# Effects of environmental change on plant performance and plant-herbivore interactions

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THIS DISSERTATION IS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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December 2014

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Dissertation submitted for the degree of Doctor of Philosophy

# Abstract

Global environmental change fundamentally affects plants and their interactions with other species, and this has profound impacts on communities and ultimately ecosystems. In order to understand the mechanisms involved, we need to elaborate on the combined effects of different global change drivers on multiple levels of plant organization, including the biochemical level (production of defence compounds), the whole organism, the population, and the plant-herbivore interaction level. This thesis investigates (1) the combined effects of factors related to climate change and habitat fragmentation on *Brassica nigra* and (2) the effects of Zn soil pollution on the heavy metal hyperaccumulator *Noccaea caerulescens* at these different levels. Common garden and greenhouse experiments with *B. nigra* applied drought stress and elevated $CO_2$ to examine climate change impacts, while crossing treatments (inbreeding and between-population outbreeding) were used to investigate habitat fragmentation effects. Heterosis was lost under drought stress, and there were several interactive effects of the experimental treatments that varied within and among populations. In a greenhouse experiment with *N. caerulescens*, plants were grown on soil with different amounts of zinc. Plants had greater herbivore resistance when grown on Zn-amended soil, and invested more in herbivore tolerance when grown on soil without added Zn. In general, the results indicate that factors related to global environmental change have complex and interactive effects on different levels of plant organization. The findings are discussed in terms of their implications for ecology, evolution and conservation.

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# Chapter 1: Introduction

## 1.1 General introduction and scope of study

Human activities exert substantial impacts on the environment, exposing natural populations to extensive changes in their living conditions, including change in climate, soil, habitat size and species interactions (Millenium Ecosystem Assessment, 2005). The main drivers from anthropogenic global environmental change include climate change, habitat fragmentation, nutrient loading, invasive species and pollution (Millenium Ecosystem Assessment 2005; compare Fig. 1.1). These global change drivers are considered to be leading to the sixth mass extinction of species (Barnosky et al. 2011). Loss of biodiversity in turn negatively affects ecosystem functioning and ecosystem services (Hooper et al. 2012).

Although the negative impacts of these global change drivers have been well characterized, their combined effects remain unclear. There is increasing evidence that the interactive effects of different global change drivers on biodiversity and species interactions cannot be predicted based on the effects of the individual components (Brook et al. 2008; Tylianakis et al. 2008). Surprising antagonistic or synergistic outcomes of interactions among multiple anthropogenic stress factors seem to be more common than additive effects, both in animals (Darling & Côté 2008) and plants (Reich et al. 2006). Conservation management focusing on only one global change driver therefore omits to capture the potentially synergistic negative consequences of anthropogenic environmental change (Brook et al. 2008). This thesis aims to investigate (1) the combined effects of global change drivers associated with climate change and habitat fragmentation on plants and plant-herbivore

interactions and (2) the effects of plant adaptation to heavy metal pollution on plant-herbviore interactions.

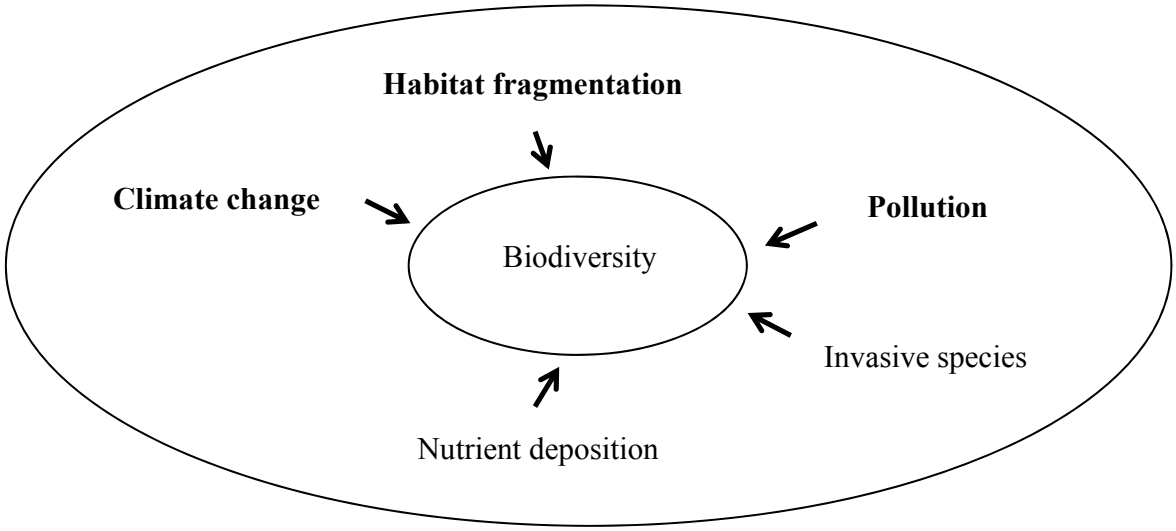


**Fig. 1.1.** The major global change drivers threatening biodiversity according to the Millenium Ecosystem Assessment (2005). **In bold:** Heavy metal pollution and the combined effects of climate change and habitat fragmentation are in the focus of this thesis.

Recently, a number of leading ecologists identified the 100 most important ecological questions for the upcoming decades (Sutherland et al. 2013). A number of these questions were concerned with the effects of global environmental change on biological systems, and 25 of them contained the terms “interaction”, or “community” (Sutherland et al. 2013). This is because the effects of global change drivers on single species can proliferate through ecosystems via species interactions and even have cascading effects on communities (Gilman et al. 2010). In order to understand how global environmental change affects communities and ultimately ecosystems, we need to examine thoroughly how it affects all levels of biological organization, including plant chemistry, growth and fitness, population, and plant-herbivore interaction levels (Peñuelas et al. 2013). Plants and insects comprise nearly half of the identified species on earth (Schoonhoven et al. 2005). The combined effects of global change

drivers on biotic interactions in general and plant-herbivore interactions in particular are being progressively more studied (Tylianakis et al. 2008), but studies investigating the interactive effects of habitat fragmentation and climate change on plant-herbivore interactions remain scarce. Global environmental changes associated with climate change, such as elevated global temperature and $CO_2$ levels as well as increased variability of precipitation, pose a major threat to biodiversity and ecosystem functioning (IPCC, 2014). Habitat fragmentation reduces population sizes and increases population isolation, which results in the loss of genetic diversity and augmented levels of inbreeding (Young et al. 1996). This compromises the ability of organisms to respond to global environmental change (Lande 1995; Frankham 2005). Heavy metal hyperaccumulating plants are able to grow on metal-contaminated soils, for example areas around mines. This adaptation to environmental change strongly affects their interactions with herbivores.

The outcomes and underlying mechanisms of plant-herbivore interactions under increasing anthropogenic pressures may not be detected in studies focusing solely on cultivated plants. Research on tritrophic interactions between plants, herbivores and their natural enemies has demonstrated that cultivated plants significantly differ from wild plants in herbivore defence mechanisms and attraction of natural enemies (Benrey et al. 1998; Chen & Welter 2003), including studies on *Brassica oleracea* (Gols et al. 2008a). Plant breeding programmes that target the decrease in (e.g. bitter-tasting) plant secondary compounds and the increase in primary metabolites, such as proteins and sugars, inevitably lead to better herbivore performance (Schoonhoven et al. 2005). Also in terms of evolutionary considerations, it is more suitable to study plant-herbivore interactions in plants that have evolved under natural selection rather than artificial selection (Gols et al. 2008a). Studying

the response of several populations of both *N. caerulescens* and *B. nigra* to global change drivers allows evaluation of the role of genetic diversity (within- and among-population differences) to buffer the negative effects of anthropogenic environmental change.

*Scope of study*

This thesis aims to investigate:

1) The combined effects of habitat fragmentation (inbreeding and between-population outbreeding) and climate change (drought and elevated $CO_2$) on different populations of *Brassica nigra*. Plant growth and fitness, the production of secondary metabolites and interactions with specialist and generalist herbivores are assessed. *Brassica nigra* is used as a model species for plants in the temperate zone.
2) The effects of Zn soil pollution on two populations of the heavy-metal hyperaccumulator *Noccaea caerulescens.* Plant growth, the production of secondary metabolites and the interactions with a generalist and specialist herbivore are studied.

# 1.2 Habitat fragmentation

## Definition and historical background

Since humans have changed from a nomadic lifestyle based on hunting to a settled lifestyle based on agriculture, they have massively altered natural landscapes. One of the most famous examples of past human transformation of the landscape and the concurrent overexploitation of the land is the example of Easter Island, which led to the collapse of the ecosystem and

human basis of existence (Diamond 2007). Extensive anthropogenic land use change was also described in ancient Greece; Plato's Critias bewails that overexploitation has left "the mere skeleton of the land" (Plato 1977). At present, natural populations are embedded in an anthropogenic matrix (McGarigal & Cushman 2002). This matrix contains not only roads and settlements, but also invasive species fragmenting native vegetation (Ghazoul 2005). Thus, habitat fragmentation is a very common phenomenon on a global scale. Habitat fragmentation is often associated with logging in tropical rainforests, but in the temperate zone, native grasslands and heathlands have been subjected to heavy fragmentation as well (Ghazoul 2005). Habitat fragmentation is defined as a process where "a large expanse of habitat is transformed into a number of smaller patches of smaller total area, isolated from each other by a matrix of habitats unlike the original" (Wilcove et al. 1986). A number of characteristics of habitat fragments therefore resemble those of oceanic islands (Fig. 1.2).

**Fig. 1.2.** Habitat fragments share some characteristics of oceanic islands. Left: Oceanic Islands: Whitsunday Islands, Queensland, Australia. Right: Fragmented Landscape: Thetford Forest, UK. Both photos are courtesy of NASA.

The four major consequences of habitat fragmentation are: a) a decrease in total habitat area, b) a decline in the sizes of the habitat remnants, c) an increase in the number of habitat remnants, d) a greater degree of isolation among the habitat remnants (Fahrig 2003). Small

habitat patches cannot maintain as many species as large habitat patches. These species-area relationships have been part of ecological theory for nearly a century (Arrhenius 1921), but it was only after McArthur and Wilson published their island biogeography theory (IBT) in 1967 that habitat fragmentation was put high on the research agenda. IBT studies species-area relationships from the point of view of isolated natural communities, which can also be applied to habitat fragments. The main question that is examined with the help of mathematical models is to explain species richness in terms of the balance between colonization and extinction, which depends on the island area and degree of isolation (MacArthur & Wilson 1967). The idea that habitat fragmentation affects species in the same way as insularity has deeply influenced the ecological literature and stimulated conservation research and management (Haila 2002). One of the most famous debates in the scientific community arising from IBT pivoted on whether single large or several small reserves ('SLOSS') were more valuable for conservation. The main conclusion was that the SLOSS solution depends on whether the reserves in question can represent a subset of the ecosystem that should be protected (Laurance 2008). In some cases, many small reserves may contain a greater variety of communities that are characteristic for an ecosystem than one single large reserve (Laurance 2008). In addition, endemic species should be maintained in small reserves spread across a large region to protect as many endemic species as possible (Dapporto & Dennis 2008).

Yet, the analogy between oceanic islands and terrestrial habitat remnants reaches its limits once we look beyond species/area relationships. Islands ("oceanic archipelagos") have specific evolutionary histories, whereas habitat remnants formed by humans have no evolutionary history (Haila 2002). Matrix effects (interactions with the surrounding

environment) and habitat connectivity are not essential on islands, but they play a major role in habitat remnants, because habitat connectivity is essential for migration between habitats and therefore deeply influences population genetics and survival (Cook et al. 2002; Laurance 2008). IBT does not consider species interactions, community composition and ecosystem processes that can be strongly affected by habitat fragmentation (Laurance 2008). However, IBT paved the way for studies on habitat fragmentation, since it alerted to the increased extinction risk due to small habitat size and habitat isolation (Laurance 2008).

# Effects of habitat fragmentation

## Ecological effects

*Effects on biodiversity and ecosystem function*

Habitat fragmentation impairs plant and animal fitness (Higgins & Lynch 2001; Theodorou & Couvet 2006) as well as species richness (Grashof-Bokdam 1997; Bruun 2000; Reed 2004). However, the negative effects of habitat fragmentation on biodiversity may be time-delayed and therefore underestimated (Krauss et al. 2010). Matrix effects, or the ability of species to live not only in habitat patches, but also in the landscape surrounding them, are likely to buffer the negative effects of fragmentation on species richness (Cook et al. 2002; Krauss et al. 2004). Six characteristics have been identified that may predict the sensitivity of a species to habitat fragmentation: population size, population variability, competitive ability and sensitivity to disturbance, habitat specialisation, abundance and biogeography (Henle et al. 2004). Correlations and interactions between these traits complicate predictions (Henle et al. 2004). As an example, competition is usually intensified in habitat remnants due to the

scarcity of resources (Laurance 2008), but competition for light and nutrients is reduced along habitat edges (Zschokke et al. 2000).

Species extinction due to habitat fragmentation is especially severe and can have cascading effects if keystone species that have major influence on ecosystem structure and function are affected (Jones et al. 1994; Soulé et al. 2005). Species interactions frequently involve processes that are essential for preserving biodiversity and ecosystem functioning, for example pollination, seed dispersal and nutrient cycling (Bond 1994; Didham et al. 1996). The effects of habitat fragmentation on species interactions shall therefore be explored in more detail below.

*Mutualistic plant-animal interactions*

Habitat fragmentation disrupts mutualistic plant-animal interactions, including pollination and seed dispersal. Examples of studies investigating the effects of habitat fragmentation on mutualistic plant-animal interactions are outlined in Table 1.1.

**Table 1.1** Case studies on the effects of habitat fragmentation on mutualistic plant-insect interactions.

| Interaction | Species groups | Habitat type | Study category | Effect | Reference |
|---|---|---|---|---|---|
| Plant-pollinator | Bumblebees, *Lythrum salicaria* | Archipelago | Experimental | Lower seed production due to fewer pollinator visits | (Ågren 1996) |
| Plant-pollinator | Butterflies | Calcareous grassland | Experimental | Lower abundance and species richness of butterflies | (Zschokke et al. 2000) |
| Plant-pollinator | Syrphid flies and *Arnica montana* | Heathland | Observational | Reduced number of flowering stems and seed production | (Luijten et al. 2000) |
| Plant-seed disperser | Ants and *Helleborus foetidus* | Scrubland, meadows | Experimental | Reduced seed dispersal | (Magrach et al. 2011) |
| Plant-seed disperser | Birds and *Juniperus thurifer* | Forest | Observational | Loss of main seed disperser reduces seed recruitment | (Santos et al. 1999) |

The impairment of plant-pollinator interactions in fragmented habitats is well-documented in the literature (reviewed in Aguilar et al. 2006). Plants occupying fragmented habitats suffer from a reduction in both the quantity and quality of pollination services. Pollinators prefer large, continuous habitats and isolated habitat patches may lie out of their foraging range (Rathcke & Jules 1993; Sih & Baltus 1987; Kearns et al. 1998; Goverde et al. 2002). Reduction of plant density in fragmented habitats and the consequent failure to attract pollinators, resulting in lower fitness and ultimately extinction is a form of **Allee effect** (Allee et al. 1949; Courchamp et al. 1999). Lower species richness and abundance of pollen vectors have been reported in fragmented habitat patches (Steffan-Dewenter & Tscharntke 1999; Zschokke et al. 2000). Several studies have established a causal link between pollinator limitation in fragmented plant populations and increased rates of self-pollination, decreased seed set, higher extinction risk (Jennersten 1988; Lamont et al. 1993; Lennartsson 2002) and reduced ability to respond to environmental change (Kery et al. 2000). Generalist pollinators frequently take over pollination services from specialist pollinators in small habitat remnants (Groom 2001), thereby mitigating the negative effects of habitat fragmentation on pollination (Bascompte et al. 2003; Ghazoul 2005). However, since generalist pollinators visit a number of different plant species, pollination with heterospecific pollen is unavoidable, which can clog stigmas and therefore negatively affects fecundity (Petanidou et al. 1998; Groom 2001). This is the reason why pollen quality is typically lower in fragmented habitats, causing even more dramatic reductions in seed-set (Kunin 1993).

Habitat fragmentation also disrupts seed dispersal (Herrera & García 2010; Wright & Duber 2001). Edge effects seem to have stronger negative effects on the abundance of seed dispersers than patch size and degree of isolation (Magrach et al. 2011). The extent to which

the disruption of plant-pollinator interactions and plant-seed disperser interactions affects plant populations and their probability of extinction depends on the specificity of the interaction, the degree of their dependence on pollination services and the importance of seeds for population maintenance (Bond 1994). We should therefore be careful when trying to draw general conclusions about the effects of fragmentation on mutualistic plant-animal interactions. For example, self-incompatible plants are more likely to suffer from pollinator limitation than self-compatible plants (Byers 1995), and plant species with the ability to survive via vegetative propagation might be able to compensate the lack of pollinators and seed dispersers (Bond 1994).

*Antagonistic plant-animal interactions*

Habitat fragmentation reduces the trophic chain length (Komonen et al. 2000) and disrupts complex food webs (Valladares et al. 2006). Species richness and the abundance of antagonistic plant interaction partners may also be lower in fragmented habitats (Steffan-Dewenter & Tscharntke 2002; Reed 2004; but see Tscharntke et al. 2002), allowing isolated plant populations to escape parasites, pathogens (Ericson et al. 1999; Colling & Matthies 2004) as well as herbivores (Zabel & Tscharntke 1998; Groom 2001) and seed predators (Johannesen & Loeschke 1996; Farwig et al. 2009). On the contrary, isolation can also lead to greater herbivore abundance, e.g. due to changes in oviposition rates (Elzinga et al. 2005) or longer time spent foraging in an isolated habitat patch (Cantrell & Cosner 1999).

Theoretical and empirical work suggest that higher trophic levels are more susceptible to habitat fragmentation (Kareiva 1987; Kruess & Tscharntke 1994; Bascompte & Solé 1998; Zabel & Tscharntke 1998). Animal species richness and abundance are lower in fragmented habitats (Steffan-Dewenter & Tscharntke 2002), but some animals use isolated habitat patches

as refuges to escape from predators (Terborgh et al. 2001) or as retreats between forays (Zschokke et al. 2000) and are accordingly more abundant in these habitat patches. The absence of large predators from habitat fragments can lead to greatly increased abundances of generalist omnivores and seed predators (Terborgh et al. 2001), which reduces seedling recruitment (Tellería et al. 1991; Díaz et al. 1999; Jules & Rathcke 1999; Donoso et al. 2003). Similarly, the lower abundance of the natural enemies of herbivores weakens the top-down control of herbivores in fragmented habitats, so that herbivore pressure on plants increases (McLaren & Peterson 1994; Kruess & Tscharntke 1994; Colling & Matthies 2004).

Few studies have assessed the effects of fragmentation on both mutualistic and antagonistic plant-animal interactions simultaneously (Groom 2001; Steffan-Dewenter et al. 2001; García & Chacoff 2007; Kolb 2008; Magrach et al. 2011). Greater pollinator visitation rates and seed dispersal were concurrent with increased seed predation in larger habitats compared to smaller habitats (Steffan-Dewenter et al. 2001; Farwig et al. 2009; Magrach et al. 2011). Increased herbivore damage can lower the attractiveness of plants to pollinators (Lehtilä & Strauss 1997). Herbivory may also reduce the number of flowering individuals, which could lower the number of pollinator visitors (Kolb 2008).

## Genetic effects

Genetic and ecological effects of habitat fragmentation cannot be completely disentangled; for example, impoverishment of genetic diversity alters species interactions and communities (Ouborg et al. 2006), and matrix effects influence pollinator behaviour and therefore gene flow and genetic diversity (Ezard & Travis 2006; Kunin 1997). Reduction of pollination services due to habitat isolation and reduction in habitat size exacerbates the genetic consequences of small population size through increased rates of inbreeding and less

gene flow between populations (e.g. Ågren 1996). An overview of the interactions between both the genetic and ecological consequences of habitat fragmentation is outlined in Fig. 1.3.

*Genetic drift and genetic variation*

There are three main potential consequences of habitat fragmentation on the structure of populations and metapopulations that elicit the genetic effects outlined below: I) reduction in the number of populations, II) reduction in population size and III) increase in between-population distance, that is increased isolation (Ouborg et al. 2006). These parameters determine how genetic drift, selection, and migration influence allele frequency in fragmented populations differently compared to a large continuous population (Theodorou & Couvet 2006). Genetic drift is the change in allele frequency due to random sampling (Wright 1931), whereas selection favours alleles that generate a fitness advantage (e.g. Lande 1995). I) A lower number of populations will reduce genetic variation and provide fewer opportunities to increase fitness via migration between populations; II) the relative strength and influence of genetic drift compared to selection on allele frequencies increases in a small population; III) a lack of connections between populations prevents gene flow via migration or dispersal, further decreasing the power of selection (Theodorou & Couvet 2006).

The immediate consequence of habitat fragmentation is a reduction in population size, which produces genetic bottlenecks, as not all the alleles from the original population will be maintained (Young et al. 1996). On the one hand, this leads to the loss of alleles, including alleles with potentially adaptive value (Lande 1995). Two meta-analyses on the effects of population size on genetic variation have demonstrated that the predicted positive correlation between population size and level of genetic diversity is common in plants (Leimu et al. 2006; Aguilar et al. 2008). The reduction of genetic diversity diminishes the efficiency of selection

and impairs the evolutionary potential of small populations (Frankham 2005). On the other hand, genetic drift leads to the random fixation of alleles, including fixation of deleterious mutations (Lande 1995), which reduces fitness and elevates extinction risk (Lynch et al. 1995; Keller & Waller 2002). In populations of small effective size, the fixation of deleterious alleles is more likely than the fixation of beneficial alleles (Whitlock 2003). This can cause a mutational meltdown, that is the accumulation of deleterious mutations and the concurrent decline in population size, ultimately resulting in extinction (Lynch & Gabriel 1990; Lynch et al. 1995).

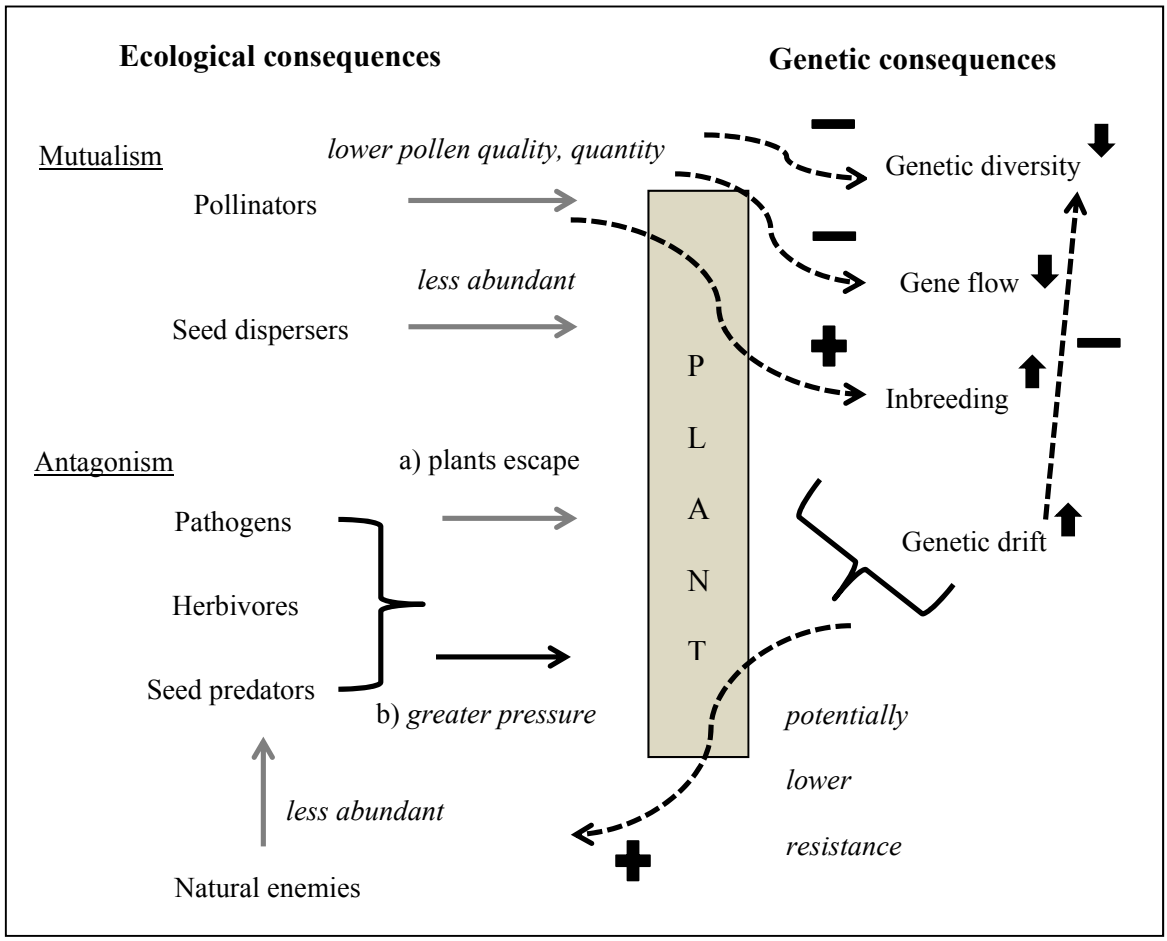


**Fig. 1.3.** Genetic and ecological consequences of habitat fragmentation. Arrows in grey denote that the interaction is weakened due to habitat fragmentation, arrows in black denote that the interaction is intensified under habitat fragmentation. Dashed lines denote interactions among the ecological and genetic consequences of habitat fragmentation. + refers to a positive effect, - refers to a negative effect.

*Inbreeding depression, outbreeding depression and heterosis*

The reduction of population size and connections among populations outlined above not only affect genetic variation and genetic drift, but also the occurrence of inbreeding depression, outbreeding depression, and heterosis. Smaller populations incur higher rates of inbreeding, that is mating between related individuals (Frankham 2005). In plants, inbreeding can occur via cross-pollination with relatives (biparental inbreeding) and via self-fertilization. Inbreeding depression is the reduction of trait values in inbred offspring compared to outbred offspring (e.g. Cheptou & Donohue 2011). Charles Darwin was the first scientist to describe inbreeding depression. When he was studying the fertilization of orchids via insects (Darwin 1862), he deliberated as to why there were manifold provisions to ensure cross-fertilization. Accordingly, Darwin conducted crossing experiments with 57 plant species, involving self-pollination, within-stock outcrossing and between-stock outcrossing (Darwin 1876). Self-fertilized plants performed worse than intercrossed plants in terms of growth and fitness. Darwin deduced that cross-fertilization is taking place to avoid inbreeding depression (Darwin 1876). In the last few decades, inbreeding depression has been demonstrated for a variety of plant traits, including germination, biomass production, survival, reproduction and herbivore resistance (reviewed in Angeloni et al. 2011). The effects of inbreeding on the production of secondary metabolites involved in herbivore defence have only been explored recently. Campbell et al. (2013) demonstrated a reduction in the concentration and variety of phenolic compounds in inbred plants. Other studies have found a reduction in volatile emissions and changed constitutive and induced volatile emissions in inbred plants, resulting in lower herbivore resistance (Delphia et al. 2009a; Kariyat et al. 2012).

**Table 1.2.** Examples for studies on the effects of between-population outcrossing.

| Species | Experimental Design | Findings | References |
|---|---|---|---|
| *Delphinium nelsonii* | 1 population, different distances: 1m, 3m, 10m, 30m | OD in fitness traits in greater crossing distance, heterosis in growth and lifespan in intermediate crossing distances | (Waser & Price 1994) |
| *Chamaecrista fasciculata* | 5 pop., each in 3 regions, distances: 100m, 1km, 100km, 2000km | Heterosis in growth- and fitness-related traits in F1 generation, F3 generation equal fitness as parents | (Fenster & Galloway 2000) |
| *Lotus scoparius* | 6 pop., between tens to hundreds of kilometers apart | OD; overall fitness of between-population crosses 40-50% of within-population crosses | (Montalvo & Ellstrand 2001) |

Habitat fragmentation also increases between-population distance and therefore isolation. The isolation of fragmented populations reduces gene flow amongst them, leading to increasing genetic differentiation between populations over time (Wright 1969; Young et al. 1996). This process may be accelerated in a heterogeneous environment, where selection may bring about local adaptation (Hufford & Mazer 2003). If gene flow occurs between genetically differentiated populations, this can lead to outbreeding depression or heterosis. Outbreeding depression (OD) is the decrease in character means in the F1 or subsequent generations following crosses between genetically distinct individuals, i.e. intraspecific hybridization (Waser & Price 1994; Hufford & Mazer 2003).

Heterosis, on the other hand, refers to an increase in character means in the F1 or subsequent generations following crosses between genetically distinct individuals (Hufford & Mazer 2003). Charles Darwin observed this phenomenon in his crossing experiments as described above, when he found that F1 plants derived from crosses between a “fresh stock” and the original stock had greater values in growth- and fitness-related traits than intercrossed

plants derived from the same stock. Additional examples for heterosis and OD are outlined in Table 1.2.

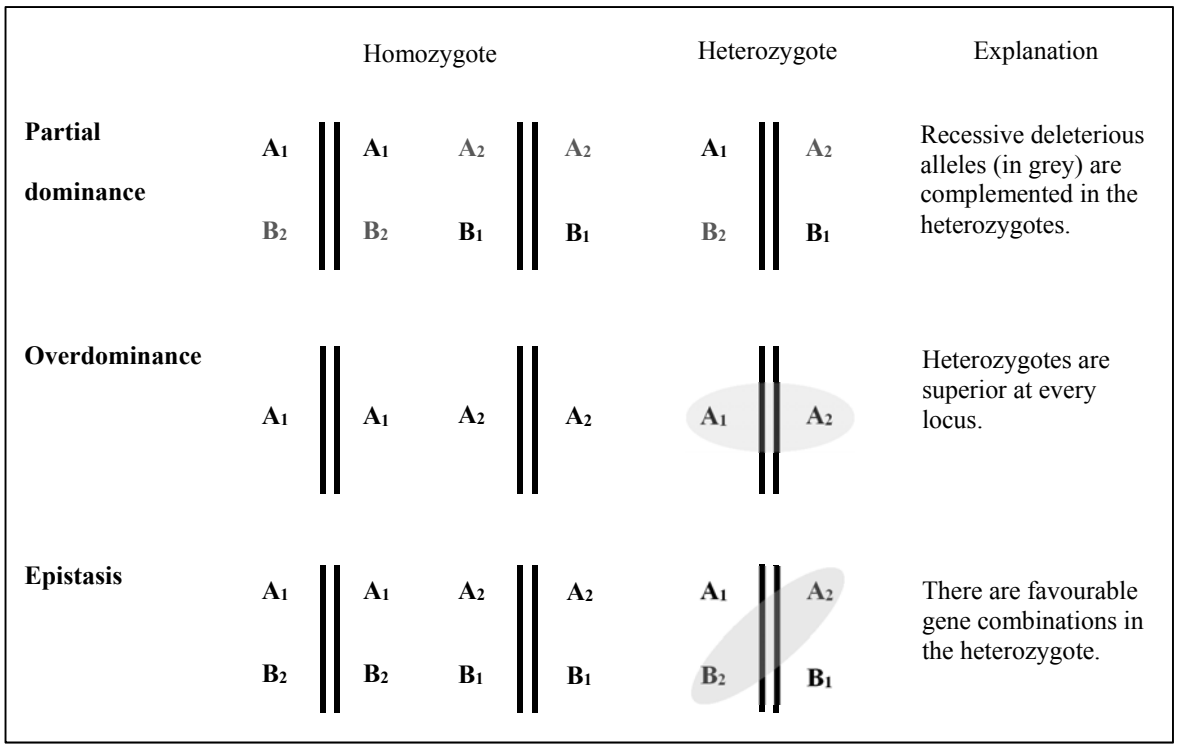


**Fig. 1.4.** Schematic drawing explaining the genetic basis of inbreeding depression (after Kristensen et al. 2010).

*The genetic basis of inbreeding depression, outbreeding depression and heterosis*

Three main mechanisms explain the genetic basis of inbreeding depression: partial dominance, overdominance and epistatic effects (see Fig. 1.4). The partial dominance hypothesis postulates that the increased expression of recessive deleterious alleles in inbred individuals renders a fitness disadvantage (Jones 1917), while the overdominance hypothesis states that heterozygotes have higher fitness than homozygotes at every locus, causing lower fitness overall in inbred individuals (East 1936). Epistatic interactions involve the fixation of unfavourable gene combinations due to inbreeding (Yu et al. 1997). Recently, an additional

mechanism for inbreeding depression has been suggested: increased DNA methylation in inbred individuals (Vergeer et al. 2012).

Observations at the phenotypic and genetic level suggested that partial dominance is the most important mechanism for inbreeding depression. If overdominance is the cause of inbreeding depression, it cannot be removed from the population without lowering the mean population fitness. If deleterious recessive alleles cause inbreeding depression, they can be removed from the population by selection (Charlesworth & Charlesworth 1999). This process is termed purging and it has been frequently demonstrated in a variety of taxa, including plants (reviewed by Charlesworth & Charlesworth (1999). In addition, it has been shown in *Drosophila melanogaster* that the majority of the observed inbreeding depression can be attributed to mutations, which again demonstrates that partial dominance should be mainly responsible for inbreeding depression (reviewed by Charlesworth & Charlesworth 1999).

The genetic basis of inbreeding depression partly explains the strong variation in inbreeding depression among families (Agren & Schemske 1993; Norman et al. 1995; Koelewijn 1998; Picó et al. 2004) and among populations (Ouborg et al. 2000; Carr & Eubanks 2002; Leimu & Fischer 2010; Bello-Bedoy & Núñez-Farfán 2011). Since individual families and populations have different deleterious recessive alleles at different loci coding for different traits, it is to be expected that they vary in their susceptibility towards inbreeding. Which and how many deleterious recessive alleles individuals carry depends on the inbreeding histories both of individual families and populations (Lande & Schemske 1985; Charlesworth & Charlesworth 1987), random genetic variation (Schultz & Willis 1995), and differences in environmental conditions among populations (Leimu et al. 2008).

Heterosis has been mainly attributed to the avoidance of inbreeding depression. Again, allele frequencies vary among populations due to genetic drift and the random occurrence of mutations (Whitlock et al. 2000). Consequently, the parent generation from different populations should have different recessive deleterious alleles, which are masked in the F1, so that they have greater fitness (Whitlock et al. 2000). Heterosis can also arise because of overdominance and epistatic effects (Edmands 2007).

