drought treatments (0.04). In multiple regressions of the effects of mother plant, stress intensity, phenotypic variation and their interaction on family means of inbreeding depression in biomass, models including phenotypic variation (CV^2) performed better than the model including only stress intensity (Table 5). After model averaging, stress intensity had a relative importance weight of 0.57, compared to one of 0.93 for CV^2. The best model based on Akaike's information criterion contained both stress intensity and CV^2. In this model, family level inbreeding depression in biomass increased with phenotypic variation ($\beta = 0.40$, $p = 0.002$, Fig. 6a) and decreased with stress intensity ($\beta = -0.22$, $p = 0.077$, Fig. 6b).

Table 5: Comparison of coefficients of determination (r^2), AICc values and AICc based likelihoods (weights) of four models testing the effects of stress intensity and CV^2 on inbreeding depression in biomass. All models include the effects of mother plant as a nuisance variable. Models are ranked from the best (lowest AICc) to the worst. Weights were computed only for models without interaction (Burnham and Anderson 2002). Importance of CV^2 = 0.93, of intensity = 0.57.

Model	r^2	AICc	weight
Mother + stress intensity + CV^2	0.48	2.66	0.55
Mother + CV^2	0.44	3.40	0.38
Mother + stress intensity + CV^2 + interaction	0.48	5.90	-
Mother	0.33	7.41	0.05
Mother + stress intensity	0.34	9.65	0.02

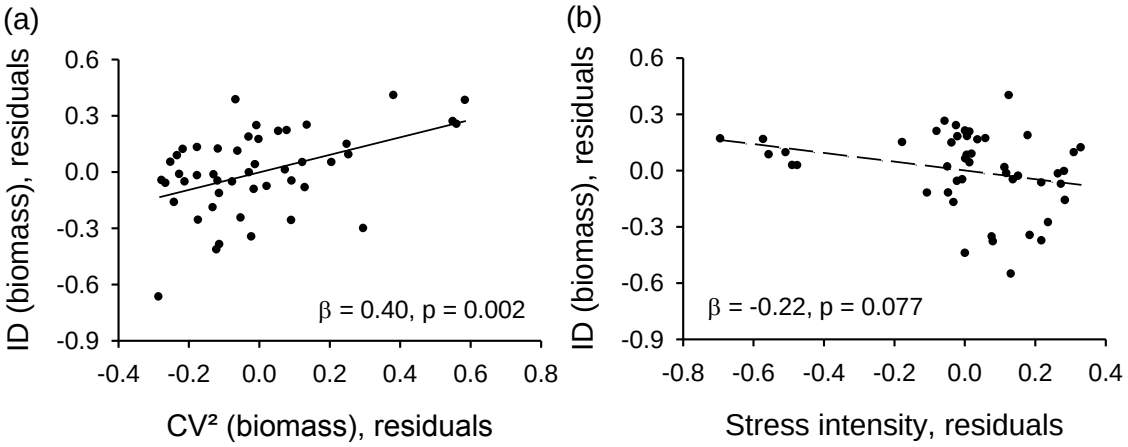

Figure 6: Partial regression plots showing the relationship between inbreeding depression in biomass and (a) phenotypic variation (CV^2) and (b) stress intensity. The relationships are based on a model containing mother plant, phenotypic variation and stress intensity as predictors (r^2 = 0.48, $p < 0.001$; see Table 5).

The two measures of size depression among cross-pollinated plants were strongly correlated (r = 0.856, p = 0.007). The effect of stress treatments on total biomass at harvest differed among size classes (stress x size class: $F_{7,140} = 3.32$, p = 0.003 and $F_{7,99} = 3.02$, p = 0.006 for the two methods). The relative size differences between small and large crossed offspring were largest in the control treatment and under strong shade, and smallest in the drought, low nutrient and light shade treatments (Fig. 7). Size depression and inbreeding depression were not correlated (r = 0.185 and r = 0.134, both p > 0.6), but size depression was stronger in environments with high phenotypic variation (CV^2; r = 0.63, p = 0.095 and r = 0.69, p = 0.058 for the classifications based on initial plant size and final biomass, respectively).

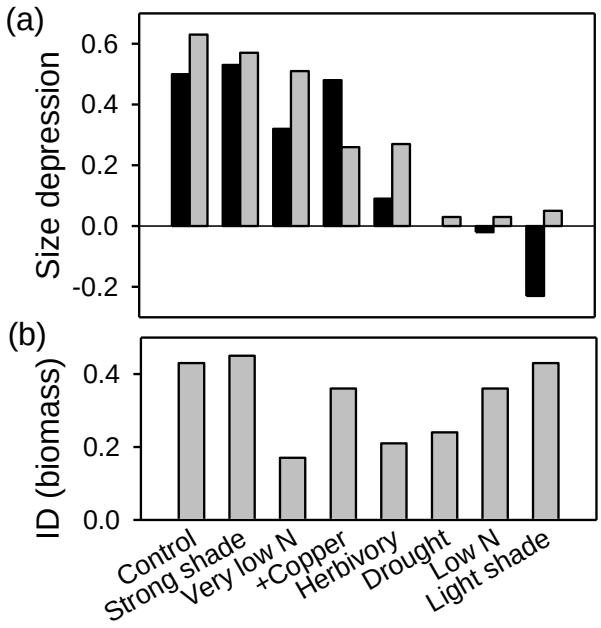

Figure 7: Effects of stress treatments on size depression (i.e. the relative size differences between small and large plants) of cross-pollinated offspring of *S. vulgaris*. The two groups of small and large plants were determined by two methods, based on initial leaf length (black bars) or based on final biomass of genotypes in the control treatment (gray bars), see text for details. Treatments are in the order of decreasing size depression. Effects of treatments on inbreeding depression are given for comparison.

Effects of field vs. garden conditions

Plants grew larger in the common garden than in the field and their survival was higher (Fig. 8a, b). The field site was therefore regarded as more stressful. In both years, average inbreeding depression was higher in the field than in the garden. However, family means of inbreeding depression did not differ between the two sites in the first year (Table 6, Fig. 9a). In the second year, inbreeding depression for all traits was significantly higher in the field site than in the more benign common garden (Table 6, Fig. 8, 9b). Phenotypic variation in biomass was also much higher in the field than in the garden (2012: $CV^2 = 0.54$ vs. 0.17, $F_{1,18} = 15.82$, $p < 0.001$; 2013: 0.97 vs 0.14, $F_{1,18} = 73.82$, $p < 0.001$), but mean inbreeding depression of the offspring of a family was not significantly related to its phenotypic variation (2012: $r = 0.33$, $p = 0.16$; 2013: $r = 0.31$, $p = 0.25$), which may, however, be due to the low power of detecting differences with only 10 families.

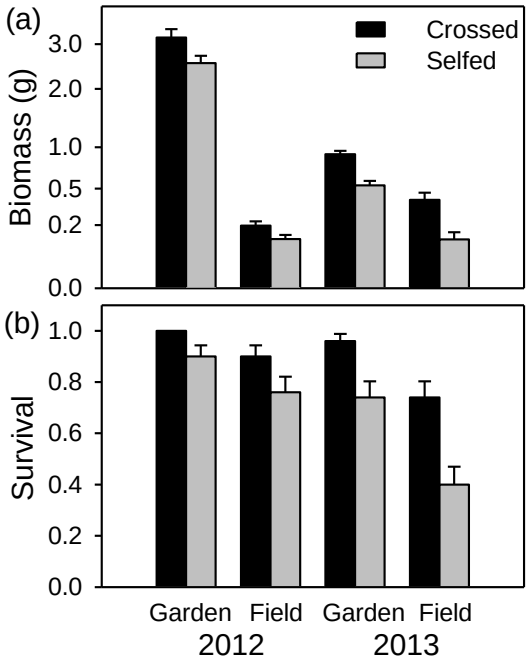

Figure 8: (a) Above-ground biomass and (b) survival of offspring of *S. vulgaris* from cross- and self-pollinations grown in a common garden and a field site for two years. Error bars indicate +1 SE. Note square-root scale for biomass.

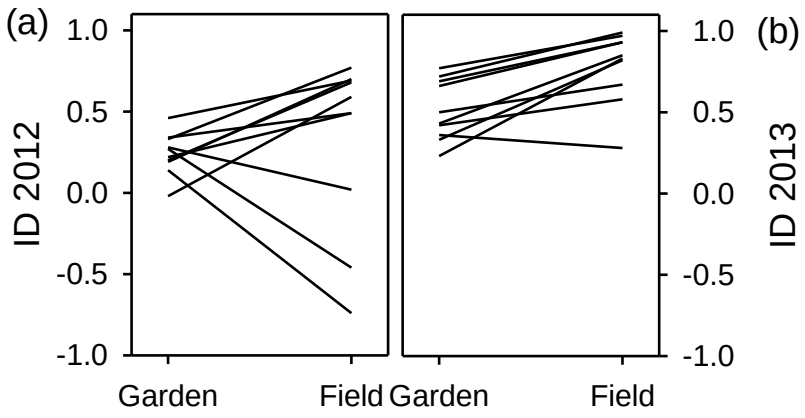

Figure 9: Inbreeding depression in the biomass per transplanted seedling for offspring of *S. vulgaris* from ten mother plants in a common garden and a field site in (a) 2012 and (b) 2013.

Table 6: Differences between populations of *S. vulgaris* grown at a field site and in a common garden in family means (10 mother plants) of inbreeding depression. F-values (1 vs. 18 df) from separate analyses of variance and deviance. **, $p < 0.01$, *, $p < 0.05$.

	2012			2013			
Fitness measure	ID garden	ID field	F-value	ID garden	ID field	F-value	
Survival	5.3	9.4	0.41	17.7	40.7	4.48	*
Biomass	18.9	26.5	0.18	41.4	68.2	8.23	*
Multiplicative fitness	24.1	32.3	0.22	51.1	78.5	9.00	**

Discussion

Inbreeding depression and stress intensity

In contrast to the predominant expectation that inbreeding depression (ID) increases in more stressful environments (Dudash 1990, Armbruster and Reed 2005), ID did not increase but slightly decreased with increasing stress intensity in the controlled stressful environments. This pattern was even stronger for reproductive traits (inflorescence mass, flowering probability) than for total biomass. Decreasing ID with stress is in contrast to the results of a meta-analysis by Fox and Reed (2011), who found a positive relationship between the number of lethal equivalents and stress intensity and suggested that exceptions to this pattern were due to low stress intensities (most likely in studies with less than 25% fitness reduction). The stress intensities in our study were very high (> 40% for the weakest stress, and up to 89% fitness reduction in the strong shade treatment), but no increase of ID with stress intensity was observed. Another possible reason for no increase of ID with stress is mortality, which can truncate size distributions in a stressful environment (Armbruster and Reed 2005). Because mostly small plants die, the magnitude of ID for size-related traits under stress may be underestimated if there is mortality. This does not hold for this study, however, as there was hardly any mortality. The results of our study thus lend some support to the hypothesis that crossed plants are more able than inbred plants to capitalize on the favorable control conditions, while under stress the performance of offspring from both cross types is poor (Cheptou and Donohue 2011), which might be called the "capable crossed hypothesis". The mother x cross interaction had a small effect on biomass compared to the stress and cross x stress effects,

and the absence of possibly confounding mother x cross x stress interactions indicates that the low number of mother plants in the design was sufficient for answering our questions.

Differences in ID among stress types

Apart from a general trend of decreasing ID with stress intensity, our greenhouse study revealed differences among stress types. While inbreeding depression did not change with increasing shade, it decreased with increasing nutrient stress. In line with our findings, crossed offspring of *Saxifraga granulata* reacted more strongly to nutrient addition than selfed offspring (Walisch et al. 2012), while there was no effect of competition or defoliation on ID. Similarly, plants of *Primula veris* from large populations reacted more plastically to nutrient addition than plants from small, probably more inbred populations (Kéry et al. 2000), whereas they did not differ in their response to competition. In the Hawaiian *Schiedea lydgatei*, ID in biomass did not differ between two fertilizer treatments, but ID in flowering age and number of flowers was found only in the high nutrient environment, not under nutrient stress (Norman et al. 1995). In three populations of *Lychnis viscaria*, ID in the number of leaves was increased by fertilization, though the pattern was less clear for ID in cumulative fitness in the second year, which differed among populations (Mustajärvi et al. 2005). However, ID does not always increase with nutrient supply. In the annual *Lupinus texensis*, nutrient deficiency increased the abortion rate of developing seeds, and proportionately more selfed than crossed seeds were aborted under nutrient-poor than under more nutrient-rich conditions (Helenurm and Schaal 1996). Similarly, in the wild gourd *Cucurbita pepo* ssp. *texana*, ID in several flower and seed traits was higher in a less fertile field site (Hayes et al. 2005). However, because both studies did not study offspring fitness, the results are difficult to compare with those of the other studies.

There are several possible explanations for the observed differences in environment-dependent inbreeding depression among stress types. It has been shown that at the cellular level, both inbreeding and environmental stress can lead to similar molecular responses, which could result in an increase of ID with stress intensity (Reed et al. 2012, Leimu et al. 2012). However, in addition to a general stress response, there are numerous specific physiological responses to different types of stress (Lichtenthaler 1998, Schulze et al. 2005, Taiz and Zeiger 2010) which can be influenced by recessive deleterious mutations.

Some studies, especially on *Drosophila*, indicate that recessive alleles involved in inbreeding depression are not generally deleterious, but only in some environments (Bijlsma et al. 1999, and references in Reed et al. 2012). As a consequence, the magnitude of ID under stress may depend on the purging history of a population. When a plant population has a history of inbreeding under specific environmental conditions, recessive alleles which are deleterious under these conditions may have become purged. In experiments, these conditions will not increase inbreeding depression, while novel conditions will, even if they are less stressful (Cheptou and Donohue 2011, Reed et al. 2012). The plants we used in our experiment originated from a population growing under unshaded, but nutrient-poor and dry conditions. Apart from being adapted to drought and nutrient deficiency, the plants were probably adapted to the loss of biomass by mowing or herbivory, as the growth of shrubs and trees at their site of origin has been prevented by mowing or grazing. Indeed, inbreeding depression was highest under the novel conditions shade, copper addition and abundant nutrient supply, and lowest under conditions like drought, herbivory, and nutrient deficiency that the population had experienced during its history. A possible objection against this interpretation of the observed pattern of ID is that purging is often not very efficient and a slow process (Glémin 2003). However, our source population has likely existed under the same conditions for a long time.

ID and stress in the field and the common garden

In contrast to the effects of stress intensity on ID in the greenhouse experiment, inbreeding depression in *S. vulgaris* was higher in the more stressful field site than in the common garden, which corresponds to the predominant expectation that selfed plants are more sensitive to stress, which we might be called the "sensitive selfed hypothesis". The difference between the results of the two experiments could be due to the longer duration of the outdoor experiment (2 years) compared to that of the greenhouse experiment (9 weeks), and the fact that mortality occurred. Other studies have found increasing ID with stress in survival, but not growth or reproduction of the survivors (Hauser and Loeschcke 1996, Sedlacek et al. 2012, but see Cheptou et al. 2000a for the opposite result).

The contrasting results of the two experiments could also be due to types of stress that were not studied in the greenhouse but occurred at the field site, like competition or interactions among various types of stress. The field site was undermined by voles, which kept some plants very small for weeks and thus increased variation. Many of the fruits of

the plants in both the common garden and the field site were consumed by caterpillars of the moth *Sideridis rivularis*. The strong random noise in the field site may have been responsible for the absence of a relationship between ID and stress in the first year (Waller 1984, Mustajärvi 2005). However, in the second year ID was significantly higher in the field site than in the garden. Plants in the field site were subject to competition which has often been found to increase ID (Schmitt and Ehrhardt 1990, Van Treuren et al. 1993, Wolfe 1993, Eckert and Barrett 1994, Daehler 1999, Cheptou et al. 2000b), but a review found no general effect on ID (Willi et al. 2007, see also Walisch et al. 2012). Simulated herbivory in our greenhouse experiment reduced ID, but has been also found to increase ID, e.g. in *Solanum carolinense* (Campbell et al. 2013). The results of other studies on the effect herbivory on the magnitude of ID have been inconsistent, as herbivory either increased (Carr & Eubanks 2002, Hayes et al. 2004) or decreased ID in fitness traits (Leimu et al. 2008), or did not affect it at all (Stephenson 2004, Kariyat et al. 2011).

Conditions in the field are less constant than in the greenhouse, and stress intensity may fluctuate over time, which might have different effects on the strength of ID than a constant stress level. Cheptou et al. (2000a) did not observe an increase of ID under constant drought in *Crepis sancta* (Asteraceae) and suggested that constantly stressful conditions have smaller effects on inbreeding depression than fluctuating stress, e.g. a periodical drought treatment (see Hauser and Loeschcke 1996). Yun and Agrawal (2014) proposed that simple environments in which less genetic pathways are required to function properly may have less ID, in contrast to more complex environments. Environments in which stress intensities fluctuate or stresses interact may be considered more complex environments and expose more and different detrimental mutations to selection than an environment characterized by a strong but continuous stress to which plants may respond and acclimatize (Lichtenthaler 1998). Selfed plants may thus react more sensitive to complex, fluctuating stresses than to continuous stresses of the same overall intensity. This may explain the high ID in the field site, but it cannot explain the decrease of ID with stress intensity in the greenhouse.

Support for the phenotypic variation hypothesis

Finally, the driver of the differences in ID among environments may not be stress intensity but phenotypic variation. An environment that increases the phenotypic

variation in a trait (CV^2) allows for more selection and may thus lead to stronger ID, which is a form of selection against inbred individuals (Waller et al. 2008). This phenotypic variation hypothesis was supported by comparisons among traits, as fitness-related traits with larger variation also showed higher ID, but it could not predict levels of inbreeding depression across stress treatments in *Brassica rapa* (Waller et al. 2008). In a multiple regression analysis based on nine animal data sets, Reed et al. (2012) found stress intensity to be the more important predictor of ID, although the importance value of CV^2 was not much lower. In contrast, in our greenhouse study, family CV^2 was a better predictor of ID in biomass than stress intensity. While ID slightly decreased with stress intensity, it was higher in environments that caused high phenotypic variation. Under strong shade, both phenotypic variation and ID were highest, while under drought, the size variation among plants was strongly reduced and there was only little ID.

The phenotypic variation hypothesis correctly predicted the changes in inbreeding depression both in the greenhouse, where inbreeding depression and variation were often reduced under stress, and in the field vs. garden experiment, where both inbreeding depression and phenotypic variation were much higher in the field site than in the common garden. However, the phenotypic variation hypothesis does not predict which environments increase or reduce phenotypic variation, and is thus not mutually exclusive of other hypotheses, like environment-dependent purging. In environments in which a population is inbred, both phenotypic variation and inbreeding depression might be reduced in subsequent generations.

An environment can influence phenotypic variation and inbreeding depression if stress intensity depends on plant size. Initial size differences among plants or differences in relative growth rates may then be either magnified or levelled out. The analysis of size depression among cross-pollinated offspring revealed that environments differed strongly in their effect on size distributions, but these effects were not responsible for the observed differences in inbreeding depression. The coefficient of size depression cannot be directly compared to the coefficient of inbreeding depression, as the exact magnitude of size depression depends on the arbitrarily chosen size classes. However, the relative differences between environments should not be strongly influenced by this classification, as can be seen in the similarity of the results of the two methods chosen.

Lineage effects

Populations or lineages within populations are known to differ in the magnitude of inbreeding depression (Picó et al. 2004, Leimu et al. 2008, Walisch et al. 2012, and references in Byers and Waller 1999, Armbruster and Reed 2005). Similarly, in *S. vulgaris* offspring of different mothers differed considerably in the amount of inbreeding depression in germination and seedling size and in their susceptibility to different stress treatments. For half of the mothers it depended on the stress treatment whether ID in multiplicative fitness was higher or lower than 0.5. This has important consequences for mating system evolution, as a coefficient of ID > 0.5 is usually regarded as necessary to overcome the twofold transmission advantage of selfing and select for selfing avoidance (like gynodioecy in *Silene vulgaris*), while ID < 0.5 should favor selfing (Charlesworth & Charlesworth 2010). An environment-dependent ID of around 0.5 is thus expected to stabilize mixed mating systems under changing environmental conditions (Cheptou and Donohue 2011). Our multiplicative estimates of lifetime ID are likely underestimates of inbreeding depression in the population of origin, as only hermaphrodites were used in our pollination experiments. In the gynodioecious *S. vulgaris*, the proportion of females in a population is known to increase after selfing, which reduces strong inbreeding (Emery and McCauley 2002), so hermaphrodites are expected to carry less genetic load than females.

Conclusions

We found that ID did not increase under various stresses in comparison to the control in a greenhouse experiment. Instead, in *S. vulgaris*, ID decreased under most controlled stress treatments, suggesting that offspring from cross pollination could more effectively use benign conditions than selfed offspring (capable crossed hypothesis). This was especially true for stresses prevalent at the site of origin (drought, nutrient deficiency and herbivory), suggesting environment-dependent purging. These treatments at the same time reduced the phenotypic variation of plant biomass, thus supporting the phenotypic variation hypothesis of Waller et al. (2008), which correctly predicted both the decrease of ID with stress intensity in the greenhouse and the higher ID in the field than in the more benign garden environment.

Chapter III

Inbreeding limits responses to environmental stress in *Silene vulgaris*

With D. Matthies, in preparation

Abstract

Plants can respond to different environmental conditions by plastically changing morphological and physiological traits and patterns of biomass allocation. To test if these responses are influenced by inbreeding, we grew clones of self- and cross-pollinated offspring of *Silene vulgaris* under eight different stress treatments, including a control, drought, copper addition, simulated herbivory, and two levels of nutrient deficiency and of shade. Four non-reproductive traits, stem length, leaf area, leaf chlorophyll content and specific leaf area (SLA), were higher in the shade treatments than in the control, and lowest under nutrient deficiency, which can be regarded as functionally appropriate responses to the different conditions. The plasticity of these four traits was lower in offspring from self- than from cross-pollination. Biomass allocation patterns changed in response to the environment in agreement with the optimal partitioning theory, but were not influenced by inbreeding. Two traits potentially involved in general stress response – leaf senescence and the proportion of leaf area that is red, a measure of anthocyanin production – were increased under copper stress and nutrient deficiency but reduced in the herbivory and shade treatments. Leaf senescence was higher and the proportion of red leaf area lower in selfed than in crossed offspring. Fluctuating asymmetry (FA) of leaves, a measure of developmental instability, differed among stress treatments, but was not generally higher under stress. Inbreeding increased only one measure of FA, and only under high stress intensities. Our findings suggest that by reducing phenotypic plasticity, inbreeding limits the ability of plants to cope with changing environmental conditions. In *S. vulgaris*, leaf fluctuating asymmetry does not serve as an indicator of environmental stress, nor of genetic stress by inbreeding.

Introduction

Phenotypic plasticity is the capacity of a genotype to express different phenotypes in different environments (Sultan 2000). As terrestrial plants cannot move when environmental conditions change, their performance under different environmental conditions depends to a large degree on their phenotypic plasticity. Some aspects of phenotypic plasticity are inevitable, passive consequences of the environment, while others are adaptive (Sultan 2003, van Kleunen and Fischer 2005). Generally, specific functionally appropriate environmental responses are regarded as adaptive, but to test if a

plastic response is really adaptive requires complex manipulative experiments (Schmitt et al. 1999, Sultan 2003). While the concept of adaptive plasticity assumes that a change in the phenotype in response to the environment may increase fitness, it may also be beneficial for a genotype to minimize within-individual variation. This capacity to buffer the development against random noise is known as developmental stability (Møller and Shykoff 1999, van Dongen 2006). Developmental noise is often not easy to distinguish from plasticity (Scheiner 1993), and sometimes even regarded as an aspect of phenotypic plasticity (Debat and David 2001).