Finally, outbreeding depression can be ascribed to two main mechanisms: I) the disruption of local adaptation and II) the loss of co-adapted gene complexes (Templeton et al. 1986; Fenster & Galloway 2000). Mechanism I) occurs when two populations have locally adapted loci, which are diluted following hybridization (Hufford & Mazer 2003). In that case, OD would occur in the F1 hybrids due to heterozygosity at the locally adapted loci (Hufford & Mazer 2003). This can be attributed to either additive or nonadditive gene action and involves genotype × environment interactions (Waser & Price 1994). Mechanism II) occurs when epistatic interactions between gene loci are disrupted due to breeding of individuals with different combinations of co-adapted gene complexes (Templeton et al. 1986; Fenster & Galloway 2000).

## 1.3 Climate change - Effects on plant performance and plant-herbivore interactions

Climate is the key factor that has led to the formation of the major biomes of the World (Hansen et al. 2001), and climate change is one of the most pressing threats to the stability of natural ecosystems and food security (Fuhrer 2006). Among the major factors associated with climate change are elevated $CO_2$ levels, changes in precipitation patterns including drought, and increased temperature (IPCC, 2013). Extreme climatic events and increased climate variability have increased in the last decades, with critical effects on plants and ecosystems (IPCC, 2014). During the summer of 2003, Europe suffered from an extreme heat wave, with precipitation lowered up to 300mm and temperatures up to 6°C above average (Tubiello et al. 2007). A combination of different factors associated with climate change has substantially increased the frequency and intensity of insect outbreaks (Fuhrer 2003; Grimm et al. 2013). The effects of climate change on plant performance and plant-herbivore interactions vary, but some general patterns emerge that are outlined below.

### Drought stress

Drought stress puts a strain on aboveground biomass production, since it decreases stomatal conductance and relative water content and therefore impedes carbon assimilation via photosynthesis (Chaves 2002; Lawlor & Cornic 2002). Drought stress also hampers nutrient uptake (Hsiao 1973; McDonald & Davies 1996) and reproduction (Conner & Zangori 1998). At the community level, drought stress can be regarded as a disturbance event that leads to local extinction or reduced competitive ability of local plant species, facilitating the establishment of invasive species (Buckland et al. 2001). Plants have developed a number of

strategies to cope with drought stress, for example earlier flowering for drought avoidance or the increased allocation of biomass to the roots for drought tolerance (reviewed in Chaves 2002).

Increased climatic variability is predicted to lead to an increased frequency and intensity of insect herbivore outbreaks due to disruption of the interactions between herbivores and their natural enemies under a more variable climate (Stireman et al. 2005; Raffa et al. 2008). Drought stress has varying influences on different herbivore feeding guilds (Hull-Sanders & Eubanks 2005; Stiling & Cornelissen 2007). For example, phloem-feeders benefit from intermediate levels of drought stress on account of the elevated concentration of primary metabolites in the phloem, whereas the effects of drought stress on leaf-chewers depend on the strength of the imposed drought stress, the chemical nature of the defence compound and the degree of feeding specialisation (Stiling & Cornelissen 2007). The growth-differentiation balance hypothesis (Herms & Mattson 1992) predicts an increase in secondary metabolite concentration under moderate water stress and a decrease under high water stress. In a study on *Brassica oleracea*, decreased levels of glucosinolates were measured under drought stress (Khan et al. 2010).

## Elevated $CO_2$

Most modern plant genera evolved during the late Oligocene and Miocene, which was characterized by a rapid decrease of atmospheric $CO_2$ concentrations to below 500ppm (Pearson & Palmer 2000; Pagani et al. 2005; Körner 2006). $CO_2$ levels before the industrial revolution were about 280ppm (Petit et al. 1999). They have risen to about 390ppm today (IPCC, 2013), and they are expected to double by 2100 (IPCC, 2013).

Elevated $CO_2$ ($eCO_2$) stimulates photosynthesis, saturation is reached at 1000ppm (Körner 2006). Several key processes in plants are substantially more efficient under $eCO_2$: water use, light use, and nutrient use (Drake et al. 1997). Reviews summarizing results from studies on plants grown under natural conditions and $eCO_2$ report a major increase in carbon assimilation, a reduction in stomatal conductance, increases in plant height and aboveground biomass, and increased yield (Ainsworth & Long 2005). Plants allocate the additional carbon to carbohydrates, such as starch and sugars (Obrist et al. 2001) for storage in their roots and rhizomes (Niklaus & Körner 2004), and to secondary carbon-based metabolites, such as phenolics (Bezemer & Jones 1998). At the community level, increased species evenness under $eCO_2$ has been reported in calcareous grassland (Niklaus & Körner 2004). However, $eCO_2$ seems to slow down succession in grasslands, which might facilitate invasion of alien species in some ecosystems (Dukes & Mooney 1999).

A recent meta-analysis indicated that herbivory increases under $eCO_2$ (Massad & Dyer 2010). However, the effects of $eCO_2$ vary among feeding guilds and levels of specialization. For example, phloem-feeders seem to be positively affected and leaf-miners and leaf-chewers seem to be negatively affected in their performance (Bezemer & Jones 1998; Stiling et al. 2003), and generalists tend to profit more from $eCO_2$ than specialists (Stiling & Cornelissen 2007; Massad & Dyer 2010). The differences between specialists vs generalists in their performance under $eCO_2$ have been largely attributed to the effects of $eCO_2$ on secondary metabolites. Quantitative defences (i.e. carbon-based defences) are increased under $eCO_2$ and specialists, although they are adapted to qualitative compounds, are not as well-adapted to quantitative defences as generalists (Massad & Dyer 2010). In addition, qualitative, nitrogen-based defence compounds might be diluted under $eCO_2$, which would again benefit generalist

herbivores (Massad & Dyer 2010). The carbon-nutrient balance hypothesis (CNBH; (Bryant et al. 1993) states that increases in carbon or nutrient supplies beyond requirements for growth can be used for increased production of carbon- or nitrogen-based defences. There is some evidence for CNBH, but only in studies testing carbon-based defence compounds (Lindroth et al. 1993; Ballhorn et al. 2011). It was demonstrated that nitrogen-based defence compounds decreased under $eCO_2$, whereas carbon-based defence compounds increased (Ballhorn et al. 2011). There are conflicting results for the effects of $eCO_2$ on the concentration of nitrogen-based defence compounds (e.g. Reddy et al. 2004; Klaiber et al. 2013), and the effects of $eCO_2$ on nitrogen -based secondary metabolites might therefore be species specific (Karowe et al. 1997).

The higher C:N ratio in plants under $eCO_2$ reduces the plant nutritional value, leading to compensatory feeding, especially by leaf-chewers (e.g. meta-analyses by Bezemer & Jones 1998; Massad & Dyer 2010). Compensatory feeding causes longer larval development times, which increases the time of exposure to natural enemies and results in higher attack rates and increased mortality due to higher parasitism rates (Stiling et al. 1999; Stiling et al. 2003; Vuorinen et al. 2004).

## Warming

Increased temperature brings about an extended growing season in the temperate zone (Menzel & Fabian 1998; Bloor et al. 2010) and greater productivity (Grimm et al. 2013). However, the positive effects of elevated temperature on plant performance are partly offset by the increased evapotranspiration and therefore lower soil moisture and nutrient uptake (Rustad et al. 2001). Nutrients also leach from the soil due to winter warming and the resulting reduction of snow cover (Grimm et al. 2013). Warming has substantial effects on

species composition; some species profit from elevated temperatures, while others lose out (Valpine & Harte 2001; Zavaleta et al. 2003).

Higher temperatures strongly affect phenology. This can result in mismatches between the phenologies of plants and insects, for example the mismatch between the hatching of the winter moth larvae and the budburst of Sitka spruce (Dewar & Watt 1992), with negative effects on herbivores. Positive effects of warming on insect phenology include the earlier spring activity of insects (Tubiello et al. 2007), increased numbers of generations (Porter et al. 1991), and increased survival rates in the winter (Patterson et al. 1999). As a consequence, more frequent insect outbreaks are predicted under elevated temperatures (Logan et al. 2003). This is supported by the trend that the number of insect species per unit area declines with increasing latitude (Gaston & Williams 1996) and higher temperatures will allow temperate insect species to shift their ranges to higher latitudes and altitudes (Bale et al. 2002). There is evidence for this phenomenon for butterflies (Parmesan et al. 1999) and from fossil leaves from the late Paleocene-early Eocene global warming period (Wilf & Labandeira 1999; Currano et al. 2010).

## 1.4 The combined effects of habitat fragmentation and climate change

### Effects of multiple global change drivers

Environmental factors associated with climate change have profound impacts on plant metabolism, performance, herbivore resistance, communities and ecosystems, as outlined above. The combined effects of multiple global change drivers can be additive, synergistic and

antagonistic (Brook et al. 2008). Simulation models have been created to predict the effects of different climate scenarios on different ecosystems (e..g Aber et al. 2001; Luo et al. 2008) and various large-scale experiments have been undertaken to assess the combined effects of different global change drivers (see Table 1.3).

**Table 1.3.** Large-scale experiments assessing the effects of multiple global change drivers.

| Name | Variables | System | Main Findings | Reference |
|---|---|---|---|---|
| Jasper Ridge Global Change Experiment | $eCO_2$, warming, precipitation, N deposition | Californean grassland | Increased net primary production for combination of all variables, strong interactive effects | (Shaw et al. 2002) |
| CLIMAITE | $eCO_2$, drought, warming | Temperate heathland, Denmark | Mainly antagonistic effects; but increased C/N ratio under $eCO_2$ remains under full treatment combination | (Larsen et al. 2011) |
| DIRECT | Precipitation, N deposition | Grassland, Silwood Park, UK | Different functional groups buffer effects of global change drivers | (Fry et al. 2013) |

A major conclusion from the theoretical and experimental work is that the combined effects of different global change drivers cannot be predicted based on the individual stressors (Tylianakis et al. 2008). Examples include the observations that $eCO_2$ alleviates some negative effects of environmental stress factors on plant performance, such as ultraviolet (UV) B, salt stress (Bray & Reid 2002; Qaderi & Reid 2005) and drought (Upretty et al. 1995; Morgan et al. 2004), whereas warming counteracts the positive effects of $eCO_2$ on plant growth (Tubiello et al. 2007). An additional complication is that the combined effects also vary among species. For example, the heather beetle *Lochmaea suturalis* is most susceptible to the combination of $eCO_2$, drought and warming (Scherber et al. 2013), while the mountain pine beetle *Dendroctonus ponderosae* benefited from a combination of drought, increased

temperature and climatic variability-induced escape from natural enemies, which has triggered frequent bark beetle outbreaks in North America (Raffa et al. 2008).

## Habitat fragmentation and climate change

Habitat fragmentation and other global change drivers frequently occur simultaneously (Staudt et al. 2013) and have combined negative effects on biodiversity (Forister et al. 2010; Mantyka-Pringle et al. 2012). The combined effects of habitat loss and climate change have been predicted to cause a reduction of 7-24% in vascular plant diversity from 1995 to 2050 (van Vuuren et al. 2006). The joint effects of land use change and climate change are likely to lead to a decline in ecosystem services (Schröter et al. 2005) and dramatically alter plant-herbivore coevolutionary dynamics (Leimu et al. 2012; Appendix 2).

There are manifold links between climate change and habitat fragmentation. Large-scale deforestation (habitat loss) releases more greenhouse gases and accelerates climate change (IPCC, 2014); it reduces transpiration and rainfall (Dickinson 1991) and aggravates the occurrences of extreme drought events associated with climate change (Hansen et al. 2001). Climate change-induced range shifts of populations (Buckland et al. 2001) could bring genetically distinct populations into contact, which increases the likelihood of outbreeding depression (Frankham et al. 2011). The strong selection pressure exerted by climate change, such as extreme climatic events, may remove genotypes from the population that are disadvantageous under extreme climatic conditions ("**selective sweep**") (Davis & Shaw 2001). This is especially likely in isolated populations (Lande 1988) and may increase rates of inbreeding in plant populations (Billington & Pelham 1991; Potvin & Tousignant 1996; Etterson & Shaw 2001). In turn, increased isolation between populations due to habitat fragmentation impedes long-distance dispersal to more suitable habitats under climate change

(Huntley 1991; Davis & Shaw 2001; Pearson & Dawson 2005). Inbreeding due to habitat fragmentation might impair the evolutionary response to climate change, as has been demonstrated for the annual plant *Brassica juncea* (Potvin & Tousignant 1996).

## Environment-dependent inbreeding depression, outbreeding depression and heterosis

In addition to inbreeding depression and heterosis, Charles Darwin also found environment-dependent inbreeding depression; that is the observation that inbreeding depression is more pronounced under stressful conditions (Darwin 1876; Cheptou & Donohue 2011; see Fig. 1.6). The difference in above-ground biomass production between inbred and outbred plants was more pronounced under stress, i.e. at high plant density (Fig. 1.5). In a meta-analysis, 76% of the analyzed studies demonstrated increased inbreeding depression in plants and animals under stressful compared to ambient conditions (Armbruster & Reed 2005). Stressful conditions that increase inbreeding depression in plants include herbivory (Carr & Eubanks 2002) and drought (Hauser & Loeschcke 1996). Why is inbreeding depression more severe under environmental stress? Explanations for this phenomenon have been mainly obtained from studies on animals, especially from *Drosophila melanogaster* and the blue mussel *Mytilus edulis*. The two main hypotheses are:

1) Conditional deleterious recessive alleles that are only expressed under specific environmental conditions are less likely to be purged from inbred populations when these specific conditions rarely occur (Bijlsma et al. 1999).

2) Inbreeding increases metabolic inefficiency (Myrand et al. 2002). Higher rates of protein synthesis have been found in homozygous mussels, and under increased temperatures, heterozygous mussels with lower rates of protein synthesis could better cope with increased metabolic demand under stressful conditions (Hawkins et al. 1987).

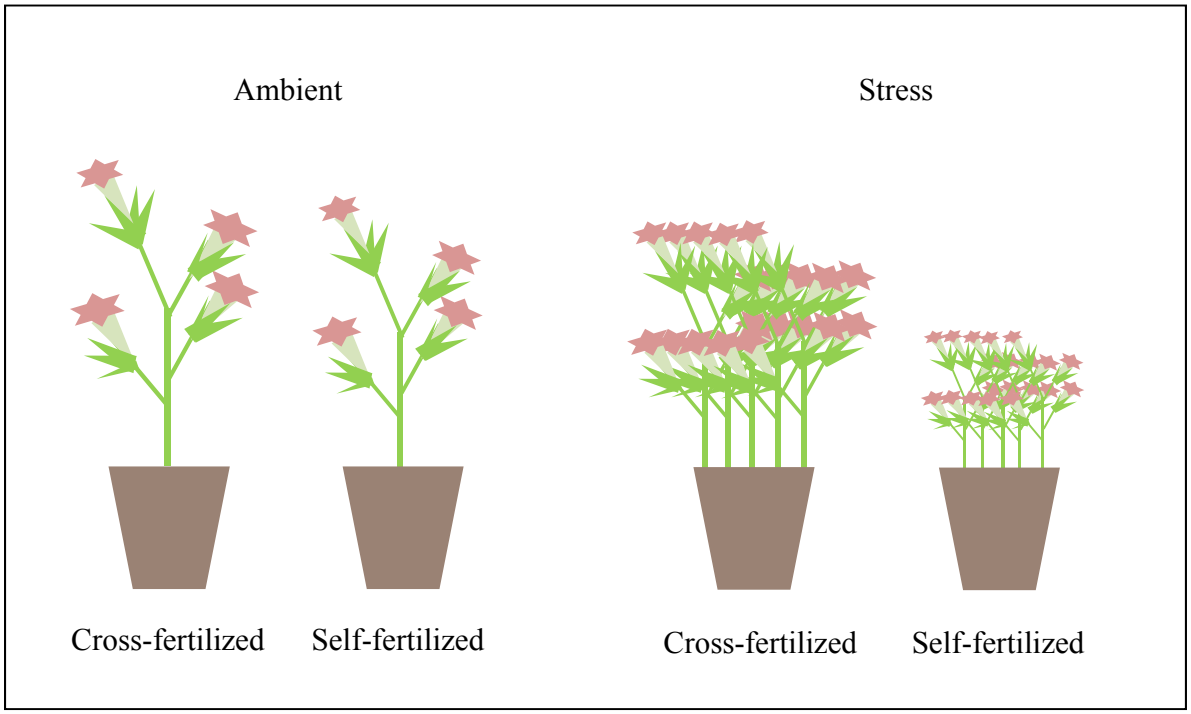


**Fig. 1.5.** Schematic drawing of Darwin's experiment with *Nicotiana tabacum*.

A third hypothesis has only recently been but forward and is related to the newly discovered role of epigenetics in inbreeding depression (Vergeer et al. 2012): Cheptou & Donohue (2013) proposed that inbreeding depression might be environment-dependent because DNA methylation strongly depends on the environment, which would explain the observed differences in inbreeding depression under different environmental conditions.

Outbreeding depression and heterosis can also be environment-dependent. Between-population outbreeding × environment interactions are usually considered in the context of

local adaptation. If the difference between the environmental conditions of outcrossed populations is high, outbreeding depression will be more likely (Frankham et al. 2011;Table 1.2). In contrast, it has been shown in *Arabidopsis thaliana* ecotypes and in several crop species that heterosis can also be increased under environmental stress (Griffing & Langridge 1963; Villa et al. 2012; Roth et al. 2013). The underlying mechanisms for between-population outbreeding × environment and heterosis × environment interactions are not clear yet, and experiments investigating the effects of environmental stress on outbreeding depression and heterosis are lacking.

# 1.5 Heavy metal pollution and hyperaccumulation

## Background

Metal pollution due to human activities is a major threat to ecosystems (Zvereva & Kozlov 2010). Heavy metals are defined as metals with a mass of 5 g/cm$^3$ and with the ability to form sulphides (reviewed by Leyval et al. 1997). The main causes for soil metal pollution are mining and smelting (Cu), electroplating (Cd), sewage sludge disposal, pesticides and fertilizers (Mogren & Trumble 2010). Zinc is used as anode in batteries and as anti-corrosion agent, e.g in electricity pylons (Al-Hiyaly & Bradshaw 1988). Zn production has increased exponentially in the last decades (Nriagu 1996). Zn compounds produced by human activities occur in the soil as zinc chloride, zinc oxide, zinc sulphate, and zinc sulphide (Mogren & Trumble 2010).

Research on plant adaptations to metal pollution was spurred in the 1950s by the discovery that adaptation to metal-polluted soils has happened at a very fast pace in response

to anthropogenic change, demonstrating contemporary evolution. At first, the focus of attention was on metal tolerant species, such as *Agrostis tenuis* and *Festuca ovina* (Antonovics et al. 1971). In the 1990s, metal-hyperaccumulating plants were increasingly studied. Whereas metal tolerance involves the reduction of metal accumulation in the above-ground plant parts, metal hyperaccumulation refers to the active accumulation of metals in the leaves (Baker 1981). There are different threshold values for different metals that define a species as a heavy metal hyperaccumulator, for example > 1% Zn, > 0.1% As, Co, Cr, Cu, Ni, Pb or Se, and > 0.01% Cd leaf dry matter (Verbruggen et al. 2009). About 450 angiosperm species meet the criterion of heavy metal hyperaccumulation, which is less than 0.2% of all angiosperm species (Rascio & Navari-Izzo 2011). Examples of well-studied heavy metal hyperaccumulators include *Noccaea caerulescens* (Pollard & Baker 1997), formerly known as *Thlaspi caerulescens*, as well as *Arabidopsis halleri* (Hanikenne et al. 2008), *Alyssum pintodasilvae* (Peterson et al. 2003; Gonçalves et al. 2006), and *Streptanthus polygaloides* (Jhee et al. 2005).

Three main mechanisms empower plants to accumulate heavy metals: (1) increased uptake of heavy metals from the soil, (2) highly effective root-to-shoot translocation of the heavy metals and (3) effective detoxification and storage of heavy metals in the leaves, mostly inside the vacuoles (reviewed by Rascio & Navari-Izzo 2011). Both metal tolerance and hyperaccumulation are based on a few major loci and probably have evolved independently in different species in the Brassicaceae (reviewed by Krämer 2010). Populations have been shown to differ in their ability to tolerate (Al-Hiyaly & Bradshaw 1988; Schat et al. 1996) and accumulate (Escarré et al. 2000; Roosens et al. 2003) heavy metals, depending on population genetic variation. The most prominent theory as to why heavy metal hyperaccumulation has evolved is the theory of herbivore defence.

## The effects of heavy metal hyperaccumulation on plant-herbivore interactions

While non-hyperaccumulators store metals in the roots, heavy metal hyperaccumulators sequester heavy metals in the leaves, rendering them highly toxic to above-ground herbivores. It has therefore been suggested that metal hyperaccumulation evolved as a defence against herbivores and pathogens (Martens & Boyd 1994). Many studies support this, demonstrating the toxic (meta-analysis by Butler & Trumble 2008) and feeding deterrent (Pollard & Baker 1997; Behmer et al. 2005) effects of several metals, including Zn, on herbivores. Related hypotheses, such as the trade-off and joint effects hypotheses, state that the evolution of heavy metal hyperaccumulation was facilitated by the combined effects of heavy metals and organic defence compounds in plant tissue on herbivore resistance (Boyd 2012). There is some evidence for a trade-off between heavy metal hyperaccumulation and the production of organic defence compounds (Tolrà et al. 2001). Few studies have assessed tolerance to herbivore damage in heavy metal hyperaccumulators, and showed increased tolerance in plants growing on Ni-rich soils (Palomino et al. 2007). Positive effects of Ni on growth (Krämer et al. 1996) and fitness (Ghasemi et al. 2014) have been reported.

Studying the effects of heavy metal hyperaccumulation on plant-herbivore interactions is important not only from an adaptive evolution perspective, but also from an ecotoxicologial point of view: the transfer of heavy metals, including Zn, through the food chain has been demonstrated (Winder et al. 1999; Green et al. 2003) and we need to understand the effects of heavy metal hyperaccumulation at the base of the food chain to be able to prevent the transfer of heavy metals through the food web (Peterson et al. 2003).

# 1.6 Experimental design

## Model species *Brassica nigra* and *Noccaea caerulescens*

The Brassicaceae (mustard) family contains 338 genera and 3709 species (Warwick et al. 2006) and is famous for its crop species grown at a global scale (Hopkins et al. 2009). Examples include oilseed rape (*Brassica napus*) and cabbage, broccoli (*B. oleracea*). Species belonging to the Brassicaceae frequently serve as model systems in plant sciences to study plant genetics and biochemistry (*Arabidopsis thaliana*), physiology and "exotic" plant traits such as heavy metal hyperaccumulation (*N. caerulescens;* reviewed by (Milner & Kochian 2008)), as well as plant-herbivore interactions (several different *Brassica* species, including *B. nigra*; reviewed by Hopkins et al. (2009).

### *Brassica nigra*

The first model plant used in this thesis is *Brassica nigra.* Its close relation to commercially used crops makes *B. nigra* a suitable model system to study plant-herbivore interactions under global environmental change as well as the implications for agriculture. While crop yield is likely to increase under $eCO_2$, there is substantial evidence that other global change drivers and threats, including pests, will change the outcome of this response (Tubiello et al. 2007). 30-40% of all crop losses can be attributed to pests (Thomas 1999), and increased pests outbreaks are predicted under climate change (Logan et al. 2003).

The *B. nigra* populations from which the plants in this study were derived are naturally fragmented and show metapopulation dynamics (Wichmann et al. 2008), which makes them a suitable model system to study the effects of fragmentation and climate change on plant performance and resistance in a metapopulation context. It has been suggested that habitat

fragmentation effects on plant-insect interactions should be studied in a metapopulation context in order to help to understand the effects of fragmentation on communities (Tscharntke & Brandl 2004). Moreover, genetic diversity is of vital importance for plant populations to cope with anthropogenic environmental change (Jump et al. 2009) and understanding the role of within- and between-population variation to cope with biotic and abiotic stress is a central aim of this study.

Another reason to choose *Brassica nigra* as model species was its mating system. *Brassia nigra* is an obligate outcrossing species and such species seem to be more vulnerable to fragmentation than self-compatible species (Honnay & Jacquemyn 2007). Inbreeding effects can therefore be expected in the F1 generation, which is more feasible for experiments with a large number of plants in the crossing design. In terms of between-population outbreeding crosses, genetic differentiation is expected over distances of 15-25m based on typical gene flow data for annual plants of small stature (Linhart & Grant 1996). The populations used in this study are 200m to 9.1 km apart.

### *Noccaea caerulescens*

The well-characterized heavy metal hyperaccumulator *Noccaea caerulescens* (Fig. 1.6) is the second model plant used in this thesis. Since the effects of heavy metal hyperaccumulation in *N. caerulescens* are well studied at the genetic, biochemical, and physiological levels, it is an interesting species to study the effects on the next level of biological organization, the interaction with herbivores. *N. caerulescens* populations differ strongly in their heavy metal hyperaccumulating capabilities (Dechamps et al. 2007), and they show trade-offs between heavy metal hyperaccumulation and herbivore resistance (Dechamps et al. 2008). These characteristics make them a suitable model system to test the trade-off and joint effects

hypotheses stating that there are interactive effects of heavy metals and organic defence compounds (glucosinolates) on herbivore resistance and these played a major role in the evolution of heavy metal hyperaccumulation (reviewed by Boyd 2012).

*N. caerulescens* is also used in phytoremediation, the removal of heavy metals from the soil via plants. Understanding the ecological effects of heavy metal hyperaccumulation is of practical/applied use, because it can assist in developing countermeasures to prevent the transfer of metals through the food chain (Peterson et al. 2003), which can seriously impair animal and human health.

**Fig. 1.6.** *Mamestra brassicae* feeding on *N. caerulescens*.

## Glucosinolates

Glucosinolates are very well-characterized plant secondary metabolites and defence compounds against herbivores that are common in Brassicales (Hopkins et al. 2009). About 132 glucosinolates have been identified to date (Agerbirk & Olsen 2012). Glucosinolates are *β*-thioglucoside *N*-hydroxisulfates that contain a variable side chain (Fahey et al. 2001). There are three main groups based on the amino acid precursor of the variable side chain: the indole glucosinolates (about 10% of all identified glucosinolates) which derive from tryptophan, aliphatic glucosinolates (50%) that are synthesized from methionine, and aromatic glucosinolates (10%), derived from phenylalanine or tyrosine (Mithen 2001). The remaining glucosinolates have other amino acids or as yet uncharacterized compounds as precursors (Hopkins et al. 2009).

Upon plant tissue damage, glucosinolates are released from the vacuole and are hydrolysed by the enzyme myrosinase (Fahey et al. 2001). Different toxic compounds can be released upon that reaction, depending on the structure of the specific glucosinolate and the characteristics of the cellular environment (Wittstock & Halkier 2002; Bones & Rossiter 2006). For example, aliphatic glucosinolates such as sinigrin produce isothiocyanates following hydrolysis (Lankau & Strauss 2008). Insect specialists on Brassicaceae are adapted to the glucosinolate-myrosinase system via different mechanisms: detoxification with specific enzymes, excretion, storage and feeding behavior (Hopkins et al. 2009). Some herbivores even use the sequestered glucosinolates as defence against their natural enemies (Müller et al. 2002). Examples of specialized herbivores include the diamondback moth *Plutella xylostella*, the larvae of the cabbage white butterfly *Pieris rapae*, the aphid *Brevicoryne brassicae* and the flea beetle *Psylliodes chrysocephalus* (Hopkins et al. 2009, compare Table 1.4).

Sinigrin (2-propenyl-glucosinolate; Fig. 1.7a,b) is the main defence compound produced by *Brassica nigra* (Lankau & Strauss 2008). Sinigrin is generally considered to be constitutive (Gols et al. 2008a), but its concentration can also increase upon attack depending on the herbivore (Traw & Dawson 2002; van Dam et al. 2004). The interactions between *B. nigra* and its herbivores are well studied. Herbivores seem to impose high selection pressure on *B. nigra* and they impair plant adaptations to other abiotic factors (Bischoff & Trémulot 2011). This makes *B. nigra* a suitable model system for this thesis, since it allows one to analyse how biotic stress imposed by the predicted insect outbreaks under climate change might impair the response to abiotic stress under climate change.

Sinalbin (p-hydroxybenzyl-glucosinolate; Fig. 1.7c) is the main defence compound produced by *N. caerulescens* (Tolrà et al. 2001). Recently a new glucosinolate has been

characterized in *N. caerulescens* populations from Southern France; glucomoringin, a rhamnose derivative of sinalbin (de Graaf et al. 2014; in press), that has previously only been found in *Moringa* spp (Moringaceae), tropical trees. This thesis describes for the first time the effects of glucomoringin and glucomoringin in combination with heavy metals on the interactions of *N. caerulescens* with a generalist and a specialist herbivore.

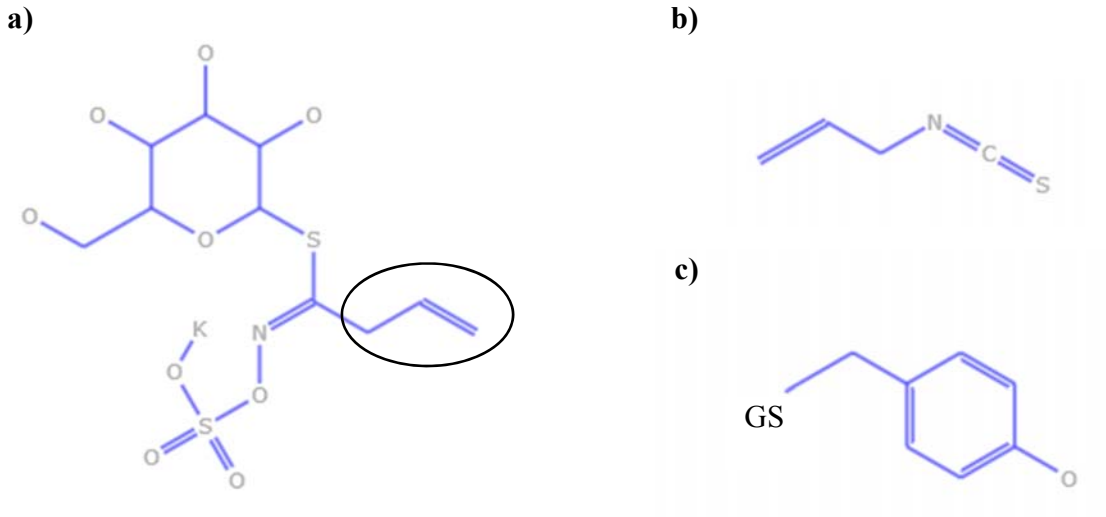


**Fig. 1.7**. Glucosinolate structure. a) Sinigrin (2-propenyl-glucosinolate). The variable side chain that characterizes sinigrin is marked with a circle. The rest of the molecule is common for all glucosinolates. b) Allyl isothiocyanate, the toxic compound that is produced from sinigrin upon release from the vacuole and contact with the enzyme myrosinase. c) Sinalbin. GS denotes the same molecule as in A except for the circled area.

Although it has been hypothesized that herbivores play a crucial role in the evolution of heavy metal hyperaccumulation, very few studies have examined the effects of heavy metal hyperaccumulation on herbivores that naturally co-occur with heavy metal-hyperaccumulating plants (Kramarz & Stark 2003). The herbivores used in the experiment with *N. caerulescens* naturally co-occur with this species and therefore allow drawing conclusions on the role of herbivore for the evolution of heavy metal hyperaccumulation.

## Research hypotheses

This thesis puts particular emphasis on plant-herbivore interactions in the context of habitat fragmentation, climate change, and pollution. Greater herbivore loads are likely in isolated and fragmented habitats (Kruess & Tscharntke 1994; Elzinga et al. 2005) and increases in the frequency and intensity of insect outbreaks are predicted due to climate change with potentially major impacts on natural (Logan et al. 2003; Stireman et al. 2005) and agricultural ecosystems (Fuhrer 2003). Heavy metal pollution in the soil is an increasing threat to natural ecosystems and heavy metal hyperaccumulating plants serve as a model system to study the effects of heavy metal hyperaccumulation on different levels of biological organization, from the plant physiology to plant-herbivore interactions to populations and ecosystems. An overview about the experiments in this thesis is given in Table 1.4. Three main experiments were conducted, one in a common garden (Chapter 2; Fig. 1.8), and two in the greenhouse (Chapter 3 and 4).

**Table 1.4**. Comparison of the experimental design for the three main experiments.

| Parameter | Chapter 2 | Chapter 3 | Chapter 4 |
|---|---|---|---|
| Species | *Brassica nigra* | *Brassica nigra* | *Noccaea caerulescens* |
| Location | NERC Centre for Ecology & Hydrology, Wallingford, Common garden experiment | NERC Centre for Ecology & Hydrology, Wallingford, Greenhouse Experiment | Department of Plant Sciences, Oxford, Greenhouse experiment |
| Crossing background | Inbred, within-population outbred, between-population outbred | Inbred, within-population outbred | - |
| Abiotic factors | Drought stress | Drought stress, $eCO_2$ | Zn in the soil |
| Biotic factors (herbivores) | Mainly specialist herbivores*: Brevicoryne brassicae*, *Psylliodes chrysocephalus*, *Ceutorhynchus quadridens* | Generalist herbivore: *Spodoptera exigua* | Generalist herbivore: *Mamestra brassicae*; Specialist herbivore: *Pieris rapae* |
| # Populations | 5 | 3 | 2 |
| # Plants | 1081 | 315 | 168 |

## Research hypotheses in Chapter 2

(1) Environmental stress (drought) increases inbreeding depression in plant traits related to growth, reproductive output and resistance, and these effects vary among and within populations.

(2) The negative effect of inbreeding on the production of plant secondary metabolites (glucosinolates) counteracts the positive effect of drought on the production of these metabolites.

(3) Environmental stress (drought) does not affect heterosis.

**Fig. 1.8.** The common garden experiment with *B. nigra* (Chapter 2).

## Research questions in Chapter 3

(1) Drought, elevated $CO_2$ and inbreeding synergistically negatively influence the plant functional traits herbivore resistance and production of glucosinolates, and they have antagonistic effects on height, biomass and the number of flowers.

(2) The effects of inbreeding, drought, and elevated $CO_2$ vary among populations.

## Research questions in Chapter 4

(1) Zn soil contamination reduces the production of glucosinolates and therefore herbivore induced responses.

(2) Zn soil contamination increases both herbivore resistance and tolerance.

(3) Herbivores can distinguish between plants grown on different soil Zn concentrations irrespective of whether they are infested by parasitoids.

(4) Sinalbin and glucomoringin have different effects on herbivore resistance against a generalist and a specialist herbivore.

# Chapter 2: Loss of heterosis and family-dependent inbreeding depression in plant performance and resistance against multiple herbivores under drought stress

Published in Journal of Ecology: **102**, 1497-1505

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Keywords: environment dependence, evolutionary ecology, habitat fragmentation, outbreeding depression, plant defence, plant-herbivore interactions, secondary metabolites

## 2.1 Summary

**1.** Inbreeding depression (ID), outbreeding depression (OD) and heterosis can occur concurrently in plant populations. ID often increases under environmental stress, but the combined effects of inbreeding, outbreeding between populations and environmental stress, such as drought, on plant performance and herbivore resistance remain unclear.

**2.** In order to determine environment-dependent and family-dependent ID, OD and heterosis we conducted a common garden experiment with plants from five populations of *Brassica nigra*. Inbred, within-population outbred and between-population outbred plant families were exposed to drought or ambient water levels. We recorded the abundance and damage caused

by specialist herbivores from contrasting feeding guilds, i.e. the phloem-feeding *Brevicoryne brassicae*, the leaf-chewing *Psylliodes chrysocephalus* and the stem-boring *Ceutorhynchus quadridens* larvae.

**3.** Drought stress had negative effects on growth, herbivore resistance and resistance against *B. brassicae* and positive effects on investments in reproductive output and plant secondary metabolites (sinigrin). We found drought stress-induced loss of heterosis for plant height and investment in reproductive output. Between-population outbred plants were more sensitive to drought stress in terms of above-ground biomass compared to within-population outbred plants.

**4.** Drought and inbreeding synergistically negatively influenced traits related to growth and reproductive output (environment-dependent inbreeding depression, EDID). There was high variation among families within populations in the degree of ID and EDID. Genetic variation in EDID could buffer the negative effects of genetic stress associated with habitat fragmentation and concurrent environmental stress. In order to capture fully the effects of both inbreeding and between-population outbreeding under stress the different spatial scales of the effects of inbreeding and between-population outbreeding should be taken into account.

**5.** *Synthesis*. Our results indicate that drought stress influences not only inbreeding depression (ID), but also heterosis. These findings shed new light on the combined effects of anthropogenic environmental change and the genetic consequences of habitat fragmentation on plants and their interactions with other organisms. Conservation programmes aiming to restore genetically degraded populations with the translocation of individuals between populations should consider environmental stress as a risk factor.

## 2.2 Introduction

Inbreeding depression (ID) and outbreeding depression (OD) – that is, reduced performance in inbred and between-population outbred individuals compared to within-population outbred individuals (Wright 1977) – have been demonstrated for plant traits related to fitness and growth (reviewed in Edmands 2007 and Angeloni *et al.* 2011) as well as for herbivore resistance (e.g. Heschel & Paige 1995; Carr & Eubanks 2002; Bello-Bedoy & Núñez-Farfán 2011). Environmental stress, such as drought, is known to increase ID (Armbruster & Reed 2005), which has been termed environment-dependent inbreeding depression (EDID, Cheptou & Donohue 2011). Yet, there is a knowledge gap about the effects of stress on OD and heterosis in natural populations. Environment-dependent outbreeding depression due to the genetic dilution of local adaptation has been demonstrated in previous studies, i.e. the offspring obtained from crosses between and genetically differentiated populations suffered from outbreeding depression (e.g Montalvo & Ellstrand 2001). In contrast, hybrids obtained from crosses between less genetically differentiated populations have been shown to perform better than the parents, showing heterosis (e.g. Fenster & Galloway 2000; but see Quilichini *et al.* 2001). Enhanced heterosis in crop species grown under stressful conditions, such as drought, have been demonstrated (Villa *et al.* 2012; Roth *et al.* 2013), but studies of the effects of environmental conditions, environmental stress in particular, on heterosis and OD in natural populations are scarce (Fenster & Galloway 2000; Edmands & Deimler 2004; Escobar *et al.* 2008; Walisch *et al*. 2012). However, ID, OD, and heterosis may occur concurrently among populations (Becker *et al.* 2006; Escobar *et al.* 2008) as a result of increasing habitat fragmentation that leads to small population sizes and greater distances between populations. We need to quantify the interactions between ID, OD, heterosis and environmental stress in

order to obtain a comprehensive understanding of how species will respond to multiple stress factors in this era of rapid anthropogenic environmental change. If the results from crop species are applicable to natural populations, we can expect between-population outbred plants from populations that are not highly genetically differentiated from each other to be more drought stress tolerant than inbred plants.

Variation in inbreeding and outbreeding effects have been found among populations (Ouborg *et al.* 2000; Carr & Eubanks 2002; Leimu & Fischer 2010; Bello-Bedoy & Núñez-Farfán 2011) and within populations (Koelewijn 1998; Picó *et al.* 2004). ID for a given trait can vary between individuals due to different histories of inbreeding in different populations (Lande & Schemske 1985; Charlesworth & Charlesworth 1987), random genetic variation (Schultz & Willis 1995), and differences in environmental conditions among populations (e.g. Leimu *et al.* 2008). Yet, little is known about the variation within and among populations in environment-dependent ID (EDID) (Bijlsma *et al.* 1999; Fowler & Whitlock 2002) or environment-dependent OD (Fenster & Galloway 2000). Family-level variation in EDID and EDOD could buffer the negative effects of simultaneously occurring environmental stress, inbreeding and between-population outbreeding, because some individuals would perform equally well or even better under these conditions.

Finally, little is known about the combined effects of drought and inbreeding or between-population outbreeding on plant defence chemistry and the corresponding effects on plant herbivore resistance. The production of plant defence compounds can be substantially influenced by drought (English-Loeb *et al.* 1997; Showler & Moran 2003; Hale *et al.* 2005; Gutbrodt *et al.* 2011) and inbreeding (Campbell *et al.* 2013), but only a few studies have investigated these effects showing mixed results. Drought and inbreeding have different

effects on herbivores from different feeding guilds (Stiling & Cornelissen 2007; Hull-Sanders & Eubanks 2005). It is therefore important to assess the effects of drought and inbreeding on plant resistance against different herbivores from different feeding guilds simultaneously to make realistic predictions about the effects of environmental stress and inbreeding on plant-herbivore interactions.

To address the knowledge gaps detailed above, we conducted a common garden experiment with 1081 individuals from inbred and within- and between-population outbred *Brassica nigra* families originating from five wild populations. *B. nigra* is frequently attacked by multiple herbivores from different feeding guilds which are differentially affected by defence compounds, i.e. glucosinolates (Newton *et al.* 2009). The following hypotheses were tested:

(1) Heterosis is maintained under environmental stress.

(2) Environmental stress (drought) increases ID in plant traits related to growth, reproductive output and resistance, and these effects vary among and within populations.

(3) The negative effect of inbreeding on the production of plant secondary metabolites (glucosinolates) counteracts the positive effect of drought on the production of these metabolites.

## 2.3 Materials and methods

### Study species and crossing design

*Brassica nigra* (L.) W.D.J. Koch, black mustard, is an annual outcrossing plant species that occurs in metapopulations on the coast, river valleys and field edges (Wichmann *et al.* 2008).

We studied plants from five *B. nigra* populations located on the coast of Dorset in the UK, and separated by up to 9.1km (see Appendix S1 and Table S1 in Supporting Information). Seeds of 20 plants were collected from each population. The seeds were sown in a greenhouse and 20 maternal plants from each population were selected at random to be grown with ample supply of water and nutrients.

To obtain the different crossing backgrounds in the F1 (IN=inbred, OUT=within-population outbred, BTW=between-population outbred plants) approximately 20-50 flowers on each plant were self-pollinated (IN) (the method to overcome self-incompatibility was taken from Tingdong *et al.* 1992) and cross-pollinated with another individual chosen at random from the same population (OUT). Individuals from each population were cross-pollinated with individuals from two other populations (BTW), with these other populations chosen at random. Two plants were always crossed reciprocally and each individual was used as pollen donor only once within a cross type.

## Common garden experiment

Seeds from the crosses were collected and sown in trays in May 2011. Fourteen days later, 20 randomly chosen F1 seedlings of each cross within each maternal plant (family), 1081 plants in total, were transplanted to pots with a soil mixture of 50% sand and 50% high-nutrient potting compost and placed in the gravel plunge beds of the common garden at the Centre for Ecology and Hydrology in Wallingford, UK. The plunge beds were split into four randomly assigned sections, each of which was hydrological isolated; two had high water tables (ambient treatment) and two had low water tables (drought treatment). In the high water table plots the water level was adjusted to reach the bottom of the flower pots allowing the roots to reach the water, whereas in the low water table plots the water level was adjusted to be 10cm

below the bottom of each pot. Five replicates per each inbred, outbred, and between-population outbred family were randomly assigned to the drought and ambient water treatments. The drought treatment was applied from June 2011; one week after the plants had been transplanted to the common garden. Pot soil moisture measurements (percentage volume of water) were taken with a soil moisture metre once every month and showed a significant difference between the treatments (Fig. S1). Herbivores colonized the plants naturally and were surveyed in July 2011 over seven days. Each plant was closely examined for herbivores on the stems and leaves and the herbivores were identified in accordance with Kirk (1992). The most frequently occurring herbivore species were the cabbage aphid *Brevicoryne brassicae* L., the flea beetle *Psylliodes chrysocephalus* L. and larvae of the stem weevil *Ceutorhynchus quadridens* (Panz.). The abundance of *B. brassicae* was assessed by measuring the length of the area that *B. brassicae* covered on the plant in cm. *P. chrysocephalus* individuals were counted and damage by *C. quadridens* was examined by counting after harvest the number of holes that the larvae had produced in the plant stem.

## Glucosinolate analysis and plant measurements

Leaf samples were taken from all plants in July. Two middle-sized leaves were sampled and immediately flash-frozen in liquid nitrogen and stored at -80°C. The samples were freeze-dried, weighed into a micro-centrifuge tube and ground. Glucosinolates were extracted and purified using the methods of van Dam *et al.* (2004) and *Kabouw et al.* (2010). Glucosinolate content and composition was analysed with high-performance liquid chromatography. Sinigrin was the main glucosinolate (> 99%) found in the leaves (see also van Dam *et al.* 2004) and therefore only sinigrin was included in the analyses.

In August 2011, plant height was measured and the number of inflorescences counted. The number of inflorescences was defined as the number of flowering stems emerging from the rosette. The percentage herbivore damage was estimated visually for all plants. The plants were cut at the root-shoot interface and dried at 70°C for at least 3 days before they were weighed to determine the above-ground biomass.

## Calculating inbreeding depression and outbreeding depression coefficients

We calculated inbreeding depression coefficients (δ) for each family according to Ågren & Schemske (1993). $\delta_I = 1 - (w_S/w_O)$, if $w_S \leq w_O$ and $\delta_O = (w_o/w_s) - 1$ if $w_s > w_o$, where $w_o$ is the mean value for within-population outcrossed families and $w_s$ is the mean value for inbred families. A positive value of $\delta_I$ indicates inbreeding depression, a negative value an inbreeding benefit. Analogously, we calculated outbreeding depression for each plant trait: $\delta_O = 1 - (w_B/w_O)$, if $w_B < w_O$ and $\delta_O = (w_B/w_O) - 1$ if $w_O > w_B$. $w_B$ is the mean value for between-population outcrossed families. A positive value of $\delta_O$ indicates OD, a negative value heterosis. We calculated the inbreeding depression coefficient and outbreeding depression coefficient for each water treatment separately ($\delta_D$ = drought stress, $\delta_W$ = ambient water treatment). In order to estimate the inbreeding depression coefficient and outbreeding depression coefficient for resistance to herbivory, we used a value for resistance which we defined as 1/(herbivore abundance or damage).

## Statistical analysis

To investigate within- and among-population variation in the effects of inbreeding, outbreeding and drought on plant performance and resistance we conducted a split plot analysis using the PROC MIXED procedure of the SAS statistical package version 9.1.

Percent damage, number of inflorescences, plant height, plant above-ground biomass, the abundance of *B. brassicae*, *P. chrysocephallus* and the damage caused by *C. quadridens* were used as response variables. Population, cross (IN, OUT, BTW), water treatment and all two- and three-way interactions were used as fixed factors. Plunge bed, plunge bed × water represented restriction errors and family and its interactions with cross and water treatment were used as random factors in the model. Family was nested within population. To test the significance of the random factors, the chi-squared test of the difference in the -2 residual log likelihood value of the respective random factor when it is included versus when it is excluded from the model was used with a 1° of freedom chi-square distribution (Littell *et al.* 2006). Percent damage, number of inflorescences, plant height, and plant above-ground biomass were square root transformed and aphid abundance, flea beetle abundance, and stem weevil damage were log-transformed to meet the assumptions of normality. We also analysed the effects of population, cross and water treatment on sinigrin concentration in the same way as described above, but using family means because the leaf samples were pooled for the chemical analysis. To detect environment-dependent inbreeding depression and heterosis we had to compare within-population outbred plants specifically with either inbred or between-population outbred plants under the different water treatements. To achieve these comparisons, we constructed separate ID and OD models that contained, respectively, only OUT vs IN or OUT vs BTW plants. The ID and OD models used the same fixed and random factors as described above and thus allowed us to analyse genetic variation in environment-dependent inbreeding depression and in heterosis.

## 2.4 Results

### Plant growth and investment in reproductive output

Between-population outbred plants were taller under the ambient water treatment and they were as tall as within-population outbred plants under drought stress ($\delta_{OD} = 0.01$, $\delta_{OW} = –0.19$; Fig. 2.1a; Table 2.1). This indicates loss of heterosis in plant height under drought stress, which was confirmed with the significant cross × treatment interaction in the separate OD model (d.f. =1,21.1, $F = 7.78$, $P = 0.011$). This is in contrast to our first hypothesis predicting the maintenance of heterosis under environmental stress. Inbred plants were shorter than within-population outbred plants in both water treatments, indicating ID irrespective of the water treatment ($\delta_{ID} = 0.16$, $\delta_{IW} = 0.21$; Fig. 2.1a; Table 2.1). The effects of drought stress on plant height varied among plant populations, as indicated by the significant population × water interaction (Table 2.1). We found genetic variation in ID and OD for plant height, as indicated by the significant family × cross interaction (Table 2.1)

Drought stress reduced the above-ground biomass of between-population outbred plants, but not the above-ground biomass of within-population outbred plants (Fig. 2.1b), suggesting that between-population outbred plants were more sensitive to drought stress in terms of above-ground biomass than within-population outbred plants, as indicated by the difference in the outbreeding depression coefficient for both water treatments ($\delta_{OD} = 0.11$, $\delta_{OW} = –0.12$) and the significant cross × treatment interaction in the OD model (df =1,17, $F = 4.50$, $P = 0.049$). Inbred plants had a lower above-ground biomass than within-population outbred plants, and the difference between inbred and within-population outbred plants was greater under drought stress ($\delta_{ID}= 0.25$; $\delta_{IW}= 0.19$), indicating EDID (Table 2.1; Fig. 2.1b), which is

in line with our second hypothesis. The significant cross × treatment interaction in the ID model confirmed EDID for above-ground biomass (d.f. = 1,13.8, $F$ = 5.92, $P$ = 0.029). The marginally significant family × cross interaction suggests that there was genetic variation in inbreeding depression and outbreeding depression for above-ground biomass (Table 2.1). The marginally significant family × water interaction indicates that the combined effects of drought stress and inbreeding or between-population outbreeding varied among families as well (Table 2.1). Specific families were more sensitive to inbreeding and between-population outbreeding under drought stress than others.

Between-population outbred plants had a higher number of inflorescences in the ambient water treatment compared to within-population outbred plants, indicating heterosis in investment in reproductive output ($\delta_{OW}$ = –0.15; Fig. 2.1c). However, there was no difference in the number of inflorescences between within- and between-population outbred plants under drought stress ($\delta_{OD}$ = 0.02; Fig. 2.1c). The loss of heterosis, as indicated by the significant cross × water treatment interactions in the full model (Table 2.1) and in the OD model (d.f. = 1,14.7, $F$ =13.23, $P$ = 0.003), is in contrast to our first hypothesis. There was slight EDID for the number of inflorescences; that is inbred plants tended to have more inflorescences than within-population outbred plants under the ambient water treatment, but they had slightly fewer inflorescences under drought stress ($\delta_{ID}$ = 0.09, $\delta_{IW}$ = –0.03). EDID for the number of inflorescences was confirmed with the significant cross × treatment interaction in the ID model (d.f. = 1,15.9, $F$ = 9.04, $P$ = 0.008). The number of inflorescences varied among populations (Table 2.1). The significant family × cross × water interaction indicates that there was genetic variation in EDID and environment-dependent heterosis (Table 2.1). Plunge bed had a significant effect on the number of inflorescences and height (Table 2.1).

## Plant herbivore resistance

Plants under drought stress had significantly higher levels of herbivore damage than plants subjected to the ambient water treatment, indicating lower herbivore resistance under drought stress (Table 2.1; Fig. 2.2a). Plunge bed significantly influenced herbivore damage (Table 2.1). There was no ID or OD in herbivore resistance under both water treatments ($\delta_{ID} = 0.02$, $\delta_{IW} = 0.00$; $\delta_{OD} = 0.01$, $\delta_{OW} = 0.00$). However, a significant family × cross interaction indicates genetic variation in the effects of inbreeding and outbreeding on resistance (Table 2.1). Herbivore resistance also tended to vary among families under different water treatments, as indicated by a marginally significant family × water interaction (Table 2.1).

The relative production of sinigrin significantly increased under drought stress (Table 2.1; Fig. 2.2b). There was no significant effect of the crossing background on the concentration of sinigrin (Table 2; $\delta_{ID} = 0.00$, $\delta_{IW} = –0.01$; $\delta_{IW} = 0.12$), although the negative value of the outbreeding depression coefficient under drought stress suggests heterosis in sinigrin production ($\delta_{OD} = –0.26$). These results partly support our third hypothesis that drought has positive effects on sinigrin production, but they do not support the notion that inbreeding has negative effects on sinigrin production.

Overall, the aphid *Brevicoryne brassicae* was significantly more abundant on drought-stressed plants compared to plants grown under the ambient water treatment, indicating lower resistance against *B. brassicae* under drought stress (Table 2.1 and Fig. 2.3a). There was no ID in aphid resistance ($\delta_{ID} = –0.03$, $\delta_{IW} = –0.01$), but there was slight OD in aphid resistance under the ambient water treatment ($\delta_{OD} = 0.06$, $\delta_{OW} = 0.14$). Resistance against *B. brassicae* varied significantly among families and it varied marginally among populations (Table 2.1).

The abundance of the flea beetle *Psylliodes chrysocephalus* varied among populations and varied significantly among plunge beds (Table 2.1). There were slightly fewer flea beetles on inbred plants, indicating an inbreeding benefit in flea beetle resistance ($\delta_{ID} = -0.11$, $\delta_{IW} = -0.15$; Fig. 2.3b). A greater number of flea beetles was recorded on between-population outbred plants compared to within-population outbred plants under both water treatments, indicating outbreeding depression in flea beetle resistance ($\delta_{OD} = 0.07$, $\delta_{OW} = 0.23$; Fig. 2.3b). The significant family × cross and marginally significant population × cross interaction indicate that the influence of the crossing background on beetle abundance varied among families and populations (Table 2.1).

The damage caused by *Ceutorhynchus quadridens* was greater on plants grown under the ambient water treatment than on drought-stressed plants (Table 2.1 and Fig. 2.3c). However, the strong plunge bed effect diminished the treatment effect (Table 2.1). Between-population outbreeding did not affect the damage caused by *C. quadridens* under drought stress ($\delta_{OD} = -0.04$), but we found greater weevil damage on between-population outbred plants compared to within-population outbred plants under the ambient water treatment, indicating outbreeding depression in weevil resistance ($\delta_{OW} = 0.18$). In contrast, inbred plants had less weevil damage in both water treatments, suggesting that there was an inbreeding benefit in weevil resistance ($\delta_{IW} = -0.20$, $\delta_{ID} = -0.14$). Families differed in their weevil resistance under different water treatments and crossing backgrounds, as indicated by the significant family × cross × water interaction for *C. quadridens* damage (Table 2.1).

We found significant and marginally significant family × cross × water interactions for percent damage ($\chi^2 = 8.5$, $P = 0.004$) and stem weevil resistance ($\chi^2 = 2.0$, $P = 0.079$) in the ID model, suggesting that there was genetic variation in EDID in resistance-related traits.

## 2.5 Discussion

Between-population outbred plants are expected to be more stress-tolerant since they have a variety of alleles derived from genetically distinct parents from different populations (review by Schnable & Springer 2013). Enhanced heterosis under stressful conditions has been demonstrated for crops (Villa et al. 2012; Roth et al. 2013) and for hybrids derived from crossbred ecotypes of *Arabidopsis thaliana* (Griffing & Langridge 1963). Our results suggest, in contrast to the results for crop species and in contrast to our hypothesis, that heterosis in growth and investment in reproductive output is lost under environmental stress. This implies that heterosis can be environment-dependent. Together with the results from previous studies showing stress-induced loss of heterosis in the number of produced flowers (Walisch *et al.* 2012) and variation in OD among different field sites (Fenster & Galloway 2000), our findings imply that genotype-by-environment interactions (G × E) (Wade *et al.* 2001) should be considered for the genetic rescue of populations not only in the context of local adaptation, but also in terms of environmental stress. The same applies to models aiming to predict the extent of OD (Frankham *et al.* 2011). The increased investment of resources in defence and putative decreased resource availability for growth and reproductive output (Herms & Mattson 1992) cannot explain our results, as there was no negative correlation between sinigrin concentration and growth or reproductive output (Table S4). A possible mechanism could be the disruption of co-adapted gene complexes (Templeton *et al.* 1990) that play an important role under drought stress, or a new combination of alleles (Fenster & Galloway 2000) that are disadvantageous under drought conditions. Alternatively, the increased abundance of specialist herbivores on between-population outbred plants could negatively influence tolerance, leading to decreased growth and investment in reproductive output.

We demonstrated environment-dependent ID (EDID) in above-ground biomass, height and number of inflorescences, indicating EDID in growth and investment in reproductive output as hypothesized. Our finding of increased ID under drought is in line with previous studies that found increased ID under natural conditions, disease and competition (Dudash 1990; Schmitt & Ehrhardt 1990; Wolfe 1993; Eckert & Barrett 1994). Other studies have not found increased ID under stress in plants (Johnston 1992; Norman *et al.* 1995). Specifically, drought has been shown to have varying effects on ID, from no effect (Nason & Ellstrand 1995; Waller *et al.* 2008) to increased ID in plant fitness traits (Cheptou *et al.* 2000) and plant mortality (Hauser & Loeschcke 1996). The lack of EDID in resistance might be due to the overall higher abundance of aphids and beetles on drought-stressed plants, which might have overridden the effects of inbreeding. We therefore did not find evidence that herbivores prefer inbred plants as has been reported previously (Hayes *et al.* 2004; Delphia *et al.* 2009a; Bello-Bedoy & Núñez-Farfán 2011). However, we found family-level variation in EDID in traits related to herbivore resistance. Family-level variation in EDID has been demonstrated in *Drosophila melanogaster* (Bijlsma *et al.* 1999; Fowler & Whitlock 2002), but not in plants. Since our experiment was conducted in a common garden, the differences among families must have occurred because of genetic variation rather than local adaptation. This implies that random genetic variation is not only an important cause of variation in ID (Schultz & Willis 1995), but also in EDID, and might therefore enable plant populations to cope with the combined effects drought and increased rates of inbreeding. An additional mechanism for EDID could be that the combined effects of drought and inbreeding have more negative effects on some families than on others due to inconsistent purging of alleles that are conditionally deleterious under drought.

Few studies have compared variation in inbreeding and between-population outbreeding effects at different spatial scales (Dudash *et al.* 1997), i.e., at both the family and population levels. We found higher variation in inbreeding and outbreeding depression within populations than between populations. Within-population variation in OD (Quilichini *et al.* 2001; Pélabon *et al.* 2005) appears to be under-represented in the literature, but our results suggest that the spatial scale of inbreeding and between-population outbreeding effects should be taken into account in future studies to capture fully the effects of both increased rates of inbreeding and between-population outbreeding.

Finally, our finding that *B. nigra* produced more sinigrin under drought conditions is in line with our hypothesis. Although some herbivores induce sinigrin production (Traw & Dawson 2002), it has not been shown yet that there is a strong causal correlation between percent damage and the concentration of sinigrin. In our experiment, all plants were damaged to some extent and thus sinigrin may have been induced in all plants. We therefore argue that the effect of drought stress is likely the main cause for the differences observed in sinigrin concentration. The greater sinigrin production may have attracted specialist herbivores, which resulted in the increase in herbivore abundance and damage caused under drought. Our results complement studies demonstrating better performance of specialist herbivores under drought conditions (Hale *et al.* 2005*;* Khan *et al.* 2010; Gutbrodt *et al.* 2011) through an increased abundance of these herbivores. However, we did not find evidence that inbreeding counteracts the positive effects of drought on the production of glucosinolates.

In conclusion, we have demonstrated that drought causes the loss of heterosis and magnifies inbreeding depression, which has serious implications for conservation in a changing climate. However, the substantial amount of family-level variation in EDID for traits

related to herbivore resistance suggests that plant populations might be able to buffer some of these negative effects. These results shed new light on the possible effects of environmental stress on small and fragmented populations that have increased rates of inbreeding and between-population outcrossing and re-emphasize the importance of genetic variation for population persistence under rapid environmental change.

## 2.6 Acknowledgements

We thank Colette Roach and the undergraduate students who helped with the experiments and Sebastian Krosse for conducting the HPLC. CEH provided greenhouse and growth facilities. We thank two anonymous reviewers for helpful comments. This study was financially supported by the British Ecological Society and the German National Academic Foundation (Studienstiftung des deutschen Volkes).

## 2.7 Data Accessibility

Data available from the Dryad Digital Repository:

http://datadryad.org/resource/doi:10.5061/dryad.36746

# 2.8 Tables and Figures

**Table 2.1.** The results of linear mixed model analyses testing for the effects of population, crossing background (inbred, within-population outbred, between-population outbred), water treatment and their interactions on herbivore resistance, the number of inflorescences and herbivore abundance in *Brassica nigra*.

| | **Inflorescences** | | | **Height** | | | **Above-ground biomass** | | |
|---|---|---|---|---|---|---|---|---|---|
| Source of variation | ***Numdf, dendf*** | ***F*** | ***P*** | ***Numdf, dendf*** | ***F*** | ***P*** | ***Numdf, dendf*** | ***F*** | ***P*** |
| **Fixed Effects** | | | | | | | | | |
| Population | 4, 12.5 | 2.97 | (*) 0.062 | 4,9.67 | 11.63 | ** | 4,15.4 | 2.01 | n.s. |
| Cross | 2, 25.5 | 1.31 | n.s. | 2,21.6 | 5.81 | ** | 2,27.1 | 2.00 | n.s. |
| Water | 1, 3.13 | 0.73 | n.s. | 1,2.53 | 0.86 | n.s. | 1,4.65 | 9.94 | * |
| Cross × Water | 2,32.4 | 8.23 | ** | 2,4.4 | 3.78 | * | 2,28.8 | 2.86 | (*) 0.074 |
| Pop × Water | 4,15.9 | 0.74 | n.s. | 4,16.2 | 5.12 | ** | 4,15.7 | 1.19 | n.s. |
| Pop × Cross | 8,25.2 | 0.99 | n.s. | 8,20.8 | 1.47 | n.s. | 8,25.9 | 1.54 | n.s. |
| Pop × Cross × Water | 8,29.7 | 0.96 | n.s. | 8,30.7 | 0.97 | n.s. | 8,26.5 | 0.17 | n.s. |
| **Random Effects** | | $\chi^2$ | | | $\chi^2$ | | | | |
| Plunge bed | | 13.7 | *** | | 6.2 | ** | | 2.2 | (*) 0.069 |
| Plant family | | 0.6 | n.s. | | 0 | n.s. | | 0.1 | n.s. |
| Fam (pop) × Cross | | 18.4 | *** | | 4.2 | * | | 2.6 | (*) 0.053 |
| Fam (pop) × Water | | 12.7 | *** | | 1.3 | n.s. | | 2.0 | (*) 0.078 |
| Fam (pop) × Cross × Water | | 2.7 | * | | 0.4 | n.s. | | 1.0 | n.s. |

The significance of the random factors was tested by the chi-squared test (d.f. = 1) of the differences in the -2 log likelihood value of that factor included vs. excluded from the model. N.S. $P > 0{\cdot}1$, (*) $P < 0{\cdot}1$, * $P < 0{\cdot}05$, ** $P < 0{\cdot}01$, *** $P < 0{\cdot}001$. Plunge bed × Water was fitted to the model as the error term, but is not presented here.

| | Percent Damage | | | *B. brassicae* | | | *P. chrysocephalus* | | |
|---|---|---|---|---|---|---|---|---|---|
| Source of variation | ***Numdf, dendf*** | ***F*** | ***P*** | ***Numdf, dendf*** | ***F*** | ***P*** | ***Numdf, dendf*** | ***F*** | ***P*** |
| **Fixed Effects** | | | | | | | | | |
| Population | 4,13.2 | 1.27 | n.s. | 4,16.4 | 2.42 | (*) 0.091 | 4,9.22 | 3.44 | (*) 0.056 |
| Cross | 2,25.2 | 0.13 | n.s. | 2,23.2 | 1.58 | n.s. | 2,21.1 | 2.09 | n.s. |
| Water | 1,2.71 | 13.35 | * | 1,2.73 | 14.87 | * | 1,2.2 | 2.12 | n.s. |
| Cross × Water | 2,26.5 | 0.03 | n.s. | 2.29.8 | 0.40 | n.s. | 2,25.9 | 0.42 | n.s. |
| Pop × Water | 4,13.8 | 1.54 | n.s. | 4,15.4 | 1.85 | n.s. | 4,9.93 | 0.63 | n.s. |
| Pop × Cross | 8,24.5 | 0.84 | n.s. | 8,21.7 | 1.37 | n.s. | 8,20.8 | 2.00 | (*) 0.097 |
| Pop × Cross × Water | 8,24.7 | 0.66 | n.s. | 8,28.5 | 1.29 | n.s. | 8,25.1 | 0.91 | n.s. |
| **Random Effects** | | $\chi^2$ | | | | | | | |
| Plunge bed | | 14.9 | *** | | 2.6 | (*) 0.053 | | 27.6 | *** |
| Plant family | | 0.9 | n.s. | | 10.3 | ** | | 0 | n.s. |
| Fam (pop) × Cross | | 4.4 | * | | 0.6 | n.s. | | 4.0 | * |
| Fam (pop) × Water | | 2.0 | (*) 0.077 | | 0.1 | n.s. | | 0 | n.s. |
| Fam (pop) × Cross × Water | | 0 | n.s. | | 0.1 | n.s. | | 0.8 | n.s. |

| | *C. quadridens* | | |
|---|---|---|---|
| Source of variation | ***Numdf, dendf*** | ***F*** | ***P*** |
| **Fixed Effects** | | | |
| Population | 4,14.7 | 2.97 | (*) 0.055 |
| Cross | 2,28.0 | 0.02 | n.s. |
| Water | 1,2.23 | 2.19 | n.s. |
| Cross × Water | 2,28.8 | 0.05 | n.s. |
| Pop × Water | 4,11.5 | 0.41 | n.s. |
| Pop × Cross | 8,25.1 | 0.36 | n.s. |
| Pop × Cross × Water | 8,28.1 | 0.56 | n.s. |
| **Random Effects** | | $\chi^2$ | |
| Plunge bed | | 8.2 | ** |
| Plant family | | 7.0 | ** |
| Fam (pop) × Cross | | 1.9 | (*) 0.084 |
| Fam (pop) × Water | | 0.3 | n.s. |
| Fam (pop) × Cross × Water | | 3.4 | * |

**Table 2.2.** The results of linear mixed model analysis testing for the effects of population, crossing background (inbred, within-population outbred, between-population outbred), water treatment and their interactions on the concentration of sinigrin in *Brassica nigra*.

| | ***Sinigrin*** | | |
|---|---|---|---|
| Source of variation | ***Numdf, dendf*** | ***F*** | ***P*** |
| Fixed Effects | | | |
| Population | 4,48 | 0.38 | n.s. |
| Cross | 2,48 | 0.18 | n.s. |
| Water | 1,48 | 4.78 | * |
| Cross × Water | 2,48 | 0.61 | n.s. |
| Pop × Water | 4,48 | 0.55 | n.s. |
| Pop× Cross | 8,48 | 1.56 | n.s. |
| Pop × Cross × Water | 8,48 | 0.74 | n.s. |

N.S. $P > 0{\cdot}1$, (*) $P < 0{\cdot}1$, * $P < 0{\cdot}05$, ** $P < 0{\cdot}01$, *** $P < 0{\cdot}001$.

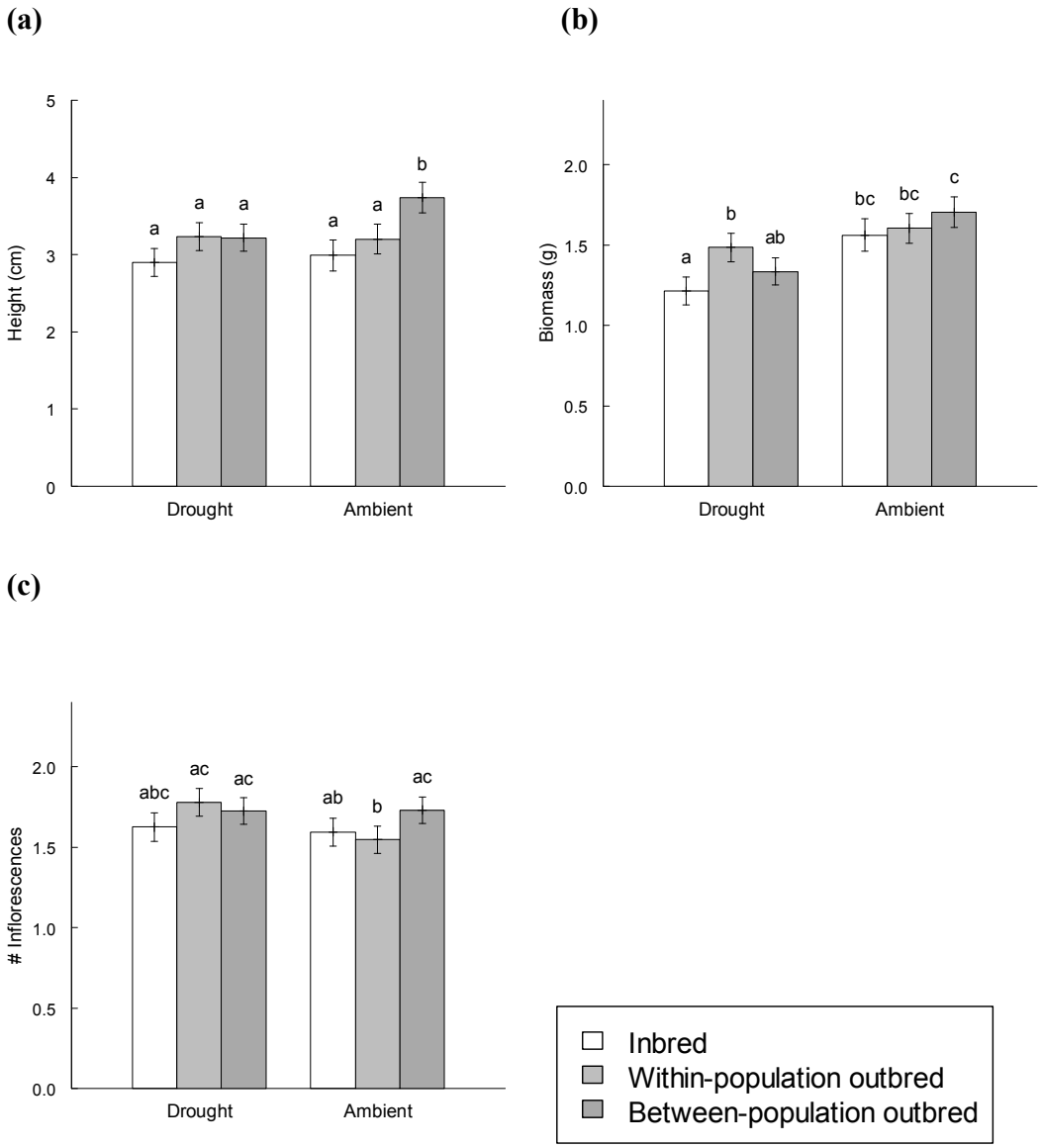


**Fig. 2.1**. Growth (height, above-ground biomass) and investment in reproductive output (number of inflorescences) in inbred, within-population outbred and between-population outbred *Brassica nigra* plants that were exposed to ambient water treatments and drought treatments. Data are LS means ± SE. Different letters denote significant differences between LS means ($P < 0.05$, Tukey's post hoc test, conducted with the same full model that was used for the analysis).