The importance of phenotypic plasticity will increase with the effects of climate change (Nicotra et al. 2010). At the same time, land use change and the fragmentation of natural habitats reduce population sizes in many plant species, and the frequency of inbreeding will increase in the remaining small and isolated populations (Ellstrand and Elam 1993, Young et al. 1996). However, little is known about the effects of inbreeding on phenotypic plasticity (Auld and Relyea 2010, Murren and Dudash 2012, Campbell et al. 2014). Inbreeding increases homozygosity in the offspring and usually reduces their fitness (Charlesworth and Charlesworth 1987), which is called inbreeding depression (ID). As phenotypic plasticity itself has a genetic basis (Scheiner 1993, Nicotra et al. 2010), it has been suggested that inbreeding and reduced genetic variation may also reduce adaptive plasticity (Kéry et al. 2000, Fischer et al. 2000, Bijlsma and Loeschcke 2012, Walisch et al. 2012). Reduced adaptive plasticity after inbreeding would reduce the performance of selfed compared to crossed offspring in some environments, which in turn would result in differences in ID among environments. Indeed, the magnitude of ID depends on the environment under which it is studied (Cheptou and Donohue 2011), but the contribution of plasticity to this pattern is not understood. ID has often been observed to increase under more stressful conditions (Armbruster and Reed 2005, Fox and Reed 2011), which suggests that inbred genotypes are more sensitive to environmental stress, while crossed genotypes are better able to maximize their fitness in different environments. A lower plasticity, but higher canalization of fitness-related traits is advantageous for an individual (fitness homeostasis; Hoffmann and Parsons 1991, Richards et al. 2006). However, the evidence for higher ID under stress is equivocal, and some studies even found the opposite pattern, i.e. reduced ID under stress (Armbruster and Reed 2005). This can be interpreted as a better ability of crossed individuals to exploit benign conditions (Cheptou and Donohue 2011). For example, some studies

reported that inbred plants draw less benefit from nutrient addition than cross pollinated plants (e.g. Kéry et al. 2000, Walisch et al. 2012, chapter II). A higher plasticity in fitness is only adaptive, if it increases overall fitness of the plants.

Most studies on environmental effects on ID in plants have investigated single species under one type of stress, which makes comparisons among studies difficult. In a controlled greenhouse experiment with *Silene vulgaris* grown under eight different stress treatments, ID did was not increased but decreased relative to the control under most stress treatments (Chapter II). In this second part of the same study our aim is to examine whether inbreeding also affects phenotypic plasticity in non-reproductive traits, and whether the effects on plasticity can explain the observed differences in ID of biomass among treatments. Few studies have reported inbreeding effects on plasticity in non-reproductive traits (Auld and Relyea 2010). In plants, plasticity in leaf length in response to competition was reduced in small populations of *Ranunculus reptans* (Fischer et al. 2000). In addition, inbreeding has been shown to affect herbivore resistance by foliar defense traits (Campbell et al. 2013, Campbell et al. 2014) and the response of photosynthesis to fertilization (Norman et al. 1995).

Inbreeding increases homozygosity and thereby increases the expression of deleterious recessive mutations, which is regarded as the predominant cause of inbreeding depression (Charlesworth and Willis 2009). Similarly, recessive mutations may potentially influence some of the diverse specific responses to different environmental stresses expressed by plants (Lichtenthaler 1998, Schulze et al. 2005, Taiz and Zeiger 2010). One major plastic response of plants to different environments is to change the allocation of resources to different organs (Sultan 2003, Poorter et al. 2012). During plant growth, resource allocation changes allometrically with plant size (Weiner 2004). In addition, plants often show increased biomass allocation to organs involved in the uptake of the limiting resource as predicted by economic models (optimal partitioning theory, Bloom et al. 1995, Shipley and Meziane 2002, Poorter et al. 2012). To increase resource uptake, plants may not only modify the fraction of biomass allocated to roots, stems or leaves, but also specific leaf area (SLA, Poorter et al. 2012) or leaf chlorophyll content (Lichtenthaler et al. 1981).

In addition to specific stress responses, plants can show general stress responses. For example, heat-shock proteins, so called stress proteins, are produced under different stress

types (Vierling 1991, Wang et al. 2004, Leimu et al. 2012). Similarly, anthocyanins in vegetative tissues are synthesized by plants under a range of stressful conditions, which is regarded as an adaptive response due to the photoprotective, osmotic and antioxidant functions of anthocyanins (Chalker-Scott 1999, Steyn 2002, Gould 2004). In addition, the controlled withdrawal of nutrients from old leaves leading to leaf death (leaf senescence) is expected to be advantageous under many different types of stress (Munné-Bosch and Allegre 2004). Inbred offspring may be expected to show a reduced plasticity in these traits. However, inbreeding itself is sometimes regarded as a genetic stress and, like environmental stress, increased the levels of stress proteins in some populations of *Lychnis flos-cuculi* (Leimu et al. 2012). Traits involved in general stress responses may thus be generally increased in selfed offspring.

While adaptive plasticity is expected to be reduced by inbreeding, non-adaptive plasticity may even increase in inbred progeny (Schlichting 1986, Fischer et al. 2000, Murren and Dudash 2012). Maladaptive phenotypic variation within and among environments may be due to reduced genetic and environmental canalization, respectively, i.e. as the reduced ability of a genotype to produce a constant phenotype in spite of genetic or environmental variation and thus to produce the optimal phenotype (Debat and David 2001, Sultan 2003). Similarly, inbreeding can be expected to increase developmental instability (Møller and Shykoff 1999, van Dongen 2006). Developmental instability is often measured by fluctuating asymmetry (FA), which is the amount of random deviations from bilateral symmetry (Palmer and Strobeck 1986). In plants, developmental instability has been shown to increase both with environmental and genetic stress, though not consistently (Palmer and Strobeck 1986, Freeman et al. 1993, Møller and Shykoff 1999). FA has been proposed as a non-destructive measure of stress intensity (e.g. Graham et al. 1993, Leung et al. 2000, but see Anne et al. 1998). As an indication of genetic stress, FA was increased after inbreeding in flower traits of *Silene diclinis* (Waldmann 1999) and *Scabiosa canescens* (Waldmann 2001), as well as in small populations of *Lychnis viscaria* (Siikamäki and Lammi 1998). However, inbreeding or increased homozygosity did not increase FA in leaf traits (Sherry and Lord 1996, Hochwender and Fritz 1999, Waldmann 1999) and the consistency of the effects of homozygosity on FA has been questioned (van Dongen 2006).

To study the effects of inbreeding on the response of plants to stress, we self and cross-pollinated *Silene vulgaris* plants and clonally propagated the seedlings from the two

pollination types. Each genotype was then grown under each of eight different stress treatments, which allows to separate genetic and environmental effects on traits (Sultan 2003). We asked the following questions: (1) Are the plastic responses of plants to various stress treatments affected by inbreeding? (2) Do both environmental stress and inbreeding increase two traits as part of a general stress response: leaf senescence and foliar anthocyanin concentrations? (3) Do environmental stress and inbreeding increase developmental instability and fluctuating asymmetry?

Methods

For information on the study species, pollination of the mother plants, germination and clonal propagation and experimental conditions in the stress experiment see Chapter II.

Measurement of plant traits

For every plant, the day when the first flower opened was noted as a measure of phenology. After nine weeks of growth, the plants were harvested. The height of a plant was measured as the length of the longest stem, and the length and width of the longest leaf was measured to give an estimate of longest leaf area (length x width). Leaf chlorophyll content of at least six randomly chosen leaves was measured using a chlorophyll meter (SPAD-502, Konica Minolta) and averaged. The SPAD-units were then transformed into chlorophyll content per leaf area (mg cm^{-2}) using the formula $y = 0.000552 + 0.000404\ x + 0.0000125\ x^2$ (Richardson et al. 2002). The roots were washed free of soil and the above-ground parts partitioned into leaves, stems, inflorescences and dead leaves. The leaves of each plant were scanned at 300 dpi to determine total leaf area, except for very large plants, for which only a random sample of leaves was scanned. All plant parts were dried at 80 °C until weight constancy (24 h) and weighed separately. From the data on leaf area and mass, specific leaf area (SLA) was calculated for each plant, and total leaf area of the very large plants was calculated from total leaf mass and SLA. From the biomass data, the allocation to roots (root mass fraction, RMF), stems (stem mass fraction, SMF), leaves (leaf mass fraction, LMF) and inflorescences (reproductive effort, here termed flower mass fraction, FMF), and the proportion of dead above-ground biomass was calculated. Chlorophyll content per leaf mass (mg g^{-1}) was calculated from chlorophyll per area and SLA.

From the images of scanned leaves, the proportion of red leaf area was determined with the software ImageJ (Rasband 2014). A color threshold in RGB color space of a red value > 50 and a green value < 70 was found to best select the leaf area perceived as red (Fig. 1a) and used for analysis, but different thresholds led to highly correlated estimates of the proportion of red leaf area. As a reaction to nutrient shortage *Silene vulgaris* produces anthocyanins, specifically cyanidin, which leads to visible changes in plant color (Ernst et al. 2000). The proportion of leaf area that is red has been found to be closely related to anthocyanin concentration (Gould et al. 2000).

Figure 1: Leaves of *S. vulgaris* showing (a) the red leaf area extracted and (b) the 21 regularly spaced landmarks used for Procrustes analysis (left leaf) and the three manually set landmarks for calculation of width asymmetry (right leaf). Each method was applied to both leaves of a pair.

Calculation of fluctuating asymmetry

For the analysis of leaf shape and fluctuating asymmetry, one healthy pair of opposite leaves per plant was collected and pressed. The leaf pair was chosen preferably from position 3 - 5 from the top to reduce shape variation due to position on the plant. Pressed leaf pairs were scanned at a resolution of 800 dpi. Curved leaves of a pair were arranged to face each other. Leaves of *S. vulgaris* are simple, and veins are often not visible except for the midrib. Thus, for shape analysis 21 landmarks were positioned regularly along the margin of each leaf (Fig. 1b) with the software LeafAnalyser (Weight et al. 2008). The first landmark marked the leaf tip; the other twenty were arranged counter-clockwise for the left and clockwise for the right leaf of a pair. With the software MorphoJ (Klingenberg 2011), shape information using these landmarks was extracted by

Procrustes superimposition. Landmarks of leaves of a pair were reflected on each other, and principal components were calculated for the symmetric components (differences between means of a leaf pair and the average over all plants) and asymmetric components (differences between the two leaves of a pair) of leaf shape.

Four different measures of fluctuating asymmetry (FA) were calculated: (1) The deviation from bilateral symmetry within each leaf ("midrib FA"), and the differences between the two opposing leaves of each pair in (2) width, (3) size and (4) shape. (1) To determine midrib FA, the width of the leaf on each side of the midrib at the widest point (Hochwender and Fritz 1999) was calculated from 3 landmarks set manually for every leaf with ImageJ (Fig. 1b). The signed R-L differences in leaf width were not normally distributed, but slightly leptokurtic (kurtosis = 1.00 and 1.31 ± SE 0.23 for the left and right leaves, respectively). This can be caused by antisymmetry, i.e. a bimodal distribution caused by either the left or right sides being enlarged in different individuals, or by differences in FA among individuals (Palmer and Strobeck 1992, Van Dongen 2006). The mean of the signed R-L differences was slightly larger than zero (2.5 pixels in the scanned images, i.e. 0.079 mm), which is usually interpreted as directional asymmetry, i.e. a unimodal distribution of one side enlarged in all individuals (Palmer and Strobeck 1992). However, as it is arbitrary which of the two opposing leaves is considered the left and the right one, the observed directional asymmetry is probably an artifact of shading during scanning of the leaves and the distribution mean was therefore subtracted from every difference (Hochwender and Fritz 1999). All remaining asymmetry will be regarded as fluctuating. The unsigned relative R-L differences of both leaves of a pair were square-root transformed and averaged.

The other three measures of asymmetry compared the two leaves of a pair. (2) To determine width FA, the difference in width between the leaves of each pair was calculated. This signed width difference between the two leaves was not normally distributed (Kolmogorov-Smirnov Test: $D = 4.10$, $p < 0.001$), but slightly leptokurtic (kurtosis = 1.17 ± 0.23), with a mean of zero. The absolute values of width asymmetry were square-root transformed for analysis. (3) To determine size FA, differences between the log-transformed area of the right and left leaf of a pair were calculated. The signed differences were leptokurtic (kurtosis = 6.75 ± 0.23) with a mean not different from 0. The unsigned differences were box-cox transformed as $(y + 0.00001)^{0.33}$ to correct for their half-normal distribution (Hochwender and Fritz 1999, Swaddle et al. 1994). (4) To

determine shape FA, a Mahalanobis FA-score was calculated in MorphoJ based on a PCA of the asymmetric component of leaf shape corrected for non-isotropic variation (Klingenberg and McIntyre 1999, Klingenberg and Monteiro 2005). A combined FA measure was calculated as the mean of all four FA measures after standardization (Leung et al. 2000).

Statistical analyses

Hierarchical analyses of variance were used to analyze the effects of mother plant, cross type, genotype, and the stress treatments on all traits. According to the rules for the analysis of hierarchical mixed models (Zar 2010), the effect of cross type was tested against the mother x cross type interaction, the effect of stress treatment was tested against the stress x mother interaction and the stress x cross interaction was tested against the stress x mother x cross interaction. To test for differences among lineages in trait values and plasticities, the effect of mother plant (random) and the mother x cross interaction were tested against the variation among the plants resulting from the crossings (= genotypes), which was, like the stress x genotype interaction, tested against the residual variation among cloned replicates. The interaction of stress treatment with mother plant and the stress x mother x cross interaction were tested against the stress x genotype interaction. For a better understanding of phenotypic plasticity (i.e. the interactive effects of stress treatment and cross or genotype on a trait), the stress treatment was partitioned into the linear effect of the mean trait value (MTV) per treatment (1 df) and the remaining treatment effect ("rest", 7 df). A significant cross x MTV interaction indicates that the slope of the regression of individual trait values on the mean trait value per treatment differs between selfed and outcrossed offspring, which is a measure of environmental sensitivity (Finlay and Wilkinson 1963, Falconer 1981). For the analysis of fluctuating asymmetry, stress intensity was calculated as 1 minus the fitness of the crossed plants in each environment, relative to the fitness of crossed plants in the control (Fox and Reed 2011). For this purpose, total biomass was used as a fitness measure, because it is assumed to be more relevant for perennial species than flowering traits and less influenced by allocation patterns or phenology (see chapter II). Data for biomass, SLA, and the proportion of dead biomass and red leaf area were log-transformed, and data for total leaf area square-root-transformed prior to analysis to achieve normally distributed residuals and homoscedasticity.

The effect of individual stress treatments on the various biomass fractions was compared with Tukey's HSD test based on the appropriate standard errors from the hierarchical ANOVA model (genotype x stress interaction). For the analysis of the phenotypic response to shade, only the plants grown in the control and the two shade treatments were analyzed. Separate ANOVAs for selfed and crossed plants were calculated for the effect of shade and genotype on total leaf area, LMF, SLA, chlorophyll content, and the proportion of leaf area that was red. From the ANOVAs the proportion of variation (total sums of squares) due to genotype, shade treatment and their interaction was calculated to compare the amount of variation among the genotypes in the two cross treatments as an estimate of developmental instability. For these analyses one genotype was excluded, because it was not represented in the control treatment.

The overall stress response was studied by a PCA using eight of the traits measured. Traits which are supposed to be influenced by stress, but not involved in stress response, like dead biomass, reproductive effort (FMF), stem mass fraction (SMF), and measures of fluctuating asymmetry, were excluded.

Most analyses were carried out with the software IBM SPSS statistics version 22. The PCA of overall stress response was calculated with the package vegan (Oksanen et al. 2015) with the software R version 3.2.1 (R Core Team 2015).

Results

Functional responses to specific stress types

Two size-related variables, which describe different aspects of plant morphology (length of the longest stem, i.e. height, and total leaf area) were strongly influenced by stress treatment (Table 1). The longest stem of plants was shorter under most stress treatments (minimum under very low N: 27.3 cm) than in the control (51.5 cm), but longest under light shade (59.2 cm, Fig. 2a). Plants grown under light shade also produced the greatest total leaf area (328 cm^2, compared to 224 cm^2 in the control and 48 cm^2 under very low N, Fig. 2b). The stems of selfed offspring were 13% shorter and their leaf area was 28% smaller than in crossed offspring. In addition, plasticity in these two traits was reduced by inbreeding: with increasing mean trait values of the environment, selfed offspring

increased their stem length and leaf area less strongly than did crossed offspring. Genotypes differed in the environmental sensitivity of stem length (Table 1).

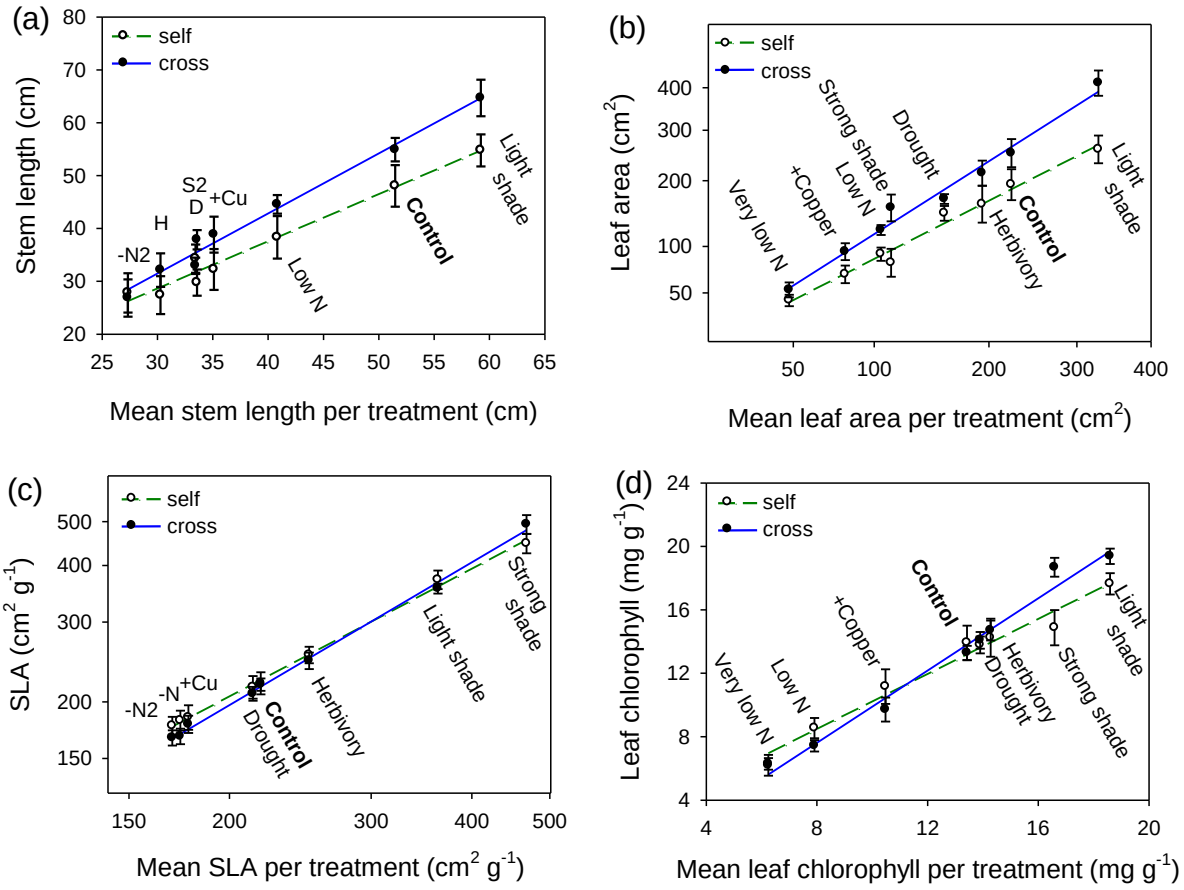

Figure 2: The relationship between trait values for selfed (dashed line) and crossed offspring (continuous line) of *S. vulgaris* and mean trait values for eight stress treatments. (a) Length of the longest stem; (b) total leaf area; (c) specific leaf area (SLA) and (d) chlorophyll content. +Cu = +Copper, D = Drought, H = Herbivory, -N = low N, -N2 = Very low N, S2 = Strong shade. Means ± 1 SE. Note square-root scale for leaf area and log-scale for SLA.

Table 1: Results of analyses of variance of the effects of mother plant, cross type (crossed vs. selfed), genotype and stress treatment on size related traits (height, i.e. length of the longest stem, and total leaf area, TLA), leaf traits (leaf area of the longest leaf, LLA; specific leaf area, SLA and leaf chlorophyll content, Chl), biomass allocation (root, leaf, shoot and flower mass fraction) and general stress response (% red leaf area and % dead above-ground biomass) of *S. vulgaris*. The effects of stress treatment were partitioned into the linear contrast of mean trait value (MTV) for all plants in that environment and the remaining effect of stress treatment (rest). ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

		Size related traits				Leaf traits						Biomass allocation								Stress response			
		Height		TLA		LLA		SLA		Chl		RMF		LMF		SMF		FMF		% red		% dead	
Source	df	F		F		F		F		F		F		F		F		F		F		F	
Mother	5	5.33	**	5.85	**	3.81	*	13.58	***	4.84	**	2.46		13.64	***	2.87	*	4.00	*	6.58	**	5.19	**
Crossed vs. selfed	1	2.42		9.83	*	10.95	*	0.79		1.78		0.18		0.63		1.68		4.30		20.81	**	3.07	
Mother x cross type	5	3.82	*	2.49		2.11		2.33		1.88		0.92		2.91	*	1.48		1.64		0.77		8.70	***
Genotype	17	3.12	***	2.09	**	3.74	***	3.00	***	3.36	***	4.80	***	1.57		5.75	***	3.50	***	3.52	***	1.21	
Stress	7	41.27	***	78.25	***	31.85	***	303.00	***	106.16	***	117.91	***	93.50	***	58.25	***	42.51	***	110.73	***	37.52	***
Mother x stress	35	2.25	**	1.91	*	1.67		1.50		0.94		1.25		1.17		1.00		2.00	*	2.28	**	3.09	***
Cross x stress	7	2.13		2.58	*	0.95		1.64		4.82	***	0.73		1.88		1.65		3.61	**	0.95		0.96	
Cross x MTV	*1*	*7.91*	**	*6.28*	*	*2.27*		*6.50*	*	*16.21*	***	*1.38*		*1.17*		*3.86*		*20.75*	***	*0.01*		*1.82*	
Cross x Rest	*6*	*1.17*		*1.96*		*0.73*		*0.83*		*2.93*	*	*0.63*		*2.00*		*1.29*		*0.75*		*1.10*		*0.82*	
Mother x cross x stress	35	0.91		0.93		0.86		1.20		1.26		1.27		0.86		1.75	*	1.00		1.19		0.46	
Genotype x stress	116	1.17		1.02		0.85		1.25		1.24		1.26		0.86		1.00		1.32	*	1.69	***	0.90	
Genotype x MTV	*17*	*1.71*	*	*0.75*		*1.11*		*1.25*		*0.83*		*1.60*		*0.86*		*1.00*		*1.75*	*	*2.09*	**	*0.80*	
Genotype x rest	*99*	*1.08*		*1.07*		*0.80*		*1.25*		*1.31*		*1.20*		*0.86*		*1.00*		*1.25*		*1.62*	**	*0.91*	
Residual	218																						

The longest leaf of a plant was smaller in selfed than in crossed offspring (4.9 vs 7.1 cm², Table 1). Results were very similar to those for total leaf area, but the cross x MTV interaction was not significant for longest leaf area. Specific leaf area (SLA) was lower under nutrient deficiency and copper stress and higher in the shade treatments than in the control (Fig. 2c, Table 1). SLA of selfed offspring increased less strongly with the mean SLA of a treatment than SLA of crossed offspring. Leaf chlorophyll content (mg g^{-1}) was lower under nutrient deficiency and copper stress than in the control, and highest under light shade (Fig. 2d, Table 1). Selfed and crossed offspring did not consistently differ in their leaf chlorophyll content. Instead, the difference between the chlorophyll content of crossed and selfed offspring increased with the mean chlorophyll content of the plants in response to a treatment, indicating lower plasticity of selfed offspring.

Biomass allocation to roots, stems, leaves and flowers was influenced by mother plant and genotype and differed strongly among stress treatments (Fig. 3, Table 1). Allocation to roots (RMF) was highest under drought and nutrient deficiency, while allocation to leaves (LMF) was highest after herbivory and in the shade. Reproductive effort, measured by the allocation of biomass to inflorescences (FMF), was highest in the control and reduced in all stress treatments.

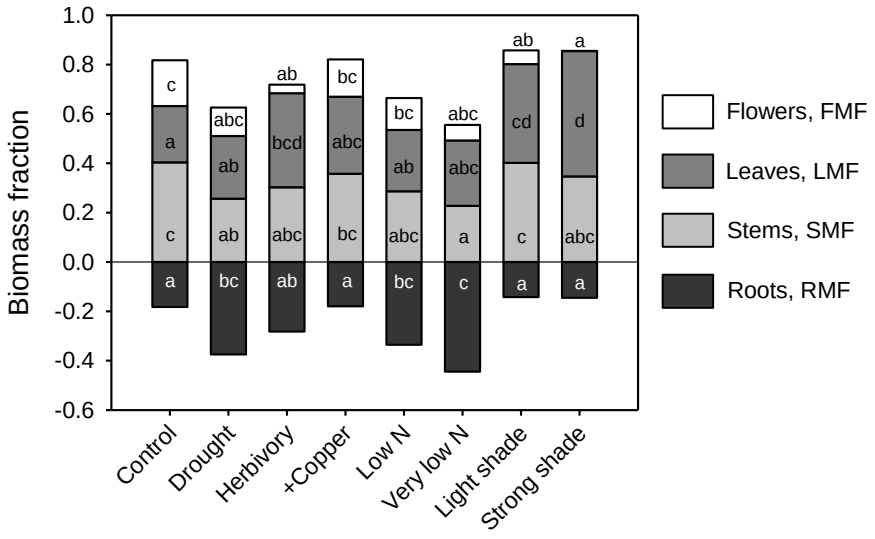

Figure 3: Allocation of biomass to roots, stems, leaves and inflorescences by plants of *S. vulgaris* grown under eight stress treatments. For each biomass fraction, bars with different letters are significantly different at the 0.05 level (Tukey's HSD).

There was no consistent effect of cross treatment on biomass allocation. However, the FMF differed between selfed and crossed offspring depending on the treatment. Crossed offspring invested more resources into inflorescences than selfed offspring in treatments with a higher FMF (linear contrast in Table 1).

Traits potentially involved in general stress response

The proportion of leaf area that was red, an indirect measure of leaf anthocyanin content, was highest under nutrient deficiency and heavy metal stress, intermediate in the control and lowest in strong shade (Fig. 4a, Table 1). Red leaf area was also generally lower in selfed offspring. Genotypes differed strongly in the plasticity of their anthocyanin response. Mean trait values for the herbivory and light shade treatments were very similar, as were those for the control and drought treatments, and the low nutrient and copper treatments (Fig. 5). Leaf senescence, the proportion of dead above-ground biomass, was highest under strong nutrient deficiency and copper stress, while it was lowest for plants under light shade and herbivory. Across all treatments, leaf senescence was higher for selfed than for crossed offspring (3.16% vs. 1.81%, Table 1, Fig. 4b), but this effect was not significant due to the low statistical power ($F_{1,5} = 3.07$, $p = 0.14$).

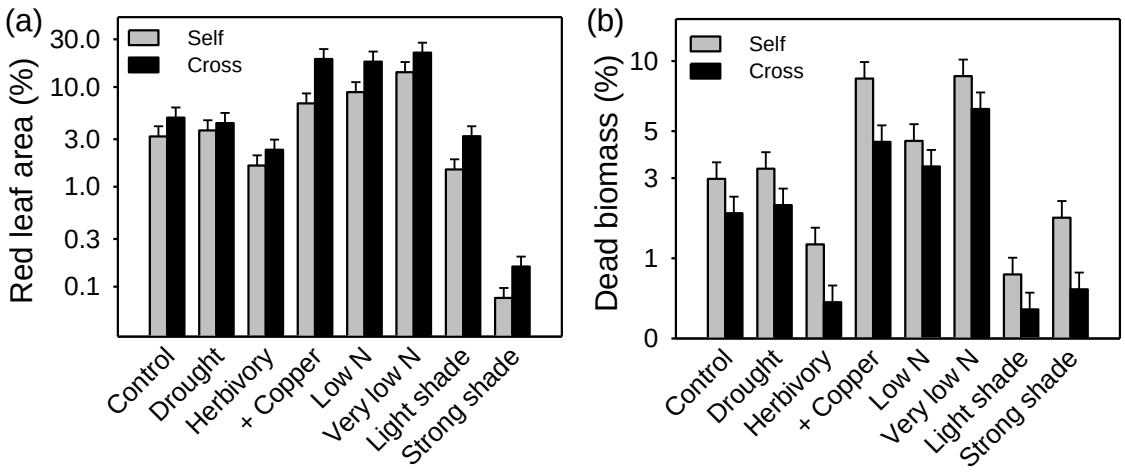

Figure 4: Effects of stress treatment and cross type on (a) the proportion of leaf area that was red, and (b) senescence, measured as the proportion of dead above-ground biomass (means ± 1 SE). Note log-scales for the dependent variables.

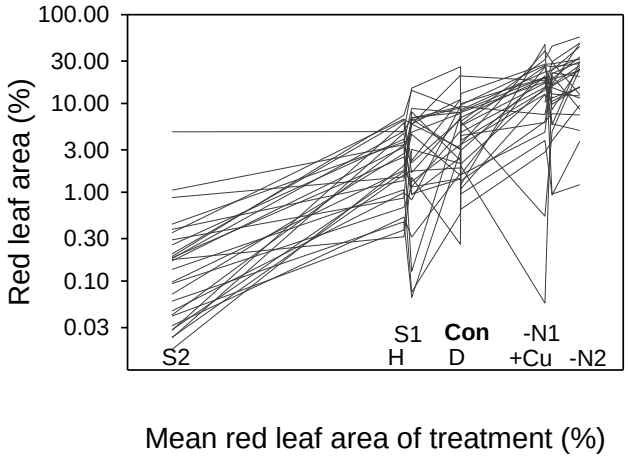

Figure 5: Reaction norm of 29 genotypes. Every line represents the proportion of leaf area that is red of one genotype under eight stress treatments, ordered by the mean trait value of all plants. Con = Control, +Cu = +Copper, D = Drought, H = Herbivory, -N1 = Low N, -N2 = Very low N, S1 = Light shade, S2 = Strong shade. Note log-scale of the y-axis.

The number of days until flowering was influenced by mother, genotype and stress treatment. The first plants flowered after 15 days, while many plants in the herbivory treatment and all under strong shade did not flower even at the end of the experiment after 60 days. Self-pollinated plants needed longer until they flowered (46.8 days vs. 44.1 days; $F_{1,21} = 5.58$, $p < 0.05$), although one self-pollinated genotype was among the first to flower. The average number of days until flowering increased with the proportion of plants not flowering per stress treatment, indicating that more plants would have flowered if the experiment had lasted longer.

Lineage effects were very strong. Offspring of different mother plants differed in all measured traits except RMF, and for some traits differed in their response to stress (Mother x stress interaction in Table 1). Even different seeds (i.e. genotypes) from the same mother and cross type differed in their trait values and sometimes also in their response to stress (Table 1).

Shade avoidance

Two stress types, shade and nutrient deficiency, were applied in two intensities, which allows for more in-depth analysis of genotype x environment interactions. We analyzed separately the response of plants to the two shade treatments, as the adaptive response to shade is especially well understood. In response to shade, some genotypes increased the

total leaf area under light shade compared to the control, but under strong shade all genotypes produced less leaf area than in the control (Fig. 6a). With increasing shade, most genotypes increased their allocation to leaves (LMF) and their SLA, but the reaction norms were steeper for crossed offspring, and there was less variation among genotypes for crossed than for selfed offspring (Fig. 6b, c, Table 2). The crossed offspring increased their chlorophyll content under light shade and held it constant under strong shade, while the reaction norms of selfed offspring differed more among genotypes than between shade treatments (Fig. 6d, Table 2). Selfed genotypes also differed strongly in their anthocyanin response to shade, whereas all crossed offspring reduced their red leaf area under strong compared to light shade (Fig. 6e, Table 2).

Table 2: Results of analyses of variance of the effects of genotype (G), light environment (E) and their interaction (G x E) on five functional traits in selfed (left) and crossed (right) offspring of *S. vulgaris*. Explained variation is based on Type I sums of squares from separate ANOVAs and the corresponding probabilities are based on 13 (G), 2 (E) and 26 (G x E) degrees of freedom and 43 and 47 residual df for selfed and crossed offspring, respectively. Significant effects are printed in bold face. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

	Selfed						Crossed					
	G		E		G x E		G		E		G x E	
Total leaf area	**29.9**	***	**30.3**	***	14.8		14.6		**34.8**	***	17.7	
LMF	13.5		**51.7**	***	10.6		**8.7**	***	**80.8**	***	2.9	
SLA	**14.1**	***	**62.7**	***	**14.0**	**	**8.1**	**	**73.7**	***	8.5	
Chlorophyll	**43.1**	***	**15.5**	***	**23.8**	*	5.0		**41.4**	***	13.4	
Red leaf area	**20.3**	***	**51.2**	***	**19.1**	***	**15.0**	***	**62.3**	***	9.1	

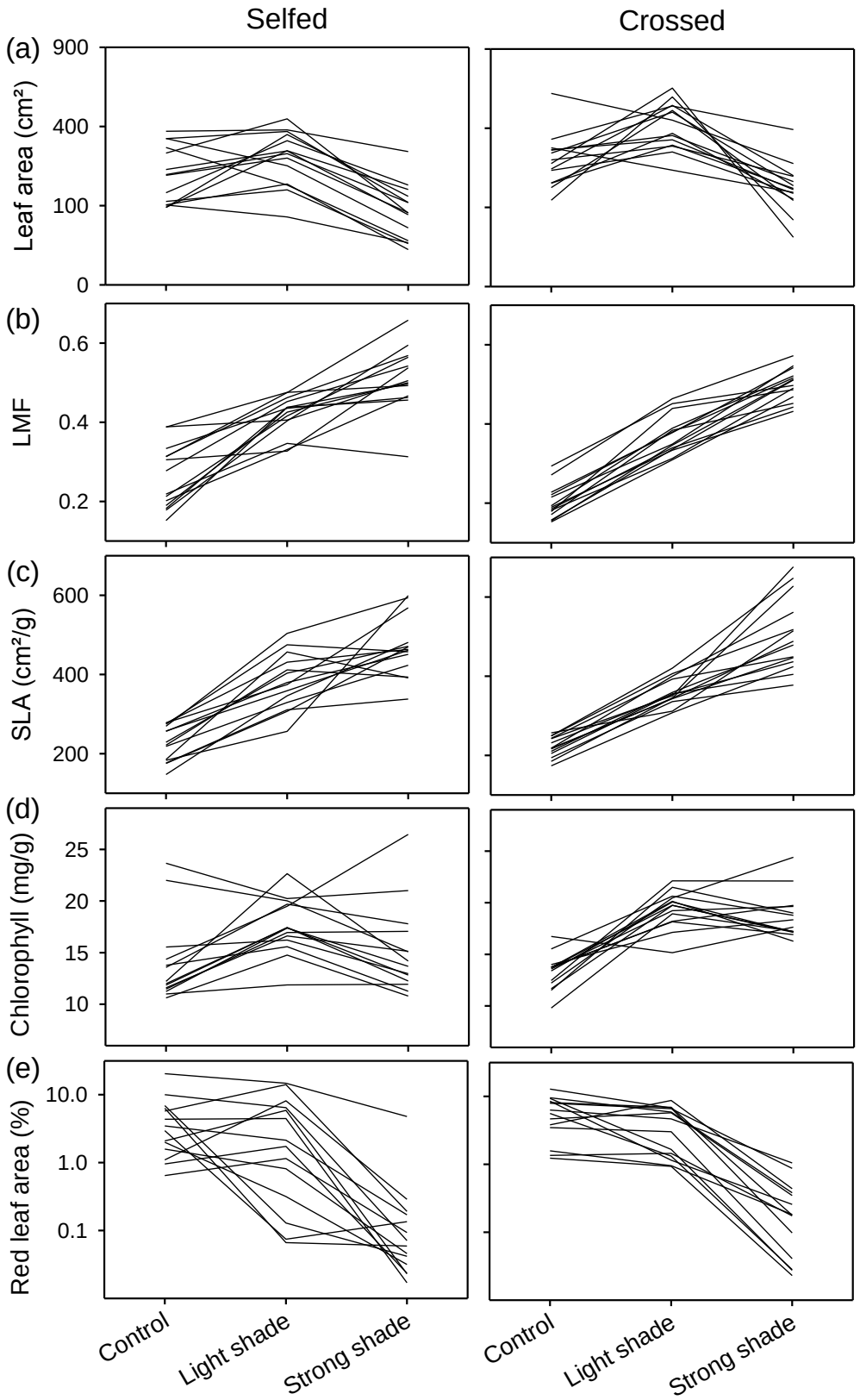

Figure 6: Norms or reaction for five traits of selfed and crossed genotypes in response to shade. Each line represents one genotype. Note square-root scale for leaf area and log-scale for red leaf area.Correlated stress responses

In a principal component analysis of eight traits involved in the response to stress, the first two principal components had eigenvalues > 1 and together explained 72% of the total variance. PC1 and PC2 were strongly influenced by the stress treatment (all $F_{7,35} > 38.66$, $p < 0.001$). The first principal component (PC1) differentiated among plastic responses to shade (high chlorophyll content, high SLA, high LMF) and responses to nutrient deficiency (high anthocyanin content, high RMF), while PC2 extracted differences in plant size and thus illustrates the response to general stress intensity (Fig. 7).

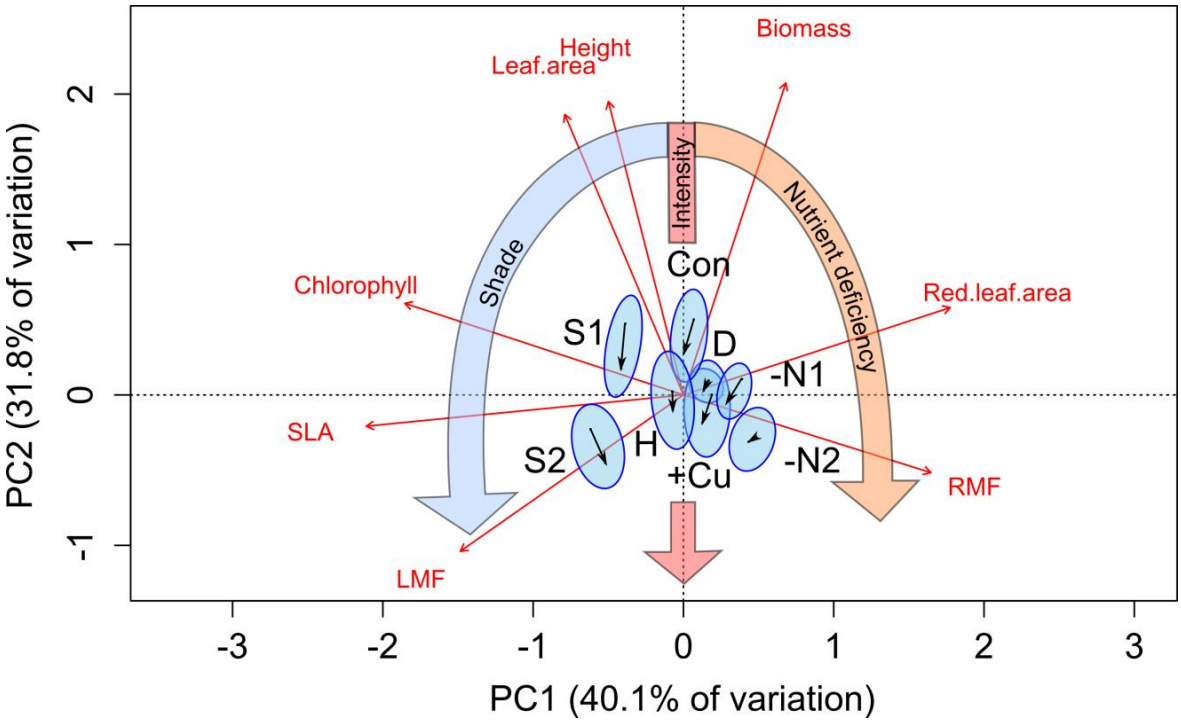

Figure 7: Principal component analysis of eight traits measured for selfed and crossed offspring of *S. vulgaris* grown under eight stress treatments. Ellipses show standard deviations per stress treatment. Con = Control, +Cu = +Copper, D = Drought, H = Herbivory, -N1 = Low N, -N2 = Very low N, S1 = Light shade, S2 = Strong shade. Small black arrows lead from centroids of crossed offspring to centroids of selfed offspring within each stress treatment and thus illustrate the effect of inbreeding. Large arrows illustrate the directions of increasing shade (left), increasing average stress intensity (center) and increasing nutrient deficiency (right).

Cross type directly influenced PC2 ($F_{1,5} = 12.46$, $p = 0.017$), because inbred plants were generally smaller than outbred plants. However, both the scores along the first axis ($F_{7,35} = 3.50$, $p = 0.006$) and the second axis ($F_{7,35} = 2.70$, $p = 0.024$) were influenced by the cross x stress interaction, indicating that selfed plants responded less plastically to

stress (PC1) and that inbreeding depression of size differed among stress types (PC2, Fig. 7).

Leaf shape and fluctuating asymmetry

In a PCA of leaf shape after removing the effects of size, position and orientation by Procrustes transformation, the first four principal components together explained 94.6% of the variation in leaf shape. PC1 was related to differences in leaf width (50% of variance, Fig. 8), while leaves became more spatulate along PC 2 (28%). PC3 differentiated plants according to leaf curvature (14%). PC 4 explained only an additional 3.1 %, describing the form of leaf tips. These first four principal components were influenced by mother and genotype, but were also significantly influenced by stress treatment (Table 3). Leaves of shade plants, for example, were more narrow and spatulate (high scores on PC1 and PC2), while leaves of plants grown under low nutrients were often curved (low PC3 scores) and had acuminate tips (high PC4 scores). However, inbreeding did not influence this plasticity in leaf shape in response to stress (no cross type x stress interaction), and only weakly affected PC1 and PC2 of leaf shape, at least in some mothers (Table 3).

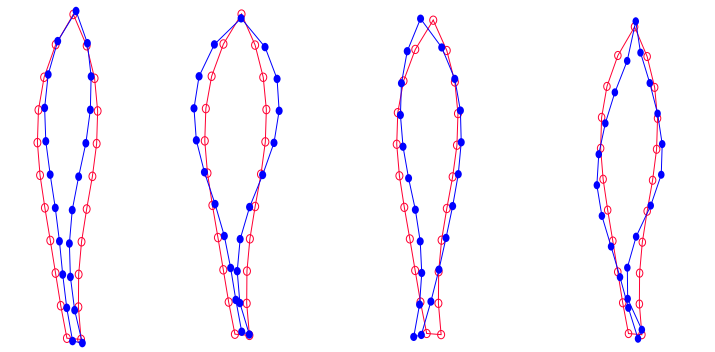

Figure 8: First four principal components of leaf shape variation after Procrustes analysis. Open circles show the average leaf shape, filled circles show the effect of the principal component.

Table 3: ANOVAs of the effects of mother plant, cross type, genotype and stress treatment on symmetric components of leaf shape, compare Fig. 8. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; +, $p < 0.10$.

		PC1		PC2		PC3		PC4	
Quelle	df	F		F		F		F	
Mother	5	2.78	+	5.66	**	3.77	*	7.30	***
Cross type	1	2.17		5.19	+	0.00		0.02	
Mother x cross	5	3.08	*	0.84		1.30		2.18	
Genotype	17	4.95	***	6.14	***	1.67	+	1.75	*
Stress type	7	3.55	**	27.39	***	1.59		3.68	**
Mother x stress type	35	1.90	**	0.81		1.58	*	1.31	
Cross x stress type	7	0.14		0.43		1.11		0.82	
Stress x mother x cross	35	1.73	*	0.96		1.15		1.14	
Stress x genotype	116	0.90		1.22		0.85		0.76	
Residual	216								

The four different measures of fluctuating asymmetry (FA) were mostly only weakly correlated ($0.04 < r < 0.10$ for five of the six combinations, $r = 0.55$ for the correlation between width FA and size FA). Most measures of FA differed among stress treatments (Table 4). However, leaves were not more asymmetric under stress, but often less asymmetric under stress than in the control (Fig. 9). Inbreeding did not directly influence FA. Only one measure of FA, the difference in the distances from the midrib of a leaf to its margin, was influenced by the interactive effects of stress intensity and cross type (Table 4). Leaves of crossed offspring were more asymmetric in the control, while leaves of selfed offspring were more asymmetric at high stress intensities, especially under strong shade (Fig. 9a). The mean standardized FA combining all FA measures was not influenced by inbreeding ($p = 0.59$), but differed among mothers and stress treatments (Table 4, Fig. 9d). It was highest in the strong shade and lowest under drought and copper stress. All measures of FA increased with SLA. This correlation was weakest for midrib FA ($r = 0.093$, $p = 0.05$) and strongest for shape FA and the mean standardized FA ($r = 0.214$ and $r = 0.222$, respectively, both $p < 0.001$). When included as a covariate in the ANOVAs, SLA slightly reduced the effects of stress treatment on all measures of FA, but the interaction effect of cross x stress intensity on midrib FA remained ($F_{1,35} = 5.43$,

p = 0.026). The average mean standardized FA per treatments was positively correlated with the average SLA per treatment (r = 0.82, p = 0.013, 7 df).