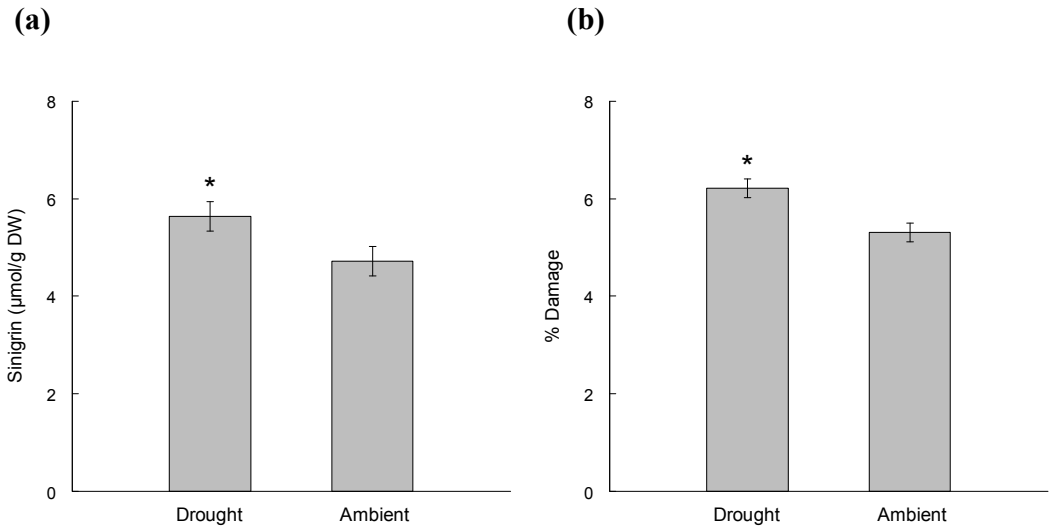


**Fig. 2.2.** Percent damage and sinigrin concentration under ambient water and drought treatments. Data are LS means ± SE. Stars denote significant differences between LS means ($P < 0.05$, Tukey's post hoc test, conducted with the same full model that was used for the analysis).

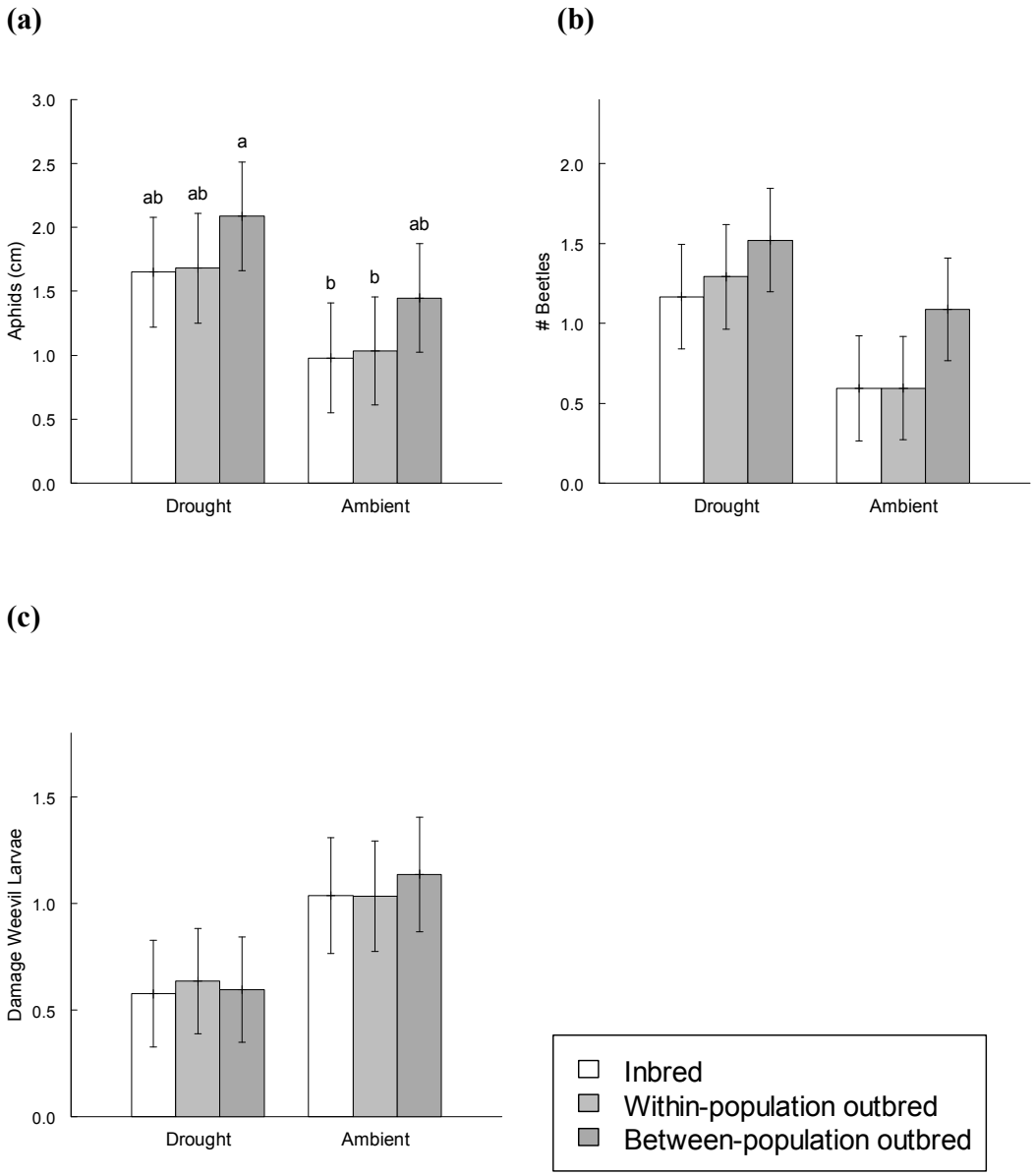


**Fig. 2.3.** Abundance of a) *Brevicoryne brassicae*, b) *Psylliodes chrysocephalus*, c) Damage caused by *Ceutorhynchus quadridens* on *Brassica nigra*. Data are LS means ± SE, a) and c) are back-transformed LS means. Different letters denote significant differences between LS means ($P < 0.05$, Tukey's post hoc test, conducted with the same full model that was used for the analysis).

# Chapter 3: Climate change and habitat fragmentation: elevated $CO_2$, drought and inbreeding have interactive effects on plant performance and herbivore resistance

Prepared for journal submission

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Keywords: global environmental change, plant-herbivore interactions, secondary metabolites, glucosinolates, environment-dependent inbreeding depression, *Brassica nigra*, *Spodoptera exigua*

## 3.1 Abstract

Climate change and habitat fragmentation are key drivers of global environmental change, yet their combined effects on plants and plant-herbivore interactions are poorly understood. Reduced genetic variation and increased inbreeding, the genetic consequences of habitat fragmentation, can modify the impact that changes in the abiotic environment have on plants

and their interactions with herbivores. We investigated the interactive effects of elevated $CO_2$ ($eCO_2$, 600 ppm), drought stress, and inbreeding on plant performance and herbivore resistance in *Brassica nigra* from three populations in a greenhouse experiment. Drought stress and $eCO_2$ had additive positive effects on the number of flowers and antagonistic effects on plant height and resistance to the generalist herbivore *Spodoptera exigua*. The concentration of defence compounds (glucosinolates) was reduced under $eCO_2$. Populations showed conformity in the measured traits under ambient conditions, but $eCO_2$ alone and in combination with inbreeding substantially increased among-population differences, while drought stress decreased among-population differences. Populations also differed in their ability to exploit the greater amount of carbon under elevated $CO_2$ in terms of growth. Drought stress and $eCO_2$ increased inbreeding depression in plants from two of the populations, whereas inbred plants from the third population performed better than outbred plants under these conditions. Our results demonstrate that not only environmental stress, but also novel environmental conditions, such as $eCO_2$, can increase inbreeding depression. We conclude that climate change and habitat fragmentation have complex and interactive effects on plant performance and their interactions with herbivores, and can lead to the expression of previously hidden phenotypes.

## 3.2 Introduction

Global environmental change, such as altered precipitation patterns, elevated levels of atmospheric $CO_2$ concentrations ($eCO_2$), and the increasing fragmentation of habitats have profound impacts on plants and plant-herbivore interactions, with potentially cascading effects on ecosystem structure and function (IPCC, 2014). Accumulating evidence shows that single-

factor experiments do not predict well the combined effects of climate change and habitat fragmentation (Tylianakis *et al.*, 2008). Whilst numerous studies focus on investigating the interactive effects of factors related to climate change (e.g. Shaw *et al.*, 2002; Tubiello *et al.*, 2007; Larsen *et al.*, 2011), few studies explore the combined effects of climate change and habitat fragmentation on plants (Leimu *et al.*, 2010). However, many plant populations are being affected concurrently by habitat fragmentation and climate change (Staudt *et al.*, 2013). Habitat fragmentation reduces population size and consequently results in reduced genetic variation and increased rates of inbreeding (Young *et al.*, 1996). Inbreeding in combination with environmental stress can cause a decrease in plant performance (Armbruster & Reed, 2005), i.e. environment-dependent inbreeding depression (Cheptou & Donohue, 2011). Among-population differences in the effects of inbreeding are well-documented (Ouborg *et al.,* 2000; Carr & Eubanks, 2002; Bello-Bedoy & Núñez-Farfán, 2011), whereas studies of among-population differences in environment-dependent inbreeding depression have been mostly limited to animal model systems (Pray *et al.*, 1994; Bijlsma *et al.*, 1999; Fowler & Whitlock, 2002) and are rare in plants (Prill *et al.*, 2014). Studies on a number of wild plant populations are required to understand the interactive effects of genetic stress associated with inbreeding and environmental factors associated with climate change, such as drought stress and $eCO_2$, on plant performance and herbivore resistance.

Understanding the influence of climate change and fragmentation on plant-herbivore interactions is essential to determine the effects of these global change factors at the level of communities and ultimately ecosystems (e.g. Pautasso *et al.*, 2010). A recent meta-analysis found that the effects of $eCO_2$ on plant-herbivore interactions are shaped by the interactions with other abiotic factors, such as drought stress (Robinson *et al.*, 2012). Few studies have

assessed the effects of inbreeding or $eCO_2$ on plant secondary metabolites that mediate plant-herbivore interactions and no study has assessed these effects in combination. Two recent studies found a qualitative or quantitative reduction in phenolic compounds in inbred plants and a corresponding decrease in herbivore resistance (Campbell *et al.*, 2013; Kalske *et al.*, 2014). Others have demonstrated lower production of volatiles in inbred plants (Ferrari *et al.*, 2006; Delphia *et al.*, 2009b) or a maladaptive change of volatile signalling in inbred plants, leading to increased herbivore damage (Kariyat *et al.*, 2012). While carbon-based defence compounds increase under $eCO_2$ conditions (e.g. Lindroth *et al.*, 1993; Ballhorn *et al.*, 2011), opposing trends have been reported for nitrogen-based defence compounds, including an increase in concentration under $eCO_2$ ( Klaiber *et al.*, 2013), a decrease (Karowe *et al.*, 1997; Himanen *et al.*, 2008), or no effect (Reddy *et al.*, 2004; Bidart-Bouzat *et al.*, 2005).

To address these knowledge gaps we studied the interactive effects of inbreeding, drought stress and extended exposure to $eCO_2$ on plant performance and herbivore resistance in a greenhouse experiment with plants from three *Brassica nigra* populations. We asked the following questions: (1) What are the combined effects of $eCO_2$ and drought stress on plant performance and resistance, including N-based defence compounds (glucosinolates)? (2) How does $eCO_2$ affect inbreeding depression? (3) How do populations vary in the interactive effects of inbreeding, drought, and $eCO_2$?

## 3.3 Materials & Methods

### Study species

*Brassica nigra* (L.) W.D.J. Koch black mustard is an annual, outcrossing, Eurasian plant species that occurs on the coast, river valleys and field edges in the UK (Stace, 1997). The *B. nigra* individuals used in this experiment came from three populations within a single metapopulation on the coastal cliffs of Dorset in the UK (Table 1; Wichmann *et al.*, 2008). The population "Durlston" is small and isolated, whereas the other two populations are of medium and large size and are less isolated (Table 3.1). All three populations grow in habitats characterized as maritime grassland, dominated by *Festuca rubra* (Wichmann *et al.*, 2008). The methods of seed collection, self-pollination and cross-pollination are described in detail in (Prill *et al.*, 2014). These produced F1 seeds that were either inbred as a result of selfing or outbred by crossing with other plants in the same population.

The beet armyworm, *Spodoptera exigua* Hübner (Noctuidae), is a generalist herbivore that was chosen for the experiment because it shows no adaptations to glucosinolates and is negatively affected by them (Müller *et al.*, 2010). It is commonly used in plant-herbivore studies in the Brassicaceae (Widarto *et al.*, 2006; Kos *et al.*, 2012).

### Abiotic treatments

The experimental design is outlined in Fig. 3.1. Inbred and outcrossed seeds from 20 maternal plants (families) per population were sown in trays in February 2012. The trays were put in greenhouse chambers at the Centre for Ecology and Hydrology in Wallingford, UK. Half of the inbred and outbred seeds from each population were randomly assigned to the $CO_2$ treatments: a) ambient ($aCO_2$, 380 ppm), b) elevated ($eCO_2$, 600 ppm). The $eCO_2$ level of 600

ppm is frequently used in climate change research (Volk *et al.*, 2000; Bloor *et al.*, 2010). Two weeks later, the plants were transplanted to pots with a soil mixture of 1/3 sand and 2/3 high-nutrient soil. Some families were lost due to insufficient germination rates, so that we obtained between 8 and 17 families for each population under each treatment. The families served as replicates within the populations. Half of the replicates from each inbred and each outbred family were randomly assigned to the water treatment: a) ambient, b) drought stress. Four randomly chosen plants with the same assigned water treatment were put in one tray. Trays were randomly distributed into the four greenhouse chambers depending on their assigned $CO_2$ treatment. All trays were watered with 1 L of water once every week and randomly redistributed and rotated between chambers with the same $CO_2$ levels each week to achieve a fully randomized factorial design. Plants were rotated among trays with the same water treatment as well. The water treatment started three weeks after the seeds had been sown: a) ambient (2 x 1000 ml/wk for each tray), b) drought stress (2 x 500 ml/wk for each tray). The amount of water was allocated to achieve similar soil moisture contents as in previous drought experiments with *B. nigra* (Prill *et al.*, 2014). After four weeks, the amount of water was increased to 2000 ml/wk for the ambient water treatment and 1000 ml/wk for the drought stress treatment to meet the increased water demand of larger plants. Soil moisture measurements with a soil moisture meter showed a significant difference between the drought stress (3.11 ± 0.68 % vol./vol.) and the ambient water treatment (33.12 ± 9.29 % vol./vol.).

## Herbivore treatment, glucosinolate analysis and plant measurements

Seven weeks after the seeds had been sown, half of the plants from each family and each water treatment were randomly assigned to the +/- herbivore treatment whereby four 2$^{nd}$ instar *S. exigua* larvae were put on each plant allocated to the + herbivore level (Fig. 3.1). All plants

were bagged to ensure that differences between herbivore treatments did not arise because of the bagging of the plants. The herbivores were removed from the plants after seven days. Three days after the herbivores had been put on the plants, the 3rd or 4th fully expanded undamaged leaf was sampled from each plant. Leaves were immediately flash-frozen in liquid nitrogen and stored at -80°C. The samples were freeze-dried, weighed into a micro-centrifuge tube and ground. Sinigrin was extracted, purified and analysed by high-performance liquid chromatography using the methods developed by van Dam *et al.* (2004) and Kabouw *et al.* (2010). Sinigrin was the main glucosinolate (> 99%) found in the leaves (see also van Dam *et al.,* 2004) and therefore only sinigrin levels were analysed statistically.

The percentage of herbivore damage was estimated by counting all leaves and those that were damaged by *S. exigua*. We measured plant height as a growth estimate. The number of flowers was counted as a fitness estimate, as done in other studies (Etterson, 2004; Ivey *et al.*, 2004), including the Brassicaceae (Conner & Zangori, 1998; Campbell *et al.*, 2006).

## Inbreeding depression coefficient

We calculated inbreeding depression coefficients (δ) for each measure in each population following Ågren & Schemske (1993). $\delta = 1 - (w_s / w_o)$ if $w_s \leq w_o$ and $\delta = (w_o / w_s) - 1$ if $w_s > w_o$, where $w_o$ is the mean value for outcrossed families and $w_s$ is the mean value for inbred families. A positive value of δ indicates inbreeding depression, a negative value an inbreeding benefit (compare Prill *et al.*, 2014). We calculated the inbreeding depression coefficient for each water treatment and $CO_2$ treatment separately. In order to estimate the inbreeding depression coefficient for herbivore resistance, we defined resistance as 1/(percentage of damaged leaves).

## Statistical analysis

To investigate the effects of inbreeding, elevated $CO_2$ and drought stress on plant resistance and performance we conducted ANOVAs with R (fully randomized factorial design). The percentage of herbivore damage, sinigrin concentration, number of flowers and plant height were response variables. Population, cross, water treatment, $CO_2$ treatment and herbivore treatment were explanatory variables. All two-, three- and four-way interactions were originally included in the model, and herbivore treatment and the three- and four-way interactions were removed to simplify the models after they showed no significant effects. Normality and homogeneity of variances were examined by visual inspection of diagnostic plots and by the Bartlett test. Outliers were removed from the model. The number of flowers was square-root-transformed and herbivore damage was log-transformed to meet the assumptions of normality.

## 3.4 Results

### Effects of $eCO_2$ and drought stress on plant performance and resistance

Drought stress and $eCO_2$ had antagonistic effects on plant height. Drought stress reduced plant height by 37 % (Table 3.2; Fig. 3.2a). Elevated $CO_2$ had a positive effect on plant height (Table 3.2; Fig. 3.2b), although the magnitude of the effect varied among populations, as indicated by the significant population × $CO_2$ interaction (Table 3.2). When grown under $eCO_2$, plants from the small Durlston population were 4 % taller, whereas plants were 25% and 40% taller in the St Adhelm's and Swyre Head populations respectively.

There were no significant differences in the number of flowers between plants in the two watering treatments under ambient $CO_2$ levels (Table 3.2; Fig. 3.3a). However, under $eCO_2$, the number of flowers increased by 45% under drought stress (by 45 %; Fig. 3.3a) compared to an increase of 30% under the ambient water treatment, indicating an additive positive effect of $eCO_2$ and drought stress on a fitness-related trait.

Elevated $CO_2$ and drought stress had antagonistic effects on herbivore damage, as indicated by the significant $CO_2$ × water interaction (Table 3.2). There was less herbivore damage under drought stress compared to the ambient water treatment, but the difference in herbivore resistance between the water treatments diminished under $eCO_2$ (Fig. 3.4a). Sinigrin concentration was significantly lower in plants grown under $eCO_2$ compared to plants grown under $aCO_2$ (Table 3.2; Fig. 3.4b).

## Among-population differences and effects of inbreeding, eCO2 and drought stress on plant performance and herbivore resistance

Overall, there was a high degree of conformity of the measured traits in among-population means under control conditions, as indicated by the low standard deviations of population means under the ambient water treatment and $aCO_2$ (Table 3.3). Among-population differences increased under $eCO_2$ and they decreased under drought stress, as indicated by the increase of the standard deviations of population means under $eCO_2$ and their decrease under drought stress (Table 3.3). Drought stress therefore counteracted the effects of $eCO_2$ on among-population differences. Inbreeding in combination with $eCO_2$ increased among-population differences under the ambient water treatment and inbreeding in combination with $aCO_2$ decreased among-population differences under the ambient water treatment (Table 3.3).

The inbreeding depression coefficient (δ) for plant height was positive for all three populations under drought stress and $aCO_2$, and for drought stress and $eCO_2$ in the small Durlston population (Fig. 3.5a), indicating inbreeding depression under these conditions. In contrast, δ was negative for the St Adhelm's and Swyre Head populations under drought stress and $eCO_2$, indicating an inbreeding benefit in plant height for these two populations and a mitigating effect of $eCO_2$ on environment-dependent inbreeding depression under drought stress (Fig. 3.5a).

Inbreeding depression in the production of flowers varied among populations, as indicated by the significant population × cross interaction (Table 3.2). Inbred plants from the Durlston population produced more flowers than outbred plants, indicating an inbreeding benefit, which was substantially increased under drought stress and $eCO_2$ alone and in combination (Fig. 3.3b; Fig. 3.5b). In contrast, the populations St Adhelm's and Swyre Head

had consistently positive inbreeding depression coefficients under all water treatments and $CO_2$ levels, indicating inbreeding depression (Fig. 3.3b).

Herbivore damage was 13 % lower in outbred plants compared to inbred plants, indicating inbreeding depression in herbivore resistance irrespective of the $CO_2$ level and drought stress (Table 3.2; Fig. 3.5c). Inbreeding depression in resistance was confirmed by the positive δ values for herbivore damage for all populations and all treatments, except for the Durlston population under the ambient water treatment and $eCO_2$, and for the St Adhelm's population under $aCO_2$ (Fig. 3.5c). Inbreeding depression in sinigrin production varied among populations, as indicated by the significant population × cross interaction (Table 3.2). The δ values for sinigrin were negative for plants from the Durlston population under $eCO_2$, indicating an increased inbreeding benefit under $eCO_2$ (Fig. 3.5d). By contrast, the δ values for sinigrin production were positive for plants from the Swyre Head population in all treatments, indicating inbreeding depression (Fig. 3.5d).

Overall, among-population differences in inbreeding depression were greater under drought stress and $eCO_2$ (Fig. 3.5a-d). Plants from the small Durlston population benefitted from inbreeding under drought stress and $eCO_2$, as indicated by a negative δ, with the only exception being height under drought stress. In contrast, for plants from the St Adhelm's population we found variation among traits in whether they showed inbreeding benefits or inbreeding depression. These inbreeding effects were more profound under drought stress and $eCO_2$ (Fig. 3.5). Inbreeding depression was greater in plants from the Swyre Head population under drought stress and $eCO_2$ compared to ambient conditions, except for plant height (Fig. 3.5).

## 3.5 Discussion

In general, we found a number of interactive effects of $eCO_2$ and drought stress on *Brassica nigra* performance. Our results therefore support the contention that the effects of $eCO_2$ on plant traits are influenced by other factors, such as soil P (Niklaus & Körner, 2004) and water availability (Robinson *et al.*, 2012). The additive positive effects of elevated $CO_2$ and drought stress on the number of flowers can be attributed to the increased water use efficiency under $eCO_2$ (Drake *et al.*, 1997; Morgan *et al.*, 2004). This may have supported drought-induced increase in allocation of resources into traits related to reproduction at the expense of vegetative growth (review by Chaves, 2002). The augmentation of drought resistance by increased nutrient and water use efficiency of plants under $eCO_2$ has been shown in the greenhouse (Ottman *et al.*, 2001; Franzaring *et al.*, 2011; Naudts *et al.*, 2013) and in a long-term grassland field study (Niklaus & Körner, 2004). This explanation is consistent with our finding that elevated $CO_2$ and drought stress affected plant height antagonistically, as the negative effect of drought stress on plant height was mitigated under elevated $CO_2$.

The difference between herbivore resistance under drought stress compared to the ambient water treatment diminished under $eCO_2$. Plants produced less sinigrin under $eCO_2$ and were consequently less toxic to the generalist *S. exigua*, yet resistance was greater under combined $eCO_2$ and drought stress. Since primary metabolites decrease under $eCO_2$ (Bezemer & Jones, 1998) and increase under drought stress (Stiling & Cornelissen, 2007), it is likely that *S. exigua* consumed less leaf material under drought stress and consumed more leaf material under the ambient water treatment and $eCO_2$ to obtain the same nutritional value, so that the effects of drought stress and $eCO_2$ partly cancel each other in terms of herbivore resistance. Our finding that sinigrin production was reduced under $eCO_2$ is in line with other

studies showing a decrease of nitrogen-based secondary metabolites (Himanen *et al.*, 2008; Ballhorn *et al.*, 2011). This response may, however, be species specific (Karowe *et al.*, 1997): some studies report the opposite effect (e.g. Klaiber *et al.*, 2013) or no effect (Reddy *et al.*, 2004). It should also be noted that the studies to date have usually exposed between 1- and 4-week-old plants to elevated $CO_2$. Since important developmental processes are initiated in very early stages of the life cycle, exposing plants to $eCO_2$ at germination, as we did in our experiment, mimics natural conditions to a greater extent.

Inbreeding depression was overall greater under drought stress and $eCO_2$ than under the ambient water treatment and $aCO_2$. This finding supports the notion that inbreeding depression increases under environmental stress (e.g. Hauser & Loeschcke, 1996; Carr & Eubanks, 2002), such as drought stress, but it also demonstrates that novel environmental conditions, such as $eCO_2$, can increase inbreeding depression (environment-dependent inbreeding depression; EDID). However, drought stress-induced EDID for height was lost under $eCO_2$, suggesting a mitigating effect of $eCO_2$ on inbreeding depression for plant growth under drought stress. Elevated $CO_2$ alone and in combination with inbreeding substantially increased the differences between the three populations, while drought stress decreased among-population differences. Schlichting (2008) suggested that trait values have low variation when one phenotype represents the optimum under previously experienced environmental conditions, which has been shown here under ambient and drought stress conditions. By contrast, trait values are unregulated when they have not been exposed to selection before and should therefore strongly differentiate under novel environmental conditions (Schlichting, 2008). Novel environmental conditions, such as $eCO_2$, and novel genetic conditions related to habitat fragmentation, such as increased inbreeding, may thus

expose previously hidden genetic variation. The among-population differences were especially evident in the ability to take advantage of the increased carbon supply under $eCO_2$ levels. These results confirm studies showing species- and genotype-specific differences in the effects of $eCO_2$ on plant traits (Ward *et al.*, 2000; Lau *et al.*, 2007; Vannette & Hunter, 2011). Inbred plants from the Durlston population performed better than their outbred counterparts under drought stress and $eCO_2$ and better than inbred plants from the other two populations in fitness- and resistance-related traits. The Durlston population is smaller and more isolated than the other two populations. Purging and exposure of recessive conditionally neutral alleles could explain these results. Small populations have been shown to suffer lower levels of inbreeding depression than large populations, but it is unclear whether this can be attributed to purging (Thiele *et al.*, 2010; Angeloni *et al.*, 2011). Nonetheless, in the long term, genetic drift counteracts selection in small populations and strongly impairs their ability to adapt to environmental change (reviewed in Willi *et al.*, 2006).

In conclusion, our results shed new light on the combined effects of climate change and habitat fragmentation on plant populations. The observation that elevated $CO_2$ and drought stress, alone and in combination, increase inbreeding depression, is worrying in the face of climate change and increased rates of inbreeding as a result of habitat fragmentation. Differences between populations in plant performance and herbivore resistance increased substantially under $eCO_2$ alone and in combination with inbreeding, suggesting exposure of recessive conditionally neutral alleles or purging. While we cannot extrapolate our results to long-term consequences of climate change and habitat fragmentation on population viability, we note the tendency for increased population differentiation under novel genetic and environmental conditions related to climate change and habitat fragmentation. More studies

are required that unravel the role of among-population genetic variation for resilience to anthropogenic environmental change.

## 3.6 Acknowledgements

We thank Setareh Mohammadin and Laura Mallada González who helped with the experiment and Sebastian Krosse who conducted the HPLC analyses. CEH provided *S. exigua* and the greenhouse facilities. This study was financially supported by the British Ecological Society and the German National Academic Foundation.

## 3.7 Tables and Figures

**Table 3.1.** *B. nigra* populations used in the greenhouse experiment. The pH, P, K, Mg, N values have been obtained from soil samples (Wichmann et al., 2008).

| Population | Size | Distance from nearest population (km) | pH | P mg/L | K mg/L | Mg mg/L | N g/100g | Grid coordinates |
|---|---|---|---|---|---|---|---|---|
| Durlston (DUL) | ~60 | 1.4 | 8.1 | 4 | 600 | 340 | 0.64 | SZ 03329, 77141 |
| Swyre Head (SWY) | ~400 | 0.15 | 7.6 | 7 | 446 | 385 | 0.79 | SY 91336, 78257 |
| St Aldhelm's (STA) | ~1000 | 0.49 | 7.6 | 20 | 670 | 347 | 0.59 | SY 95912, 75780 |

**Table 3.2.** The results of ANOVA testing for the effects of population, crossing background, water treatment, $CO_2$ and their interactions on plant height, number of flowers, unit damage and sinigrin concentration of *Brassica nigra*.

| | ***Height*** | | | ***Flowers*** | | |
|---|---|---|---|---|---|---|
| Source of variation | ***d.f.*** | ***F*** | ***P*** | ***d.f.*** | ***F*** | ***P*** |
| Population | 2 | 11.69 | < 0.001 | 2 | 0.94 | n.s. |
| Cross | 1 | 1.34 | n.s. | 1 | 0.01 | n.s. |
| Water | 1 | 158.95 | < 0.001 | 1 | 0.10 | n.s. |
| $CO_2$ | 1 | 41.02 | < 0.001 | 1 | 52.47 | < 0.001 |
| Population × Cross | 2 | 1.82 | n.s. | 2 | 8.61 | < 0.001 |
| Population × Water | 2 | 1.20 | n.s. | 2 | 0.03 | n.s. |
| Population × $CO_2$ | 2 | 6.18 | 0.002 | 2 | 0.06 | n.s. |
| Cross × Water | 1 | 0.01 | n.s. | 1 | 0.33 | n.s. |
| Cross × $CO_2$ | 1 | 2.73 | 0.099 | 1 | 0.16 | n.s. |
| Water × $CO_2$ | 1 | 0.74 | n.s. | 1 | 3.76 | 0.054 |
| Error | 281 | | | 277 | | |

| | ***Damage*** | | | ***Sinigrin*** | | |
|---|---|---|---|---|---|---|
| Source of variation | ***d.f.*** | ***F*** | ***P*** | ***d.f.*** | ***F*** | ***P*** |
| Population | 2 | 0.17 | n.s. | 2 | 3.19 | 0.044 |
| Cross | 1 | 5.11 | 0.026 | 1 | 1.95 | n.s. |
| Water | 1 | 25.62 | < 0.001 | 1 | 0.03 | n.s. |
| $CO_2$ | 1 | 0.00 | n.s. | 1 | 18.40 | < 0.001 |
| Population × Cross | 2 | 0.07 | n.s. | 2 | 3.23 | 0.042 |
| Population × Water | 2 | 1.91 | n.s. | 2 | 0.35 | n.s. |
| Population × $CO_2$ | 2 | 3.47 | 0.035 | 1 | 1.03 | n.s. |
| Cross × Water | 1 | 0.05 | n.s. | 2 | 0.04 | n.s. |
| Cross × $CO_2$ | 1 | 0.78 | n.s. | 1 | 0.08 | n.s. |
| Water × $CO_2$ | 1 | 6.81 | 0.010 | 1 | 1.51 | n.s. |
| Error | 108 | | | 187 | | |

**Table 3.3.** Standard deviations of population means of the measured traits under the different treatment combinations. IN = inbreeding, OUT = outbreeding, W = water treatment, D = drought treatment, A = ambient $CO_2$, E = elevated $CO_2$.

| Cross | Water | $CO_2$ | Flowers | Height | Sinigrin | Resistance |
|---|---|---|---|---|---|---|
| OUT | W | A | 1.84 | 13.28 | 6.56 | 0.04 |
| IN | W | A | 0.89 | 4.28 | 4.57 | 0.02 |
| OUT | D | A | 2.17 | 5.64 | 3.12 | 0.05 |
| IN | D | A | 5.97 | 8.09 | 8.47 | 0.03 |
| OUT | W | E | 3.20 | 23.35 | 9.19 | 0.10 |
| IN | W | E | 10.98 | 18.15 | 11.67 | 0.06 |
| OUT | D | E | 7.19 | 13.72 | 5.00 | 0.02 |
| IN | D | E | 6.07 | 13.73 | 6.98 | 0.01 |

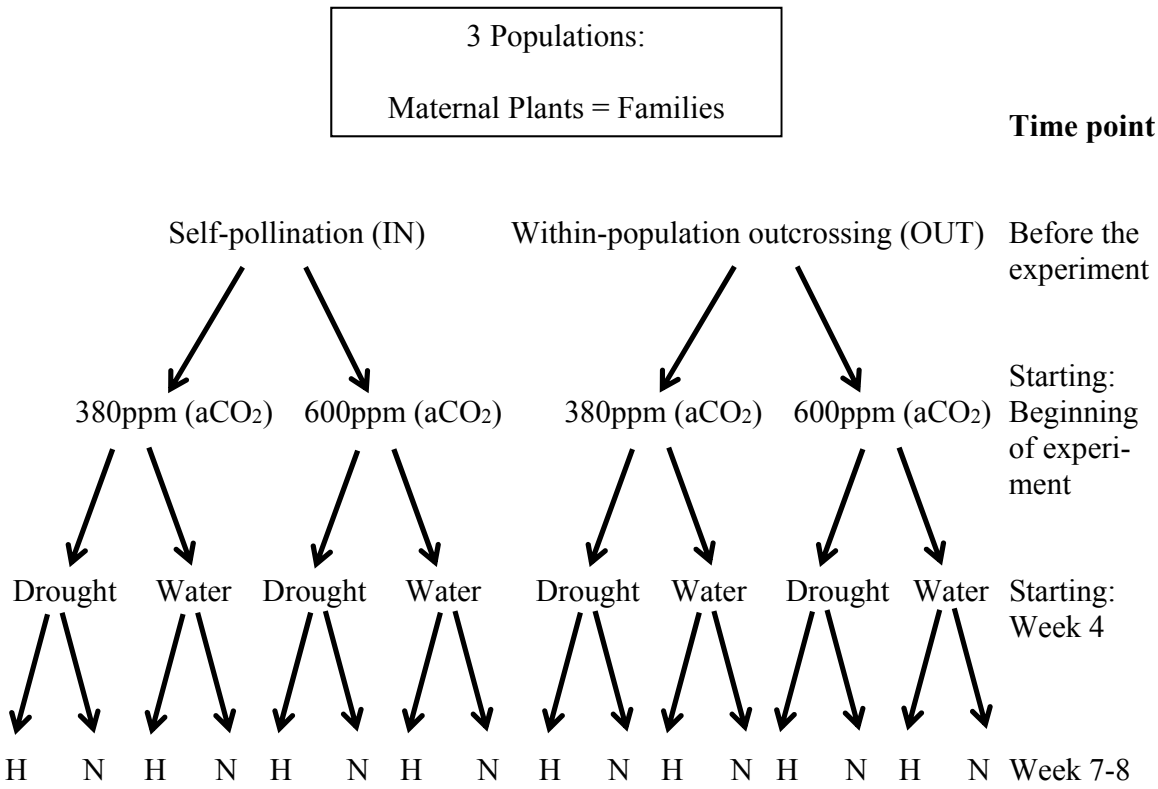


**Fig. 3.1.** Diagram of the experimental design. Crossing treatment: IN and OUT, $CO_2$ treatment: $aCO_2$ and $eCO_2$, water treatment: Drought (D) and ambient (W), herbivore treatment: Herbivore on the plant (H) and no herbivore on the plant (N).