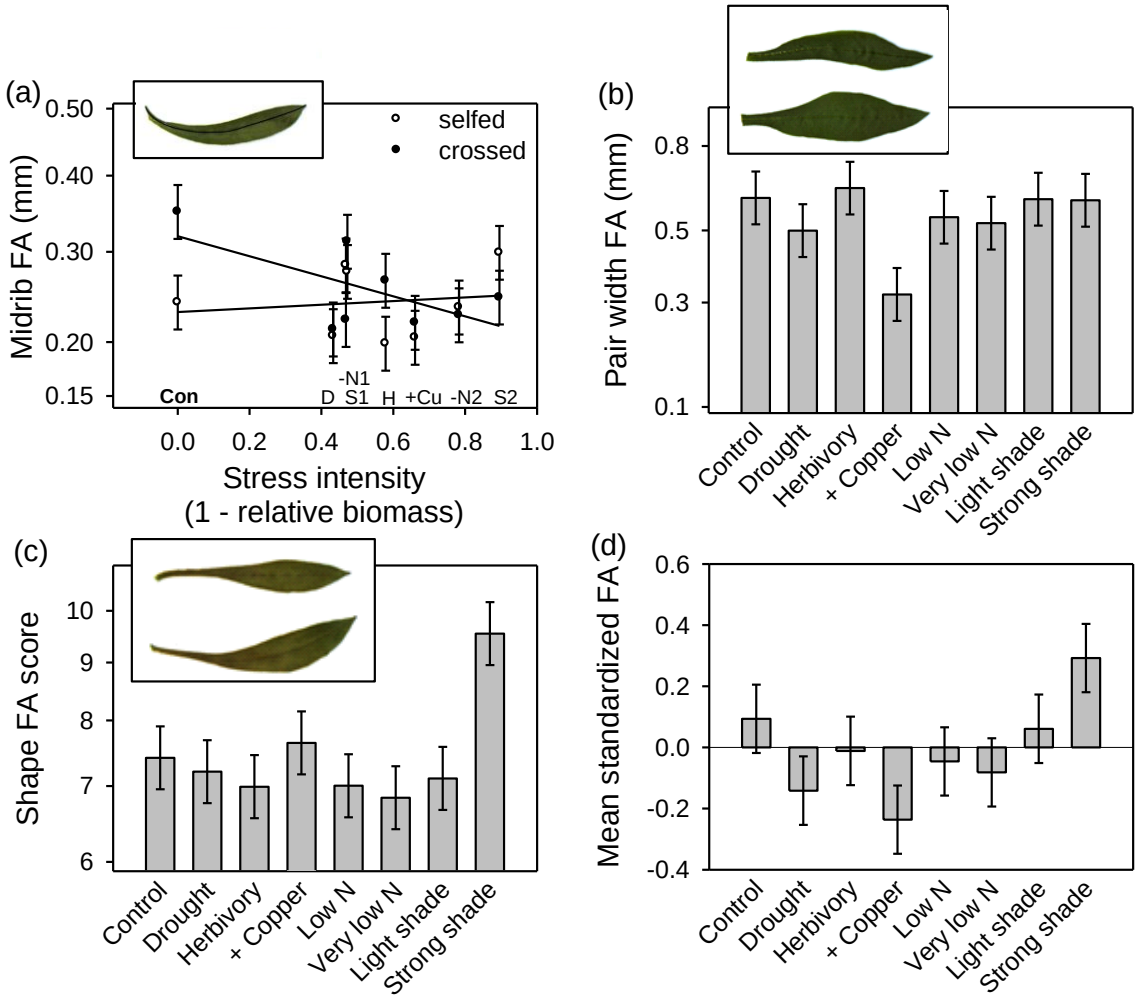

Figure 9: The influence of eight different stress treatments on four measures of fluctuating asymmetry (FA) in a leaf pair of selfed and cross-pollinated S. vulgaris. (a) Effects of stress intensity and cross treatment on midrib FA (the difference in distances from the midrib of a leaf to its margins); (b, c) Effects of stress treatments on (b) width FA (difference in leaf width between the two leaves of a pair); (c) shape FA (difference in leaf shape between the two leaves of a pair after Procrustes transformation of 21 landmarks); and (d) mean standardized FA based on four different measures. Means ± SE; Con = Control, +Cu = +Copper, D = Drought, H = Herbivory, -N1 = Low N, -N2 = Very low N, S1 = Light shade, S2 = Strong shade. Note square-root scale in (a) and (b) and log scale in (c). Scans show leaf pairs of the plants which were most asymmetric according to the respective method; the midrib was accentuated in (a).

Table 4: Results of analyses of variance of the effects of cross type and stress treatment on various measures of fluctuating asymmetry (FA). The effect of stress treatment was partitioned into a linear contrast (stress intensity) and the remaining effect of stress treatment. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

		Midrib FA		Width FA		Size FA		Shape FA		Mean FA	
	df	F		F		F		F		F	
Mother	5	2.43		1.29		0.92		5.83	**	3.94	*
Cross type	1	0.58		0.94		2.25		1.97		0.33	
Mother x cross	5	0.92		0.50		0.67		1.63		1.53	
Genotype	17	0.93		1.00		1.20		1.26		1.02	
Stress type	7	2.39	*	2.75	*	1.18		5.63	***	3.99	***
Intensity	1	2.21		0.80		1.00		6.75	*	0.26	
Rest	6	2.42	*	3.07	**	1.27		5.46	***	4.62	***
Mother x stress type	35	0.90		1.68		0.76		0.90		0.70	
Cross x stress type	7	1.79		2.21		0.50		0.86		0.68	
Cross * intensity	1	5.41	*	0.03		0.65		1.76		0.52	
Cross x rest	6	1.18		2.58	*	0.47		0.71		0.71	
Stress x mother x cross	35	0.91		0.58		1.55	*	0.88		0.94	
Stress x genotype	116	0.92		0.99		1.10		1.26		1.16	
Error	216										

Discussion

Inbreeding and environmental sensitivity

In *Silene vulgaris*, inbreeding had strong effects on many of the studied traits and their plasticity in response to different stress types. Across eight different stress treatments, phenotypic plasticity of selfed offspring was reduced for the size-related traits stem length and leaf area, and the leaf traits chlorophyll content and specific leaf area (SLA). The chosen stress types required very different responses, and it is not always possible to distinguish between adaptive and non-adaptive responses (Sultan 2000, van Kleunen and Fischer 2005). However, arranging the treatments by their mean trait values (MTVs) can help to compare individual plasticity with the average response of the population (Finlay and Wilkinson 1963) and allows to fit linear or polynomial reaction norms over very different treatments (Via et al. 1995). Stem length and total leaf area were smaller in the nutrient deficiency and drought treatments than in the control, which may be a

consequence of reduced plant size under stress. However, these phenotypic differences can also be regarded as a functionally appropriate response to reduce water loss via transpiration. In contrast, both stem length and leaf area were increased under light shade, which is part of the typical shade avoidance response. The shade avoidance syndrome describes a correlated response of elongated stems, larger leaves with higher SLA, less chlorophyll per leaf area, increased apical dominance and accelerated flowering (Smith and Whitelam 1997), which has been shown to be adaptive (Schmitt et al. 1999). The pattern of mean trait values expressed by all *Silene vulgaris* plants thus is the expected adaptive response to these stresses. Offspring from self pollination had a reduced environmental sensitivity of stem length and leaf area, i.e., in relation to MTVs, self pollinated plants increased stem length and leaf area less than the population mean, which suggests a reduced adaptive plasticity in these traits. In both traits, selfed and crossed plants did not differ at low MTVs, but differed considerably at high MTVs in the control and shade treatments.

In addition, offspring from self-pollination had a reduced environmental sensitivity in SLA and chlorophyll content. In contrast to the size-related traits stem length and leaf area, the reaction norms of SLA and chlorophyll content did not diverge, but were crossed at intermediate MTVs. High SLA and chlorophyll content in the shade are expected to be adaptive according to the shade avoidance syndrome, whereas low SLA and low chlorophyll content are expected to be adaptive under nutrient deficiency and drought. This is illustrated by the suite of traits in bog vegetation ("peinomorphism"), of small and thick leaves with a high C:N ratio (Greb 1957, Chapin 1980), and by comparisons of leaf traits of species across different habitats (Reich et al. 1999). Selfed offspring were thus less well adapted under both shade (large MTV) and nutrient deficiency (small MTV).

Few other studies have addressed the effect of inbreeding on phenotypic plasticity in non-reproductive traits, and the results are equivocal. Plasticity in plant response to herbivory was reduced after inbreeding in *Solanum carolinense* (Campbell et al. 2014). Inbred plants showed a reduced ability to upregulate defense-related phenolics after damage, and a reduced production of phytohormones. Moreover, in response to simulated herbivory, the plasticity of leaf and root growth was reduced after inbreeding, but the plasticity of stem growth and biomass was increased compared to crossed offspring, which was interpreted as a consequence of the altered hormone production (Campbell et al. 2014). In *Schiedea lydgatei*, inbreeding reduced the plasticity in photosynthesic carbon assimilation

and stomatal conductance in response to fertilization (Norman et al. 1995) and in comparisons of plants from large and small, probably more inbred, populations or *Ranunculus reptans*, plants from small populations had a reduced phenotypic plasticity in leaf length in response to competition, which can be regarded as maladaptive (Fischer et al. 2000). In *Echinacea angustifolia*, many functional traits influencing photosynthesis and resource uptake were reduced after inbreeding, potentially affecting the response of the plant to stress (Kittelson et al. 2015). However, differences in plasticity were not studied.

In other studies, inbreeding did not affect phenotypic plasticity (O'Halloran and Carr 2010, Murren and Dudash 2012). However, the studied traits are not involved in stress response and the observed plasticity was not regarded as adaptive. In *Mimulus ringens* grown at different moisture levels, inbreeding barely influenced phenotypic plasticity in floral and size-related traits (O'Halloran and Carr 2010). In *Mimulus guttatus*, inbreeding affected the plasticity in stem diameter of plants transplanted to native and novel sites (Murren and Dudash 2012). In *Phlox drummondii* grown under six stress treatments, inbreeding influenced phenotypic plasticity in some traits (plant height, root to shoot ratio, days to flower and capsule production), but without any clear pattern. Plasticity was either increased or reduced after selfing, and the changes differed among generations of inbreeding (Schlichting and Levin 1986). However, in the analysis it was not differentiated whether traits increased or decreased under certain environments, which makes it impossible to distinguish developmental instability from adaptive responses.

Biomass allocation

According to the optimal partitioning theory, plants increase their allocation of biomass to organs involved in the uptake of the limiting resource (Bloom et al. 1995, Sultan 2003, Poorter et al. 2012). As expected, *S. vulgaris* increased the allocation to roots (root mass fraction, RMF) in the drought and nutrient deficiency treatments, while under shade the leaf mass fraction (LMF) was increased. The simulated herbivory treatment, in which the above-ground biomass was removed after five weeks of growth, reduced the stem mass fraction (SMF) and increased LMF, which can be regarded as attempt to quickly compensate for the loss of photosynthetic tissue (Stowe et al. 2000). However, in contrast to height, leaf area, SLA and chlorophyll content, the plasticity of biomass allocation in *S. vulgaris* was not affected by the cross type. This is in agreement with the results of a

recent meta-analysis on allocation in plants, which found that plants adjust organ morphology more than allocation patterns. For example, across species groups SLA responded more strongly to shade than LMF (Poorter et al. 2012).

Inbreeding and traits related to general stress response

The proportion of leaf area that is red is a good estimate of leaf anthocyanin content (Ernst et al. 2000, Gould et al. 2000). Leaf anthocyanin concentrations are increased in response to various stresses, including UV radiation, drought, nutrient deficiency and heavy metal contamination (Chalker-Scott 1999, Gould 2004), but the causalities are not completely resolved, and in *Arabidopsis thaliana* it has been shown that not anthocyanin itself, but intermediate products from anthocyanin synthesis help the plants against nutrient and light stress (Misyura et al. 2013). While some authors have emphasized the antioxidant function of anthocyanins and regarded higher concentrations under drought as adaptive (Chalker-Scott 1999, Gould 2004), Steyn et al. (2002) argued that anthocyanins have predominantly a photoprotective function, and that nutrient-deficient – but not drought-stressed – plants produce more anthocyanins because they are more sensitive to photostress. In *S. vulgaris*, the proportion of leaf area that is red was not increased under drought, but increased in response to nutrient deficiency, which corresponds to the expectation of Steyn et al. (2000), but also under copper stress. Increased reddening of leaves under heavy metal stress has been found in other studies (Fernandes and Henriques 1991, Gould 2004), but may also be an indirect effect of nutrient limitation, as root growth is limited under copper excess (Fernandes and Henriques 1991). *S. vulgaris* leaves were less red in the shade, as is expected because the induction of anthocyanin production requires high light intensities (Steyn et al. 2002) and because anthocyanins can reduce the amount of light available for photosynthesis (Chalker-Scott 1999). In contrast to most other studied traits, the proportion of red leaf area was strongly influenced by the genotype x stress interaction. Different genotypes showed different reactions to treatments with similar mean trait values. This suggests that there is not one optimal trait value (level of pigmentation) per environment, as for SLA or chlorophyll content, but that there may be different physiological processes leading to similar proportions of leaf area that are red.

Like the production of leaf anthocyanins, leaf senescence is regarded as an adaptive response to many stresses, especially to nutrient deficiency and drought. Senescence does

not simply mean tissue mortality, but is a highly regulated process to withdraw nutrients from old, less effective leaves, and reduce water loss which is especially advantageous under nutrient deficiency and drought (Chapin 1980, Munné-Bosch and Allegre 2004). We measured the proportion of above-ground biomass that was dead, and as this consisted mainly of leaves we regard it as an estimate of leaf senescence. In *Silene vulgaris*, leaf senescence showed a response very similar to that of anthocyanins. The dead biomass increased under copper stress and nutrient deficiency compared to the control, but not under drought. The lack of an increase of senescence in the drought may be due to the very constant way in which drought was applied, which allowed the plants to acclimate by increasing their RMF, and reducing their stem length and total leaf area. and were rarely wilting. The reduced senescence after herbivory can be due to the fact that after resprouting, leaves were on average younger. However, a reduced senescence after herbivory has be found by other studies and can be regarded as adaptive, because functional leaves are essential for regrowth (Stowe et al. 2000). Similarly, the very low senescence in the strong shade can be regarded as essential to maximize photosynthesis with the given resources.

Selfed offspring produced less anthocyanin than crossed offspring in all treatments. This may be a disadvantage in environments where the production of anthocyanins helps to face stress, but it may also be advantageous in the shade, where anthocyanins have increased metabolic costs (Chalker-Scott 1999). The plasticity of anthocyanin production was not influenced by inbreeding. Thus, the general reduction of anthocyanin by inbreeding cannot be consistently interpreted as positive or negative. Instead, it suggests that one of the steps in the synthesis chain of red pigments may be disrupted by selfing (compare Misyura et al. 2013), or that selfed plants cannot afford the metabolic costs of anthocyanin production. In contrast, offspring from self-pollination tended to show more senescence than offspring from cross pollination in all stress treatments. This is surprising, as overall the response of read leaf area and senescence to the eight stress types was similar. The reduced anthocyanin concentration in selfed offspring may be partly responsible for the increased leaf senescence in selfed offspring. Anthocyanins are known to have antioxidant functions (Gould 2004), and a disturbed balance between reactive oxygen species and antioxidants increases leaf senescence (Munné-Bosch and Allegre 2004). In addition, the inbreeding effects on anthocyanins and senescence may be due to a disturbed hormonal regulation. The plant hormones ABA and jasmonic acid are

involved in the regulation of leaf senescence (Munné-Bosch and Allegre 2004), and the production of ABA and IAA, as well as the upregulation of jasmonic acid production after wounding were reduced by inbreeding in *Solanum carolinense* (Campbell et al. 2014), which would however lead to reduced, not increased, senescence. Finally, more dead biomass in inbred offspring may be a non-adaptive result from a disturbed development that cannot be separated from adaptive senescence. Independent of the mechanism, the results suggest that inbreeding affected both traits in a way that is maladaptive at least under some of the studied conditions, without influencing their plasticity.

Response to increasing shade

The comparison of plasticity in multiple traits in response to two levels of shade suggests that plants were able to acclimate to light shade, whereas growth under strong shade was severely limited. The chlorophyll content was not further increased from light to strong shade, the stem length and total leaf area were reduced because plants remained very small, and leaf senescence, which was minimized under light shade, increased under strong shade. All crossed genotypes showed similar reaction norms, which is an indication of environmental canalization (Scheiner 1993, Debat and David 2001). In contrast, the variation among selfed genotypes in response to stress was larger, as indicated by the often significant genotype effects and genotype x environment interactions in selfed offspring, which can be interpreted as increased developmental instability or reduced genetic canalization (Debat and David 2001).

Leaf shape changed in response to shade. In response to shade, *S. vulgaris* had narrower and more spatulate leaves, irrespective of their size. Similar to our observations, shade leaves of *Arabidopsis thaliana* develop a longer petiole and a narrower blade, which is regarded as part of the shade avoidance syndrome, as it allows a better leaf positioning (Tsukaya 2005). This shows that leaf shape, together with functional traits like SLA and chlorophyll content, is an active response to shade to increase light harvesting. Leaf shape in *S. vulgaris* differed among mother plants and genotypes. Similarly, families and plants within families of *Impatiens capensis* differed in leaf shape, but the cross type did not influence leaf shape (Mitchell-Olds and Waller 1985).

Fluctuating asymmetry

Fluctuating asymmetry (FA) is widely used as a measure of developmental instability, and is usually expected to increase with both environmental and genetic stress (Palmer and Strobeck 1986, Freeman et al. 1993, Møller and Shykoff 1999). In *S. vulgaris*, FA in leaf traits was not consistently influenced by stress intensity or by inbreeding. The classical measure of FA in leaves is the R-L difference between the two sides of a leaf (Palmer and Strobeck 1986, Hochwender and Fritz 1999). This was the only measure of FA that was influenced by inbreeding, and it was increased in selfed offspring compared to crossed offspring only under strong stress. Similarly, in the rare African bird *Turdus helleri*, the increase of FA with inbreeding was found only in stressed individuals living in disturbed habitats (Lens et al. 2000). Many recent studies report that the correlation between reduced heterozygosity, developmental instability and FA is not as strong as often supposed (Leamy and Klingenberg 2005, Van Dongen 2006). Even if developmental instability increases with homozygosity or stress, this may not translate into an increase of FA (Anne et al. 1998, Van Dongen 2006).

Interestingly, FA did also not increase with environmental stress. Three of the measures of FA differed among treatments, but were not correlated. The low correlation among different measures of FA is not unusual (e.g. Sherry and Lord 1996; Waldmann 1999, Leung et al. 2000). Estimates of FA based on single traits have been shown to poorly reflect developmental instability (Palmer and Strobeck 1986), in contrast to measures combining FA from multiple traits (Leung et al. 2000, Van Dongen 2006). The mean standardized FA has a higher power of detecting real differences in asymmetry (Leung et al. 2000). In *S. vulgaris*, this measure was increased under strong shade, but similar to the control or even lower in the other treatments, which is in contrast to the expectation that FA increases under stress (Graham et al. 1993, Leung et al. 1996, Møller and Shykhoff 1999). Similarly, FA was not increased in the legume *Glycine max* under salinity stress, which was interpreted as a result of the adaptation of plants to osmotic stress (Anne et al. 1998). In *S. vulgaris*, adaptation to certain types of stress may explain the low FA under drought, herbivory and nutrient deficiency, as these are typical conditions in the population of origin. However, adaptation cannot explain the low FA in the copper treatment. Heavy metal stress often increases FA in plant leaves (Mal et al. 2002), and although some populations of *S. vulgaris* occur on contaminated soils and have evolved

tolerance to heavy metals, especially copper (Schat and Ten Bookum 1992) this is not the case for our study population.

Leaf shape FA, the difference in shape between two opposing leaves, was the only measure of FA that increased with stress intensity, but this was mainly due to the high shape FA under strong shade. It was suggested that leaf FA under shade may not be a consequence of developmental instability but an attempt to reach light (Waldmann 1999) or adjustment to microenvironmental variation (Debat and David 2001). However, all measures of FA were reduced under light shade and increased only under strong shade, a treatment which increased developmental instability and reduced canalization in SLA, chlorophyll and other functional traits (see above). We thus suppose that the increased FA under strong shade reflects developmental instability. The positive correlation between SLA and FA suggests that FA in leaves may become especially visible under conditions where leaves have to grow large and thin and meristematic activity is high. In contrast, under types of stress that lead to small and thick leaves, developmental instability may not become visible as FA in leaf traits. In comparison to leaves, flowers are expected to be more developmentally stable (Mitchell-Olds and Waller 1985, Waldmann 1999 and citations therein, but see Møller and Shykhoff 1999), and symmetry in flower traits may even be adaptive by increasing pollination success (Møller 2000). In agreement with this expectation, FA was higher after inbreeding in flower traits of *Scabiosa canescens* (Waldmann 2001), and flowers of *Lychnis viscaria* from smaller populations and more stressful conditions were more asymmetric (Siikamäki and Lammi 1998). In contrast, most studies report no negative effect of homozygosity or inbreeding on FA in leaf traits (Sherry and Lord 1996, Hochwender and Fritz 1999, Waldmann 1999, Vaupel and Matthies 2012).

Correlated stress responses

Similar to shade avoidance, the observed responses to nutrient deficiency consisted of correlated changes in many traits, which at least partly will have been adaptive. As shown by the principal component analysis (PCA) of plant traits, *Silene vulgaris* was smaller under all types of stress in comparison to the control (lower PC2 scores) but showed different correlated responses to nutrient deficiency and shade. Nutrient deficiency resulted in a combination of higher allocation to roots, lower SLA, less chlorophyll and more anthocyanins (high PC1 scores), whereas shade resulted in higher allocation to

leaves, higher SLA, more chlorophyll and less anthocyanins (low PC1 scores). Plants exposed to the copper, herbivory and drought treatments had intermediate PC1 scores. Photosynthetic tissue lost by herbivory can be compensated by the quick production of large leaves with a high SLA and high chlorophyll content (Stowe et al. 2000), and *S. vulgaris* after resprouting had short stems, but nearly as much total leaf area as control plants, with even higher SLA and chlorophyll content. Copper in the soil reduces root growth more than shoot growth, and the leaves may show chlorosis or reddening (Fernandes and Henriques 1991). The plants thus face nutrient deficiency without a compensation by increased RMF. Correspondingly, copper-stressed *S. vulgaris* were characterized by a similar RMF as control plants, but by reduced SLA, lower chlorophyll concentration and increased red leaf area as well as higher leaf senescence. In contrast, drought plants increased their allocation to roots but did not share the other responses to nutrient deficiency.

Selfed offspring were not only smaller (low PC2 scores), but also showed less plastic responses to most types of stress, as manifested in the cross x stress type interaction of PC1 scores: PC1 scores of selfed offspring were lower under conditions of nutrient deficiency and higher in the shade.

Conclusions

Inbreeding influenced not only plant size, but also the response to shade and nutrient deficiency. In particular, inbreeding reduced the environmental sensitivity in four non-reproductive functional traits. However, this did not result in a generally increased inbreeding depression in biomass under stressful than benign conditions. ID in biomass was highest under benign conditions and in the shade (Chapter II), and the reduced plasticity in functional traits may be partly responsible for the reduced fitness of inbred offspring under these conditions. In the face of climate change and land-use intensification, conditions for many species change, but do not necessarily become more stressful. For plants, which are sessile organisms, phenotypic plasticity is an important mechanism to cope with changing environmental conditions (Nicotra et al. 2010), and a reduced phenotypic plasticity because of inbreeding may thus further increase the threat to plants in small and fragmented populations.

Inbreeding increased developmental instability in some traits, as illustrated by greater variability in the responses to shade among selfed than crossed genotypes. However, fluctuating asymmetry (FA) of leaf traits, a widely used predictor of stress, was not influenced by inbreeding. FA was also not consistently increased under stress, but only under treatments which resulted in a higher SLA, which suggests that developmental instability leads to higher FA in leaves when meristematic activity is higher. FA in leaf traits may thus not be a suitable non-destructive measure of genetic and environmental stress. FA of other traits like flower shape, whose symmetry is under stronger selection, could be better indicators of developmental instability.

Chapter IV

Effects of inbreeding on the interactions between a hemiparasite and hosts of different quality

With D. Matthies, in preparation

Abstract

Inbreeding depression (ID) depends on the environment and is often expected to be higher in more stressful environments. However, there are also studies suggesting that under some stress types ID is reduced compared to benign environments, in which the cross pollinated plants are more capable of exploiting favourable conditions (capable crossed hypothesis) or that ID increases not with stress intensity of an environment, but with its effects on phenotypic variation (phenotypic variation hypothesis). We tested these hypotheses with a special type of biotic stress, the quality of host species for the hemiparasite *Rhinanthus alectorolophus*. Selfed and open pollinated parasites from two populations were (1) grown with 13 potential host species and (2) with 15 four-species mixtures differing in the number of legumes and of host functional groups. Parasites grown with the best host (a grass) were more than eight times larger than with the poorest host (a legume) or without a host, and parasite size differed strongly with different host species within functional groups. Neither the size, nor the relative growth rate of the host species could explain much of the variation in host quality. In the mixtures, parasite biomass increased with the number of legumes and of functional groups. Inbreeding depression differed among host species and mixtures. In experiment 1 ID was highest in parasites grown with good hosts and declined with stress intensity, supporting the capable crossed hypothesis. In the second experiment, ID was not influenced by stress intensity, but was highest in mixtures of hosts from only one functional group and lowest in mixtures containing three functional groups. Neither of the experiments supported the phenotypic variation hypothesis. Host species, especially grasses and forbs, were considerably suppressed in their growth by the parasite. However, inbreeding did not influence the effect of the parasites on host biomass. In the mixtures, the presence of the parasite changed the competitive balance and increased size inequality among the four hosts. However, the effects could not be predicted from the suitability of a species as a host for *R. alectorolophus* in experiment 1.

Introduction

Inbreeding, i.e. the mating between close relatives, usually reduces the fitness of offspring (Darwin 1878, Husband and Schemske 1996), a phenomenon that is called inbreeding depression (ID). However, the magnitude of ID can differ among environments (Cheptou

and Donohue 2011). It is often assumed that ID is stronger under more stressful conditions because inbred plants are more sensitive to stress (Darwin 1868, Dudash 1990, Frankham et al. 2010, Reed et al. 2012), which may be called the sensitive selfed hypothesis (Chapter II). Although in the majority of studies ID has been found to be higher in stressful environments, i.e. in environments that reduce average fitness compared to more benign environments (see reviews by Armbruster and Reed 2005, Fox and Reed 2011), this pattern is not very consistent. Some studies found no effect of stress on ID while others even found higher ID under benign conditions (Norman et al. 1995, Armbruster and Reed 2005, Waller et al. 2008). ID may decrease with stress intensity if offspring resulting from cross pollinations is better able to exploit good conditions, but offspring from both cross types have similarly low fitness under stressful conditions (capable crossed hypothesis; Cheptou and Donohue 2011, Chapter II). Another hypothesis, the phenotypic variation hypothesis, posits that differences in ID among environments may not be due to differences in stress intensity, but due to the effects of an environment on phenotypic variation (Waller et al. 2008). This hypothesis is based on the reasoning that inbreeding depression represents selection against selfed offspring and its magnitude should thus be influenced by the opportunity for selection in an environment, measured as the squared coefficient of variation (CV^2, Crow 1958). The effect of CV^2 in an environment on ID should be tested as a null-model before searching for more complex hypotheses (Waller et al. 2008).

Most existing studies on environment-dependent inbreeding depression compare ID between only two environments (Armbruster and Reed 2005, Reed et al. 2012). The few existing studies which compare the effects of more than one stress treatment on inbreeding depression in one plant species suggest that there may be differences among stress types. While some types of stress reduce ID, others may increase or not affect ID in the same species (Waller et al. 2008, Walisch et al. 2012, Chapter II). A very special biotic stress which might potentially influence ID is the quality of the host species for hemiparasitic plants. Root-hemiparasites have green leaves and produce some carbohydrates by their own photosynthesis. However, they attach to the host roots via specialized organs called haustoria (Kuijt 1969, Rümer et al. 2007) and obtain water, nutrients and assimilates from other plant species (Heide-Jørgensen 2008, Těšitel et al. 2011). Hemiparasites may use a wide range of host species (Weber 1976, Gibson and Watkinson 1989, Westbury 2004), but species differ strongly in their quality as hosts for

the parasite (de Hullu 1984, Gibson and Watkinson 1991, Hautier et al. 2010). The host species thus represent a very important part of the environment of the parasites and their reproductive success depends strongly on their host species. A good host species that supports growth of the parasite can be regarded as a benign environment, whereas a poor host species limits parasite growth and represents a stressful environment. Inbreeding can influence the infection success of parasitic plants (Gonzáles et al. 2007), but to our knowledge nothing is known about the effects of inbreeding on the ability of hemiparasites to grow with hosts of different quality.

Inbreeding has been proposed to be especially detrimental to parasites. The evolution of new resistant host genotypes may require a rapid counteradaptation by the parasites, which is facilitated by outcrossing (Gemmill et al. 1997, Agrawal and Lively 2001, Gonzáles et al. 2007). However, for parasitic plants selfing can be a means to assure reproduction if cross-pollination fails. Reproductive assurance is especially important for short-lived monocarpic species like *Rhinanthus* spp. (Barrett 2002, Charlesworth and Charlesworth 2010). All *Rhinanthus*-species are annuals, and some of them are known to be self-compatible and to have a mixed mating system (Kwak 1979, Oja and Talve 2012). In regularly selfing species, at least a part of the genetic load of recessive deleterious alleles is likely to be removed by selection (purging) or to become fixed by genetic drift, which results in reduced inbreeding depression after enforced selfing in short-lived, self-compatible species compared to long-lived and self-incompatible species (Husband and Schemske 1996, Angeloni et al. 2011). The importance of inbreeding for species of *Rhinanthus* in increasing, as suitable habitat for *Rhinanthus* is decreasing across Europe due to agricultural intensification. The remaining populations are often small and fragmented (Blažek and Lepš 2015). Habitat fragmentation and reduced population size increase genetic erosion and the frequency of inbreeding (Ellstrand and Elam 1993, Young et al. 1996).

We used *Rhinanthus alectorolophus* (Orobanchaceae) as a model system to study the effects of inbreeding and stress by poor host quality on hemiparasites. Host quality is to some degree expected to increase with host size, as host root biomass may be correlated with the number of haustorial connections (e.g. Keith et al. 2004) and faster growing hosts may provide more resources and support larger parasites (Hautier et al. 2010). However, hemiparasites also compete with their hosts for light (Matthies 1995) and larger hosts cause more shading. Moreover, some plant species can defend themselves against

an attack by root hemiparasites, e.g. through lignification of the roots, local dieback at the point of infection, or through accumulation of phytoalexins (Govier 1966, Cameron et al. 2006, Heide-Jørgensen 2008). Different functional groups of plants have been found to differ in their quality as hosts, although there can also be considerable differences in host quality among species within functional groups (Marvier and Smith 1997, Rowntree et al. 2014). For *R. alectorolophus*, legumes are mostly good hosts because of their high nitrogen content due to their symbiosis with rhizobia. In addition, legumes have never been reported to block haustoria of *Rhinanthus* (Westbury 2004, Cameron et al. 2006). Grasses have been reported to be good host species for *Rhinanthus* (Keith et al. 2004, Cameron et al. 2006, Cameron and Seel 2007) and to only partially block haustoria (Rümer et al. 2007, Hautier et al. 2010). However, considerable differences in the quality of grass species as hosts for *Rhinanthus angustifolius* have been found in a study involving a large number of species (de Hullu 1984). In contrast, most forbs (i.e. non-leguminous dicotyledons) studied so far are poor hosts, and many of them block haustoria (Cameron et al. 2006).

In natural populations, hemiparasites rarely use only single host species, but mixtures of potential host species. Because different host species supply parasites with different compounds (Govier et al. 1967), growth of parasites may be stronger if they use multiple host species simultaneously (Marvier 1998a, but see Matthies 1996). In experimental grassland communities, biomass and reproduction of *R. alectorolophus* increased with functional diversity of the potential hosts, and total fitness of the parasites increased with the number of species in experimental grasslands (Joshi et al. 2000). The presence of good host species in a community may increase parasite biomass, but the effects of individual species in a mixture are not easy to predict as host species preferred by the parasites are more likely to be suppressed by competing species (Gibson and Watkinson 1991, Matthies 1996).

Parasitic plants can considerably reduce the growth of their hosts (Fürst 1931, Matthies 1995, 1996, Matthies and Egli 1999, Westbury 2004, Ameloot et al. 2005). In natural communities, grasses and legumes are most strongly suppressed by parasites, whereas forbs often benefit from the presence of hemiparasites (Davies et al. 1997, Ameloot et al. 2005). The presence of parasites usually reduces the biomass of the host community, but this effect often decreases with the number and functional diversity of species present, because stronger growth of more resistant species may compensate the lower biomass of

the more susceptible host species (Joshi et a. 2000, Ameloot et al. 2005). However, it is not known how inbreeding influences virulence in hemiparasites.

The total productivity of a host-parasite system is often lower than the productivity of the hosts grown without parasites (Matthies and Egli 1999, Ameloot et al. 2005, Hautier et al. 2010). This reduction of total productivity has been attributed to the lower resource-use efficiency of the parasites (Matthies 1995, Ameloot et al. 2005) or to the reduction of host photosynthesis by the parasite (Cameron et al. 2008), and it is also predicted by growth models in which the parasite reduces the relative growth rates of the host species (Hautier et al. 2010).

We grew offspring from selfed and open-pollinated *Rhinanthus alectorolophus* autotrophically, with 13 potential host species from different functional groups, and with 15 different four-species mixtures. We address the following questions: (1) Does *R. alectorolophus* show inbreeding depression (ID) after self-pollination? (2) Does the magnitude of ID differ among hosts and host mixtures? Specifically, does ID increase or decrease with host quality, or increase with hosts that increase phenotypic variation? (3) Does inbreeding affect the suppression of host growth by the parasite? (4) Is host quality influenced by host size, host growth rate or functional group, or by the number of legume species or of host functional groups in a mixture? (5) Does the presence of the parasite influence the competitive balance among the host species in a mixture?

Methods

Study species

Rhinanthus alectorolophus (Scop.) Poll. (Orobanchaceae) is an annual root hemiparasite, which grows in nutrient-poor to fertile meadows throughout Europe (Hartl 1974). *R. alectorolophus* is not a rare species, but declining in many parts of Europe due to agricultural intensification (Blažek and Lepš 2015) and is endangered in some German states (e.g. Garve 2004, Raabe et al. 2010). Flowers of *Rhinanthus*-species are pollinated by long-tongued bees (Kwak and Jennersten 1986), but are self-compatible and the plants thus have a mixed mating system (Oja and Talve 2012). Capsules open only at the apex and slowly scatter the winged, mostly wind-dispersed seeds (Hartl 1974, Westbury 2004). *Rhinanthus* is a facultative parasite that can flower and produce seeds even without a

host, but remains smaller than when grown with a host. Seeds require several weeks of cold stratification. In the field germination begins with the growth of the radicle in early winter, but the leaves only emerge above ground in spring (Westbury 2004, ter Borg 2005). Haustoria are formed when the roots of the parasite come in contact with host roots. Once a successful parasite-host connection has been established, the parasites grow faster and their leaves turn dark-green (ter Borg and Bastiaans 1973, Hartl 1974).

Host species in the two experiments

Two experiments were conducted in which single *R. alectorolophus* plants were grown in pots together with single host plants of one of 13 different species (experiment 1) or with different combinations of four host plants (experiment 2).

Experiment 1: We grew *R. alectorolophus* with 13 species that are known to differ in their quality as hosts for the parasite. Parasites grown with poor hosts were assumed to grow in a stressful environment. To differentiate among the effects of host functional group and stress intensity, we aimed to include good and poor hosts from each functional group (grasses, legumes and non-leguminous forbs) in the experiment. All chosen host species naturally occur together with *R. alectorolophus*. Seeds of the host species were obtained from a commercial supplier (Appels Wilde Samen, Darmstadt). We included four grasses: *Lolium perenne*, *Dactylis glomerata* and *Trisetum flavescens*, which have been found to be good hosts, while *Anthoxanthum odoratum* is known to be a poor host for *R. alectorolophus* (Hautier et al. 2010, D. Matthies, unpubl.). Five legumes were included: *Trifolium pratense*, *Lotus corniculatus* and *Medicago sativa* as good hosts (D. Matthies, unpubl.), and *Onobrychis viciifolia* because it often co-occurs with *R. alectorolophus.* Its suitability as a host is not known. *Anthyllis vulneraria* was included because it proved to be a poor host for *R. alectorolophus* in a pilot study. Four non-leguminous forbs were also included in the experiment: *Sanguisorba minor* (Rosaceae) and *Taraxacum officinale* (Asteraceae), which are relatively good hosts (D. Matthies, unpubl.), and *Plantago lanceolata* (Plantaginaceae) and *Leucanthemum vulgare* (Asteraceae), which are poor hosts for *Rhinanthus* (D. Matthies, unpubl.; Cameron et al. 2006). In the following, will refer to the host species only by their genus.

Experiment 2: We created 15 different mixtures of host species, each consisting of four different species (Table 1). The mixtures were compiled from the 13 host species, using

the following criteria: Each species was used in a similar number of mixtures, and mixtures differed in two features that were likely to influence the quality of the mixture for *R. alectorolophus*, the number of functional groups (1 - 3) and the number of legumes (0 - 4).

Table 1: The 13 species used as hosts for *Rhinanthus alectorolophus* in experiment 1 and the composition of the 15 different mixtures of hosts in experiment 2. Vertical lines separate groups of three mixtures differing in the number of legumes (4 – 0), and shading illustrates differences in the number of functional groups (FG) per mixture (white = 1 FG, light grey = 2, dark grey = 3 functional groups).

No. legumes:		4			3			2			1			0		
No. functional groups:		1	1	1	2	2	2	2	3	3	3	3	2	2	1	1
Mixture number:		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
Lolium perenne	Grasses				1						1		1			1
Dactylis glomerata										1		1		1		1
Trisetum flavescens									1				1	1		1
Anthoxanthum odoratum							1				1		1			1
Trifolium pratense	Legumes	1	1	1	1					1			1			
Lotus corniculatus		1	1	1		1	1	1								
Medicago sativa		1	1		1	1			1		1					
Onobrychis viciifolia			1	1	1		1	1				1				
Anthyllis vulneraria		1		1		1	1		1	1						
Taraxacum officinale	Forbs					1		1							1	
Plantago lanceolata												1		1	1	
Sanguisorba minor								1		1				1	1	
Leucanthemum vulgare									1		1	1			1	

Pollination, germination and growth

In June 2012, 60 *Rhinanthus alectorolophus* plants with flower buds but no open flowers in two populations near Großalmerode, Germany, were covered with nylon mesh bags to prevent insect pollination of the flowers. Population A (Rösberg) was a large, continuous meadow population with high densities of *R. alectorolophus* and population B (Eisenberg) was a low density population growing along a field lane. The distance between the two populations was 2 km. Seeds from the 28 remaining bagged plants and from 53 open-pollinated plants nearby were collected in July. The seeds from each plant were counted, weighed, and mean seed mass was calculated to estimate inbreeding effects on seed mass. For germination, parasite seeds were pooled per population and pollination treatment. Seeds were incubated in Petri dishes for three days at room temperature and kept for 8 weeks at 5 °C for cold stratification. They were then kept at fluctuating temperatures (20/10 °C, 12 h of light) until cotyledons emerged.

Host seeds were germinated in Petri dishes at room temperature and seedlings kept in the fridge at 4 °C (12 h light) until seeds of all species had germinated. Mean seed mass, mean fresh weight of a seedling after 10 days of germination and the number of days until 50% of the live seeds had germinated (t_{50}) were determined per host species as traits possibly influencing host quality (Table 2). Seed mass of the host species may correlate negatively with host quality, as species with larger seeds have been reported to be more competitive and stress resistant (Leishman et al. 2000). Seedlings were planted in 0.9 L pots filled with sand and loam (1:1) and kept in a greenhouse. They were fertilized with 10 ml of 8 gL^{-1} solution of a commercial fertilizer (N:P:K = 14:7:14%; Hakaphos Gartenprofi, Compo, Wien) and watered sparely from above to stimulate root growth close to the surface. The size of the host plants and especially their root system is known to have a strong effect on parasite establishment and performance (Keith et al. 2004). To determine the size and the root : shoot ratio of the host species at the start of the experiment, one plant of each host species was harvested above ground on day 16, 20, 27, 38 and 51 after planting. The roots were carefully washed free of soil and shoots and roots were dried separately for 24 h at 80 °C and weighed. The average root : shoot ratio of the five measurements per host species over time was used as a covariate to predict host quality (Table 2).

Table 2: Overview of the host species and their attributes. Fresh mass of seedlings was determined 10 days after germination; t50 = days until 50% of finally germinating seeds had germinated (i.e. unfolded their cotyledons); R : S = root : shoot ratio at the time of planting the parasite; RGR = relative growth rate, estimated as the slope of the regression of log aboveground biomass on days after planting.

Host species	Seed mass (mg)	Fresh mass (mg)	t50 (days)	R : S	RGR
Lolium perenne	1.96	13.23	5.67	0.52	0.068
Dactylis glomerata	1.17	7.77	6.38	0.55	0.072
Trisetum flavescens	0.21	2.45	5.10	0.48	0.072
Anthoxanthum odoratum	1.19	4.05	8.30	0.44	0.072
Trifolium pratense	1.75	21.95	4.86	0.71	0.072
Lotus corniculatus	1.26	12.90	5.14	0.89	0.076
Medicago sativa	2.23	26.84	5.44	0.65	0.068
Onobrychis viciifolia	15.51	136.85	2.00	0.85	0.061
Anthyllis vulneraria	2.98	31.66	0.90	0.46	0.067
Taraxacum officinale	0.52	6.33	4.84	0.99	0.066
Plantago lanceolata	2.03	14.65	5.16	0.52	0.072
Sanguisorba minor	4.68	14.46	11.33	0.63	0.072
Leucanthemum vulgare	0.29	2.97	4.99	0.46	0.073

When the hosts were two weeks old, one parasite seedling each was planted at 1 cm distance to single hosts or, for mixtures, in the centre of the four host individuals, each 1 cm from the parasite. If parasites died during the first two weeks after planting they were replaced. The first experiment consisted of 15 replicates for each of the 56 combination of host, population, and pollination treatment (840 overall), plus 15 control pots per host species grown without parasites (195 overall). The second experiment was smaller, with only five replicates per mixture x population x pollination (300), plus eight control pots per host mixture grown without parasites (120).

Pots from both experiments were transferred from the greenhouse to flower beds in an experimental garden of the University of Marburg after two weeks, when the parasite seedlings had established themselves. Plants were watered when necessary, and their positions in the flower beds randomized regularly. During the growth period, pots were fertilized twice with 20 ml of 8 g L^{-1} commercial fertilizer. Parasites and host plants where harvested above ground after 9 weeks, when most of the parasites had finished

flowering. The height of the parasites was measured and the number of flowers counted, and for every fourth parasite the leaf chlorophyll content of two randomly chosen leaves was measured using a chlorophyll meter (SPAD-502, Minolta) and averaged. The SPAD-units were then transformed into chlorophyll content (mg cm^{-2}) using the formula $y = 0.000552 + 0.000404\ x + 0.0000125\ x^2$ (Richardson et al. 2002). Parasites and host plants where dried separately for 24 h at 80 °C and weighed.

Data analysis

The effects of population and pollination type on mean seed mass were analyzed with an ANOVA using the 81 open or self pollinated plants as replicates.

Experiment 1: Analyses of variance with Type I sums of squares were used to study the effects of population, pollination treatment, and the host species on measures of plant performance. Population was regarded as a fixed effect as seeds from only two parasite populations were used, which differed in size and density. In a second step, the effect of host species (13 df) was split into the contrasts autotrophy vs. hosts (1 df), functional group (2 df) and the remaining host effect (within functional groups, 10 df). In the ANOVA tables, interactions involving the contrasts are not shown, as none of them were significant. Data for biomass, height and number of flowers were log-transformed prior to the analysis to achieve homoscedasticity and normally distributed residuals. The binary variables early and late mortality were analyzed by generalized linear models with a logit link and binomial errors (analysis of deviance), using the same model structure as in the ANOVA models (analysis of deviance, Quinn and Keough 2002).

From the five early measurements and the final harvests of the above-ground biomasses of hosts grown without parasites, relative growth rates were calculated for each host species as the slopes of the increase in log-transformed biomass over time. Log-linear functions described the growth of the host species very well ($r^2 > 0.976$ for all 13 species), and their slopes differed among host species (species x time: $F_{12,234} = 3.12$, $p < 0.001$). The estimated relative growth rates were used as predictors of parasite biomass to test the hypotheses of Hautier et al. (2010).

Experiment 2: The effects of host mixture, pollination type and parasite origin on parasite performance were studied by ANOVAs. However, the population of origin had no effect on any of the studied variables, and population was removed from the analysis.

In the ANOVAs, the effect of host mixture was split into the linear contrasts number of legume species (0 - 4) and number of functional groups (1 - 3), and a rest. These contrasts are not completely independent, as all mixtures of four legumes consisted of only one functional group and mixtures containing three functional groups could not contain more than two legumes. However, the number of functional groups and of legumes in the mixtures had been designed to be as independent as possible (Table 1), and the order of the two contrasts did not qualitatively change any of the results.

To test how strongly properties of the mixtures influence mixture quality for the parasite, multiple regressions were performed, relating mean parasite biomass per mixture to the following predictors: mean productivity of a mixture without the parasite, the number of functional groups and of legumes in a mixture, and the mean quality of the four host species as estimated in the single host experiment. The best possible model based on Schwartz's information criterion (BIC) was selected using the package leaps version 2.9 with the software R version 3.2.1 (R Core Team 2015).

To analyze the effects of the intensity of stress on inbreeding depression, stress intensity was calculated as one minus the biomass of the open-pollinated (presumably crossed) parasites per host species, relative to the biomass of the crossed plants grown with the best host (Fox and Reed 2011). It thus is equal to 1 – host quality. Biomass was chosen as a fitness measure instead of survival, because it was assumed to better reflect host quality. Stress intensity thus is a quantitative variable with one value per combination of population and host species. It was used as a linear contrast in ANOVAs and explains a part of the population x host interaction.