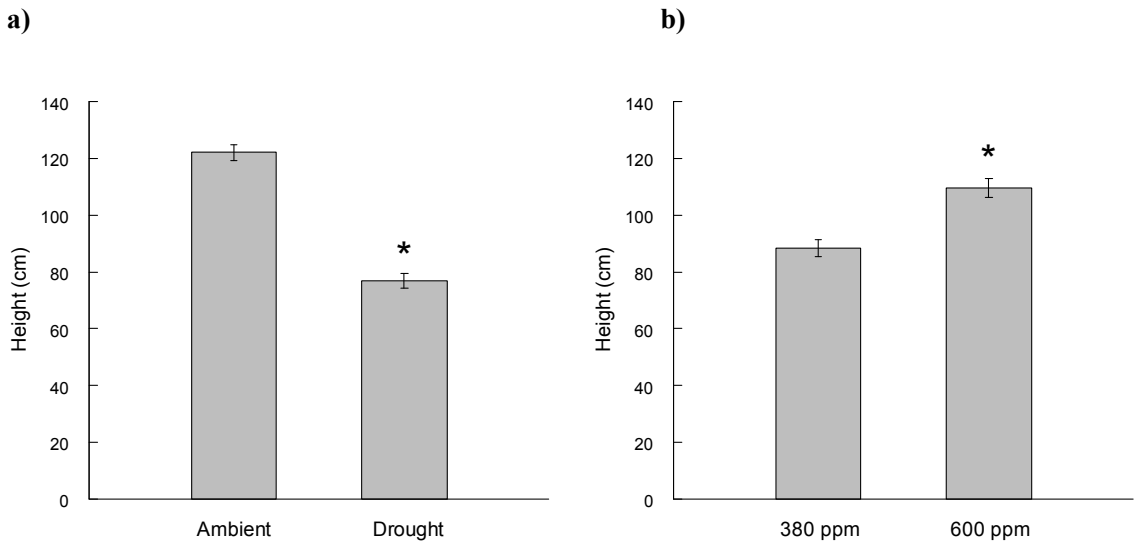


**Fig. 3.2.** The effects of a) water treatment and b) $CO_2$ levels on plant height. Stars denote significant differences between means ($P < 0.05$, Tukey's *post hoc* test).

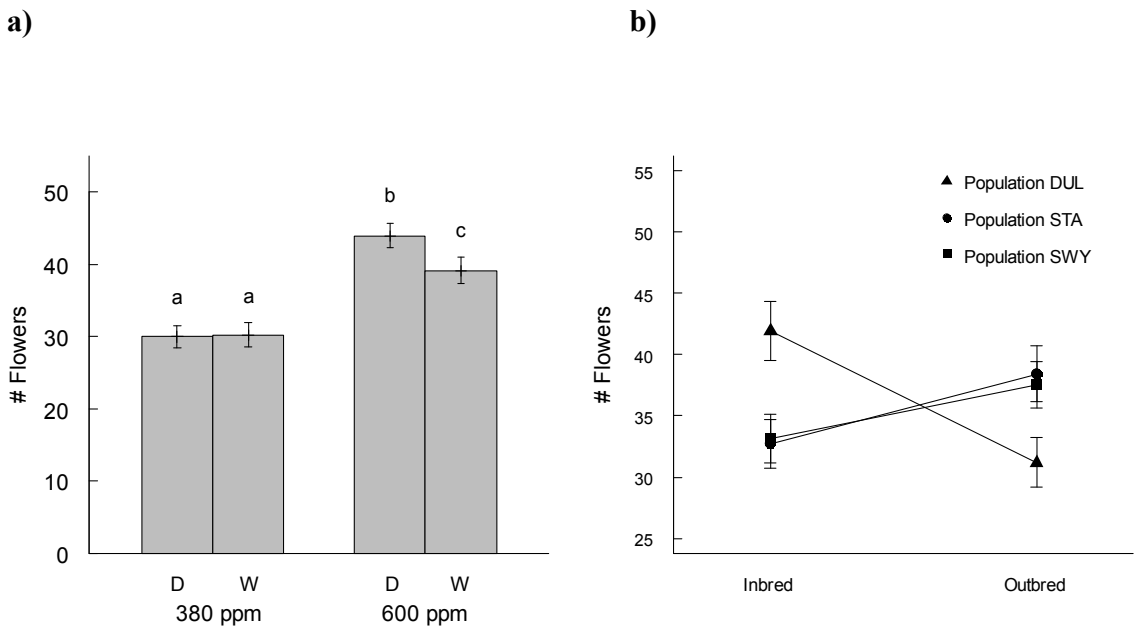


**Fig. 3.3.** Effects of elevated $CO_2$, drought and inbreeding on the number of flowers. Number of flowers a) under different water treatments (D = drought, W = ambient) and $CO_2$ levels, b) of inbred and outbred plants from all three different populations. Letters denote significant differences between means ($P < 0.05$, Tukey's *post hoc* test).

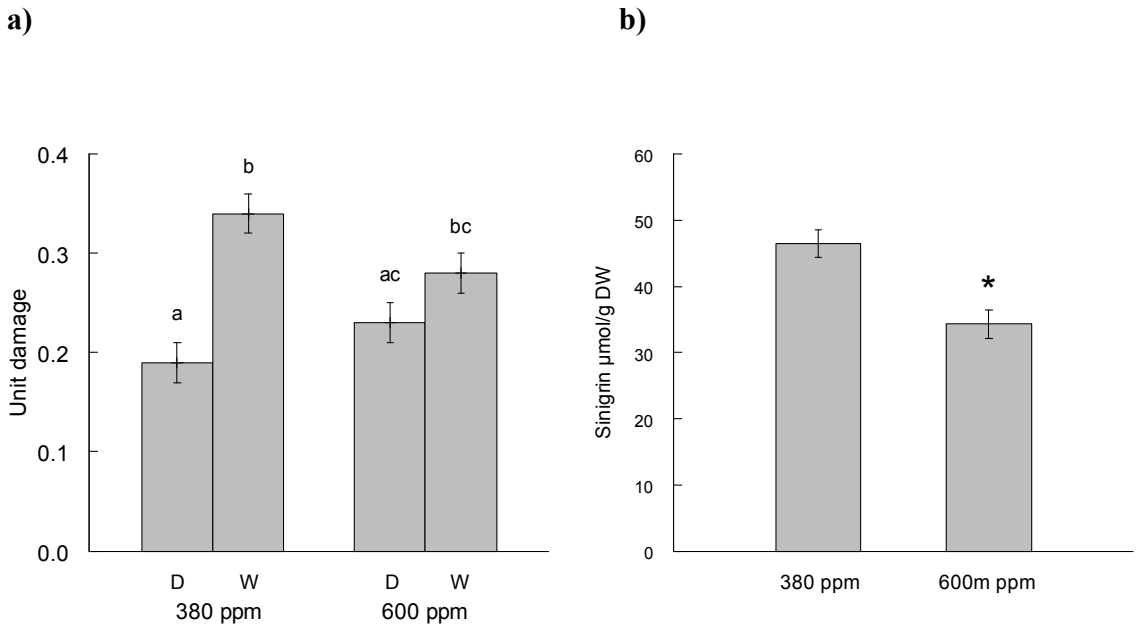


**Fig. 3.4.** The effects of $CO_2$ levels and water treatment on resistance traits. a) Herbivore damage under the different $CO_2$ and water treatments (D = drought, W = ambient), b) sinigrin concentration under ambient and elevated $CO_2$ levels. Letters and stars denote significant differences between means ($P <$ 0.05, Tukey's *post hoc* test).

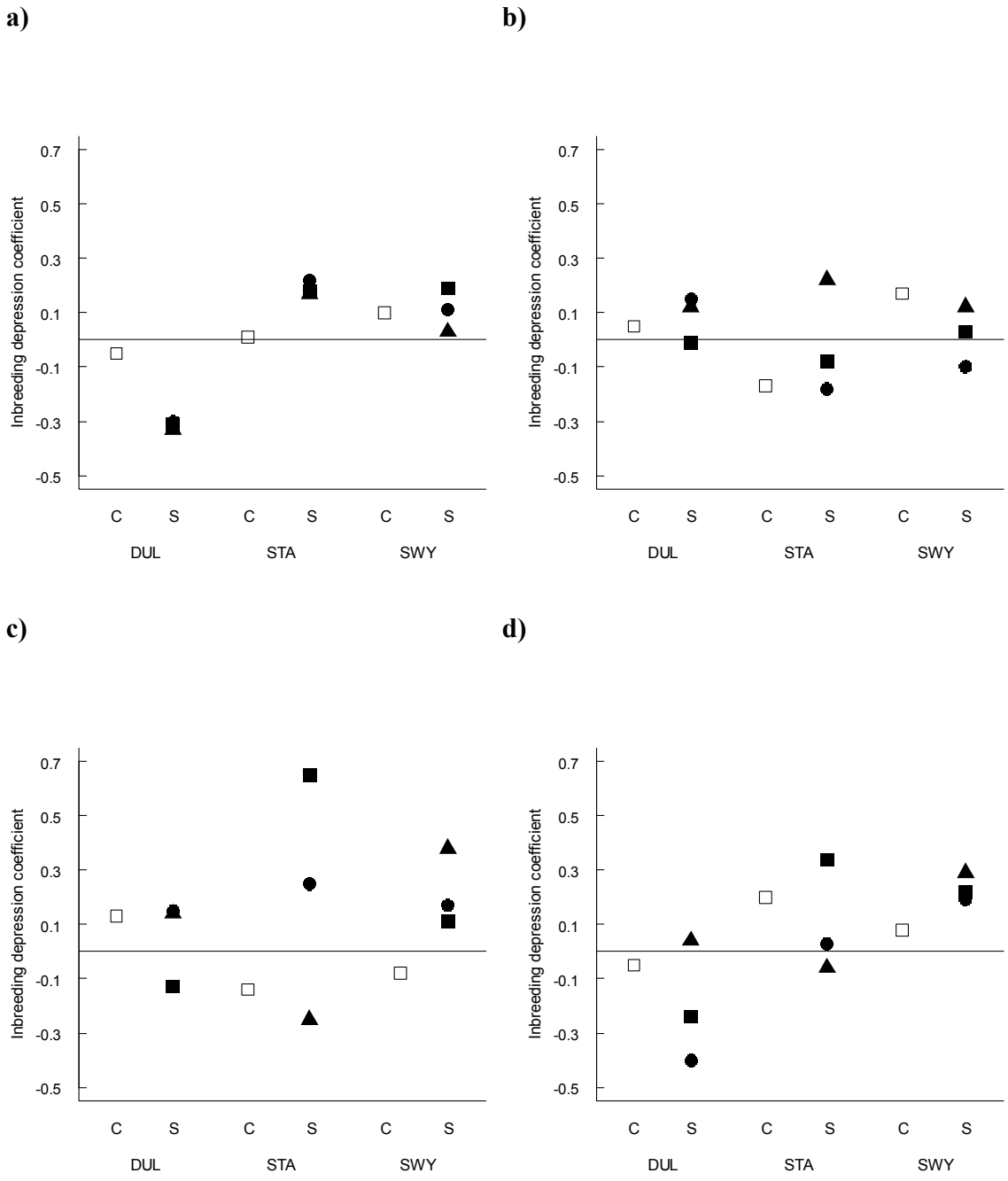


**Fig. 3.5**. The inbreeding depression coefficients in **a)** plant height, **b)** number of flowers, **c)** herbivore resistance and **d)** sinigrin production from the populations Durlston (DUL), St Adhelm's (STA), Swyre Head (SWY) under control conditions (C = Control; ambient water treatment and ambient $CO_2$ levels), and under stress conditions (S = stress conditions; ■ = $eCO_2$, ambient water treatment; ▲ = $aCO_2$, drought; ● = $eCO_2$, drought)

# Chapter 4: Mixed herbivore defence strategies in the zinc hyperaccumulator *Noccaea caerulescens*

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Prepared for journal submission

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Keywords: heavy metal hyperaccumulation, plant-herbivore interactions, *Noccaea caerulescens*, resistance, tolerance, secondary metabolites

## 4.1 Summary

- The toxic and feeding deterrent effects of plant heavy metal hyperaccumulation on herbivores are well known, but the effects of heavy metal hyperaccumulation on other aspects of plant-herbivore interactions, such as constitutive and induced glucosinolate production as well as tolerance to herbivores and the role of parasitoids have been rarely studied.
- We investigated the effects of Zn concentration in the soil (no Zn, medium Zn [2000mg/kg soil], and high Zn [5000mg/kg soil]) on the interactions with *Noccaea caerulescens* with a generalist (*Mamestra brassicae*) and a specialist (*Pieris rapae*) herbivore using plants from two populations that differ in their glucosinolate

compounds. Herbivore resistance and tolerance were determined in a no-choice experiment, and induced defence was determined with a feeding choice experiment and glucosinolate analyses. We examined if parasitoid infestation by *Hyposoter ebeninus* changed the feeding choice of *P. rapae* larvae between plants grown on different Zn soil concentrations.

- We found that Zn soil addition increased herbivore resistance. In turn, plants grown on non-Zn-contaminated soil were more tolerant to herbivore damage. Zn soil contamination did not affect constitutive and induced glucosinolate concentration. However, glucosinolate production was reduced in plants grown on Zn-contaminated soil after the herbivore treatment, indicating that there is a trade-off between Zn hyperaccumulation and glucosinolate production, either because herbivore induced responses last longer in non-Zn-treated plants or due to changes in glucosinolate production during the plant life cycle. Parasitoid infestation diminished the effects of heavy metal hyperaccumulation on herbivore resistance, as herbivore damage was equalized across all Zn treatments in the choice experiment with *P. rapae* and its parasitoid *Hyposoter ebeninus*.
- Our findings indicate that herbivore-induced responses and tolerance may have facilitated the evolution of heavy metal hyperaccumulation, and that top-down pressure could weaken the selection pressure on the production of organic defence compounds.

## 4.2 Introduction

Heavy metal hyperaccumulating plants can grow in soil polluted with substantial quantities of heavy metals, such as Zn, Cd and Ni, that would be toxic to other plant species (Rascio & Navari-Izzo 2011). Several explanations have been put forward as to why heavy metal hyperaccumulation has evolved, including metal tolerance, drought resistance, and defence against natural enemies. The elemental defence hypothesis states that heavy metal hyperaccumulation evolved as a defence against natural enemies, such as herbivores, and regards heavy metals as inorganic defence compounds (Martens & Boyd 1994). There is ample evidence for the toxic (Butler & Trumble 2008) and feeding deterrent ( Pollard & Baker 1997; Behmer et al. 2005) effects of several metals, including Zn, on herbivores. A meta-analysis by Butler & Trumble (2008) demonstrated that leaf-chewing and leaf-mining herbivores in particular suffered reduced survival, weight, growth and fecundity after they had been feeding on heavy metal hyperaccumulators growing on metal-rich soils.

The more recent trade-off hypothesis postulates that inorganic and organic (secondary metabolites) defence compounds have combined positive effects on plant herbivore resistance, which lead to a reduced production of organic defence compounds when plants accumulate heavy metals (Boyd 2007). The trade-off hypothesis regards organic defence compounds as agents that facilitated the evolution of heavy metal hyperaccumulation by enhancing the defensive effect of inorganic defence compounds (Boyd 2012b). Few studies have attempted to test this hypothesis. Reductions in the concentration of organic defence compounds following Zn hyperaccumulation have been observed within a single species (Tolrà et al. 2001) and when comparing a metal hyperaccumulating species with a closely related non-

metal hyperaccumulating species (Davis & Boyd 2000), indicating that there might be a trade-off between elemental and organic defence compounds.

Most studies of herbivory in heavy metal hyperaccumulators have used herbivore damage to estimate plant resistance. The effects of heavy metal hyperaccumulation on other aspects of plant-herbivore interactions, such as induced defence, tolerance to herbivores and plant-herbivore-parasitoid interactions have been studied in much less detail. The dependence of heavy metal hyperaccumulators on heavy metals as defence compounds against pathogens has resulted in reduced ability to induce disease resistance (reviewed by Fones & Preston 2013), but it is unclear whether this can be applied to induced defence against herbivores as well. Only two studies analysed the effects of heavy metal hyperaccumulation on the induction of organic defence compounds and subsequently on herbivory, and reported no change (Davis et al. 2001) and even a reduction (Asad et al. 2014) in organic defence compounds.

Herbivore tolerance, the other major defence strategy in plants alongside resistance, has been studied by Palomino et al. (2007) who reported that herbivore damage was compensated for significantly better in plants growing in Ni-enriched soils than plants growing on soils without Ni. The effects of organic defences on higher trophic levels, such as parasitoids, are well studied and show how these higher trophic levels can mediate herbivore impacts (Harvey et al. 2011). It is unknown, however, how heavy metal accumulation affects these interactions. Parasitoids can change the behaviour of their hosts (Adamo et al. 1995) and may therefore influence the feeding choice of their hosts among plants grown on different soil concentrations of heavy metals.

Here we investigated the effects of Zn-contaminated soil on plant herbivore resistance and tolerance against generalist and specialist herbivores in two populations of *N. Caerulescens*. The Zn-accumulating *Noccaea caerulescens* is one of the model species for heavy metal hyperaccumulation. The main organic defence compound produced by *N. caerulescens* is sinalbin, a glucosinolate. Recently, a new glucosinolate has been characterized in specific *N. caerulescens* populations from Southern France; glucomoringin, a rhamnose derivate of sinalbin (de Graaf et al. 2014; in press), that has not been found in the Brassicaceae before. It is as yet unknown how glucomoringin affects herbivores.

We addressed the following research questions:

1) Does Zn soil contamination reduce the production of glucosinolates and therefore herbivore induced responses?
2) How does Zn soil contamination affect herbivore resistance and tolerance?
3) Can herbivores distinguish between plants grown on different soil Zn concentrations and does this feeding choice change when they are parasitized?
4) Do sinalbin and glucomoringin have different effects on herbivore resistance against a generalist and a specialist herbivore?

# 4.3 Materials & Methods

### Study species and Zn treatment

*Noccaea caerulescens* (J. & C. Presl) F.K. Mey (Brassicaceae) is a self-compatible, short-lived perennial, metal hyperaccumulator of Zn, Cd, and Ni that occurs in Scandinavia as well as central and western Europe (http://data.gbif.org/welcome.htm). It grows on metal-

contaminated and uncontaminated soils (MacNair 2007). Two populations of *N. caerulescens* were used in the experiment: "Ganges"; St. Felix, Southern France, and "Prayon"; Belgium. Both populations are located close to previous Pb/Zn-mining areas (Robinson et al. 1998; Meerts & Grommesch 2001) and have similar capacities for Zn hyperaccumulation (Lombi et al. 2000). The generalist herbivore *Mamestra brassicae* L. (Noctuidae) feeds on several plant families, including the Brassicaceae (Popova 1993), whereas the more specialist herbivore *Pieris rapae* L. (Pieridae) has a host range within the Brassicaceae and is able to tolerate glucosinolates (Schoonhoven et al. 2005).

Seeds from the Ganges and Praypon populations were sown in trays with sand in November 2010. The trays were watered every day. Fourteen days after seedling emergence, the plants were randomly assigned to trays with potting compost containing different concentrations of added Zn (as ZnO): a) control (no Zn addition), b) medium (2000mg/kg soil) and c) high (5000mg/kg soil) Zn content. These concentrations are comparable to those observed in these populations, and they have been applied in previous studies on Zn hyperaccumulation in *N. caerulescens* (Lombi et al. 2002; Noret et al. 2007). The trays were put in a growth chamber (18°C/22°C, 16-hour light, 8-hour dark cycle) and watered twice each week. Four weeks after germination, the plants were moved from the growth chamber to a glasshouse (minimum night temperature 15°C/ minimum day temperature 21°C, no additional lights).

## No-choice experiment

A no-choice experiment was conducted to determine the effects of Zn soil contamination on glucosinolate production as well as herbivore resistance and tolerance. Initially we had 30 replicate plants per population per Zn treatment, but due to mortality the final number of

replicates ranged between 24 and 29, and there were only 13 replicates for the high Zn treatment for the Prayon population. *Pieris rapae* larvae were reared on cultivated cabbage (*Brassica oleracea*) leaves before they were put on the experimental plants. *Mamestra brassicae* larvae were reared on the semi-synthetic Hoffman's tobacco hornworm diet (Smith 1966). The larvae from both herbivore species were in the 3rd instar when they were put on the plants in February 2011, three months after the seeds had been sown.

To estimate plant size, the number of leaves was counted and the rosette diameter was measured before the herbivores were put on the plants. The plants were put in plastic bags with small holes to prevent herbivore escape. The herbivores were removed from the plants after one week and their survival was assessed (dead or alive). The overall leaf area and the damaged leaf area were measured in square millimetres with graph paper and percent damage was calculated. Two weeks after the end of the herbivore treatment, the rosette diameter was measured and the leaves were counted again to obtain estimates of plant growth.

## Choice experiments

Choice experiments were conducted at the same time as the no-choice experiment to investigate whether herbivores distinguish between plants with varying metal contents and avoid feeding on plants with high metal content. They were also done to examine the effects of herbivore induction and parasitoid infestation on generalist and specialist herbivore preference. Middle-aged leaves of similar size were chosen for the experiment and the experiments were conducted in petri-dishes. We conducted two choice experiments with *M. brassicae*; one with non-induced leaves (Zn choice experiment) and one with induced leaves (induction choice experiment). In the induction choice experiment, the larvae were presented with leaves from induced (damaged) vs non-induced (undamaged) plants from the same

population under the same Zn treatment. Induced leaves were collected from plants that had been used in the no-choice experiment with *P. rapae*. Specialist herbivores are known to induce the production of plant secondary metabolites (van Zandt & Agrawal 2004; Viswanathan et al. 2005) and the aim of the experiment was to determine if this is also the case in heavy metal hyperaccumulators. In the Zn choice experiment, the larvae were presented with one (non-damaged) leaf from plants of the same population from each control vs medium vs high Zn treatment. We did not use high Zn leaves from the Prayon population, because this population produced very few leaves under the high Zn treatment. Each treatment was replicated ten times in the Zn choice experiment and five times in the induction choice experiment.

In the Zn choice experiment with *P. rapae*, the same procedure was used as in the Zn choice experiment with *M. brassicae*. In addition, to test if being parasitized affects the ability of the larvae to choose between plants grown on different amounts of Zn in the soil, randomly selected half of the larvae were parasitized by *Hyposoter ebeninus* Gravenhorst (Ichneumonidae). *H. ebeninus* is an endoparasitoid specialized on *Pieris* (Harvey et al. 2010) and commonly used in plant-herbivore-parasitoid experiments (e.g. Kos et al. 2012). The adults were allowed to mate and then females were selected for the experiment. *P. rapae* larvae were presented individually to a parasitoid on a damaged leaf until oviposition (parasitoid infestation) occurred. In all leaf choice experiments, the larvae were allowed to feed on the leaves for two hours. Then the amount of damaged leaf area was determined with the help of graph paper.

## Glucosinolate analysis

Leaf samples were taken before the herbivore treatment and 4 weeks after the herbivore treatment. Because of the small leaf size, seven middle-sized leaves were taken from each plant for the glucosinolate analysis. Leaves were stored at -80°C immediately after sampling. The samples were freeze-dried, weighed into a micro-centrifuge tube and ground. Sinalbin and glucomoringin were extracted and purified using the methods developed by van Dam et al. (2004) and Kabouw et al. (2010). Glucosinolate content was analysed with high-performance liquid chromatography-photodiode array (HPLC-PDA) as outlined by de Graaf et al. (2015).

## Statistical analyses

For the no-choice experiment, ANOVA was conducted using R (3.1.1) to test for the effects of the Zn treatment on plant growth and plant herbivore resistance. Damaged leaf area, percent damage, rosette diameter and number of leaves before and after the herbivore treatment, sinalbin and glucomoringin concentration were used as response variables. Population and Zn treatment were used as explanatory variables for the response variables that were measured before the herbivore treatment, and population, Zn treatment and herbivore treatment were used as explanatory variables for the response variables that were measured after the herbivore treatment. All two- and three-way interactions were included in the model. Damaged leaf area and sinalbin concentration were log-transformed to meet the assumptions of the analyses. Plant survival under the different Zn treatments was analysed with a chi-square test. The survival of *P. rapae* larvae was analysed with a general linear model (GLM) containing

square-root transformed survival as response variable and population and treatment as well as their interaction as explanatory variables.

Tolerance was defined as the slope of the regression between herbivore damage and plant growth (Strauss & Agrawal 1999). Positive slopes indicate overcompensation, slopes with values around zero indicate full compensation and negative slopes indicate undercompensation in response to herbivory (Strauss & Agrawal 1999). The lower or more negative the slope, the lower is herbivore tolerance. Tolerance was therefore defined as a norm of reaction under different Zn levels in the soil (Simms 2001). To investigate the effects of the Zn treatment and population on tolerance, ANCOVA was conducted with the number of leaves after herbivory as response variable and Zn treatment and population as explanatory variables and damaged leaf area as covariate as well as all two-way and three-way interactions according to the analyses used in previous studies on tolerance (Mauricio et al. 1997; Tiffin & Rausher 1999). A significant interaction between Zn treatment and damaged leaf area would indicate differences in tolerance among Zn treatments.

For the choice experiment, repeated measures ANOVA was conducted using SAS statistical package version 9.1 to investigate the effects of the Zn treatment, population identity and induction on herbivore feeding preferences. Tukey *post-hoc* tests were conducted to determine significant differences between treatments. Damaged leaf area, percentage damage and sinalbin were log-transformed and leaf number and glucomoringin concentration were square-root-transformed to meet the assumptions of the analysis.

## 4.4 Results

### No-choice experiment

*Effects of Zn on plant growth and herbivore resistance*

Before herbivory, plants from both populations produced significantly more leaves and had greater rosette diameters in the control and medium Zn treatments than in the high Zn treatment (Table 4.1; Fig. 4.1), suggesting that Zn addition slows down growth. The greatest number of leaves was produced by plants from the Prayon population under the control Zn treatment (Fig. 4.1a). Plants from the Ganges population had greater rosette diameters than plants from the Prayon population irrespective of the Zn treatment (Table 4.1; Fig. 4.1b). Survival rates differed between the populations under the high Zn treatment: 43 % of the plants from the Prayon population and 87 % of the plants from Ganges populations survived in the first three months under the high Zn treatment ($\chi^2 = 6.08$, *d.f.* =1, $P = 0.014$). At the end of the experiment, 23 % of the plants from the Prayon population and 70 % of the plants from the Ganges population had survived under the high Zn treatment ($\chi^2 = 7.00$, *d.f.* =1, $P = 0.008$), compared to 83 % and 94 %, respectively, in the control suggesting that plants from the Ganges population had greater Zn tolerance than plants from the Prayon population.

Both herbivores caused significantly more percent damage in the control compared to the medium and high Zn treatments (Table 4.2; Fig. 4.2a,b), indicating greater herbivore resistance in plants grown on Zn-contaminated soil. Plants from the Ganges population showed higher percent damage than plants from the Prayon population, indicating lower resistance in the Ganges population (Table 4.2; Fig. 4.2a). The generalist herbivore *Mamestra*

*brassicae* caused more percent damage than the specialist herbivore *Pieris rapae* (Table 4.2; Fig. 4.2b).

*Effects of Zn on glucosinolates and herbivore performance*

Plants from the Prayon population produced in general nearly ten times more sinalbin (mean 16.10 µmol/g DW ± 0.54 SE) than plants from the Ganges population (1.90 µmol/g DW ± 0.53 SE; Table 4.1). Before the herbivore treatment, the sinalbin concentration was significantly affected by the Zn treatment (Table 4.1), but there were not enough data points to support this finding in the Tukey *post-hoc* test (Fig. 4.3a). After the herbivore treatment, plants from both populations had higher concentrations of sinalbin in the control compared to the high Zn treatment (Fig. 4.3a,b), supporting the trade-off hypothesis.

Glucomoringin was only found in plants from the Ganges population (mean 29.11 µmol/g DW ± 0.79 SE). Before herbivory, plants did not substantially differ in the production of glucomoringin (Fig. 4.3c). After herbivory, plants produced less glucomoringin under both Zn treatments compared to the control Zn treatment (Fig. 4.3c). These results are also in line with the trade-off hypothesis.

The survival of *P. rapae* was significantly lower under the medium (survival rate 22 %, $P = 0.001$) and high Zn treatments (9 %, $P = 0.001$) compared to the control (58 %), which confirms the toxic effect of Zn soil contamination on herbivores.

*Plant tolerance to herbivores*

The significant Zn × damage interaction for the number of leaves after herbivory indicated that the regression of growth on damage was different for different Zn treatments (Table 4.3). Since population and its interactions were not significant, they were omitted in further analyses. The regression of the number of leaves on damage was positive under the control Zn

treatment (Fig. 4.4), so the greater the damage, the higher the number of leaves, indicating high tolerance and overcompensation. Under the medium and high Zn treatment, there was no significant relationship between herbivore damage and number of leaves (Fig. 4.4). To test whether this lack of a relationship could be attributed to the fact that there were lower levels of damage under the medium and high Zn treatment compared to the control Zn treatment, a new data set was constructed comprising plants from all three Zn treatments that suffered "high damage" (damaged leaf area > 600mm$^2$, $n$=58). 600mm² are equivalent to 6% invertebrate herbivore damage, the mean level of invertebrate herbivore damage recorded in field experiments in general (Scherber et al. 2006) and field experiments with *N. caerulescens* (Noret et al. 2007) in particular. The average amount of leaf material in each *N. caerulescens* plant was 10000mm². Using this data set, the regression of damage on the number of leaves was again not significant ($F_{1,55}$ = 0.38, $P$ = 0.542, $r^2$ = -0.011), indicating that the positive regression of the number of leaves on damage under the control Zn treatment cannot be attributed solely to the fact that there was greater damage under the control Zn treatment.

Based on these results, we conclude that the investments in resistance, tolerance and glucosinolate production differed among the different Zn treatments. In the control, there was high glucosinolate production and, yet, high herbivore damage and therefore low resistance. However, there was also high tolerance (overcompensation for herbivore damage) under the control Zn treatment. Under the medium Zn treatment, there was lower glucosinolate production at the later time point of sampling, higher resistance, but lower tolerance compared to the control. Under the high Zn treatment, there was the same level of resistance and the same level of tolerance as under the medium Zn treatment.

### Choice experiments

In the Zn choice experiment, the generalist herbivore *M. brassicae* caused significantly more damage on leaves from plants from both populations grown under the control (mean damage 24.95 ± 5.45 SE mm², $n$=20) and medium Zn treatment (22.95 ± 5.45 mm², $n$=20) compared to the high Zn treatment (4.81 ± 6.06 mm², $n$=16) ($F_{2,32}$=4.72, $P$=0.016). In the induction choice experiment, *M. brassicae* preferred the non-induced leaves (mean damage 12.33 ± 2.01 mm², $n$=21) to the induced leaves (5.70 ± 2.06 mm², $n$=20) ($F_{2,14}$=5.31, $P$=0.037). There was no significant difference among Zn treatments, as indicated by the non-significant treatment × induction interaction. This suggests that Zn addition to the soil did not affect herbivore induction.

*P. rapae* larvae that were not parasitized preferred feeding on leaves from plants grown under the control compared to the medium and high Zn treatment (control Zn: mean damage 9.29 ± 1.86 mm²; medium Zn: 2.29 ± 1.86 mm²; high Zn: 1.14 ± 1.86 mm²). Parasitized larvae consumed less leaf material than non-parasitized larvae, thereby equalizing the differences between the Zn treatments (control: mean damage 4.11 ± 1.00 mm²; medium Zn: 2.17 ± 1.00 mm²; high Zn treatment 1.31 ± 1.00 mm²) ($F_{2,36}$=7.73, $P$=0.009).

## 4.5 Discussion

We found evidence for the elemental defence hypothesis, which states that the accumulation of heavy metals increases herbivore resistance (Martens & Boyd 1994). Herbivore resistance was greater after the heavy metal hyperaccumulating *N. caerulescens* had been grown on soil with greater Zn content. The initially slower growth of plants grown on metal-contaminated soil is set off by the lower herbivore damage they face when accumulating heavy metals. This

indirectly confirms the hypotheses that attribute herbivory as a factor facilitating the evolution of heavy metal hyperaccumulation (Martens & Boyd 1994; Boyd 2012). We found no substantial differences in glucosinolate production at the beginning of the experiment, but after the herbivore treatment, the production of glucosinolates was reduced in plants grown on Zn-contaminated soil, which supports the trade-off hypothesis (Boyd 1998). Sinalbin has been shown to decrease in plant shoots when *N. caerulescens* is grown on Zn-contaminated soil (Tolrà et al. 2001) and lower levels of glucosinolates were reported in *N. caerulescens* populations from metal-contaminated habitats compared to non-metal-contaminated habitats (Noret et al. 2007). The reason why the reduction in the glucosinolate concentration occurred only at the later time point of sampling could be that the trade-off between the production of organic defence compounds and metal hyperaccumulation occurs at a specific time point in the life cycle of *N. caerulescens*. This is confirmed by the fact that the concentration of glucomoringin was generally reduced at the second sampling time point. The relative content of different glucosinolate compounds have been shown to change during the life cycle of the perennial metal hyperaccumulator *Thlaspi praecox* (Pongrac et al. 2008), which is related to *N. caerulescens*. It is possible that differences in herbivore induction between plants grown on different concentrations of Zn produced the trade-off, even though the results from the induction choice experiment suggest that initially heavy metal hyperaccumulators invest more in organic defence compounds upon herbivore attack irrespective of the amount of Zn in the soil. The induced response could, however, last longer in plants grown on soil without added Zn. A sole dependence on heavy metals as defence compounds therefore seems to be limited to pathogen defence (Fones & Preston 2013), and does not apply to herbivore defence. A previous study reported the lack of the induction of organic defence compound production

after simulated herbivory in a Ni-hyperaccumulator (Davis et al. 2001), but plants were grown on Ni-contaminated soil only and not on non-contaminated soil.