Mean values of log-transformed biomass data were back-transformed before calculating stress intensity and inbreeding depression (ID). ID was calculated for every combination of population of origin and host species as 1 minus the relative fitness of the inbred vs. that of the outbred (open-pollinated) individuals: $\delta = 1 - (w_i/w_o)$. When inbred plants performed better than outbred plants, ID was calculated as $\delta = (w_o/w_i) - 1$ to keep all values between 1 and -1 (Ågren and Schemske 1993).

To analyze the effects of phenotypic variation on inbreeding depression, the opportunity for selection (CV^2) was calculated as the squared coefficient of variation for biomass of selfed and outcrossed individuals for each combination of population and host species (experiment 1) or for each host mixture (experiment 2). The separate CV^2-values for

selfed and crossed offspring were then averaged within each combination of parasite population and host species (experiment 1) or within each mixture (experiment 2; Waller et al. 2008, Reed et al. 2012). The effect of average CV^2 on ID was determined by linear regressions for each experiment.

The suppression of a host species or mixture by the parasite was calculated as the percent reduction of host species biomass compared to control plants grown without parasites. To compare the mean suppression of individual host species by the parasite when grown individually and when grown in mixtures, the suppression of each individual species in each mixture was calculated and averaged across mixtures. A linear regression was performed to compare the mean suppression of each species by the parasite in the two experiments.

To test the effect of the parasite on size inequality among hosts, the coefficient of variation (CV) for the untransformed biomasses of the four host species of a mixture was calculated per pot. The effect of parasite presence on the CV of host biomass was tested in an ANOVA including mixture and parasitization as factors.

All statistical analyses, if not stated otherwise, were performed with the software IBM SPSS Statistics for Windows, Version 21.0. (Armonk, NY).

Results

The mean seed mass of the sampled *R. alectorolophus* plants was 4.77 ± 0.12 mg and did not differ significantly between the populations ($F_{1,77}$ = 2.67, p > 0.1) nor between pollination treatments ($F_{1,77}$ = 3.59, p = 0.062), with a tendency of selfed seeds (5.01 mg) to be larger than seeds from open pollinations (4.53 mg). The populations did not differ in their response to self pollination ($F_{1,77}$ = 1.14, p = 0.29). After eight weeks of cold stratification and 67 days of alternating temperatures, 63% of selfed seeds and 48% of open pollinated seeds had germinated.

Parasite performance with single host species

Of the planted seedlings, 12 % died in the first weeks and had to be replaced. This early mortality differed among populations of origin, pollination treatments and host species (Table 3). More seedlings died from the Rösberg (14.8%) than from the Eisenberg

population (8.8%), and among Rösberg plants, more selfed seedlings died than seedlings resulting from open pollination (19.0% vs. 10.5%). Early mortality of the parasites was influenced by host functional group (Table 3), and was higher for parasites grown with grasses (15.5%) than for those with forbs (12.9%) or legumes (8%). The higher the root : shoot ratio of a host species at the time of planting was, the fewer parasite seedlings died ($quasiF_{1,783} = 5.78$, $p = 0.016$). When the root : shoot ratio of the hosts was included in the model, it explained 22% of the total effect of the hosts (sums of squares), but the functional group of the host species still significantly influenced early parasite mortality ($quasiF_{2,783} = 3.44$, $p = 0.033$).

Table 3: Results of analyses of deviance and variance of the effects of population of origin, pollination type (selfed vs. open) and host species on early mortality of seedlings, mortality until harvest, and biomass at harvest of the parasite *R. alectorolophus*. Stress intensity is the proportional reduction in the biomass of open-pollinated parasites from each population grown with a certain host in comparison to the performance with the best host. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; +, $p < 0.10$.

	Early mortality				Mortality			Biomass		
Source of variation	df	Mdev	qF		Mdev	qF		MS	F	
Population	1	7.16	10.77	**	7.00	5.95	*	1.56	7.90	**
Pollination type	1	1.98	2.98	+	1.64	1.40		1.72	8.75	**
Host species	13	1.33	2.00	*	2.20	1.87	*	5.46	27.72	***
No host vs. host	*1*	*0.00*	*0.00*		*7.58*	*6.45*	*	*6.83*	*34.66*	***
Functional group	*2*	*3.93*	*5.95*	**	*1.80*	*1.52*		*4.59*	*23.31*	***
Within FG	*10*	*0.94*	*1.42*		*1.74*	*1.48*		*5.50*	*27.91*	***
Population x pollination	1	5.05	7.60	**	2.52	2.14		2.80	14.19	***
Population x host species	13	1.63	2.45	**	0.88	0.75		0.34	1.71	+
Pollination x host species	13	1.50	2.25	**	0.93	0.79		0.17	0.85	
Pop. x host species x poll.	13	1.27	1.91	*	1.99	1.69	+	0.43	2.20	**
Stress intensity x poll.	*1*	*3.84*	*5.78*	*	*6.96*	*5.92*	*	*3.23*	*16.37*	***
Rest	*12*	*1.06*	*1.59*	+	*1.57*	*1.34*		*0.20*	*1.02*	
Residual	539-783	0.66			1.18			0.20		

Of the surviving parasites 28% died until harvest. Mortality of established seedlings was higher for seedlings from the Rösberg (33.1%) than for those from the Eisenberg population (24.8%), but did not depend on pollination type (Table 3). Late mortality was

higher among parasites grown autotrophically than among parasites grown with a host (45% vs. 27%), but there were no differences among host species (Table 3).

At harvest, parasite biomass was strongly correlated with parasite height ($r^2 = 0.91$) and number of flowers ($r^2 = 0.85$). Parasites from the Rösberg population were larger than those from the Eisenberg population (0.185 g vs. 0.152 g, Table 3). Offspring from selfed plants was smaller than offspring from open-pollinated plants. However, this inbreeding depression depended on the population of origin (Table 3), with 39% ID for Rösberg plants and no ID (-3%) for parasites from the Eisenberg population. In addition, inbreeding depression decreased with stress intensity for biomass (Fig. 1), early and late survival (Table 3). Stress intensity (i.e. 1 - host quality) was calculated per population x host species combination, because host quality differed among populations (see above). When included as a linear contrast in the ANOVA, the interaction of stress intensity x cross type explained most of the three way interaction population x pollination type x host species (Table 3). In contrast, differences in the average opportunity for selection (CV^2) did not explain differences in inbreeding depression ($r^2 = 0.024$, $p > 0.4$). Phenotypic variation in parasite biomass (CV^2) was not correlated with stress intensity ($r^2 = 0.008$, $p > 0.6$), but increased with the early mortality per host ($r^2 = 0.117$, $p = 0.075$).

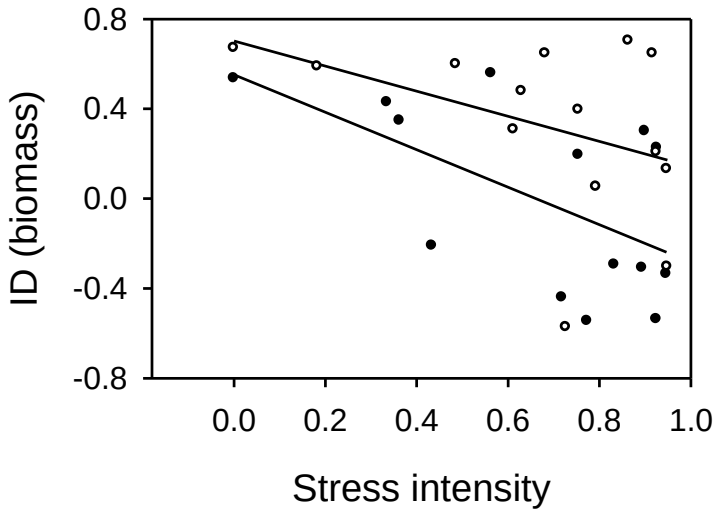

Fig. 1: Effects of stress by poor host quality (i.e. 1 – relative biomass of crossed parasites per host) on inbreeding depression of biomass in the hemiparasite *R. alectorolophus* from two populations of origin. Open symbols and dashed line: population Rösberg; filled symbols and continuous line: population Eisenberg.

Parasites were significantly larger when grown with a host, but parasite biomass differed strongly depending on host species (Table 3, Fig. 2a). When different attributes of the

host species where included as predictors in the analysis of parasite biomass they explained part of the effects of host species. Significant predictors of parasite biomass were mean log seed mass of the hosts (13.7% of host SS, i.e. 4.7% of total SS) and speed of seed germination (13.5% of host SS), as host species with larger seeds and faster germination were poorer hosts. In addition, parasite biomass was higher with host species that had high relative growth rates (5.7%), and large biomass at the time of planting of the parasite (2.2%) and at harvest (2.3%).

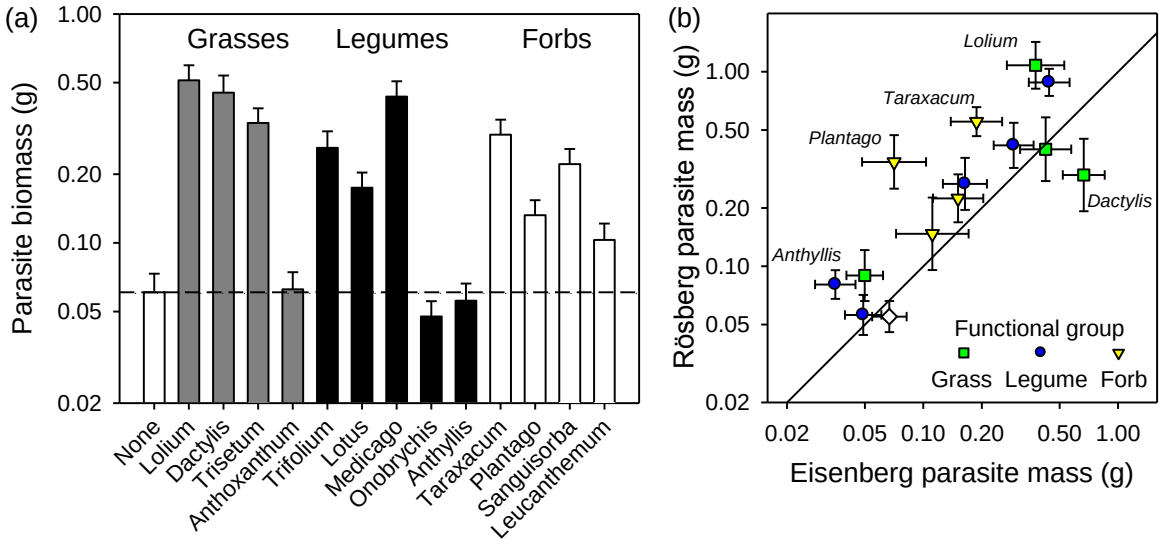

Fig. 2: (a) Mean biomass of the parasite *Rhinanthus alectorolophus* grown with various hosts, and (b) effects of host and population of origin on the size of parasites resulting from open pollination. Different symbols indicate host functional groups, diamond = without host. If parasites from both populations produced the same biomass with a host, points would fall on the diagonal line in (b). The names of host species that differed substantially in their quality for parasites from the two populations are indicated in the graph. For full host names see Table 1. Bars indicate + 1 SE. Note log-scale of the axes.

The biomass of the parasite *R. alectorolophus* was on average much higher with the four grass species (0.292 g) than with the forbs (0.179 g) or the legumes (0.143 g) used in the experiment (Table 3). Grown with the best host, the grass *Lolium*, parasites were eight times as large as parasites without a host, whereas grown with the poorest host *Onobrychis*, parasites were even smaller than without a host (Fig. 2a). However, the effect of the hosts on the parasite depended also on parasite population of origin (population x host interaction in Table 3). Plants of *R. alectorolophus* from the Rösberg population were more than twice as large as plants from the Eisenberg population when

grown with *Anthyllis*, *Plantago*, *Taraxacum* and *Lolium*, while parasites from the Eisenberg population grew larger with *Dactylis* (Fig. 2b).

Leaf chlorophyll content of the parasites differed strongly among host species ($F_{13,112}$ = 5.92, $p < 0.001$) and among functional groups ($F_{2,112}$ = 5.42, $p < 0.01$). It was higher for parasites grown with grasses (0.86 mg cm^{-2}) or legumes (0.84 mg cm^{-2}) than for those grown with forbs (0.60 mg cm^{-2}). In addition, leaf chlorophyll content increased with parasite biomass ($r^2 = 0.35$). To test if the effect of host species on parasite chlorophyll content was solely due to their effects on parasite size, parasite biomass was included in the model as a covariate (Table 4). Adjusted for parasite biomass, chlorophyll content was lower for parasites grown with forbs (0.61 mg cm^{-1}) and grasses (0.70 mg cm^{-1}) than for those grown with legumes (0.94 mg cm^{-1}), but there were also strong differences among species within functional groups (Table 4). Size adjusted leaf chlorophyll content was highest for parasites grown with *Trifolium*, *Lotus* or *Medicago* and lowest for those grown with *Taraxacum* or *Plantago* (Fig. 3). Inbreeding did not affect parasite chlorophyll content.

Table 4: ANCOVA of the effects of parasite biomass, population of origin, pollination type (selfed vs. open) on leaf chlorophyll content of the parasite *R. alectorolophus*. ***, $p < 0.001$; +, $p < 0.10$.

	Leaf chlorophyll			
Source of variation	Df	MS	F	
Log parasite biomass	1	13.418	125.38	***
Population	1	0.321	3.00	+
Pollination type	1	0.070	0.65	
Host species	13	0.649	6.07	***
No host vs. host	*1*	*0.045*	*0.42*	
Functional group	*2*	*1.799*	*16.81*	***
Within functional groups	*10*	*0.479*	*4.48*	***
Population x pollination	1	0.006	0.06	
Population x host species	13	0.121	1.13	
Pollination x host species	13	0.161	1.51	
Pop. x poll. x host species	12	0.075	0.70	
Residual	107	0.107		

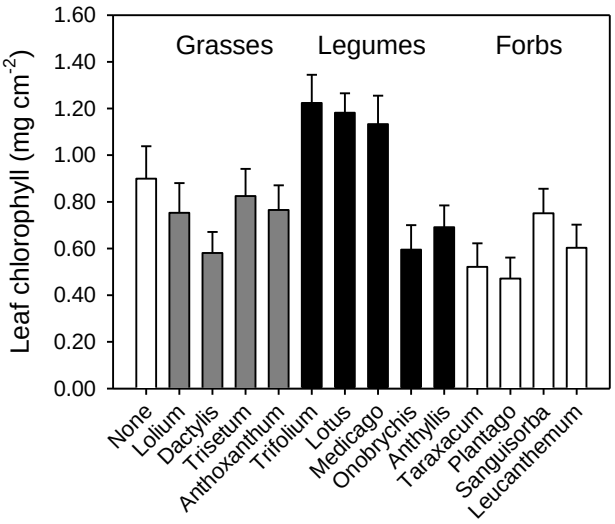

Fig. 3: Leaf chlorophyll content of the hemiparasite *R. alectorolophus* grown without a host and with 13 different host species. Fills indicate host functional groups: gray = grasses; black = legumes; white = non-leguminous forbs. Bars show means adjusted for the biomass of the parasites, see Table 4. Error bars indicate + 1 SE.

Parasite performance with mixtures of host species

Of the seedlings planted, 32% died in the first two weeks and were replaced. This early mortality was not influenced by host mixture ($quasiF_{14,270}$ = 1.12, p > 0.1) or by pollination treatment ($quasiF_{1,270}$ = 0.21, p > 0.5). Of the established parasites 44% died until harvest. Mortality was higher for offspring from selfed plants (52%) than for offspring from open-pollinated plants (37%, $quasiF_{1,270}$ = 5.50, p = 0.02), but did not differ among host mixtures.

In contrast, parasite biomass differed strongly among mixtures (Table 5, Fig. 4a). Parasite biomass increased with the productivity of a mixture measured in the controls without the parasite ($F_{1,134}$ = 20.07, p < 0.001, Fig. 4b), which in turn increased with the number of legumes (14% increase in productivity per added legume, r^2 = 0.79, p < 0.001). Host quality for the parasite increased with the number of functional groups and with the number of legumes in a mixture (contrasts in Table 5), and these effects remained significant even when adjusted for differences in productivity. The best mixture (M4, s. Table 1) included three legumes and a suitable grass, while the poorest mixtures were those containing only forbs and grasses (M13 – M15, Fig. 4a).

Table 5: ANOVA of the effects of pollination type (selfed vs. open) and host species mixture on the biomass of *R. alectorolophus*. The effect of mixture was partitioned into the three linear contrasts number of legumes in the mixture, number of functional groups, and the residual variation among mixtures. ***, $p < 0.001$; *, $p < 0.05$; +, $p < 0.10$.

		Parasite biomass		
Source of variation	df	MS	F	
Pollination	1	0.114	0.53	
Mixture	14	1.421	6.61	***
Number of legumes	*1*	*6.671*	*31.06*	***
Number of functional groups	*1*	*3.874*	*18.03*	***
Rest	*12*	*0.779*	*3.63*	***
Pollination x mixture	14	0.335	1.56	+
Pollination x number of legumes	*1*	*0.110*	*0.513*	
Pollination x number of functional groups	*1*	*1.072*	*4.991*	*
Rest	*12*	*0.292*	*1.359*	
Residual	136	0.215		

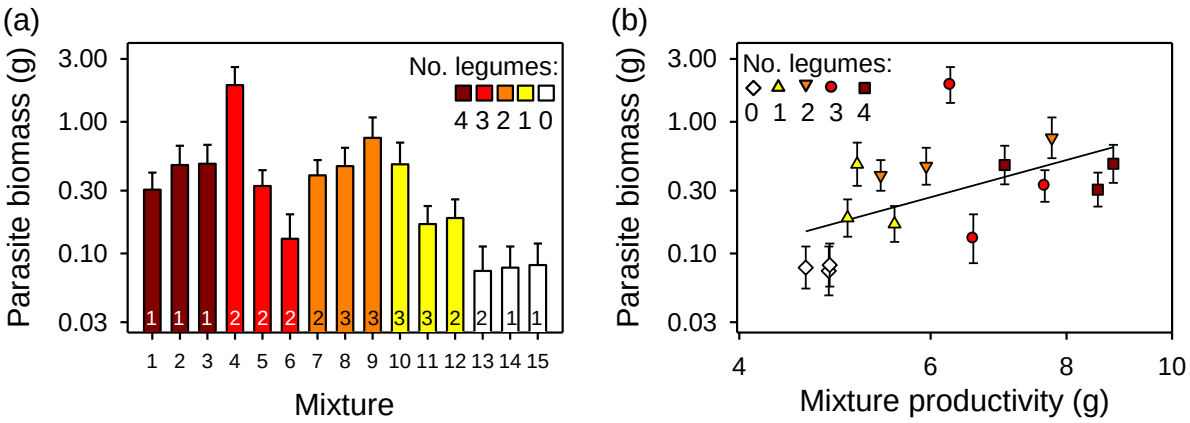

Fig. 4: Biomass of the parasite *R. alectorolophus* grown with 15 four-species mixtures. (a) Effects of different host mixtures on parasite biomass. Colors show the number of legumes (0 - 4) and numbers at the base of the bars show number of functional groups per mixture, see Table 1. (b) The relationship between parasite biomass grown with various mixtures of hosts and the productivity of the mixture grown without the parasite. Error bars indicate ±1 SE. Note log-scales for biomass.

The effect of pollination type on parasite biomass depended on the host mixture (Table 5). ID decreased with the number of functional groups in a mixture (Fig. 5, see linear contrast in Table 5). These differences in ID were due to the reduced size of selfed parasites in mixtures with fewer functional groups, whereas the biomass of open

pollinated parasites did not change with the number of host functional groups. The magnitude of ID did not depend on stress intensity ($r^2 = 0.014$, $p > 0.6$). Moreover, differences in the average opportunity for selection (CV^2) could also not explain differences in inbreeding depression ($r^2 = 0.002$, $p > 0.8$).

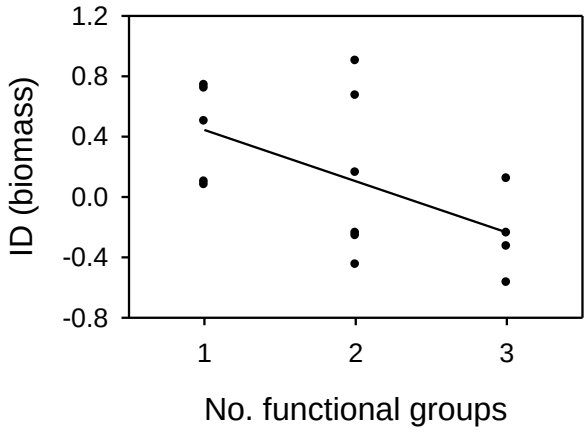

Fig. 5: Effect of the number of functional groups per mixture on inbreeding depression of biomass in the hemiparasite *R. alectorolophus*.

In single regressions, the quality of a host mixture could not be predicted from the quality of the hosts present, as measured in the single host experiment (exp. 1). Neither the mean host quality of the four host species in the mixture ($r = 0.085$), nor the quality of the best ($r = 0.22$) or poorest host present in the mixture ($r = -0.425$, $p = 0.11$) explained a substantial amount of variation in mixture host quality. In multiple regressions including as possible additional predictors the number of legumes and of functional groups, and the productivity of each mixture without the parasite, the model ($r^2 = 0.66$) with the lowest BIC contained as predictors the number of functional groups ($\beta = 0.27$) and of legumes in the mixture ($\beta = 0.90$), and the mean host quality of the four host species in the mixture ($\beta = 0.42$).

The effects of individual hosts in the mixtures on parasite biomass are not easy to separate. The presence of some species in a mixture had strong effects on parasite biomass (Fig. 6a). For example, the presence of *Medicago* and *Trifolium* had strong positive effects on parasite size, while that of *Plantago* had strong negative effects. This ranking of host quality is in contrast to the host quality estimated in the single species experiment (Fig. 1). However, the mixture composition was not random and the presence of species in mixtures was not independent of the number of legumes and the number of functional groups, both factors which strongly increased parasite size. When the effect of

the presence of a species in a mixture was tested in a linear model in addition to these two variables, the additional effects of host species identity were very different from the effects estimated without the two covariates (Fig. 6b).

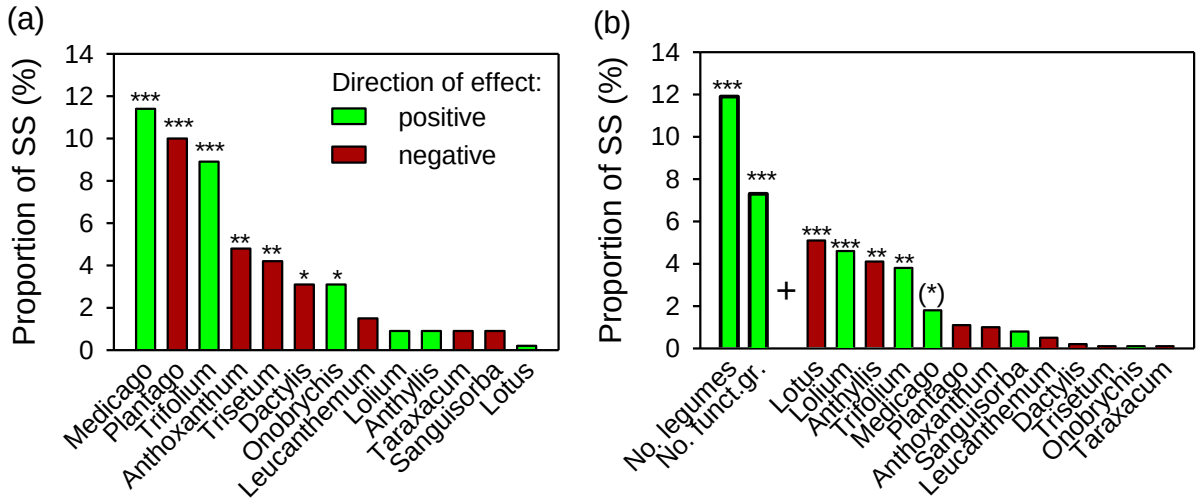

Fig. 6: (a) Results of separate analyses of variance of the effect (percentage of the total sums of squares explained) of the presence of each host species in the mixtures on the biomass of the parasite *R. alectorolophus*. (b) Percentage of the total sums of squares explained by the number of legumes, the number of functional groups, and by the effect of the presence of each species in a mixture in addition to the 19.1% explained by the number of legumes and the number of functional groups, based on separate ANOVAs. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; (*), $p < 0.10$.

Effects on host biomass

In both experiments, host biomass was reduced by the parasites. In experiment 1, host suppression increased with parasite size (Fig. 7a; $r^2 = 0.69$, $p < 0.001$). A ten-fold increase of parasite biomass increased host damage by 45%. However, the host species and functional groups differed in their tolerance of parasitism (host x parasite interaction in Table 6). The grasses in the experiment were on average suppressed by 37%, the forbs by 21%, but the legumes only by 9%. Two legume species, *Lotus* and *Trifolium*, supported parasites of medium size without being negatively affected (Fig. 7a). When a parasite was present, the effect on the biomass of the host species differed between parasites from the two populations (population x host interaction, Table 7). This interaction remained significant when log parasite biomass was included as a covariate ($F_{12,506} = 1.85$, $p = 0.038$). The pollination treatment of the parasites did not influence host biomass ($p > 0.20$, Table 7), suggesting no inbreeding effects on parasite virulence.

Table 6: Analysis of variance of the effects of species identity and parasitism by *R. alectorolophus* on the biomass of 13 different host species. The effect of the host species is split into the effect of host functional group and the effect of species within functional group (rest). ***, $p < 0.001$; **, $p < 0.01$.

	df	MS	F	
Host species	12	1.696	54.7	***
Functional group	*2*	*0.029*	*0.9*	
Rest	*10*	*2.029*	*65.2*	***
Parasite vs. control	1	1.483	47.7	***
Parasite x host species	12	0.116	3.7	***
Parasite x functional group	*2*	*0.303*	*9.7*	***
Parasite x rest	*10*	*0.078*	*2.5*	**
Residual	729	0.031		

Table 7. ANOVA of the effects of host species, the population of origin of the parasite *R. alectorolophus* and parasite pollination type (selfed vs. open) on the biomass of 13 different host species. Only pots with parasites were included in the analysis. ***, $p < 0.001$; *, $p < 0.05$.

	df	MS	F	
Host species	12	1.402	38.41	***
Parasite population	1	0.067	1.84	
Pollination type	1	0.056	1.52	
Host species x parasite population	12	0.070	1.91	*
Host species x pollination type	12	0.024	0.67	
Population x pollination type	1	0.020	0.54	
Host x population x pollination type	12	0.022	0.59	
Residual	508	0.037		

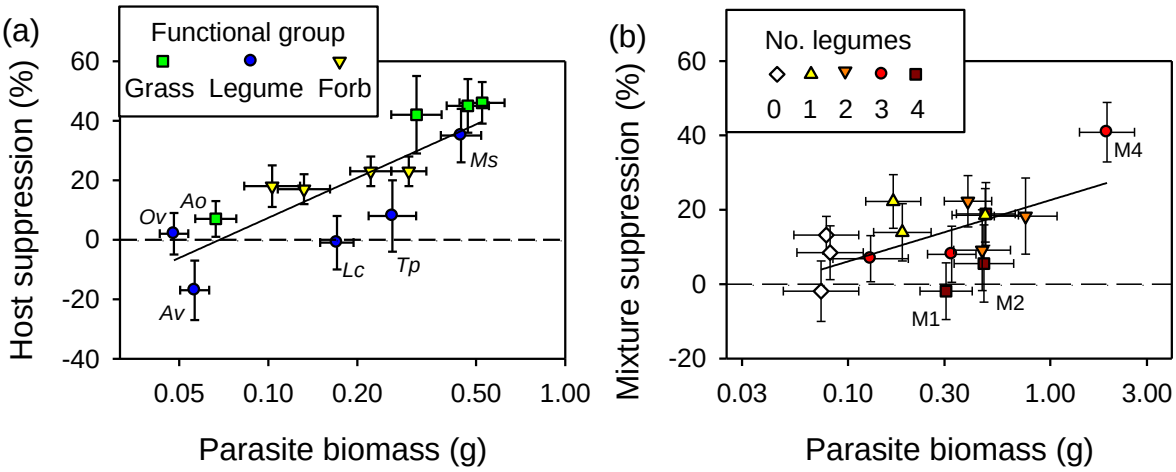

Fig. 7: Effects of parasite biomass on the suppression of the hosts, i.e. the reduction of host biomass by the parasite relative to controls without the parasite. (a) Single host species in experiment 1 and (b) species mixtures in experiment 2. Ao = *Anthoxanthum*; Av = *Anthyllis*; Lc = *Lotus*; M1 – M4 = mixture 1 – 4; Ms = *Medicago*; Tp = *Trifolium*; compare Table 1. Error bars indicate ± 1 standard error. Note log-scales for biomass.

In experiment 2, the mixtures differed substantially in their biomass. Independent of parasite size, mixture biomass increased with the number of legumes, but not with the number of functional groups (Table 8). Host suppression increased with parasite size (Fig. 7b; $r^2 = 0.37$, $p = 0.020$). A ten-fold increase of parasite biomass increased host damage by 16.5%. However, mixtures differed in their tolerance towards parasites (mixture x parasite interaction in Table 8). Two of the mixtures containing only legumes (M1 and M2) supported parasites of medium sizes without being suppressed (Fig. 7b), but there was no general effect of the number of legumes in a mixture on host suppression (linear contrast in Table 8).

To analyse the effect of parasite pollination type, a further analysis containing only pots with parasites was carried out. The pollination type of the parasite did not influence the suppression of the host mixtures ($F_{1,137} = 0.01$, $p > 0.9$).

The total productivity per pot was not consistently influenced by the presence of a parasite in experiment 1 ($F_{1,729} = 0.85$, $p = 0.36$), but the effect of the parasite differed depending on the functional group of the host species ($F_{2,729} = 5.47$, $p = 0.004$). Pots with grasses produced 12.8% and pots with forbs 5.2% less biomass when a parasite was present, whereas pots with legumes produced 5.7% more biomass (hosts + parasite). In

experiment 2, the presence of a parasite reduced total productivity per pot by 3.1% ($F_{1,257}$ = 3.4, p = 0.066).

Table 8: ANOVA of the effects of host mixture and parasitism by *R. alectorolophus* on the biomass of 15 different host mixtures. The effect of host mixture was partitioned into the linear contrasts number of legumes, number of functional groups per mixture (see Table 1), and the remaining variation among mixtures. ***, p < 0.001; *, p < 0.05.

	df	MS	F	
Mixture	14	0.207	26.06	***
Number of legumes	*4*	*0.494*	*62.15*	***
Number of functional groups	*2*	*0.088*	*11.11*	***
Residual variation among mixtures	*8*	*0.093*	*11.76*	***
Parasite vs. control	1	0.313	39.44	***
Parasite x host species mixture	14	0.016	2.04	*
Parasites x number of legumes	*4*	*0.014*	*1.74*	
Parasites x number of functional groups	*2*	*0.005*	*0.65*	
Parasites x residual variation among mixtures	*8*	*0.020*	*2.54*	*
Residual	257	0.008		

Influence of the parasite on individual species in the host mixtures

The coefficient of variation (CV) of the biomass of the four host species in a pot, a measure of size inequality, differed among mixtures ($F_{14,257}$ = 7.83, p < 0.001). The CV was largest in mixture 7 (111 %) and lowest in mixture 1 (63%), with an average of 76.5%. The presence of a parasite increased the average CV to 83% ($F_{1,257}$ = 5.18, p = 0.024), and this effect of parasitization did not differ among mixtures ($F_{14,257}$ = 1.17, p = 0.303).

The four species in a mixture were suppressed to a different degree by the parasite. The most strongly suppressed hosts in the mixtures were *Sanguisorba* (32.5%), *Trifolium* (29.2%), *Medicago* (27.5%) and *Lolium* (25.7%), whereas the biomass of *Trisetum* (-0.4%) and *Anthyllis* (-10.3%) was not suppressed by *R. alectorolophus* (Fig. 8). The average suppression of a species in the mixtures was not strongly correlated with the suppression of the same species when grown alone with a parasite in experiment 1 (r^2 = 0.16, p = 0.17). The three grass species *Lolium*, *Dactylis* and *Trisetum* that were good

hosts in exp. 1 were suppressed much less in the mixtures, whereas *Trifolium* was suppressed more in the mixtures than when grown alone (Fig. 8).

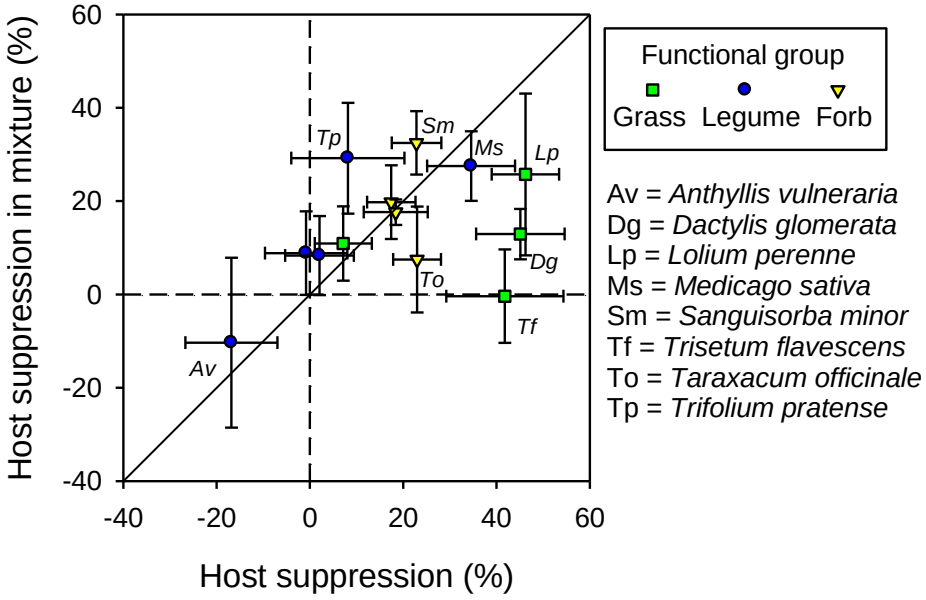

Fig. 8: Comparison of the suppression of host species by the parasite when grown alone or in four-species mixtures. Different symbols indicate host functional groups. The diagonal line indicates equal suppression of a species alone and in mixtures. Error bars indicate ± 1 standard error.

Discussion

Inbreeding depression in *Rhinanthus alectorolophus*

Overall, *Rhinanthus alectorolophus* expressed only little inbreeding depression (ID). The early traits seed mass and germination, but also mortality until flowering showed no ID at all. This corresponds to the expectation for frequently selfing angiosperms, which on average show 0 to 5% ID in early traits and juvenile survival, but 21% ID in growth and reproduction, and is attributed to a history of purging strongly deleterious, early acting mutations (Husband and Schemske 1996). In addition, ID in *R. alectorolophus* may have been underestimated because inbred offspring was compared with offspring from open pollinations, which may have partly resulted from geitonogamous self-pollination (de Jong et al. 1993). The fact that with some hosts ID in early traits and ID in biomass tended even to be negative is probably the result of a maternal effect of resource reallocation to a lower number of pollinated flowers in the bagged plants (Zimmerman

and Pyke 1988, Knight et al. 2006), which is indicated by the slightly larger seeds and better germination after self- than open pollination.

The two sampled parasite populations differed in their size. In parasites from the smaller population (Eisenberg), ID averaged across all host species was absent even in final biomass. Plants from small populations often show less ID than plants from large populations (Angeloni et al. 2011), which can be due to purging in small populations (but see Byers and Waller 1999, Glémin 2003) or due to an increased genetic load as a consequence of genetic drift (Keller and Waller 2002, Angeloni et al. 2011). Plants from the Eisenberg population were on average smaller than plants from the Rösberg population, which lends support to a higher genetic load in the Eisenberg population.

Inbreeding depression and stress by poor host quality

The magnitude of inbreeding depression differed among parasites grown with different host species (exp. 1) and among parasites grown with different mixtures (exp. 2). In experiment 1, ID increased with the quality of a species as host for *R. alectorolophus*. As poor hosts can be regarded as stressful environments for the parasite ID decreased thus with stress intensity. In contrast, ID was not affected by host quality in exp. 2 but decreased with the functional diversity of a mixture.

It has been proposed to test the phenotypic variation hypothesis as a null-model before discussing the effects of stress intensity on inbreeding depression (Waller et al. 2008). This hypothesis suggests that an environment may increase ID not because it is stressful, but because it increases the amount of phenotypic variation among individuals. It is expected that an environment that increases phenotypic variation increases the opportunity for selection (CV^2) and thus inbreeding depression, the selection against selfed offspring (Waller et al. 2008). The phenotypic variation hypothesis has only rarely been tested. In *Brassica rapa* grown under salinity and density stress, ID was higher for traits that were more variable, but for a given trait, ID was not consistently higher for treatments that increased CV^2 (Waller et al. 2008). Mixed support for the hypothesis is provided by a multiple regression analysis of nine animal data sets, in which ID often increased with CV^2, but stress intensity was a better predictor of ID (Reed et al. 2012) and a study on *Drosophila melanogaster* raised under four selection regimes, in which stress intensity and CV^2 were correlated and ID increased with both (Long et al. 2013). In a

study of *Silene vulgaris* under eight different stress treatments, CV^2 was a better predictor of ID in biomass than stress intensity, and ID in biomass increased with CV^2 but slightly decreased with stress intensity (Chapter II). In contrast to these studies, the phenotypic variation hypothesis did not explain any differences in ID in *R. alectorolophus* among host species or mixtures, and the CV^2 of parasite biomass was not correlated with stress intensity.

In experiment 1, ID was highest when parasites were grown on good hosts and lowest for parasites grown with poor hosts or autotrophically. This is in contrast to the prevalent expectation that ID increases with stress and supports the alternative hypothesis that crossed plants can use favorable conditions better than selfed plants (capable crossed hypothesis, Cheptou and Donohue 2011, Chapter II). This pattern has been found in other plants in response to different nutrient levels (Norman et al. 1995, Kéry et al. 2000, Walisch et al. 2012, Chapter II) and suggests that under resource-limiting conditions, the performance of inbred and crossed offspring is similarly poor, whereas ID increases under good conditions because crossed plants have a higher phenotypic plasticity or higher relative growth rates.

A range of mechanical and chemical defense mechanisms have been observed in different host species (Cameron et al. 2006, Rümer et al. 2007, Heide-Jørgensen 2008). However, the effects of host species on the growth of *R. alectorolophus* were not influenced by pollination type, suggesting that inbreeding did not influence the ability of the parasites to overcome specific resistance mechanisms. In contrast, in the holoparasite *Tristerix aphylla*, which grows endophytic in South American cacti, inbreeding reduced many early fitness traits, including infection success (Gonzáles et al. 2007). It has been suggested that outbreeding is especially important for parasites, because they have to overcome defenses evolving in different host genotypes (Gemmill et al. 1997, Agrawal and Lively 2001, Gonzáles et al. 2007). However, little is known about the genetics of the host-parasite coevolution in *Rhinanthus*. Different resistance mechanisms have been described for various species (e.g. Cameron et al. 2006, Rümer et al. 2007), but a reciprocal transplant experiment found no local adaptation between *Rhinanthus* and its host *Agrostis capillaris* from different populations (Mutikainen et al. 2000). It has been suggested that generalist hemiparasites will not show strong local adaptation to specific hosts, because they do not differ from their hosts in their evolutionary potential and migration rate and have a relatively low virulence. However, parasites from different

populations differed in biomass and in their effect on the host plant (Mutikainen et al. 2000). In our study the quality of the individual species as hosts for parasites from the two studied populations differed, which suggests different adaptations in the two parasite populations, although they are only 2 km apart from each other. Similarly, *Rhinanthus* from two different populations differed in their growth with identical genotypes of *Hordeum vulgare* (Rowntree et al. 2011). There is thus substantial evidence for genetic variation in the response of hemiparasites to the same host species, which may potentially be affected by inbreeding.

When grown with mixtures of host species, inbreeding depression in *R. alectorolophus* decreased with the number of functional groups in a mixture. Host species from the three different functional groups are less closely related and differ in physiological and morphological traits. Hosts from different functional groups may provide different resources to the parasites. The hemiparasite *Odontites verna* received mostly carbohydrates from the grass *Hordeum vulgare*, but nitrogenous compounds from the legume *Trifolium repens* (Govier et al. 1967). In addition, functional groups are assumed to differ in their defense mechanisms (Rümer et al. 2007). The two studied grasses show some lignification of their roots in response to parasite haustoria, and the forbs *Leucanthemum vulgare* and *Plantago lanceolata* successfully block haustoria by suberisation of the cell walls and local cell death, respectively (Rümer et al. 2007). The buffering of inbreeding depression by functional diversity of hosts suggests that the deficits of inbred *R. alectorolophus* may be better compensated if hosts from different functional groups are available.

Differences in host resistance

Several studies have found that legumes were especially good hosts for *Rhinanthus*, while forbs were mostly poor hosts (Westbury 2004, Cameron et al. 2006, Rümer et al. 2007, Rowntree et al. 2014). However, the legumes in our study were poorer hosts than the grasses. This was due to two legumes, *Anthyllis* and *Onobrychis*, that were especially poor hosts. These two species and the grass *Anthoxanthum* have to be regarded as a non-hosts for the studied parasite populations, because they did neither increase parasite biomass nor chlorophyll content and their growth was not suppressed by the parasite. Both *Anthyllis* and *Onobrychis* have thick roots and partly suberized roots (Kutschera 1960) which *Rhinanthus* may be unable to penetrate. Although legumes are not reported

to block haustoria of *Rhinanthus* (Rümer et al. 2007), *Trifolium incarnatum* did not serve as a host for *Odontites verna* (Govier 1966). Other studies have also found that some legume species are poor hosts for hemiparasites (Marvier and Smith 1997), but most studies have only used legumes which are good hosts species. The ability to overcome host defenses may differ even among closely related species. In contrast to our study, where it did not serve as a host, *Anthoxanthum odoratum* supported medium sized, though smaller than expected, *Rhinanthus minor* parasites in a study using multiple grass hosts (Hautier et al. 2010). Moreover, *Plantago lanceolata* and *Leucanthemum vulgare*, which are known to completely block haustoria and to be very poor hosts for *R. minor* (Cameron et al. 2006, Rowntree et al. 2014), were suitable hosts in our study, at least for one parasite population.

Fertilization effects of legumes

The strong growth of hemiparasites with legumes is usually attributed to the high nitrogen content of legumes (de Hullu 1984, Gibson and Watkinson 1991, Westbury 2004) or the quality of nitrogen supplied (Jiang et al. 2008). Physiological studies have shown that the quality of nitrogen compounds from leguminous symbiotic nitrogen fixation is not superior to other forms of soil bound nitrogen (Jiang et al. 2008), and it was suggested that the good host quality of legumes is due to the well-developed haustoria formed on these species by *Rhinanthus*. The high chlorophyll content of *R. alectorolophus* grown with the three good leguminous hosts suggests a high nitrogen supply by the hosts, as leaf chlorophyll content is a good predictor of leaf nitrogen content (O'Neill et al. 1984, Wu et al. 2007). However, in spite of their presumably good nitrogen supply, parasites grown with these legumes were not larger and did not have more flowers than parasites grown with grasses. Similarly, leaf nitrogen content, but not biomass of the hemiparasite *Castilleja wightii* was higher, when grown with two legumes than with two non-legume hosts (Marvier 1998a). *R. alectorolophus* grown with legumes may have received more nitrogen, but less carbohydrates from their hosts, as observed for the hemiparasite *Odontites* (Govier et al. 1967), and thus were more dependent on their own photosynthesis.

In the host mixtures it was possible to separate the direct effect of legumes as good hosts from their indirect effect of nitrogen fixation. Although two of the legumes did not serve as hosts for *R. alectorolophus* in exp. 1, the presence of one of them (*Onobrychis*) in a

mixture increased parasite biomass. In addition, pot biomass increased strongly with the number of legumes in the mixture, which suggests a fertilization effect. Fertilization has been shown to increase parasite size under conditions of low competition (Matthies and Egli 1999, Westbury 2004, Pennings and Simpson 2008, Těšitel et al. 2015), but high levels of fertilizer can lead to the competitive exclusion of hemiparasites (Fürst 1931, Matthies 1995, Hejcman et al. 2011), especially at the seedling stage when they still rely on their own photosynthesis (Těšitel et al. 2010, 2011).

Host traits that correlate with host quality

The quality of a species as a host for *R. alectorolophus* changed with the life stage of the parasite. For example, early mortality of parasites grown with grasses was particularly high, but parasite biomass was highest when grown with grasses. High early mortality was partly due to a low root : shoot ratio of the host species at the time of planting, which is difficult to explain. We suppose that drought affected early seedling survival, especially in pots with host species with a large transpiration due to their low R : S ratio. Alternatively, more and longer roots may increase the probability that parasite roots come into contact with them (Westbury 2004), but early mortality of *R. alectorolophus* was even higher in the mixture experiment, although root availability will have been higher with four than with a single host per pot. In addition, competition for light is particularly harmful to hemiparasites at early stages of development before attachment to a host makes them less dependent on their own photosynthesis (Těšitel et al. 2010, 2011). However, host species were still small when *R. alectorolophus* seedlings were planted and competition was unlikely. The hemiparasitic life-form has been suggested to be a successful strategy for annual species to establish in dense meadow vegetation (Westbury 2004). However, the influence of size and allocation patterns of the host species at the time of planting on the probability of establishment of hemiparasites are still poorly understood. Reproductive success in hemiparasites can be strongly enhanced by proximity to the host roots at planting (Keith et al. 2004) and early attachment to a host (Svensson and Carlsson 2004). In *R. alectorolophus*, the variation in final biomass (CV^2) was higher with host species which led to a higher early mortality, which suggests that a large part of the size variation of hemiparasites at harvest was still due to differences in access to a host in the early establishment phase.