Our findings thus imply that mixed defence strategies are maintained in heavy metal hyperaccumulators, presumably due to varying herbivore pressure. Plants under the control Zn treatment allocated resources both to resistance (higher glucosinolate production) and tolerance, which reduced herbivore damage and the negative effect of herbivory on fitness (definition of tolerance by Pilson (2000)). Herbivores can select for mixed defence strategies in plants (Carmona & Fornoni 2013) and in natural plant populations, both resistance and tolerance are commonly maintained at intermediate levels (Leimu & Koricheva 2006; Núñez-Farfán et al. 2007). Tolerance and the production of organic defence compounds may be maintained in heavy metal hyperaccumulators, since inorganic defence compounds do not serve as universal weapons against all natural enemies. Herbivores can cause substantial damage in heavy metal hyperaccumulators especially early in the life cycle (Huitson & MacNair 2003), which may also be the reason for the greater concentrations of glucosinolates under the medium and high Zn treatment at the first sampling time point. Herbivores that attack heavy metal hyperaccumulators do not seem to be able to taste or smell the metals, since they fed on the offered leaf material when it was presented to them in our choice experiments, including leaves from plants grown on soil with high Zn concentrations, as has been shown for desert locusts and Ni-hyperaccumulating plants (Behmer et al. 2005). Moreover, some herbivore species have adapted to host plants containing high tissue metal concentrations (reviewed by Boyd 2009), and phloem-feeders (Boyd & Martens 1999) as well as generalist herbivores (Noret et al. 2005) have not been deterred by heavy metal-containing plant tissue, but by organic defence compounds. Our induction choice experiment also

indicated that generalists are strongly deterred by the induced defence response. A change of investment in tolerance under different environmental conditions (Simms 2001) could have played a role in the evolution of heavy metal hyperaccumulation because tolerance could enhance the defence effect of Zn accumulation, as has been suggested for organic defence compounds (Boyd 2012b).

Parasitoids seemed to equalize the differences between Zn treatments on specialist herbivore damage. This finding implies that the top-down pressure exerted by the natural enemy is as strong as the bottom-up pressure exerted by the inorganic defence compounds. Parasitoid infestation also equalized survival rates of leaf-chewing butterfly larvae that fed on plants grown on Ni-amended or control soil (Martens & Boyd 1994). Accordingly, the top-down pressure could offset the advantage of inorganic defence against herbivores when the herbivores are weakened or die early after parasitoid attack, which could reduce the selection pressure on the production of volatiles that attract parasitoids specialized on herbivore Brassicaceae specialists (Santolamazza-Carbone et al. 2014) in metal hyperaccumulators. This could also serve as an additional explanation for the reduced production of glucosinolates in plants grown on Zn-contaminated soil. In turn, the accumulation of heavy metals in herbivores after feeding on heavy-metal-containing plant tissue can have negative effects on their natural enemies (Boyd & Wall 2001; Vickerman & Trumble 2003), which could weaken top-down pressure and therefore counteract the reduction of organic defence compounds in the plants.

Sinalbin seemed to cause greater herbivore resistance than glucomoringin as indicated by the differences in herbivore resistance in plants from the glucomoringin-producing Ganges vs the non-glucomoringin-producing Prayon population. However, the overall greater damage in plants from the Ganges population could also be attributed to the fact that their leaves were

thinner and therefore more palatable than leaves of plants from the Prayon population (personal observation). Here only leaf-chewing herbivores were assessed, but glucomoringin might serve as efficient defence compound against phloem-feeding herbivores, such as aphids, which have been shown to be unaffected by high metal concentrations in the leaves (Jhee et al. 2005). Since glucomoringin is more water-soluble than sinalbin due to the additional sugar, it can probably be distributed greater distances in the soil and may therefore serve as efficient defence compound against root herbivores and pathogens (de Graaf et al. 2015). Glucosinolates increase in roots compared to shoots upon metal hyperaccumulation in *N. caerulescens* (Tolrà et al. 2001). More experiments above-ground and below-ground are therefore necessary that carefully assess the effects of glucomoringin on herbivores in feeding trials.

We conclude that mixed defence strategies, including herbivore tolerance and induced defence responses, are maintained in heavy metal hyperaccumulators, which may have facilitated the evolution of heavy metal hyperaccumulation. Top-down pressure could weaken the selection pressure on the production of glucosinolates in heavy metal hyperaccumulators. We suggest that herbivore tolerance and potential trade-offs between metal hyperaccumulation, herbivore defence, tolerance and plant fitness should be quantified in terms of metabolic costs in future studies on heavy metal hyperaccumulation.

# 4.6 Tables and Figures

**Table 4.1.** The results of ANOVA testing for the effects of Zn content in the soil and population identity and their interactions on the number of leaves, rosette diameter and sinalbin concentration before the herbivore treatment.

| | ***Number of leaves*** | | | ***Rosette diameter*** | | |
|---|---|---|---|---|---|---|
| Source of variation | ***Df*** | ***F*** | ***P*** | ***Df*** | ***F*** | ***P*** |
| Zn | 2 | 42.92 | *** | 2 | 26.07 | *** |
| Population | 1 | 7.75 | ** | 1 | 87.73 | *** |
| Zn × Pop | 2 | 5.84 | ** | 2 | 2.15 | n.s. |
| Error | 139 | | | 138 | | |

| | ***Sinalbin*** | | |
|---|---|---|---|
| Source of variation | ***Df*** | ***F*** | ***P*** |
| Zn | 2 | 3.611 | * |
| Population | 1 | 224.77 | *** |
| Leaves | 1 | 0.17 | n.s. |
| Zn × Pop | 2 | 0.78 | n.s. |
| Error | 119 | | |

**Table 4.2.** The results of ANOVA testing for the effects of Zn content in the soil, herbivore species (generalist *Mamestra brassicae* and specialist *Pieris rapae*), population (Ganges and Prayon) and their interactions on damaged area, percent damage, sinalbin concentration, glucomoringin concentration, rosette diameter and number of leaves.

***Percent damage***

| Source of variation | ***Df*** | ***F*** | ***P*** |
|---|---|---|---|
| Zn | 2 | 76.97 | *** |
| Herbivore | 1 | 6.35 | * |
| Population | 1 | 12.50 | *** |
| Leaves | 1 | 0.85 | n.s. |
| Zn × Herbiv | 2 | 0.70 | n.s. |
| Zn × Pop | 2 | 2.22 | n.s. |
| Herbiv × Pop | 1 | 0.04 | n.s. |
| Zn × Herbiv × Pop | 2 | 1.76 | n.s. |
| Error | 103 | | |

***Sinalbin after herbivory***

| Source of variation | ***Df*** | ***F*** | ***P*** |
|---|---|---|---|
| Zn | 2 | 16.33 | *** |
| Herbivore | 1 | 1.15 | n.s. |
| Population | 1 | 53.58 | *** |
| Leaves (after herbivory) | 1 | 0.07 | n.s. |
| Zn × Herbiv | 2 | 0.15 | n.s. |
| Zn × Pop | 2 | 0.10 | n.s. |
| Herbiv × Pop | 1 | 0.03 | n.s. |
| Error | 33 | | |

**Table 4.3.** The results of ANCOVA testing for the effects of Zn addition to the soil, population, damaged leaf area and their interactions on the number of leaves after herbivory.

| | Number of leaves | | |
|---|---|---|---|
| Source of variation | Df | F | P |
| Zn | 2 | 11.67 | *** |
| Population | 1 | 0.05 | n.s. |
| Damaged area | 1 | 1.09 | n.s. |
| Pop × Zn | 2 | 0.69 | n.s. |
| Pop × Damage | 1 | 0.59 | n.s. |
| Zn × Damage | 2 | 3.46 | * |
| Zn × Pop × Damage | 2 | 0.01 | n.s. |

**a)** **b)**

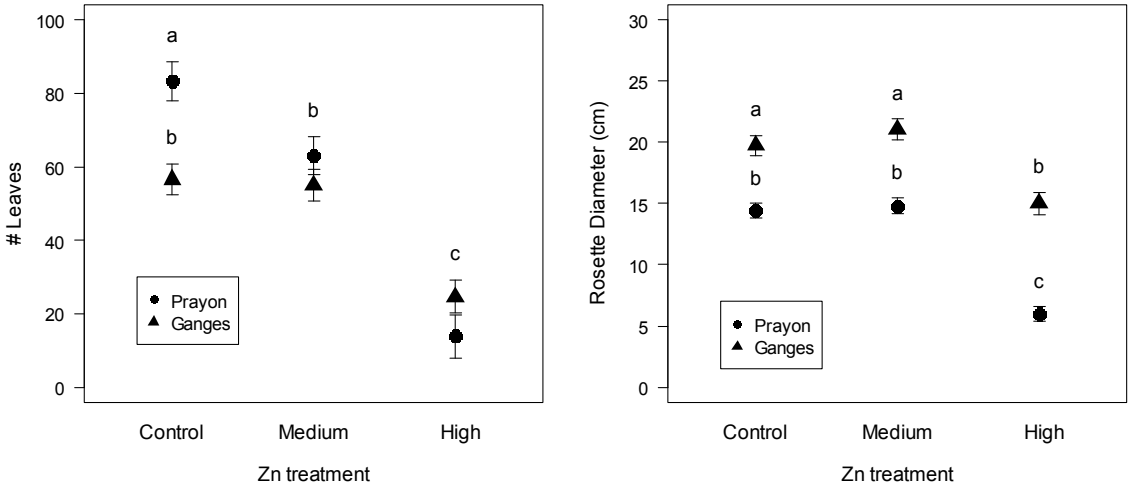



**Fig. 4.1**. Plant growth in both populations under the different Zn treatments. a) Number of leaves, b) rosette size in the populations Prayon and Ganges.

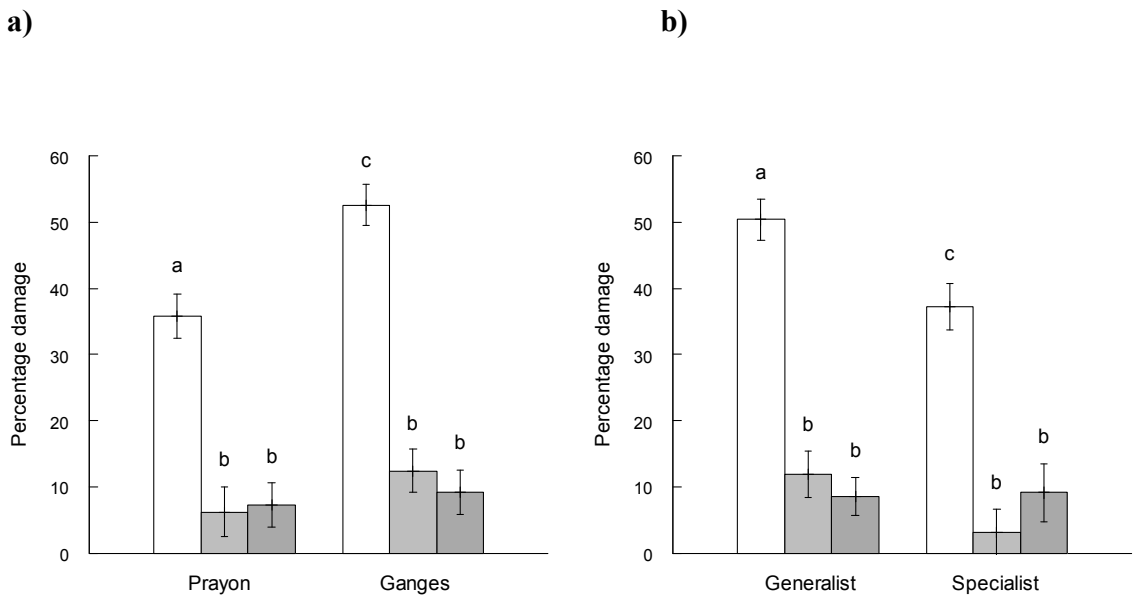



**Fig. 4.2.** Differences in percentage damage under the different Zn treatments a) among both populations and b) caused by the generalist (*M. brassicae*) and specialist herbivore (*P. rapae*). White: control Zn, grey: medium Zn, dark grey: high Zn treatment.

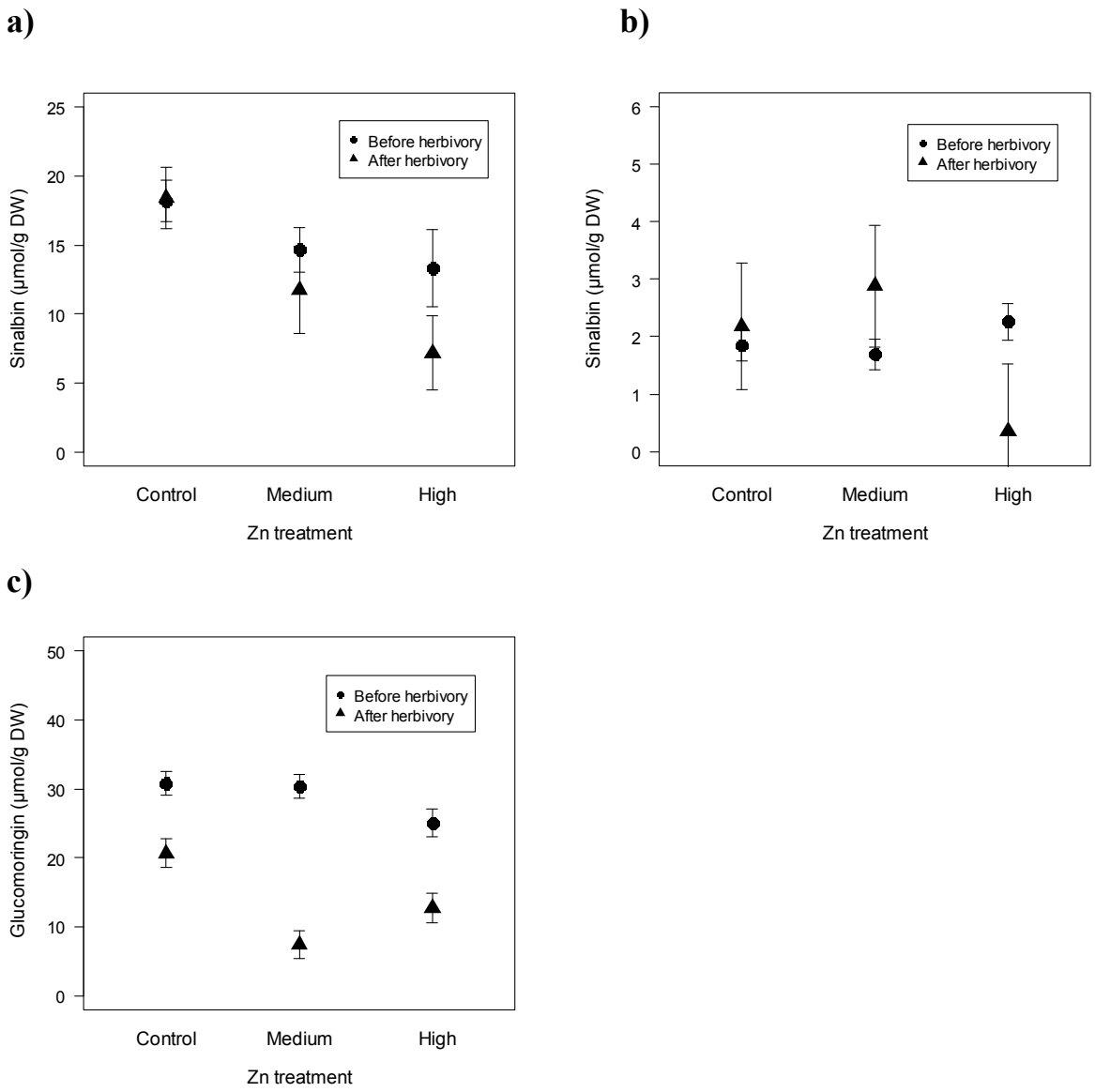



**Fig. 4.3**. Glucosinolate concentration before and after the herbivore treatment.a) Sinalbin concentration in plants from the Prayon and b) Ganges population.c) Glucomoringin concentration in the Ganges population.

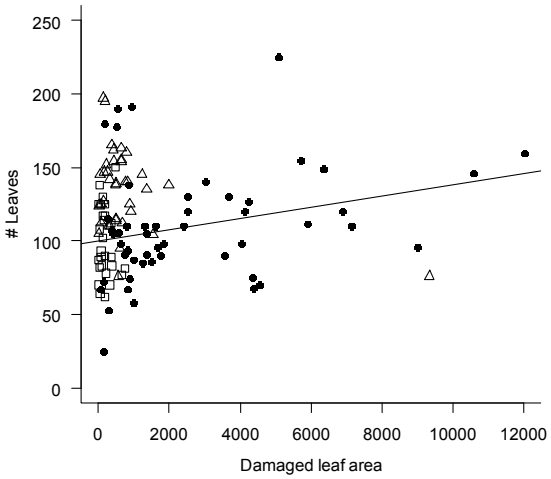



**Fig. 4.4.** Regression of the number of leaves on herbivore damage a) under the control (filled dots), medium (triangles), high (squares) Zn treatment. (The slope is the regression in the control treatment. ($F_{1,49}$=6.51, P=0.014, $r^2$ = 0.100,). slope (m) = 0.10)

# Chapter 5: General Discussion

This chapter will discuss the findings from the experiments carried out and reported in this thesis (chapter 2, 3, and 4) and bring them together in a broader context of ecology, evolution, and conservation management. The preceding methodological discussion elaborates on experimental and statistical methods.

## 5.1 Methodological discussion

### Measurements and design

*Trait measurements*

The measurement of the production of plant defence compounds was central to the characterisation of plant-herbivore interactions in all three experiments. The accuracy of the used methods is therefore essential. In both experiments with *Brassica nigra*, sinigrin was extracted, purified and analysed by high-performance liquid chromatography using the methods developed by van Dam *et al.* (2004) and Kabouw *et al.* (2010). The observed increase in the concentration of sinigrin under the drought stress treatment in the common garden experiment could also be interpreted as a dilution effect, because the plants were smaller under the drought treatment and thus the same amount of sinigrin would lead to a higher concentration. To determine if this was the case the relationship between the amount of sinigrin and the aboveground biomass was tested using a regression analysis. The result was not significant ($F = 1.47$, $P = 0.23$, $r^2 = -0.14$,). This demonstrates that the higher concentration of sinigrin under the drought treatment cannot be attributed to a dilution effects due to smaller plant size. Furthermore, if aboveground biomass is added as covariate to the mixed model for

sinigrin, it is not significant ($P = 0.56$) and does not change the outcome of the model stating that the concentration of sinigrin significantly increases under the drought treatment (compare chapter 2.8).

Additional leaf measurements, such as specific leaf area, the C:N ratio, nutrient content and leaf toughness would have been useful to obtain mechanistic explanations of the results found in all three experiments. In the greenhouse experiment with *B. nigra*, the effects of drought and elevated $CO_2$ on the production of sinigrin could not explain the diminished difference in resistance between the drought and water treatment under elevated $CO_2$. Previous studies have demonstrated that elevated $CO_2$ increases the C:N ratio and therefore lowers the nutrient content of leaves, which causes compensatory feeding and decreases herbivore resistance (Massad & Dyer 2010). At the same time, elevated $CO_2$ increases leaf toughness (Robinson et al. 2012), and elevated $CO_2$ and drought both decrease specific leaf area and therefore increase leaf thickness (Volk et al. 2000; Vuorinen et al. 2004). Herbivores tend to avoid thicker and tougher leaves (Poorter et al. 2009). The increased leaf thickness and toughness under $eCO_2$ might have counteracted the compensatory feeding due to decreased nutritional value under elevated $CO_2$, which could explain why there was increased herbivore resistance under elevated $CO_2$ even though the defence compounds had been diluted under elevated $CO_2$.

Similarly, leaf characteristics such as thickness and toughness could have helped to elucidate the observed differences among populations in terms of herbivore resistance and growth in the experiment with N. *caerulescens*. Leaves of plants from the Prayon population appeared thicker and tougher than leaves of plants from the Ganges population, which could

have contributed to the increased herbivore resistance in plants from the Prayon population under the control Zn treatment.

It would also have been useful to assess the effects of inbreeding on leaf and root characteristics. Inbreeding depression in the density of leaf trichomes (Kariyat et al. 2013), leaf growth (meta-analysis by Angeloni et al. 2011) and root growth (Koelewijn 1998; Campbell et al. 2014) have been demonstrated. A reduction of the density of leaf trichomes due to inbreeding could have played a role in the observed inbreeding depression in herbivore resistance in the greenhouse experiment with *B. nigra*. In the experiment with *N. caerulescens*, the reduced leaf growth in plants from the Prayon population could indicate inbreeding depression in this population. Unfortunately, there is no data available of inbreeding history in this population. The seeds were allocated on a population-basis and several seeds could have been obtained from the same family or from different families.

Information on root biomass would have complemented the results for aboveground biomass in the common garden experiment with *B. nigra*, since the increased allocation of resources to the roots under drought stress is a crucial adaptive strategy under drought stress (Chaves 2002). There may have been interesting interactions between inbreeding and drought stress. However, assessing the root biomass was not feasible given the large number of plants.

*Experimental design*

In the common garden experiment, the among-population crosses were chosen randomly. Because populations vary in their genetic background, as demonstrated in the mixed model and variance components analyses (see below), the results for the between-population outcrossed families could have differed substantially depending on whether the crossing partners were genetically compatible or not. However, the distances between the populations

used in the common garden experiment ranged from small (about 300-500m) to middle (2-5km) and large (7km). Using geographic distance between populations as covariate in the analysis did not reveal any significant effects of geographic distance between crossing partners in terms of the extent of heterosis or the loss of heterosis in growth and the number of inflorescences under drought stress. Seed dispersal among *B. nigra* populations in Dorset has been shown to be possible throughout these distances in a previous study (Wichmann et al. 2009). Furthermore, the disruption of co-adapted gene complexes only affects the F2 or subsequent generations, not the F1 generation (Hufford & Mazer 2003). Genetic incompatibility is therefore presumably not the sole reason for the loss of heterosis under drought stress, as outlined in the discussion in chapter 2.5. Alternative explanations for the loss of heterosis could involve new combinations of alleles in between-population outbred families that are disadvantageous under drought conditions or the fact that specialist herbivores were more abundant on between-population outbred plants and therefore reduced plants growth and investment in reproductive output under the drought treatment.

In the greenhouse experiment with *B. nigra*, the effect of the experimental treatments (elevated $CO_2$, drought stress, inbreeding) on plant-herbivore interactions was only assessed for one generalist leaf-chewing herbivore. Other herbivores from different feeding guilds or different levels of specialization may have responded differently. For example, phloem-feeding aphids are usually positively affected by elevated $CO_2$ (Bezemer & Jones 1998). Brassicaceae specialist leaf-chewing herbivores have been shown to be deterred by increased effectiveness of specific glucosinolates under elevated $CO_2$ (Klaiber et al. 2013; Landosky & Karowe 2014). It would also have been interesting to examine the combined effects of elevated $CO_2$, drought stress and inbreeding on plant-herbivore-parasitoid interactions to

understand the effects of factors related to climate change and habitat fragmentation at the community level. Drought stress and elevated $CO_2$ have complex effects on parasitoid behaviour and performance and are difficult to predict (Thomson et al. 2010). The reduced concentration of sinigrin under elevated $CO_2$ might result in reduced attraction of natural enemies and therefore lead to poorer top-down control, as demonstrated by Vuorinen et al. (2004). The among-population differences in sinigrin production and the differences in the magnitude of inbreeding depression in sinigrin production could also have led to different levels of parasitoid infestation and different effects of inbreeding on parasitoid infestation in the three different populations.

## Statistical methods

*Effect sizes*

While the mixed model analysis in chapter 2 allows the determination of the significance of the applied experimental factors (water treatment, crossing background, population identity) in terms of *P*-Values, it does not allow the quantification of the magnitude of the effects. The graphic presentation of least squares means to fulfil journal requirements makes it also more difficult to determine the magnitude of the effect. Percentages or proportions of raw data help to visualize the findings to a greater extent and shall therefore be given in the following section for the most important findings of chapter 2.

Under the drought treatment, there was no difference in height and the number of inflorescences (0%) between the within-population outbred and between-population outbred plants. Under the water treatment, between-population outbred plants were 37% taller and

produced 31% more inflorescences than within-population outbred plants. Similarly, between-population outbred plants were 11% taller and produced 6% more inflorescences than inbred plants under the drought treatment, and they were 31% taller and produced 17% more inflorescences than inbred plants under the water treatment. These proportion calculations confirm the results obtained from the mixed model in chapter 2: Heterosis in plant growth and investment in reproductive output was evident under the water treatment, but it was lost under drought stress. Since plants were not substantially smaller under the drought treatment, the increased difference between within- and between-population outbred plants in terms of height cannot be attributed to the fact that plants were generally larger under the water treatment and therefore the effects were more pronounced.

The difference between the drought and water treatment in terms of stem weevil damage was large (40% more stem weevil damage under the water treatment compared to the drought treatment), yet the standard error was very large and the effect was therefore not significant in the mixed model analysis. The minor inbreeding benefit in flea beetle abundance, as indicated by the negative inbreeding depression coefficient, is confirmed by the percentage calculations. There were 6% and 11% more beetles on within-population outbred plants compared to inbred plants under the water and drought treatment, respectively.

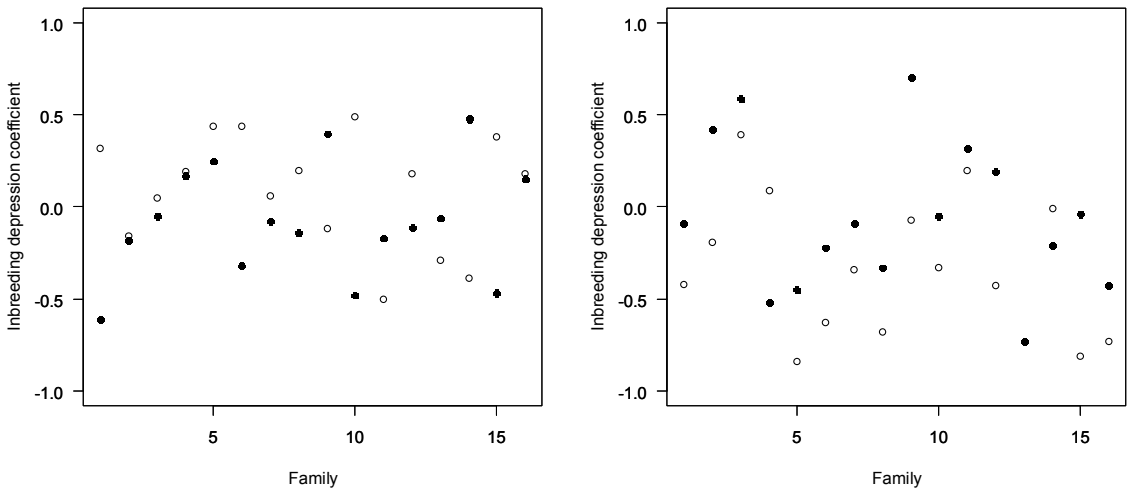
*Genetic variation in environment-dependent inbreeding depression*

The significant family × cross × water treatment interaction found for the number of inflorescences, herbivore resistance and stem weevil damage in the ID mixed model in chapter 2 indicated that there was genetic variation in environment-dependent inbreeding depression in these traits. Graphs that depict the reaction norms for inbreeding depression under different environmental conditions are appropriate to visualize genetic variation in environment-

dependent inbreeding depression (Waller et al. 2008). Fig. 5.1 also demonstrates genetic variation in environment-dependent inbreeding depression in the number of inflorescences, stem weevil abundance, and herbivore resistance.

Alternatively, variance partitioning analyses can be used that quantify the proportion of variance that was explained by variation within and among populations. Variance components analysis was conducted with the restriction maximum likelihood (REML) method that helps to accommodate for unbalanced designs (Littell et al. 2006). The variance component analysis revealed that 27% of the variation in the number of inflorescences can be explained by variation among families, while 7% of the variation in the number of inflorescences can be explained by variation among populations. 21% of the variation in stem weevil damage can be explained by variation among families, while 7% of the variation in in stem weevil damage can be explained by variation among populations. In terms of herbivore resistance, 17% of the variation was explained by variation among families, and only 3% of the variation was explained by variation among populations.

**a)** **b)**



**c)**

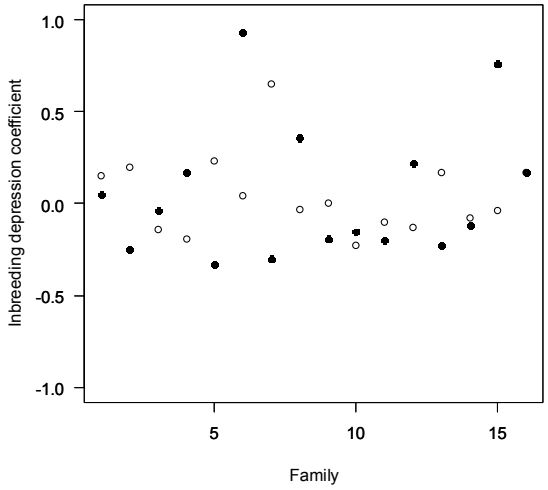



**Fig. 5.1.** The inbreeding depression coefficients for different families for a) the number of inflorescences, b) stem weevil damage, c) herbivore resistance. Filled circles denote the inbreeding depression coefficients under the water treatment, empty circles denote the inbreeding depression coefficients under the drought treatment.

These results confirm the genetic variation in environment-dependent inbreeding depression and they suggest that inbreeding should be assessed at the population level due to the large variation in environment-dependent inbreeding depression within populations. However, it should be noted that families were nested within populations in the analyses. There were 10 replicates per family per crossing background per treatment, and there were 16 maternal plants from which both inbred and within-population outbred families were obtained. In contrast, there were only 5 populations, and population was used as a fixed factor in the mixed model analysis. Larger among-family than among-population variance can therefore be expected simply because there were more families than populations, and the mean of several families was the trait mean value for one population, so the difference between variations within vs among populations should be interpreted with caution.

As a final consideration, the large variation among families might be a problem because it violates the assumptions of a number of statistical tests. However, the advantage of the REML method used here is that it does not rely on the assumption that the variances are equal and is therefore commonly used in variance components analyses in quantitative genetics (Fry 1992), including variance components analyses examining variation in inbreeding depression (Waser et al. 1995; Shaw et al. 1998).

## 5.2 Implications for ecology

### Biotic homogenization and the rise of novel communities

The findings of this thesis have a number of implications for ecology, which might be best considered in terms of biotic homogenization as a result of anthropogenic environmental change. There is ample evidence that the human transformation of the biosphere is leading to biotic homogenization and ultimately novel communities (McKinney & Lockwood 1999). Biotic homogenization refers to the increase in conformity among communities (McKinney & Lockwood 1999) and can be measured with the help of different diversity indices on different levels of biological organization, such as the taxonomic, genetic, and functional level (Olden et al. 2004). **Taxonomic homogenization** refers to the increase in similarity in the abundance of specific species, which has often been demonstrated as a consequence of invasive species (Mckinney & La Sorte 2007; Magee et al. 2008), but also as a consequence of climate change (Britton et al. 2009). **Genetic homogenizatio**n denotes the impoverishment of genetic diversity, for example due to founder effects and genetic bottlenecks (Olden et al. 2004), but also because of intraspecific and interspecific hybridization (Ellstrand & Elam 1993; Ellstrand

& Schierenbeck 2000). **Functional homogenization** refers to the loss of specific traits in the community, such as the decline in the abundance of specialists and increasing dominance by generalists, as well as their interactions (Clavel et al. 2011). Does global environmental change cause the functional homogenization in terms of generalist herbivory? Evidence from paleoecology (Blois et al. 2010) and current species distributions (Dynesius & Jansson 2000) reveals that stable climatic conditions favour specialists, and unstable climatic conditions favour generalist species. Indeed, generalist herbivores seem to profit from current climate change (Massad & Dyer 2010), while specialists are negatively affected by the combination of climate change and habitat loss (Stefanescu et al. 2011). In the greenhouse experiment with *Brassica nigra*, there was greater generalist herbivore damage under $eCO_2$ in combination with the ambient water treatment, possibly due to the dilution of glucosinolates (compare Table 5.1). In the experiment with *Noccaea caerulescens*, the generalist herbivore *Mamestra brassicae* tended to be less susceptible to Zn than the specialist *Pieris rapae* in the experiment with *N. caerulescens*. One specialist herbivore, the stem weevil *Ceutorhynchus quadridens*, was also less abundant under drought stress in the common garden experiment. By contrast, very few generalist herbivores were recorded in the common garden experiment. *B. nigra* produced more sinigrin under drought stress, which was the cause (attraction) or consequence (induced response) of the increased abundance of two specialist herbivore species under drought stress. Glucosinolates in general (Giamoustaris & Mithen 1995) and sinigrin in particular (Lankau 2007) usually deter generalist herbivores and attract specialist herbivores.

**Table 5.1.** Overview of the effects of the abiotic stress factors imposed in the three main experiments on glucosinolate production and plant resistance against specialist and generalist herbivores. (+ positive effect, - negative effect, 0 no effect, S=specialist herbivore, G=generalist herbivore)

| Experiment | Abiotic stress/novel conditions | Effect on glucosinolates | Species | Effect on resistance |
|---|---|---|---|---|
| Common garden *B. nigra* | Drought stress | + | *Brevicoryne brassicae* (S)<br>*Psylliodes chrysocephalus* (S)<br>*Ceutorhynchus quadridens* (S) | -<br>-<br>+ |
| Greenhouse *B. nigra* | Drought stress<br>Elevated $CO_2$<br>Drought + $eCO_2$ | 0<br>-<br>- | *Spodoptera exigua* (G) | +<br>+<br>+/- |
| Greenhouse N. caerulescens | Zinc | - | *Mamestra brassicae* (G)<br>*Pieris rapae* (S) | +<br>+ |

*Pieris rapae* adult females may have been attracted by the increased sinigrin production from drought-stressed plants as well and chose to oviposit on them, as indicated by the greater abundance of *P. rapae* larvae, another specialist herbivore, on these plants (Table 5.2). In the greenhouse experiment, sinigrin production slightly increased under drought stress, but the effect was not significant, possibly because the imposed drought stress was either too weak or too strong to increase the production of more sinigrin, as stated by the growth-differentiation hypothesis (Herms & Mattson 1992).

The attraction of specialist herbivores due to the increased production of glucosinolates under drought stress may counteract functional homogenization in terms of generalist herbivory, because specialist herbivores induce plant secondary metabolites to a greater extent than generalist herbivores (van Zandt & Agrawal 2004; Viswanathan et al. 2005) and generalists avoid induced plants. This was also demonstrated in the choice experiment with the generalist *M. brassicae* that avoided induced plant leaves irrespective of the Zn treatment. By contrast, elevated $CO_2$ may weaken induced responses elicited by

specialist herbivores since it dilutes nitrogen-based defence compounds, which was the case in the greenhouse experiment with *B. nigra*.

Genetic and functional homogenization are partly linked. Qualitative variation in the glucosinolate-myrosinase system affects generalist herbivores to a greater extent than specialist herbivores (Kliebenstein et al. 2002). If there is genetic homogenization, there will be less variation in chemical plant defence and therefore less efficient defence against generalist herbivory (compare also section 5.3.2), which would increase functional homogenization in terms of generalist herbivory. Genetic homogenization was applied here as experimental treatment (inbreeding and between-population outbreeding). Between-population outbred plants had greater specialist herbivore abundances than within-population outbred plants, which could be attributed to the disruption of co-adapted gene complexes (Templeton et al. 1986; Hufford & Mazer 2003) that are advantageous for herbivore resistance. Since too few generalist herbivores were recorded on the experimental plants, no assertions can be made in terms of the effects of intraspecific hybridization on resistance against generalist herbivores. However, the loss of heterosis under drought stress in the common garden experiment demonstrates that genetic homogenization via intraspecific hybridization in this case and concurrent global environmental change can have unforeseen effects on plant fitness. This should be taken into account in conservation efforts such as species translocations (Weeks et al. 2011) and corridors linking habitat fragments that have been shown to have positive effects on functional group diversity (Renton et al. 2012). Genetic homogenization due to inbreeding (increased homozygosity) will be discussed in more detail in section 5.3.1.

In sum, the evidence for functional homogenization under environmental change in terms of specialist vs generalist herbivores was mixed in all three experiments. In the following section, the consequences of functional and genetic homogenization with respect to plant performance and plant-herbivore interactions shall be explored.

*Effects of functional homogenization*

First, specialist or coevolved species may be more threatened under rapidly changing environmental conditions, because they are also in the danger of extinction when their coevolved partner goes extinct (Dunn et al. 2009). The phenomenon of coextinction of host plants and their specialised herbivores was evident in the Cretaceous-Paleogene (K/T) mass extinction about 65 million years ago (Labandeira et al. 2002). The example of *C. quadridens* in the common garden experiment illustrates that coevolved species or specialists do not only suffer when their partner or host goes extinct, but also when they change their physical, chemical, or physiological traits under new environmental conditions. *Ceutorhynchus assimilis*, a close relative of *C. quadridens*, is attracted by sinigrin (Smart & Blight 1997). However, *C. quadridens* larvae caused more damage in plants subjected to the ambient water treatment under which the plants produced less sinigrin, suggesting that drought stress either increased the concentration of primary metabolites, so that the larvae obtained more nutrients by causing less damage or that more quantitative defence compounds were produced under drought stress that negatively affected *C. quadridens* performance. Well-known examples of the disruption of close species interactions due to environmental change are phenological mismatches (Dewar & Watt 1992). On the other hand, if specialist or coevolved species profit from a specific trait that is amplified under environmental change, it is likely to increase in abundance under environmental change. Herbivores that are adapted to utilise the heavy-metal

containing tissue of heavy metal hyperaccumulators would be relatively more abundant in heavy metal-polluted habitats. The shift in the balance between species because some profit and others lose out under environmental change is likely to make community networks less complex. In the common garden experiment, flea beetles were more abundant on drought-stressed plants and consumed all flowers, thereby reducing the number of flowering individuals. This would negatively affect not only the fitness of the plant, but also specialist pollinators, which have a more restricted range of host plants than generalist pollinators. This could be exacerbated by the fact that specialist pollinators are not able to track climate change to the same extent as generalist pollinators (DeLucia et al. 2012).

Second, niche theory states that greater specialist diversity leads to more efficient resource exploitation than greater generalist diversity (Clavel et al. 2011), and this has been demonstrated experimentally by Finke & Snyder (2008). The observed specialist herbivore abundances in the common garden experiment exemplify the concept of complementary niche partitioning of specialists and demonstrate the impoverishment of communities if specialists disappear under global environmental change: *P. chrysocephalus* fed on the flowers, *B. brassicae* on the phloem, *P. rapae* larvae on the leaves, *C. quadridens* larvae in the stem of *B. nigra*.

Third, the different specialist herbivores that were occupying different niches on the plant had different natural enemies. Parasitoids (wasps) feeding on butterfly larvae and ladybirds feeding on aphids were the most abundant natural enemies. However, no differences were detected in the abundance of natural enemies under the different water treatments and crossing backgrounds (Table 5.2), presumably because there were too few data. Another possibility is that while their prey was more abundant on drought-stressed plants, the greater

amounts of consumed glucosinolates in herbivores feeding on drought-stressed plants may have been toxic for their natural enemies, as has been demonstrated for ladybirds (Francis et al. 2001), and *Pieris* caterpillars (Gols et al. 2008b). Release from natural enemy pressure under climate change has been predicted (Logan et al. 2003) and can exacerbate herbivore outbreaks or the dominance of a few generalist herbivores. In the experiment with *N. caerulescens*, *P. rapae* caused substantial plant damage under the control Zn treatment, which was reduced to a great extent when the herbivores had been attacked by a parasitoid. These results emphasize that there are manifold interdependencies between plants, herbivores, and natural enemies, which are strongly altered under global environmental change.

**Table 5.2.** The results of ANOVA determining the effects of the water treatment, crossing background, and population and their two- and three-way interactions on the abundance of *P. rapae* larvae and the abundance of natural enemies.

| | Natural enemies | | | *P. rapae* | | |
|---|---|---|---|---|---|---|
| Source of variation | Df | F | P | Df | F | P |
| Water | 1 | 0.00 | n.s. | 1 | 6.27 | * |
| Cross | 2 | 0.36 | n.s. | 2 | 0.20 | n.s. |
| Pop | 5 | 0.66 | n.s. | 5 | 1.09 | n.s. |
| Water × Cross | 2 | 0.06 | n.s. | 2 | 0.83 | n.s. |
| Water × Pop | 5 | 1.06 | n.s. | 5 | 1.43 | n.s. |
| Cross × Pop | 9 | 0.82 | n.s. | 9 | 0.24 | n.s. |
| Water × Cross × Pop | 8 | 0.43 | n.s. | 8 | 0.82 | n.s. |

NS $P > 0{\cdot}1$, (*) $P < 0{\cdot}1$, * $P < 0{\cdot}05$, ** $P < 0{\cdot}01$, *** $P < 0{\cdot}001$.

Natural enemies were combined from ladybirds, ladybird larvae, spiders and parasitoid wasps, since there were not enough data points to obtain individual results.

*Long-term effects: The rise of novel communities*

In the long term, novel environmental conditions including the release from specialist herbivore pressure can lead to the formation of novel plant and insect communities for a

geologically transient period. The rise of transient novel plant communities in North America about 12 000 years ago was attributed to a combination of warming, the retreat of ice sheets and release from herbivore pressure by large mammals due to their extinction (Gill et al. 2009). Present effects of the release from herbivore pressure facilitates the range shift of native species under climate change (Munier et al. 2010), but it also helps plant invasive species to gain ground (Lu et al. 2013). Novel plant communities are currently reported in woodlands and grasslands also because of other environmental change factors, including eutrophication (Keith et al. 2009; Newton et al. 2012) and habitat fragmentation (Dupré & Ehrlén 2002; Krauss et al. 2004). Plant community surveys in Wisconsin across a 50-year time frame revealed that common generalist plant species became dominant in all habitats, while rare species went locally extinct, resulting in an increase in habitat similarity (Rooney et al. 2004). Habitats contaminated with heavy metals in the soil are likely to become dominated by heavy metal hyperaccumulating plants that attract a limited number of herbivores, as demonstrated in the experiment with *N. caerulescens*, and further add to the impoverishment of communities (Nesterkov & Vorobeichik 2009; Babin-Fenske & Anand 2011). Communities that are characterized by the dominance of a few species (low evenness) have been shown to be less resilient to environmental change (Wittebolle et al. 2009), including species invasions (Turnbull et al. 2010).

## 5.3 Implications for evolution

### 5.3.1 The evolution of self-fertilization

Theoretical considerations predict that alleles promoting self-fertilization will increase in frequency in a population, even if they do not alter mean fitness (Fisher 1941), or if inbreeding depression (ID) remains below a certain threshold (Uyenoyama & Waller 1991). The high variation among families in ID, as has been demonstrated here in the common garden experiment and in other studies (Koelewijn 1998; Picó et al. 2004), could promote the spread of alleles promoting self-fertilization even in populations with high average ID, since some families are not affected by inbreeding depression and even show inbreeding benefits (Thiele et al. 2010). Family-level variation in ID may hence have a stronger influence on mating system evolution than population-level variation in ID (Kelly & Tourtellot 2006).

Self-fertilization can be a suitable strategy for plants under specific circumstances, for example for reproductive assurance due to pollinator and mate limitation, and due to time-limitation in ephemeral habitats (Snell & Aarssen 2005). Habitat fragmentation and climate change increase the frequency of these circumstances and may therefore individually and in combination promote self-fertilization. Reproductive assurance is important under habitat fragmentation, since fragmentation in general causes a decline in pollination services (pollinator limitation) and reduces population size and gene flow between habitat patches due to isolation (mate limitation) (Aguilar et al. 2008; Eckert et al. 2010). Climate-change-induced range shifts of plant species lead to smaller population sizes at the leading and contracting edges (Jump & Penuelas 2005), which has been linked to increased frequencies of self-compatibility at range margins (Baker 1955; Moeller & Geber 2005). A combination of

habitat fragmentation and climate change promote a rise in the number of ephemeral habitats, such as through large-scale landscape disturbance and disruption of the growing season due to extreme drought events (IPCC, 2014) This limits the time available for the completion of the life cycle and so favours annual and selfing species (Snell & Aarssen 2005). High frequencies of selfing species have been reported in habitats with short growing seasons, such as cultivated habitats and habitats with wet seasons followed by drought (Snell & Aarssen 2005). Biogeographic studies have demonstrated the evolution of selfing in ephemeral and more stressful habitats (Vasek 1964; Solbrig & Rollins 1977).

Nonetheless, inbreeding depression acts against the evolution of selfing by lowering its fitness advantage (Carr & Eubanks 2014). Selfing populations and species accumulate more putatively deleterious alleles than outcrossing populations and species (Hazzouri et al. 2013; Wright et al. 2013) and purifying selection is less effective in selfing species (Slotte et al. 2013). Moreover, inbreeding depression can increase in combination with environmental stress (Armbruster & Reed 2005), as has been shown in this thesis in both experiments with *B. nigra*. Does this make the evolution of self-fertilization less likely under global environmental change? Not necessarily, for two main reasons. First, self-fertilization should be favoured when purging reduces the genetic load caused by highly deleterious recessive alleles (Lande & Schemske 1985). In the $CO_2$ experiment, the population with the smallest population size, Durlston, showed an inbreeding benefit in several traits under drought stress, whereas the other two populations with greater population sizes suffered inbreeding depression. This suggests that the inbreeding load in this population may have been purged under drought stress. Two highly-cited meta-analyses (Byers & Waller 1999; Crnokrak & Barrett 2002) demonstrate that purging does occur, albeit inconsistently, in natural populations, especially in

annual plant species (Byers & Waller 1999). Self-compatible plants are likely to have lower ID rates than self-incompatible plants, because deleterious recessive alleles may have been purged already (Lande & Schemske 1985; Husband & Schemske 1996). The inconsistent nature of purging can be attributed to the fact that purging depends on several conditions, including the genetic basis of ID (partial dominance vs overdominance) (Charlesworth & Charlesworth 1999; Wright et al. 2007), the number and severity of deleterious recessive alleles (Crnokrak & Barrett 2002), and gene flow between populations (Kramer et al. 2008). Another reason why environment-dependent inbreeding depression does not necessarily counteract the evolution of self-fertilization is genetic variation. The greenhouse experiment with *B. nigra* demonstrated that populations vary in the combined effects of inbreeding and factors related to climate change. Some populations benefitted when all three factors came together, others lost out. A recent meta-analysis demonstrated that the evolution of self-fertilization does lead to faster extinction, but it also provides opportunities for species divergence and speciation (Wright et al. 2013).

As a final consideration, inbreeding depression tends to be greater in fitness-related traits compared to non-fitness-related traits in animals (Wright et al. 2007), but there is no evidence for this relationship in plants (Ellmer & Andersson 2004), so that there might be less selection pressure against self-fertilization. In Darwin's crossing experiments with *B. oleracea*, inbred plants outperformed within- and between-population outbred plants in terms of aboveground biomass accumulation, but within- and between-population outbred plants outperformed inbred plants in terms of fitness (Darwin 1876). There was greater ID and EDID in growth-related traits in the common garden experiment with *B. nigra*, and there was greater ID and EDID in fitness- and resistance-related traits in the greenhouse experiment. In

conclusion, global environmental change may alter local selection pressures that have a strong influence on breeding systems and, due to their different nature, promote the variability of breeding systems (Linhart & Grant 1996), so that in some cases the evolution of self-fertilization may be favoured under global environmental change.

### 5.3.2 Adaptive evolution under global environmental change

Plants can cope with global environmental change via three main options: migration, phenotypic plasticity and evolutionary adaptation (e.g. Pertoldi et al. 2007). Evidence for the range shifts of plants to higher altitudes due to climate change are accumulating, and include plants in the Alps (Grabherr et al. 1994) and in Scandinavia (Kullman 2002). However, many plant species are unable to disperse fast enough to track climate change (Huntley 1991; Davis & Shaw 2001). Habitat fragmentation further aggravates dispersal to novel habitats (Collingham & Huntley 2000; Honnay et al. 2002).

When the same genotype leads to different phenotypes in different environmental conditions, this is termed **phenotypic plasticity** (Pigliucci 2001). **Passive** phenotypic plasticity refers to a response due to resource limitation, whereas **active** phenotypic plasticity usually entails an active change in resource allocation (van Kleunen & Fischer 2005). It could be argued that the lower height or lower above-ground biomass and the concurrent maintenance of the number of flowers or inflorescences in the drought stress treatment compared to the ambient water treatment in both experiments with *B. nigra* is an example of an active plastic response, because it may reflect an active change of resource allocation, as outlined by Nicotra et al. (2010). Phenotypic plasticity is **adaptive** when the plastic response positively affects plant fitness and **maladaptive** when the plastic response negatively affects

plant fitness (van Kleunen & Fischer 2005). Phenotypic plasticity or microevolution in response to climate change usually contains one of two strategies: a) avoiding or escaping the new conditions, b) tolerating or exploiting new or increased resources to which the population is currently not well-adapted (Franks & Hoffmann 2012). Drought stress could select for avoidance strategies that include low water use efficiency, fast development, and early flowering, as has been demonstrated in *B. rapa* (Franks 2011). Another type of strategy involves drought tolerance, such as decreased above-ground biomass and late flowering, which have been demonstrated in a long-term experiment in *Plantago lanceolata* (Ravenscroft et al. 2014). Both strategies were observed in all three experiments: a) drought tolerance in the experiments with *B. nigra* with decreased above-ground growth and b) the exploitation of elevated $CO_2$ by increased above-ground growth in the greenhouse experiment with *B. nigra* as well as the use of Zn in the soil as herbivore defence compound in the experiment with *N. caerulescens*.

It is, however, difficult to disentangle the contributions of phenotypic plasticity and evolution (Franks 2011 and references therein), especially because studies rarely address both in one experiment. In addition, it has been demonstrated that both phenotypic plasticity and genetic differences contribute to differentiation among populations or ecotypes both in animals (James et al. 1997) and plants (Wilczek et al. 2009). In a recent meta-analysis, the majority of plant responses to climate change have been attributed to phenotypic plasticity (Franks et al. 2014). The different reaction norms for different genotypes observed here in all three experiments are likely to be attributable to a combination of genetic variation in phenotypic plasticity and genetic differences due to different selection pressures at the varying population locations as well as differences in microclimate within populations. However,

since in all three experiments plant responses were measured in the same generation at the same time point, no conclusions can be drawn in terms of adaptive evolutionary responses (Franks et al. 2014). The discussion below will therefore be limited to phenotypic plasticity, genetic variation in phenotypic plasticity, and genetic variation as implied by among-population and among-family differences in the reaction norms (Fig. 5.2).

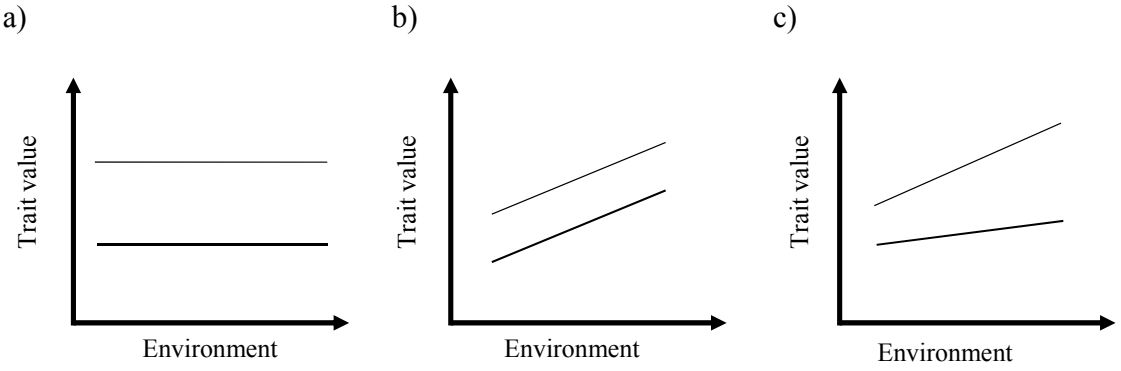



**Fig. 5.2.** Schematic explanation of reaction norms and their meaning in terms of genetic variation and phenotypic plasticity adapted from Pigliucci (2001). Lines refer to the trait values of different genotypes along an environmental gradient. a) Genetic variation among genotypes b) genetic variation among genotypes, and plasticity for both genotypes, c) genetic variation, plasticity, and genetic variation for plasticity among genotypes.

*Genetic variation in plant performance and resistance*

The results of the experiments in this thesis emphasize the importance of genetic variation for coping with co-occurring global environmental stress factors – both biotic and abiotic - as proposed by Davis & Shaw (2001). Among-population or among-family differences were especially evident in traits related to aboveground growth, fitness, and resistance, as indicated by the significant effects of population or family on the abundance of the different herbivore species in the common garden experiment and on plant height, number of flowers or inflorescences in both experiments with *B. nigra* (compare Fig. 5.2a). Some families or populations were more resistant to the different herbivore species than others, and families or populations were taller and produced more inflorescences or flowers than others under both

water treatments. In the experiment with *N. caerulescens*, the Prayon population had greater herbivore resistance than the Ganges population, whereas the Ganges population had greater above-ground growth. Although genetic diversity has not been directly assessed in this thesis, the different trait values of different families and populations indirectly propose that different genotypes showed different responses under drought stress, elevated $CO_2$ or Zn contamination in the soil. Genetic diversity is crucial for stress resilience under global environmental change; as has been demonstrated for genetically diverse seagrass populations (*Zostera marina*) under extreme temperature increases (Reusch et al. 2005). Considerable genetic differentiation in responses to climate has been found within natural populations of wild crop relatives, herbs, and tree species (Cobb et al. 1994; Jump et al. 2006; Ravenscroft et al. 2014), presumably due to the strong selection pressure imposed by (micro)climatic stress during germination and seedling establishment (Linhart & Grant 1996). Two studies on *Arabidopsis thaliana* genomes from accessions across Europe showed that single-nucleotide polymorphisms were associated with changes in climatic conditions (Fournier-Level et al. 2011; Hancock et al. 2011).

The among-population differences in traits related to herbivore resistance were substantial in all three experiments. Both *N. caerulescens* populations showed quantitative and qualitative differences in the production of glucosinolates (sinalbin vs glucomoringin production), as well as different levels of resistance against a generalist and specialist herbivore. Although the *B. nigra* populations in the greenhouse experiment did not vary substantially in herbivore damage, they varied substantially in the production of sinigrin (quantitative diversity). While only one generalist herbivore was used in this experiment, it is likely that the differences in sinigrin production may affect resistance against other herbivore species. In the common garden experiment, the abundance of the different specialist

herbivores varied among populations, indicating among-population variation in resistance against specific specialist herbivores. The major among-population differences observed here are in line with a recent study demonstrating greater numbers of polymorphisms in *Arabidopsis thaliana* genes subject to biotic selection compared to genes subject to abiotic selection (Colautti et al. 2012), and a meta-analysis reporting that quantitative trait loci (QTL) under biotic selection had greater effect sizes than QTL under abiotic selection (Louthan & Kay 2011). An explanation for these results could be that biotic interactions, such as herbivory, occur predominantly in patches, whereas abiotic conditions, such as temperature, usually change gradually over large distances (Colautti et al. 2012). High among-population genetic variation in plant defence compounds has been attributed to the strong variation in herbivore pressure among different sites and populations (Moore et al. 2013), as has been the case for *B. nigra* populations in Central Europe (Bischoff & Trémulot 2011). Herbivores may therefore be agents of diversifying selection (Mithen et al. 1995). An artificial selection experiment with *A. thaliana* accessions exposed to different species of aphids (Züst et al. 2012) as well as the high polymorphisms found in genes encoding glucosinolates in different populations of *B. oleracea* with varying herbivore pressure support this notion (Mithen et al. 1995). Genetic variation in resistance traits, as demonstrated here in *B. nigra*, and in *N. caerulescens*, hence seems to be of major importance due to locally divergent herbivore loads. Consequently, the predicted rise in herbivore outbreaks as a result of climate change and the exposure of plant populations with previously unknown herbivore species due to the range shift of insects under climate change may be partly buffered by the high genetic variation in plant herbivore resistance traits as a result of diversifying selection in the past.

*Plasticity and genetic variation in phenotypic plasticity in plant performance and resistance*

Genotype × environment interactions are fundamental for adaptive evolution under global environmental change (Jump et al. 2009). Species and populations with high genetic variation in phenotypic plasticity are more likely to cope with changing environmental conditions, as imposed by climate change (Nicotra et al. 2010). Selection pressure on plants for phenotypic plasticity as a response to climate change is likely to be high (Gutschick & BassiriRad 2003). A recent meta-analysis confirmed that plants show a greater degree of phenotypic plasticity than animals, possibly due to their sessile nature (Murren et al. 2014). In all three experiments, there were plastic responses to changing environmental conditions (drought stress, elevated $CO_2$, Zn addition to the soil), as well as high variation among families or populations under different combinations of environmental stress and/or crossing backgrounds, indicating genetic variation in phenotypic plasticity (compare Fig. 5.2b,c). For example, in the experiment with *N. caerulescens*, the Ganges population had greater plasticity under Zn addition in terms of glucosinolate production than the Prayon population. In the greenhouse experiment with *B. nigra*, one population had greater plasticity in terms of growth under elevated $CO_2$ than the other two populations. Plants from the population Swyre Head took greater advantage of the increased carbon supply than the other two populations. In a field situation, if these three populations came into contact because of range expansion, plants from the population Swyre Head would presumably outgrow the other two populations under elevated $CO_2$. These results indicate that different environments produce different selection pressures and benefit different genotypes (Linhart & Grant 1996), and lead not only to among-population genetic variation in average trait values, but also to genetic variation in phenotypic plasticity.

It is remarkable that the *B. nigra* populations differed in plasticity in terms of growth under elevated $CO_2$ even though they had not been exposed to elevated $CO_2$ before. Novel environmental conditions, such as elevated $CO_2$, may expose "hidden" parts of reaction norms, that is genetic variation that comes into play when a genotype is exposed to new environmental conditions, so that selection can act on new reaction norms and change them in terms of slope and curvature (Ghalambor et al. 2007; Schlichting 2008) (compare Fig. 5.3). This so called "cryptic" genetic variation accumulates in different genotypes over time, because selection does not act on it while the genotypes experience the normal range of environmental conditions, which is termed "**canalization**" (reviewed in Ghalambor et al. 2007). These hidden reaction norms could play a major role in evolutionary diversification (Schlichting 2008) and the adaptation to novel climatic conditions that have no modern analogue (Williams et al. 2007).

Cryptic genetic variation may be especially important when sudden environmental changes occur that require fast adaptation or otherwise will lead to extinction. Examples include plant adaptation to heavy metal soil pollution via heavy metal tolerance (not heavy metal hyperaccumulation) within 20-50 generations (Shaw & Etterson 2012) and plant adaptation to drought stress within less than two decades (Ravenscroft et al. 2014). Genotypes that are adapted to previously "ambient" and stable conditions could then be lost due to novel environmental conditions ("**selective sweep**") (Jump & Penuelas 2005). Studies using genetic mapping with *A. thaliana* accessions or ecotypes have demonstrated that relatively few genomic regions are required for local adaptation and adaptive differentiation in highly different environments (Ågren et al. 2013).

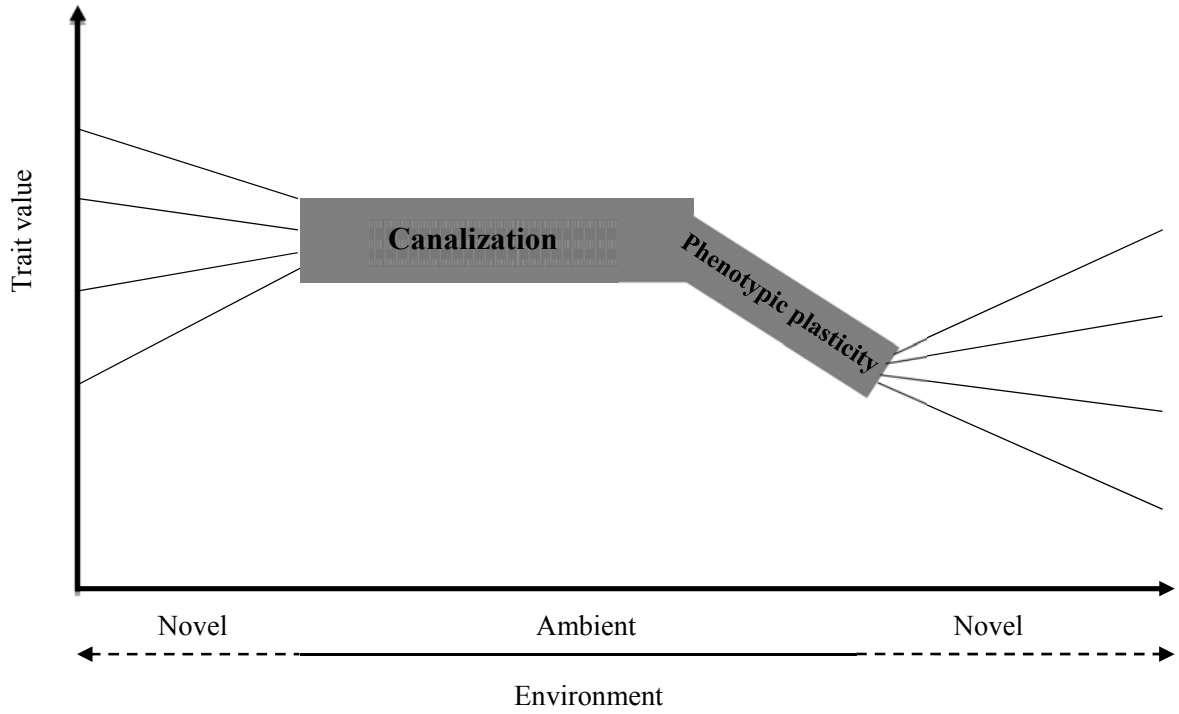



**Fig. 5.3.** Reaction norm depicting trait values or phenotypes under ambient and novel environmental conditions. Adapted from Ghalambor et al. (2007) and Schlichting (2008).

Species may thus be able to adapt to novel environmental conditions faster than predicted based on their known phenotypes under previously experienced environmental conditions, when they accumulated cryptic genetic variation in the past that is by chance advantageous under the novel environmental conditions (compare Fig. 5.3). Interestingly, inbreeding and therefore increased homozygosity may assist here by exposing conditionally neutral recessive alleles that are of neutral effect under ambient conditions and render an advantage under novel environmental conditions. This could have been the case in the Durlston population in the greenhouse experiment with *B. nigra*. Inbred plants from this population performed better under drought stress and elevated $CO_2$ compared to outbred plants. Considering the discussion on self-fertilization in section 5.3.1, one can conclude that under specific circumstances,

inbreeding (including self-fertilization) may be advantageous under rapidly changing environmental conditions: if the novel conditions strongly limit mate and pollinator availability, if the new habitats are characterized by strong disturbance and favour short life cycles, if there is effective purging, and if inbreeding leads to the exposure of conditionally neutral recessive alleles that render an advantage under novel environmental conditions.

## 5.4 Implications for conservation management

### Maintaining biodiversity

Ecologists aim to expand the focus of the effects of global environmental change on plants from model organisms such as *Arabidopsis thaliana* to a variety of wild plant species in order to understand resilience and adaptation to this change (Hendry et al. 2011). *Brassica nigra* and *Noccaea caerulescens* are related to *A. thaliana*, but the plants used here were obtained from wild populations with strongly varying characteristics and represented only a small fraction of the immense biodiversity among plants, but findings from evolutionary ecology in general and from this thesis can be applied to tackle problems in conservation management.

In conservation biology, there are two paradigms that Ouborg et al. (2006) described as the ’conservation genetics’ paradigm vs the ‘habitat quality’ paradigm. In the **conservation genetics paradigm**, loss of biodiversity is attributed to small population size and isolation from other populations, whereas in the **habitat quality paradigm** loss of biodiversity is regarded as the result of habitat quality changes, i.e. changes of biotic and abiotic characteristics of habitats that populations are unable to adapt to (Ouborg et al. 2006). These different paradigms have direct implications on conservation management; after all they

determine the measures that conservation should focus on. Should they a) try to maintain habitat quality that fits the "climate envelope" of species by either altering habitat quality or translocating species to more suitable habitats? Or should they b) try to keep (effective) population size as large as possible and promote gene flow between populations?

This thesis investigated the effects of both changes in habitat quality and population genetics characteristics. It demonstrated that the treatments related to habitat quality – drought stress and elevated $CO_2$ – had stronger effects on plant performance and resistance than treatments related to genetics – the inbreeding and between-population outbreeding crossing treatments. The combination of changes in habitat quality and changes in population genetic structure tended to have greater negative effects on plant performance and resistance than habitat quality changes on their own, which is in accordance with studies demonstrating increased inbreeding depression under environmental stress (Cheptou & Donohue 2011). This implies that a) and b) strategies should be combined to maintain biodiversity under global environmental change. It also makes a strong case for shifting the habitat-quality-orientated view to a population-oriented view, as suggested by Ouborg et al. (2006), since the effects of inbreeding and abiotic stress associated with climate change alone and in combination or the effects of Zn pollution strongly varied among populations. The major within- and among-population variation in traits related to plant growth, fitness, and herbivore resistance also implies that conservation efforts should concentrate on maintaining a variety of populations with sufficiently large population sizes that allow for the protection of genetic diversity. However, it should also be noted that small population size and the concurrent increase in inbreeding is not in every case a disadvantage and can even be beneficial under specific circumstances, as outlined above.

The findings in this thesis underline the importance of considering plant responses to habitat fragmentation and global environmental change in a population or metapopulation context. Fundamental population processes, such as migration, colonization and local adaptation need to be incorporated in experimental and theoretical studies of plant population dynamics in a changing environment (Husband & Barrett 1996), including plant-herbivore interactions in fragmented habitats (Tscharntke & Brandl 2004). The structure and history of metapopulations are of major importance to predict the likelihood of adaptive evolution (Bell & Gonzalez 2011) and can help to make decisions on conservation measures. The high among-population variation found in the experiments with *B. nigra* demonstrates the greater resilience of genetically diverse populations against global environmental change and underlines the importance of conservation efforts to protect biodiversity not only at the species level, but also at the population level.

This could also help to tackle the SLOSS (single large or several small reserves) debate. Chapter 2 and 3 demonstrated that families and populations vary in the combined effects of environmental stress and inbreeding. As a result, many small reserves with a variety of locally adapted families and populations may be more likely to cope with the combined effects of inbreeding and environmental stress. If the reserves are connected, gene flow can occur between habitat patches, so that genetic diversity is maintained and high levels of inbreeding are prevented. However, facilitating gene flow between populations could lead to outbreeding depression (Edmands 2007). While it is unclear whether loss of heterosis under drought stress would also be apparent in the second or subsequent generations, hybrid breakdown, the loss of co-adapted gene complexes resulting in lower fitness, only occurs in the second or subsequent generations (Hufford & Mazer 2003). This may further decrease trait

values. Conservation managers could test between-population hybridization for two generations in plants populations at a small scale before allowing between-population hybridization among reserves (Edmands 2007). However, in the long-term, genetic diversity and the prevention of accumulating homozygous deleterious recessive alleles may be more important to survive under increasing environmental variation than lower trait values for a limited number of generations.

# Chapter 6: General Conclusions

## Summary

The guiding question in this thesis was: How does global environmental change affect plant populations in terms of their performance and their interactions with herbivores? Chapter 2 and 3 assessed the combined effects of factors related to climate change and factors related to habitat fragmentation on plant performance and herbivore resistance. Chapter 4 examined the effects of an adaptation to environmental change (greatly increased soil Zn) on plant growth and plant-herbivore interactions. All three experiments used a variety of plant populations to account for among-population diversity and to mimic natural conditions.

Chapter 2 looked at the combined effects of drought stress and inbreeding or between-population outbreeding on plant performance and resistance. Inbred, within-population and between-population outbred *Brassica nigra* offspring (5 populations) obtained from hand-pollinations were grown in a common garden and exposed to natural herbivory. Half of the plants received a drought stress treatment. Drought stress caused the loss of heterosis and reduced herbivore resistance. It also increased inbreeding depression in plant traits related to growth, reproductive output and resistance. There was high variation among populations and families in the effects of drought stress, inbreeding, and a combination of the two. These findings imply that within- and among-population genetic variation are vital for resilience to global environmental change and that population translocations are not always advantageous under global environmental change.