Based on a simplified growth model, parasite biomass has been hypothesized to increase with host growth rates (Hautier et al. 2010). In a study with nine grass species as hosts for *R. alectorolophus*, parasite biomass increased with host absolute growth rate, but the explained variance differed among growth models (0.17 – 0.34) and it was suggested that differences in host resistance to parasites influence the strength of this correlation (Hautier et al. 2010). In our study, which included three functional groups of hosts with presumably different resistance mechanisms (Rümer et al. 2007), host relative growth rates explained less than 6% of the variation in host quality among species (i.e. parasite biomass). A better predictor of parasite biomass was host seed mass (13.7% explained variation), as plants with smaller seeds were better hosts. Similarly, it can be calculated from the data in Hautier et al. (2010, their Table 1 and Fig. 4) that in their experiment the biomass of *R. alectorolophus* decreased with log-transformed seed mass of nine grass species ($r^2 = 0.15$). Large-seeded species are known to be more resistant against a variety of stresses (Leishman et al. 2000), which is partly due to the lower RGRs of large-seeded species, but also due to a negative correlation between seed mass and specific root length (i.e. the root length per unit root dry mass; Reich et al. 1998, Wright and Westoby 1999). The two poorest hosts in our study, the legumes *Anthyllis* and *Onobrychis*, had very thick roots and few fine roots. The roots of *Onobrychis* are known to be especially thick and strongly suberized (Kutschera 1960). The negative correlation between parasite biomass and seed mass may thus be the result of a lower specific root length of species with large seeds. Shorter, more resistant host roots may for roots of the parasites reduce both the probability of encountering a host root (Westbury 2004, Keith et al. 2004) and of successfully forming a haustorial connection (Kuijt 1969).

Host quality in mixtures

Parasites grew larger in host mixtures than with single hosts. In two mixtures they grew larger than with the best single host, and even with the poorest host mixture they were larger than when grown autotrophically. However, in the mixtures containing no or only one legume (M10 - M15), parasites were smaller than with most single hosts, which was due to the low productivity and the resulting competition for resources. In studies that have investigated the effect of host diversity independent of host number, the hemiparasite *Melampyrum arvense* was not larger in mixtures than with the three host species separately (Matthies 1996), whereas the hemiparasite *Castilleja wightii* was larger

in two species mixtures than when grown with two host species separately (Marvier 1998a) and *Rhinanthus minor* benefited in one of three combinations of two hosts from a mixed diet (Rowntree et al. 2014). In our study, mixture quality increased with the number of functional groups, which suggests a complementary effect of different host functional groups, as found for *Odontites* (Govier 1967). A positive effect of the number of functional groups on parasite biomass was also found in experimental grassland communities (Joshi et al. 2000). However, the best mixture for *R. alectorolophus* contained only two functional groups (three legumes and one grass). A mixture of a grass and a legume also supported the largest *Castilleja* (Marvier 1998a) and *Rhinanthus* (Rowntree et al. 2014) and increased net reproductive rate in *Rhinanthus serotinus* (Svensson and Carlsson 2004).

The contribution of single species in a mixture to parasite biomass could not be clearly determined. Overall, the presence of *Medicago* and *Trifolium* had the strongest positive effects on the biomass of *R. alectorolophus* in a mixture, which is in agreement with the fact that they were also good hosts in single host experiments. However, *Trisetum* and *Dactylis* were similarly good hosts, but their presence decreased parasite size in mixtures. This could be a consequence of the fact that the composition of mixtures was not random and we included these species only in mixtures with less than two legumes, which had a much poorer nutrient supply. When the number of legumes and functional groups in the mixtures was included in the model, the presence of *Lolium* and *Trifolium* had positive additional effects on parasite biomass, which can be explained by their good host quality, but *Lotus* had a strong negative effect, which is hard to explain. The effect of a host species in a mixture apparently depends on its quality as a host, but also on the identity of the other hosts, their quality as hosts, their competitive ability and their contribution to the nutrient status and is not easy to predict in mixtures of more than two species.

Host suppression and competition

Hemiparasites can influence competition among species, as the preferred host species are suppressed by the parasites and thus become less competitive (Gibson and Watkinson 1991, Matthies 1996, Marvier 1998a). The presence of *R. alectorolophus* increased the size inequality among hosts, as shown by the higher coefficient of variation in the biomass of the four hosts in all mixtures.

The suppression of a species by the parasite in the mixtures was different from what would be expected by its suppression in the single host experiment. In the mixtures, the parasite could use resources from up to four instead of only one host, and the suppression of each species is thus expected to be lower than in the single host experiment. The grasses *Lolium*, *Dactylis* and *Trisetum* were much less suppressed in mixtures than in the single species experiment. In contrast, *Trifolium* was suppressed more in mixtures than when grown alone, although it was only one of four hosts, which suggests that it was preferred to other species in most mixtures. This is consistent with the results of two-species mixtures grown with one parasite, in which often the legume was suppressed more strongly than the grass (Gibson and Watkinson 1991, Matthies 1996). In contrast, most community studies found that grasses were suppressed especially strongly and their proportion in grasslands reduced, whereas forbs did profit from the presence of a parasite (Ameloot et al. 2005, Davies et al. 1997, Marvier 1998b).

The total above-ground productivity per pot was not consistently reduced by the presence of a parasite. Most studies found a reduction of total productivity when a parasite was present (Matthies and Egli 1999, Ameloot et al. 2005, Hautier et al. 2010). *R. alectorolophus* reduced the total productivity when grown with single grasses and in the four-species mixtures. However, some legumes were very tolerant of parasitism and supported large parasites without being harmed, and in these cases the parasite even increased the total biomass per pot. This contradicts the prediction of Hautier et al. (2010) that the presence of a parasite always reduces total productivity. This assumption is an important part of their model of parasitic growth which regards parasite biomass simply as a function of host biomass. The additional photosynthesis of hemiparasites can contribute substantially to parasite biomass (Seel et al. 1993, Těšitel et al. 2011), and our results suggest that parasite biomass production may outweigh the damage to the hosts, especially when nutrient supply allows for sufficient parasite photosynthesis. However, hemiparasites usually reduce the below-ground biomass of their hosts more strongly than the above-ground biomass (Matthies 1995), so that total biomass might not have been increased by the presence of the parasite.

Conclusions

The performance of the hemiparasite *R. alectorolophus* was reduced by inbreeding, especially when grown with good host species. Because growth with poor hosts is a stress

for the parasite, this finding contradicts the common assumption that ID is especially severe under stressful conditions. The phenotypic variation hypothesis could also not explain differences in ID among parasites grown with different hosts. We suggest that in plants, in which small differences in relative growth rates can lead to large size differences, higher resource levels increase ID if no specific stress responses are affected by inbreeding.

Inbreeding is expected to become more important for *Rhinanthus* spp., as many natural populations of these species have become small and fragmented and the area of potential habitat is reduced by the intensification of agriculture. The experiment using mixtures of hosts showed that the negative effects of inbreeding may be buffered by diverse host communities. When grown with four different hosts, inbred parasites were on average not smaller than offspring from open pollination, and ID decreased with the number of host functional groups. This supports the view that for hemiparasites like *R. alectorolophus* the diversity of its communities is of particular importance (Marvier and Smith 1997, Joshi et al. 2000).

Both parasite performance with individual host species and the damage to these host species differed between parasites from the two study populations. This indicates genetic variation in the adaptation to individual hosts and in host-specific virulence. However, inbreeding did not affect specific host-parasite interactions.

Chapter V

Synthesis

Synthesis

The aim of this thesis was to study the relationship between the magnitude of inbreeding depression (ID) and environmental stress. More specifically, I asked the following questions: (1) Does inbreeding depression (ID) differ among environmental conditions? (2) Does ID generally increase or decrease with the intensity of stress (sensitive selfed vs. capable crossed hypothesis)? (3) Does ID increase in environments which increase phenotypic variation? For this purpose I used two different study systems, the perennial herb *Silene vulgaris* grown under different types of abiotic stress in a greenhouse (Chapter II and III) and the annual hemiparasite *Rhinanthus alectorolophus* grown with hosts of different quality (Chapter IV).

Inbreeding depression and stress intensity

In all experiments, inbreeding had negative consequences for plant performance. Even in the annual *R. alectorolophus*, which is frequently self-pollinating, some fitness components were reduced after selfing. In addition, in all experiments the magnitude of ID differed among environments, which confirms the generality of environment-dependent inbreeding depression (Armbruster and Reed 2005, Cheptou and Donohue 2011). However, inbreeding depression did not increase with the stress intensity of an environment (measured as its negative effect on the performance of crossed plants), neither in *Silene vulgaris* grown under abiotic stress (Chapter II) nor in *Rhinanthus alectorolophus* grown with hosts or host mixtures of different quality for the parasite (Chapter IV). When the strength of ID was influenced by stress intensity, ID did usually decrease, not increase with stress intensity (Fig. 1). The same pattern was observed in the legume *Anthyllis vulneraria* grown under different types and intensities of abiotic stress, an experiment which resulted from my pilot studies (see Chapter I) and was carried out by Finn Rehling during his B.Sc. thesis to answer some of the questions which arose during my work with *Silene vulgaris* (Rehling 2014). ID increased with stress intensity only in the field vs. garden experiment with *S. vulgaris* (Chapter II). Taken together, these results refute the predominant assumption that ID generally increases with stress intensity (e.g. Frankham et al. 2010, Fox and Reed 2011, Reed et al. 2012). Instead, the effect of stress intensity on ID differs among stress types, and at least some types of stress reduce

ID relative to more benign conditions, which I call the capable crossed hypothesis (see Chapter I, Fig. 1).

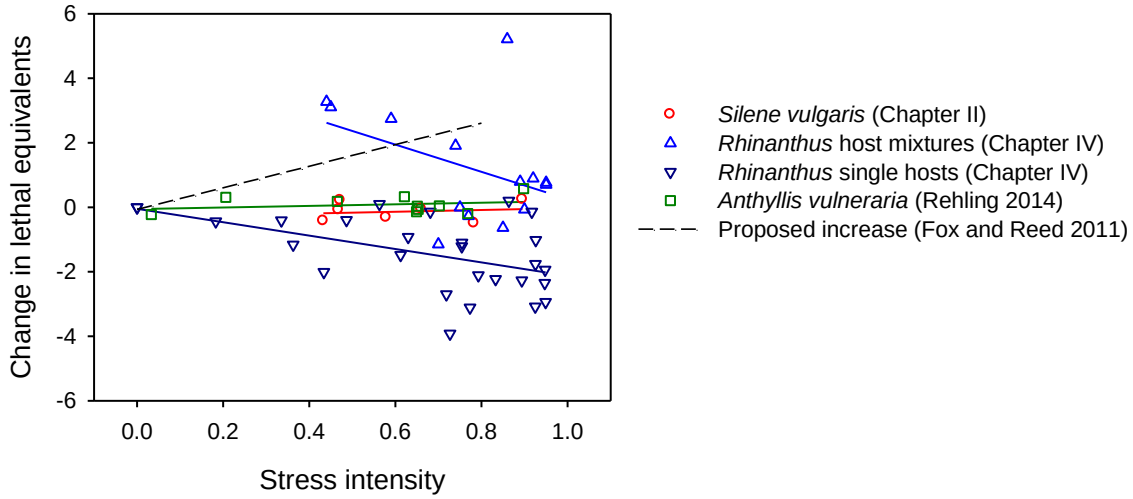

Figure 1: The effect of stress intensity (i.e. proportional reduction of the biomass of crossed plants per treatment, relative to the control) on the magnitude of inbreeding depression in four experiments; stress was applied as abiotic stress treatments in the greenhouse for *Silene vulgaris* (Chapter II) and *Anthyllis vulneraria* (Rehling 2014) and as stress by host species and species mixtures differing in host quality for *Rhinanthus alectorolophus* (Chapter IV). The dashed line shows the proposed increase of ID with stress intensity from the meta-analysis of Fox and Reed (2011).

In addition to the stress hypothesis, various other hypotheses and explanations have been put forward to explain differences in ID among environments (Box 1). These various hypotheses are not mutually exclusive, and some of them are even related. The two hypotheses predicting how ID changes with stress, i.e. whether it increases or decreases with stress (sensitive selfed vs. capable crossed, see Chapter I) are not included here, because they describe patterns and can be caused by several of the mechanisms presented in Box 1. For example, the sensitive selfed pattern can be found if ID generally increases under stress (H3), as well as when it increases only under certain types of stress (H4-7). Similarly, the capable crossed pattern is most likely to be caused by resource limitation (H6), but can also be explained by neutral hypotheses (H1-2).

The relevance for *Silene vulgaris* of the explanations presented in Box 1 has been discussed in Chapter II. The general relevance of the individual hypotheses should be tested in a meta-analysis of the existing studies, where possible. In addition, further experiments designed specifically to test some of these hypotheses could help to increase our understanding of the processes underlying environment-dependent ID.

Box 1: Overview of hypotheses on the potential effects of different environments on the magnitude of inbreeding depression.

Neutral hypotheses:

1. **Phenotypic variation hypothesis.** ID is selection against selfed offspring and thus increases in any environment that increases phenotypic variation, measured as the opportunity for selection (CV^2) independent of cross type.

2. **Size-dependent stress hypothesis.** Stress intensity or relative growth rates depend on plant size. ID thus increases in environments that increase size differences among plants independent of their inbreeding level.

Stress intensity hypothesis:

3. Inbred offspring is more sensitive to stress, or stress increases the expression of genetic load. ID thus increases linearly with the stress intensity of an environment, measured as the mean reduction of fitness in crossed offspring.

Stress concept related hypotheses:

4. **Physiological stress hypothesis**. Not the level of stress measured as the overall reduction in fitness, but the magnitude of physiological stress at the cellular level affects ID:
    a. Fluctuating stress increases ID more than constant stress.
    b. ID is increased under alternating stresses, which prevent acclimation.
    c. ID is increased under stresses causing mortality, not just size reduction.

Stress type related hypotheses:

5. **Environmental-complexity hypothesis.** ID is more likely to be affected by stresses which require more genes for a response.

6. ID is reduced under **resource limitation stress** (N, water, light), because all plants remain small, whereas under favourable conditions crossed plants are better able to exploit the high levels of resources (=> **capable crossed hypothesis**).

7. **Environment-dependent purging.** ID is stronger under novel stresses than under conditions the plants have already experienced, because alleles that cause ID under those conditions have already been purged.

Phenotypic variation hypothesis

In addition to reduced ID under some stresses, we found some support for the phenotypic variation hypothesis. Environments that increased phenotypic variation also increased ID in *Silene vulgaris* (Chapter II), but there was no correlation between ID and CV^2 in the two experiments with *Rhinanthus alectorolophus* on the effects of various single hosts and host mixtures (Chapter IV). Similarly, an analysis of the data of Rehling (2014) showed that ID was also not correlated with phenotypic variation in *Anthyllis vulneraria* under different stress treatments.

The phenotypic variation hypothesis was initially intended as a null hypothesis that should be tested prior to other, more complex hypotheses (Waller et al. 2008). The varying results of the studies thus suggest that the magnitude of ID in *R. alectorolophus* is not influenced by the effects of individual host species on phenotypic variation in parasite biomass, and the reduction of ID with stress thus supports the capable crossed hypothesis. In contrast, the pattern of ID in *S. vulgaris* grown under various abiotic stress treatments can partly be explained by the effects of the treatments on CV^2.

Alternatively, the differences between the studies may be due to the fact that in *S. vulgaris* CV^2 and ID were calculated within families, whereas in the experiments with *R. alectorolophus* and *A. vulneraria* seed families were pooled. Inbreeding is expected to reduce the variation within inbred lines, but to increase the variation among lines (Falconer 1981), and the phenotypic variation within families or within populations may therefore be related differently to ID. In addition, the CV^2 of a trait in an environment can have different causes which may influence ID in different ways. An environment may increase the variation among plants (i) by increasing random variation, which should not increase ID, because variation will not be larger among selfed plants. (ii) An environment may increase variation by affecting specific stress response mechanisms which have a genetic basis. This would lead to a correlation between ID and CV^2, but not exclude other, physiological explanations (e.g. H3-5 in Box 1). Finally, (iii) an environment may increase CV^2 by increasing size hierarchies among plants, which would cause an increase of ID with CV^2 independent of stress. This size-dependent stress hypothesis (H2 in Box 1) may thus be regarded as a specification of the phenotypic variation hypothesis.

The size-dependent stress hypothesis is based on the concept of dominance and suppression under conditions of intraspecific competition, which assumes that under

competition, initial size differences between selfed and crossed plants will increase with time, because the smaller (selfed) plants face stronger competition than the larger (crossed) plants (Weiner 1985, Schmitt and Erhardt 1990, Cheptou et al. 2001, Yun and Agrawal 2014). I suggest to extend the hypothesis to include stress types other than competition whose effects differ between small and large plants (Chapter II). For example, if a fixed amount of nutrients or water is applied to pots in a greenhouse, larger plants will receive proportionally less resources than smaller plants, and initial size differences (including ID) will be reduced. The effect of an environment on CV^2 and especially on size hierarchies can both increase or decrease size differences and thus magnify or level out effects of recessive deleterious alleles on plant performance.

Outlook – relevance of the different hypotheses

There is substantial evidence for an increase of inbreeding depression under stress (Armbruster and Reed 2005, Fox and Reed 2011, Reed et al. 2012), and some physiological mechanisms have been identified that make selfed offspring more sensitive to stress or increase the expression of genetic load under stressful conditions (Reed et al. 2012). However, the experiments presented in this thesis show that an increase of ID with stress is not a general phenomenon. These studies are the first that applied a large number of stress treatments to the same plant species and investigated their effects on ID. A study of *Drosophila melanogaster* under a large number of stress treatments also found no general effect of stress intensity (Yun and Agrawal 2014).

I suggest that many of the mechanisms presented in Box 1 may simultaneously shape ID. The assumption of an increase of ID under stress is based on a physiological stress concept (H4, H5), which may often differ from the evolutionary stress concept which measures stress intensity simply as a reduction of fitness. In addition, plants and animals may react differently to stress. Because plants are very plastic, increased nutrient or water supply may lead to large size differences under benign conditions (capable crossed hypothesis, H6 in Box 1), and other effects of an environment on CV^2 and size differences may further obscure a possible effect of stress on inbreeding depression. In future studies of environment-dependent inbreeding depression, the various hypotheses listed in Box 1 should be tested to understand the underlying mechanisms.

Implications for conservation biology

Inbreeding depression can contribute to the extinction of rare species in small and fragmented populations (Gilpin and Soulé 1986, Hedrick and Kalinowski 2000, Keller and Waller 2002, Frankham 2005). An increase of ID under more stressful conditions has been regarded as an additional threat to small populations if environmental conditions deteriorate. Furthermore, plants in ex-situ conservation programmes that are usually kept in small populations are prone to lose genetic diversity due to genetic drift (Schaal and Leverich 2004, Vitt and Havens 2004, Ensslin et al. 2011, Lauterbach et al. 2012). An important aim of the ex-situ cultivation of rare plants is the eventual creation of natural populations in the field. However, the success of the reintroduction of plants into the wild that have been cultivated ex-situ may be jeopardized if the plants face a sudden increase of inbreeding depression in the more stressful natural sites (Ralls et al. 1988, Crnokrak and Roff 1999, Havens et al. 2004, Frankham et al. 2010, Ensslin et al. 2011).

The effects of stress intensity on ID after ex-situ cultivation have rarely been tested. The monocarpic herb *Cynoglossum officinale* showed a decline of genetic diversity with the duration of cultivation in Botanic Gardens (Ensslin et al. 2011). However, the offspring of the plants did not show inbreeding depression after artificial crosses, even after the plants were substantially stressed by cutting off all leaves (Sandner 2009), which suggests that after purging during ex-situ conservation, ID will not necessarily increase under stressful conditions.

Based on the results of this thesis, I suggest that inbreeding depression will not generally increase and threaten rare species under more stressful conditions. Under constant conditions of nutrient deficiency of water shortage, ID may even be reduced. However, some conditions should be expected to potentially increase ID. These include field conditions which are more unpredictable and fluctuating than garden conditions (Chapter II, but see Angeloni et al. 2011) and novel conditions, which the plants have not experienced yet (H7 in Box 1, Chapter II, Cheptou and Donohue 2011, Yun and Agrawal 2014), although more studies are needed to test these hypotheses. Generally, negative effects of inbreeding on phenotypic plasticity in non-reproductive functional traits (Chapter III, Norman et al. 1995, Kéry et al. 2000, Fischer et al. 2005, Walisch et al. 2012, Campbell et al. 2014) may increase ID in any new environment, and reduce the ability of rare plants to cope with the effects of climate change (Nicotra et al. 2010) and to

establish after reintroduction in the wild, where conditions are different, but not necessarily more stressful than the ex-situ conditions. The threat of increased ID under changed conditions supports the recommendations for ex-situ collections (Havens et al. 2004, Ensslin et al. 2015) that inbreeding should be avoided by keeping populations large, and that conditions should be kept as close to the natural conditions as possible.

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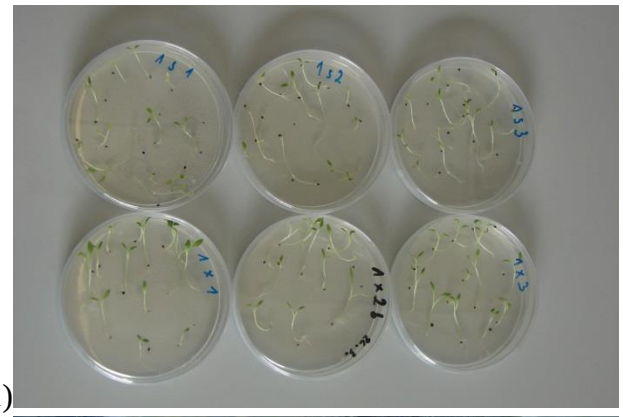
(1) 

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Plate 1: Clonal propagation of *Silene vulgaris*. (1) Sterile germination of seedlings; (2) North facing cultivation room with cultures; (3) Seedlings in shoot-inducing medium (BAP); (4) Seedlings with multiple shoots after shoot-induction; (5) After clonation – each glass contains shoot cuttings from one single seedling; (6) Clones after root induction in hormone-free medium; (7) Rooted clones for planting; (8) Growth under high humidity in the climate chamber for hardening.

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Plate 2: *Silene vulgaris* plants in the greenhouse after four weeks of stress. (1) Control; (2) Drought; (3) Simulated herbivory; (4) Heavy metal (+ copper); (5) Low nutrients; (6) Very low nutrients; (7) Light shade; (8) Strong shade; (9) Arrangement of stress treatments in the greenhouse.

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Plate 3: Experiment with *Silene vulgaris*. (1) Hermaphroditic flower on the 1st day, male; (2) Hermaphroditic flower on the 2nd day, female; (3) Malformed flowers of inbred genotype 5s1; (4) Red leaves under nutrient deficiency; **Field vs. garden experiment:** (5) Field site; (6) Common garden population; **Capsule herbivory by *Sideridis rivularis*:** (7) Caterpillar feeding on capsule; (8) Capsules destroyed by herbivory.

Plate 4: Experiments with *Rhinanthus alectorolophus*. (1) Inflorescence; (2) Bagged and open-pollinated plants in the field; (3) *Rhinanthus* seedling after planting in the center of four hosts (M5); (4) Single host experiment in the greenhouse; (5) Single host and mixture experiment in the common garden; **Growth of the parasite with:** (6) No host; (7) A poor host (*Anthyllis*); (8, 9) Good hosts (*Leucanthemum*, *Lolium*); (10, 11) A good mixture (M2); (12) A poor mixture (M15).

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Erklärung

Ich versichere, dass ich meine Dissertation

“ The effects of inbreeding and stress on plant performance”

selbständig und ohne unerlaubte Hilfe angefertigt habe und mich keiner als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe. Diese Dissertation wurde in der jetzigen oder einer ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

Marburg / Lahn, den 15.01.2015

Tobias Sandner