Chapter 3 examined the combined effects of elevated $CO_2$, drought stress, and inbreeding on plant performance and resistance against the generalist herbivore *Spodoptera exigua*. Inbred and outbred *B. nigra* plants from three populations were subjected to the

abiotic treatments in a controlled greenhouse environment. Elevated $CO_2$ and drought stress increased inbreeding depression in two populations and led to inbreeding benefits in the third population. In general, among-population differences decreased under drought stress and increased under elevated $CO_2$ and inbreeding, suggesting that elevated $CO_2$ and inbreeding expose hidden phenotypes. One can infer from the results in Chapter 3 that populations differ in their response to environmental change and this cannot be predicted based on their performance under ambient conditions.

Chapter 4 focused on a different model system; *Noccaea caerulescens*, a heavy metal hyperaccumulator. Plants from two populations were grown on three different soil zinc concentrations and the effects on plant performance and resistance were determined. The addition of Zn to the soil deterred generalist (*Mamestra brassicae*) and specialist (*Pieris rapae*) herbivores and impaired their performance. Infestation with a parasitoid (*Hyposoter ebeninus*) weakened *P. rapae* larvae and equalized their choice of plant leaves obtained from different Zn treatments. Herbivore induction of secondary chemicals equalized the choice by *M. brassicae* among plant leaves obtained from different Zn treatments. Plants grown under the control Zn treatment compensated for the substantially higher damage with high tolerance, whereas plants grown under the medium and high Zn treatment had high resistance due to the Zn addition. These findings indicate that herbivory may have played a role in the evolution of heavy metal hyperaccumulation and that heavy metal hyperaccumulators apply different defence strategies (resistance vs tolerance) depending on the heavy metal content in the soil. Furthermore, herbivore induction and parasitoid attack increase herbivore resistance in plants grown on soil without additional Zn, suggesting that higher trophic levels could weaken selection pressure on glucosinolate production in heavy metal hyperaccumulators.

## Directions for future research

Global environmental change fundamentally alters all levels of biological organization, from the level of genes through physiologies, organisms, populations, interactions, communities, habitats, ecosystems, and the biosphere (compare IPCC, 2014). Consideration of these different levels of biological organization is essential to gain an understanding of the effects of global environmental change on ecosystem services that form the basis of existence for humans (Fig. 6.1). The results from all three experiments demonstrate that the effects of global environmental change should be analyzed on different levels of biological organization.

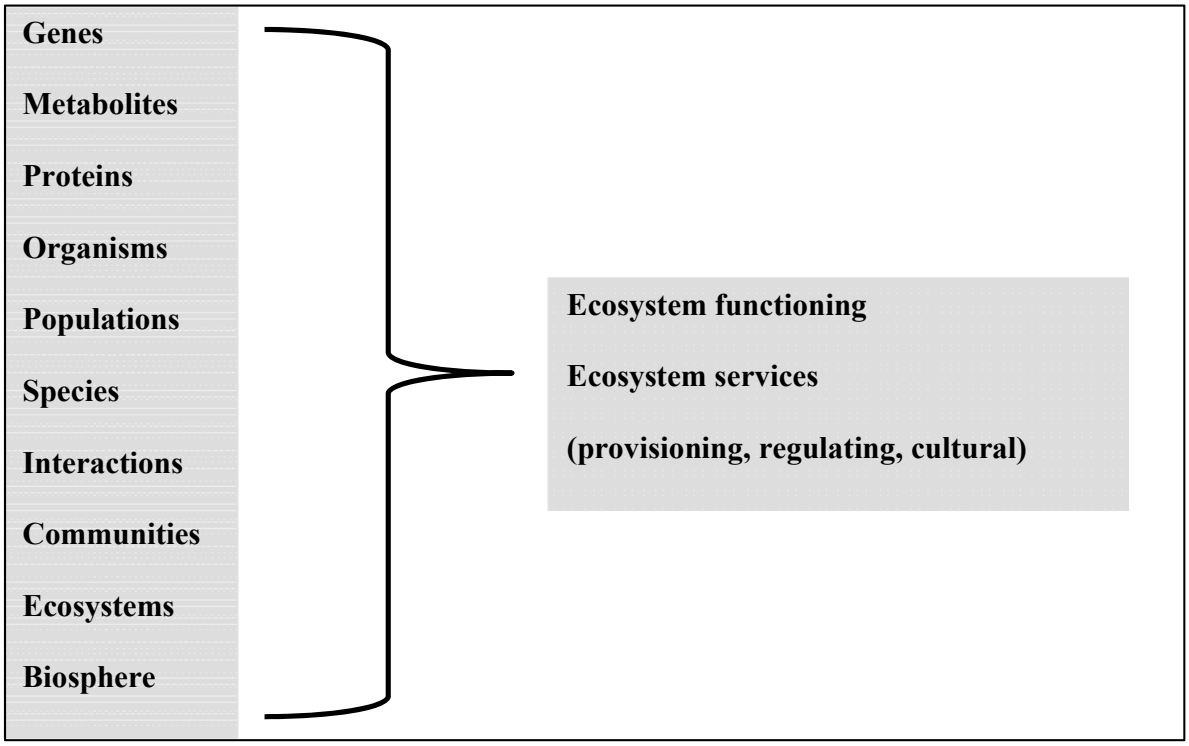



**Fig. 6.1.** Levels of biological organization and their contribution to the provisioning of ecosystem services.

This thesis demonstrated that different global change drivers interact in ways that cannot be predicted based on their individual effects (Tylianakis et al. 2008). The observed within- and among-population differences and the varying responses among generalist and specialist herbivores under different treatments related to global environmental change demonstrated that genetic diversity and functional diversity are vital to withstand the negative effects of global environmental change. Species interactions are of great significance and should play a major part in ecological research and conservation efforts, since they are the basis of communities and ultimately ecosystems and the effects of environmental change proliferate and cascade through them.

Future research should aim to portray the natural environment in experimental setups that reflect the natural environment more closely in space and time, as well as in terms of species networks and functional traits. In terms of space, studies could be conducted that assess the effects of multiple factors related to environmental change belowground (e.g. Eisenhauer et al. 2012) and/or in multi-species experiments (van Kleunen et al. 2014). In terms of time, artificial selection experiments could be conducted with multi-generation exposure to different factors related to environmental change, such as disturbance (Fakheran et al. 2010) or exposure to elevated $CO_2$. Studies uniting the dimensions of space and time contain, for example, the combination of landscape (abiotic) data with molecular markers in different populations that help to identify adaptive genetic variation (Hoffmann & Willi 2008; Manel et al. 2010). These data could also be linked with species distribution models (e.g. Kearney & Porter 2009) that aim to predict future species distributions under global environmental change. Species networks or species interactions (van der Putten et al. 2010) and functional traits (Nicotra et al. 2010) can also provide additional guidance in future

species distribution models (Blois et al. 2013). Finally, the application of *omics* techniques allows the identification of candidate genes, metabolites and traits that are important for resilience against global environmental change (Reusch & Wood 2007; Kunin et al. 2009). Ecological research that addresses the aspects outlined above can give more accurate recommendations for policy and conservation, including land planning and habitat restoration that help to maintain ecosystem functioning and ecosystem services.

# Acknowledgements

I would like to thank everyone at the Department of Plant Sciences who supported my work. Very special thanks go to my supervisors Roosa Leimu and James Bullock for their guidance and support. I would like to thank CEH that provided greenhouse and common garden facilities. Many thanks go to Nicole van Dam and her group at the University of Nijmegen. I also would like to thank Pedro Bota for the support in my lab work, and thanks to the undergraduate students who helped in the common garden experiment, especially to Colette Roach. Thanks to my colleagues Costas, Maria, Clare, Kirsty, and Maurice for the refreshing scientific and non-scientific discussions. This research was funded by the German National Academic Foundation and the BBSRC (Biotechnology and Biological Sciences Research Council).

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# Appendix 1: Supplementary Information for Chapter 2

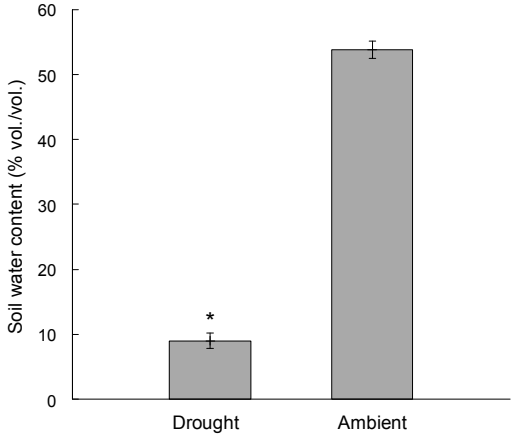
**Supporting Information**

**Appendix S1. Experimental system**

**Table S1:** Locations of *Brassica nigra* populations on the coast of Dorset, UK.

| Location | Abbreviation | Latitude | Longitude |
|---|---|---|---|
| St Aldhelm's | STA | 50.5801742 | 2.0581027 |
| Peveril Point | PEV | 50.6065898 | 1.94687641 |
| Durlston | DUL | 50.5939758 | 1.95432195 |
| Kimmeridge | KIM | 50.6060619 | 2.12894768 |
| Swyre Head | SWY | 50.6039542 | 2.12378408 |

**Figure S1:** Soil moisture in the drought and ambient water treatments.




Soil water content measured in the plant pots in the drought and ambient water treatments. Data are means ± SE. Stars denote significant differences between means ($P < 0.05$, Tukey's post hoc test).

## Appendix S2. ID and OD models

## Table S2. OD Models

The results of separate linear mixed models testing for the effects of population, crossing background (within-population outbred vs. between-population outbred), water treatment and their interactions on the number of inflorescences, plant height and above-ground biomass in *Brassica nigra*.

| | **Inflorescences** | | | **Height** | | | **Aboveground biomass** | | |
|---|---|---|---|---|---|---|---|---|---|
| Source of variation | ***Numdf, dendf*** | ***F*** | ***P*** | ***Numdf, dendf*** | ***F*** | ***P*** | ***Numdf, dendf*** | ***F*** | ***P*** |
| **Fixed Effects** | | | | | | | | | |
| Population | 4,13.0 | 1.46 | n.s. | 4,16.6 | 10.82 | *** | 4,12.4 | 1.82 | n.s. |
| Cross | 1,15.4 | 1.11 | n.s. | 1,16.8 | 5.45 | * | 1,11.5 | 0.03 | n.s. |
| Water | 1,3.86 | 1.42 | n.s. | 1,3.03 | 1.90 | n.s. | 1,6.05 | 11.24 | * |
| Cross × Water | 1,14.7 | 13.23 | ** | 1,21.1 | 7.78 | * | 1,17.0 | 4.50 | * |
| Pop × Water | 4,15.6 | 0.70 | n.s. | 4,17.1 | 4.59 | * | 4,17.0 | 1.34 | n.s. |
| Pop × Cross | 4,15.1 | 1.01 | n.s. | 4,15.4 | 1.73 | n.s. | 4,10.4 | 0.46 | n.s. |
| Pop × Cross × Water | 4,14.0 | 1.03 | n.s. | 4,17.4 | 1.41 | n.s. | 4,15.5 | 0.13 | n.s. |
| **Random Effects** | | $\chi^2$ | | | $\chi^2$ | | | $\chi^2$ | |
| Plunge bed | | 6.2 | ** | | 1.0 | n.s. | | 0.1 | n.s. |
| Plant family | | 2.3 | 0.065 | | 0.2 | n.s. | | 0.1 | n.s. |
| Fam (pop) × Cross | | 8.3 | ** | | 0.7 | n.s. | | 0.2 | n.s. |
| Fam (pop) × Water | | 7.1 | ** | | 0.6 | n.s. | | 0.1 | n.s. |
| Fam (pop) × Cross × Water | | 1.3 | n.s. | | 0.2 | n.s. | | 2.0 | (*) 0.079 |

The significance of the random factors was tested by the chi-squared test (d.f. = 1) of the differences in the -2 log likelihood value of that factor included vs. excluded from the model. Plunge bed × Water was fitted to the model as the error term, but is not presented here. N.S. $P > 0{\cdot}1$, (*) $P < 0{\cdot}1$, * $P < 0{\cdot}05$, ** $P < 0{\cdot}01$, *** $P < 0{\cdot}001$.

## Table S3. ID Models

The results of separate linear mixed models testing for the effects of population, crossing background (inbred vs. within-population outbred), water treatment and their interactions on the number of inflorescences, above-ground biomass, percent damage and damage caused by *Ceutorhynchus quadridens* in *Brassica nigra*.

| | **Inflorescences** | | | **Aboveground biomass** | | | **Percent Damage** | | |
|---|---|---|---|---|---|---|---|---|---|
| Source of variation | ***Numdf, dendf*** | ***F*** | ***P*** | ***Numdf, dendf*** | ***F*** | ***P*** | ***Numdf, dendf*** | ***F*** | ***P*** |
| **Fixed Effects** | | | | | | | | | |
| Population | 4,13.1 | 3.01 | (*) 0.058 | 4,14.9 | 3.01 | (*) 0.053 | 4,11.8 | 1.24 | n.s. |
| Cross | 1,13.4 | 0.86 | n.s. | 1,14.0 | 2.76 | n.s. | 1,11.8 | 0.02 | n.s. |
| Water | 1,2.69 | 1.92 | n.s. | 1,4.29 | 4.28 | n.s. | 1,2.9 | 10.00 | (*) 0.053 |
| Cross × Water | 1,15.9 | 9.04 | ** | 1,13.8 | 5.92 | * | 1,524 | 0.01 | n.s. |
| Pop × Water | 4,12.3 | 1.57 | n.s. | 4,14.6 | 0.65 | n.s. | 4,13.2 | 1.22 | n.s. |
| Pop × Cross | 4,13.3 | 1.15 | n.s. | 4,14.1 | 1.39 | n.s. | 4,11.6 | 0.58 | n.s. |
| Pop × Cross × Water | 4,14.4 | 0.69 | n.s. | 4,13.4 | 0.12 | n.s. | 4,525 | 0.57 | n.s. |
| **Random Effects** | | $\chi^2$ | | | $\chi^2$ | | | $\chi^2$ | |
| Plunge bed | | 11.8 | *** | | 2.0 | (*) 0.079 | | 14.5 | *** |
| Plant family | | 0.3 | n.s. | | 1.5 | n.s. | | 0.1 | n.s. |
| Fam (pop) × Cross | | 5.9 | ** | | 6.6 | ** | | 14.7 | *** |
| Fam (pop) × Water | | 2.9 | * | | 2.9 | * | | 13.6 | *** |
| Fam (pop) × Cross × Water | | 0.9 | n.s. | | 0.1 | n.s. | | 8.5 | ** |

The significance of the random factors was tested by the chi-squared test (d.f. = 1) of the differences in the -2 log likelihood value of that factor included vs. excluded from the model. Plunge bed × Water was fitted to the model as the error term, but is not presented here. N.S. $P > 0{\cdot}1$, (*) $P < 0{\cdot}1$, * $P < 0{\cdot}05$, ** $P < 0{\cdot}01$, *** $P < 0{\cdot}001$.

| | *C. quadridens* | | |
|---|---|---|---|
| Source of variation | ***Numdf, dendf*** | ***F*** | ***P*** |
| **Fixed Effects** | | | |
| Population | 4,11.3 | 2.28 | n.s. |
| Cross | 1,6.15 | 0.15 | n.s. |
| Water | 1,2.36 | 1.51 | n.s. |
| Cross × Water | 1,13.4 | 0.02 | n.s. |
| Pop × Water | 4.7.34 | 0.25 | n.s. |
| Pop × Cross | 4,5.5 | 0.81 | n.s. |
| Pop × Cross × Water | 4,13.5 | 0.46 | n.s. |
| **Random Effects** | | $\chi^2$ | |
| Plunge bed | | 5.7 | ** |
| Plant family | | 3.2 | * |
| Fam (pop) × Cross | | 2.1 | (*) 0.074 |
| Fam (pop) × Water | | 0.2 | n.s. |
| Fam (pop) × Cross × Water | | 2.0 | (*) 0.079 |

## Appendix S3. Trait correlations

**Table S4.** Pearson's correlations between sinigrin (GS) and growth- and fitness-related plant traits.

| Cross | Treat-ment | GS-Inflor | GS-Damage | GS-Height | GS-Weight |
|---|---|---|---|---|---|
| IN | D | 0.16 | -0.24 | 0.09 | 0.17 |
| IN | W | -0.08 | -0.44 (*) | 0.31 | -0.06 |
| OUT | D | -0.14 | -0.65* | -0.21 | 0.49(*) |
| OUT | W | -0.69* | -0.29 | -0.37 | 0.18 |
| BTW | D | 0.08 | -0.12 | 0.06 | 0.02 |
| BTW | W | -0.65* | -0.39 | -0.43 | -0.41 |

IN = inbred, OUT = within-population outbred, BTW = between-population outbred, D = drought treatment, W = ambient water treatment.

NS $P > 0{\cdot}1$, (*) $P < 0{\cdot}1$, * $P < 0{\cdot}05$, ** $P < 0{\cdot}01$, *** $P < 0{\cdot}001$.

# Appendix 2: Plant-herbivore coevolution in a changing world

Entomologia
Experimentalis et Applicata

DOI: 10.1111/j.1570-7458.2012.01267.x

14TH INTERNATIONAL SYMPOSIUM ON INSECT-PLANT INTERACTIONS

# Plant-herbivore coevolution in a changing world

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Accepted: 30 March 2012

*Key words*: chemical coevolution, diffuse coevolution, climate change, geographical mosaic of coevolution, habitat fragmentation, herbivory, invasive species, plant defence, species interaction

**Abstract**

Current anthropogenic environmental change causes rapid loss of biodiversity. Although the effects of the main causes of this loss (habitat fragmentation, climate change, and invasive species) on single species have been widely studied, the effects on species interactions are poorly understood. In particular, we do not yet understand how these phenomena affect the evolutionary processes that impact species interactions. Coevolution is a dominant process that organizes the web of life: most species are involved in at least one coevolved interaction. Due to rapid human modification of landscapes it is important to understand how subsequent changes in biotic and abiotic environment and in the level and distribution of genetic variation, as well as changes in population structures, influence the elements of the coevolutionary process. In this review, we synthesize recent development of theoretical work on the coevolution of interacting species with conservation genetics and the impact of anthropogenic environmental changes on single species to address the potential effects of habitat fragmentation, climate change, and invasive species on plant-herbivore coevolution.

## Introduction

Current anthropogenic environmental change is causing a rapid loss of biodiversity. Decline and loss of species due to habitat destruction and fragmentation, climate change, invasive species, overexploitation, and pollution is evident (Millennium Ecosystem Assessment, 2005). Our understanding of the ecological and evolutionary processes and mechanisms associated with this loss of biodiversity is, however, still highly inadequate. Moreover, although studies on the impact of the main anthropogenic drivers of the current diversity loss on single species are accumulating exponentially, we still know little about their impact on species interactions. Yet, focusing on species interactions and their diversity is undoubtedly crucial for conservation of biodiversity. The diversity of life has not only resulted from the diversification of species but also from the diversification of interactions among them. Coevolution, the reciprocal evolutionary change of interacting species, is a major force shaping patterns of adaptation, influencing diversification of interacting species, and ultimately generating biological diversity (e.g., Janzen, 1980; Thompson, 1994). Consequently, understanding how anthropogenic environmental change influences the coevolutionary process and dynamics is crucial.

It is evident that to be able to evaluate how the anthropogenic environmental change influences antagonistic coevolutionary interactions we need to study interspecific interactions across large spatial and temporal scales. Given the rapid rate of human modification of landscapes it is crucial to understand how associated changes in abiotic and biotic environment and genetic variation, as well as different configurations of population structure influence the elements of the coevolutionary process (Thompson, 1994). Mutations, gene flow, genetic drift, and extinction and colonization dynamics, which can all be influenced by human modification of landscapes, shape the genetic structure of coevolving species and contribute to the shifting geographical mosaic of coevolution by altering spatial distribution of alleles and traits (trait remixing: Thompson, 2005). Combining the theoretical work on coevolution and conservation genetics and the impact of the anthropogenic environmental change on single species can

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provide a framework for understanding some of the mechanisms behind the current biodiversity loss. Here, we attempt to outline how habitat fragmentation, climate change, and invasive species potentially influence plant-herbivore coevolution.

## Plant-herbivore coevolution

Together, insect herbivores and their host plants comprise a major part of total terrestrial diversity. Since the seminal paper by Ehrlich & Raven (1964), coevolution between plants and their insect herbivores has been accepted as a major force responsible for their diversification. Ehrlich & Raven's (1964) model for insect–plant coevolution suggested a continual arms race between plants and insect herbivores feeding on them: evolution of new chemical defences allows plants to escape from herbivore damage and consequently increase in abundance. This is followed by subsequent counter adaptation of insect herbivores to the novel plant defence, and so on. There is still little direct empirical evidence to support the hypothesis that diversification may be attributed to plant defence and herbivore counter defences as suggested by Ehrlich & Raven (1964). Probably the best evidence comes from a few short-term empirical studies (Berenbaum, 1983; Berenbaum et al., 1986; Berenbaum & Zangerl, 1998) and recent phylogenetic studies testing the macroevolutionary hypothesis on escalation of chemical defence and subsequent diversification (Farrell & Mitter, 1994; Becerra, 2003; Agrawal et al., 2009; Becerra et al., 2009; Futuyma & Agrawal, 2009; Rasmann & Agrawal, 2011).

Whereas the early work on coevolution focused on local populations, it has become clear that, to understand how coevolution organizes diversity across complex landscapes, coevolving interactions need to be investigated across the species' distribution ranges (Thompson, 1994, 2005). The geographical mosaic theory of coevolution (GMTC) provides a framework for studying how the coevolutionary process is driven by geographical variation in species interactions (Thompson, 1994, 1999, 2005). According to this theory, variation in species interactions among local populations forms the raw material for the coevolutionary processes that take place over larger geographical scales. This selection mosaic favours different evolutionary trajectories in different populations and communities. Selection will be reciprocal only in those communities where the interacting species occur and impose selection on each other, i.e., the coevolutionary hotspot. In coevolutionary coldspot the interacting species can at times be absent or the interaction between species may not lead to reciprocal selection. In addition, variable selection on the interaction between species is expected across geographical mosaic, because specialization and adaptation to local conditions is likely to vary among populations and communities (Thompson, 1994).

Although geographical variation in the intensity of coevolving interactions has been reported across a variety of systems (e.g., Benkman, 1999; Pellmyr, 2003; Laine, 2009; Pauw et al., 2009), very few studies have investigated geographical variation of coevolving plant-herbivore interactions and, in particular, the role of chemical defence in creating such variation (but see Berenbaum & Zangerl, 1998, 2006; Muola et al., 2010). In a recent study, Muola et al. (2010) reported spatial variation in the phenotypic selection that two specialist herbivores, *Euphranta connexa* (Fabricius) and *Abrostola asclepiadis* (Denis & Schiffermüller), exert on their host plant *Vincetoxicum hirundinaria* Medicus. Variation in the strength of the interactions between the herbivores and their host plant was associated with the among-population variation in plant chemistry (Muola et al., 2010). Berenbaum & Zangerl (1998) have also shown that, although the defensive chemicals of the wild parsnip, *Pastinaca sativa* L., and the counter defence of the parsnip webworm, *Depressaria pastinacella* (Duponchel), matched in most populations, phenotype frequencies of plants and herbivores mismatched significantly in one of the populations. This finding demonstrates that, instead of a continuous arms race, evolution of defence, and counter defence may fluctuate in space and time due to frequency-dependent selection, consequently contributing in generating a geographical mosaic.

## Environmental change and genetic interactions

Spatial variation in the strength and direction of coevolving interactions, i.e., a selection mosaic, can be illustrated by genotype × genotype × environment (G × G × E) interactions in the fitness of the interacting species (Thompson, 2005). The outcome of coevolutionary interactions depends on the genotypes of the interacting species and the environment the interaction occurs in. Although the outcome of a coevolutionary interaction is hypothesized to depend on environmental factors (e.g., Blanford et al., 2003), so far only a few studies have demonstrated such three-way interactions in coevolving host-parasite interactions (Laine, 2007; Tetard-Jones et al., 2007; Vale & Little, 2009; Byrne & Rigling, 2011), whereas corresponding data from plant-herbivore interactions is virtually lacking. Understanding how anthropogenic environmental changes, such as habitat fragmentation, climate change, and invasive species, alter G × G × E interactions provides a framework for assessing and predicting the effects of these phenomena on coevolutionary dynamics.

Basically, any factor that affects either all or one or two of the components of the interaction matrix is likely to modify the coevolutionary interactions between a plant and its herbivores. However, the direction and magnitude of modification of the interactions is likely to differ between the three major anthropogenic environmental changes.

Anthropogenic environmental changes commonly result in declines in population sizes and associated reductions in genetic variation (e.g., Lande, 1998). For example, habitat fragmentation directly reduces population size, whereas climate change may reduce the size of optimal habitats and consequently population sizes. Population sizes are also small when species are first introduced to new areas, or migrate to track climate change. Because the occurrence of a coevolutionary interaction requires that plant defences and herbivore counter defences have a genetic basis for selective responses to occur, any changes in levels and distribution of genetic variation are likely to affect plant-herbivore coevolution. Therefore, the genetic consequences of reduced population size are likely to influence plant-herbivore coevolution in a number of ways. The loss and fixation of alleles due to random genetic drift may result in reduced genetic variation in plant defence and herbivore counter defence in small populations. Moreover, reduced genetic variation negatively affects the adaptive potential of populations and the likelihood of local adaptation (Pertoldi et al., 2007), which is a central component of a geographical mosaic of coevolution (Thompson, 2005). Theoretical work suggests reduced adaptive evolution for small populations (Robertson, 1960; Hill, 1982). This idea is supported by a meta-analysis that demonstrated lack of local adaptation for small plant populations (Leimu & Fischer, 2008).

Increased inbreeding is another major genetic consequence of reduced population size and particularly well studied in the fragmentation context (e.g., Ellstrand & Elam, 1993; Young et al., 1996; Angeloni et al., 2011). Inbreeding effects on the fitness of plants and insects are well characterized (e.g., reviewed in Angeloni et al., 2011), but the effects on traits that are important for plant–insect interactions, such as defence and counter defence, are less well understood. Of the few studies that have investigated the effects of plant inbreeding on plant-herbivore interactions, some have found inbreeding depression in plant resistance and tolerance to herbivore damage (e.g., Strauss & Karban, 1994; Carr & Eubanks, 2002; Ivey et al., 2004; Stephenson et al., 2004), whereas others have not (e.g., Núñez-Farfán et al., 1996). As inbreeding effects on traits like plant defence are likely to vary among populations depending on population genetic structure and history (e.g., Leimu et al., 2008), inbreeding can have major effects on the spatial dynamics of coevolving species. Furthermore, inbreeding may influence coevolutionary dynamics by affecting the traits involved in local adaptation or by modifying the constraints of trait evolution. For example, unfavourable gene combinations could be fixed due to inbreeding and increased genetic drift (Kristensen et al., 2010). Furthermore, genetic correlations can be influenced by inbreeding, if different genetically correlated traits are affected in different families, or if inbreeding decreases or increases variation in one of the traits, but not in the other. Finally, the magnitude of inbreeding effects depends on environmental conditions (I × E) and can be more severe under stressful conditions (Dudash, 1990; Bijlsma et al., 1999; Kristensen et al., 2002, 2010; Armbruster & Reed, 2005). In general, if inbreeding occurs in either the herbivore or the plant or in both, it may modify the I × I × E interactions, and consequently influence the coevolutionary dynamics.

Anthropogenic environmental change can also affect G × G interactions by altering the patterns of migration and gene flow. Firstly, migration affects the spread of resistant and susceptible plant genotypes as well as that of herbivore genotypes and, therefore, shapes the outcome of the coevolutionary interaction (Gandon et al., 1996; Lively, 1999). Secondly, increased gene flow may lead to outbreeding depression in traits important for the interaction, if individuals from previously isolated and genetically differentiated populations come into contact and coadapted gene complexes break down (Templeton et al., 1986). These negative effects may, however, only be apparent after few generations of inter-population outbreeding due to heterosis, which results in 'hybrid vigour' in the first generations (e.g., Edmands, 1999). The few studies investigating the effects of between-population crosses on plant resistance and herbivore performance have found both evidence for outbreeding depression in herbivore resistance (Leimu & Fischer, 2010) and no effects of between-population outbreeding on resistance or herbivore performance (Muola et al., 2011).

In addition to altering genotype frequencies of the interacting species, anthropogenic environmental change can affect coevolutionary dynamics by causing variation in the abiotic environment and the structure and composition of the communities in which the species coevolve, thus modifying the environmental component of the G × G × E interactions. The ecological and evolutionary outcome of intraspecific interactions often vary spatially due to variation in the abundance of other interacting species in the community. Coevolution between interacting species has been shown to be influenced by copollinators (Thompson & Pellmyr, 1992; Thompson & Cunningham, 2002), alternative host plants (Zangerl & Berenbaum, 2003), competitors (Benkman, 1999; Benkman et al., 2001; Siepielski &

Benkman, 2004), and natural enemies such as parasitoids (Berenbaum & Zangerl, 2006; Craig et al., 2007). Moreover, changes in the structure and diversity of plant communities are likely to influence pairwise coevolution. Interactions between plants can influence the evolution of defence traits. For example, if competition alters environmental variables such as resource availability, interactions with herbivores are likely to be affected (reviewed in Agrawal et al., 2006). Moreover, diversity of plant communities can directly affect the abundance of herbivore populations and the structure and diversity of herbivore communities, thereby influencing competition between herbivores and consequently the levels of herbivory the plants experience (Agrawal et al., 2006). Plant community diversity can also influence interactions with herbivores by affecting levels of genetic variation within species: plant community diversity has been shown to correlate positively with intraspecific genetic variability (Vellend & Geber, 2005). Although there is more support for the hypothesis that evolutionary forces rather flow from bottom-up than top-down in terrestrial plant-herbivore-parasitoid interactions (Price, 1997), changes in upper trophic levels may also influence the coevolution between plants and herbivores (Craig et al., 2007). Given that interactions between a particular plant and an herbivore seldom occur independently from other interactions, diffuse coevolution has been suggested as an alternative mode for pairwise coevolution (e.g., Hougen-Eitzman & Rauscher, 1994; Iwao & Rausher, 1997). However, there is still little empirical evidence supporting the idea that plant-herbivore coevolution is diffuse rather than pairwise (e.g., Hougen-Eitzman & Rauscher, 1994; Iwao & Rausher, 1997). Furthermore, whether coevolution is pairwise or diffuse depends on how specialized the herbivores in question are (Leimu & Koricheva, 2006). Likewise, the impact of the anthropogenic environmental change on plant-herbivore coevolution depends on how specialized the herbivores are: a monophagous herbivore is likely to be more strongly influenced by changes in host plant defence than oligophagous or polyphagous herbivores. Although we focus here on pairwise coevolution, it is clear that we need to acknowledge the impact of other biotic interactions and the abiotic environment when planning and conducting empirical studies. Below, we discuss the potential impact of fragmentation, climate change, and invasive species on the G × G × E interactions and plant-herbivore coevolution.

## Habitat fragmentation and plant-herbivore coevolution

Populations of most species occur across their distribution in habitat patches that vary in size and distance to each other, and are surrounded by a matrix of unsuitable habitat. The GMTC reflects this geographical structure of populations of interacting species. However, habitat destruction and fragmentation alter the scale and patterns of this patchiness, and are therefore likely to influence the geographical mosaic of coevolution of interacting species, and the extent to which this dynamic process affects diversity.

Habitat fragmentation can reduce population sizes of both the host plant and the coevolving herbivores. Small populations are more prone to demographic and environmental stochasticity resulting in a greater risk of extinction (Lande, 1993; Saccheri et al., 1998; Westermeier et al., 1998). For highly specialized plant-herbivore interactions with a tight coevolutionary linkage, loss of host-plant populations due to habitat destruction or fragmentation may lead to coextinction of the herbivore populations even if they are not directly affected by fragmentation (Koh et al., 2004a,b). Furthermore, for coevolving herbivores whose populations follow metapopulation dynamics, increased isolation of host-plant populations may hinder re-colonization after a local extinction. Finally, as discussed above, genetic variation is commonly reduced in small fragmented populations due to increased impact of genetic drift, which also influences genotype frequencies and can, thus, affect G × G × E interactions. These interactions can be further influenced by increased inbreeding in small populations, although the negative effects of inbreeding, i.e., inbreeding depression, depend on the inbreeding history of the populations (Leimu et al., 2008). Small populations may suffer from lower levels of inbreeding depression, if they have been small and inbred for several generations and deleterious alleles have been purged (e.g., Angeloni et al., 2011). Inbreeding can affect coevolution directly by resulting in inbreeding depression expressed as suboptimal values of traits central for an interaction, or indirectly by resulting in reduced genetic variation and, thus, a poorer ability to respond to selection.

The effects of fragmentation on species interactions and coevolution depend largely on the effects of fragmentation on migration and gene flow. In general, the migration rates of insects are often greater than those of their sessile host plants. Therefore, changes in gene flow due to fragmentation may disproportionately affect the relative contribution of gene flow to the genetic variation within the populations of the coevolving species. Such disproportionate changes in gene flow and the level of genetic variation are then likely to affect the responses to selection imposed by the interacting species, and consequently coevolutionary dynamics. Gene flow can either speed up or restrict coevolutionary dynamics (Gomulkiewicz et al., 2000).

Reduced gene flow can increase genetic divergence of populations and consequently strengthen local adaptation (Thompson, 2005). However, some gene flow is required to fuel the level of genetic variation in a population and to maintain enough genetic variation for selection to act upon and for local adaptation to occur (Gandon & Michalakis, 2002; Stockwell et al., 2003; Hoeksema & Forde, 2008).

The GMTC acknowledges that populations are genetically differentiated and connected by varying levels of gene flow, and therefore provides an ideal theoretical framework for testing fragmentation effects on coevolving interactions and their diversity. Recent theoretical work has demonstrated how the relative strength of gene flow, genetic drift, and selection drives parasite local adaptation (Gandon & Nuismer, 2009). Gene flow and genetic drift affect the level of genetic variation available for reciprocal selection to act upon, but they can also affect the outcome of coevolving interaction by introducing traits or alleles shaped by selection in one population into another (Nuismer et al., 1999; Gomulkiewicz et al., 2000). However, we lack empirical studies on the relative role of selection, drift, and gene flow on coevolutionary dynamics. Such studies would be important in particular for understanding how fragmentation affects coevolution.

We suggest that future research on the consequences of the genetic changes of habitat fragmentation on plant-herbivore coevolution should concentrate on the following predictions:

- Moderate reduction in gene flow increases local adaptation and therefore geographical variation in the outcome of interactions. This creates new coevolutionary hotspots or strengthens existing ones, and accelerates diversification. This is particularly true for large populations where gene flow has homogenized genetic variation among populations.
- Strong reduction in gene flow, reduced genetic variation due to drift, and inbreeding, alone or in combination with each other, constrain local adaptation and, therefore, reduce geographical variation in the outcome of interactions. This cools down hotspots and slows down differentiation.
- Reduced genetic variation reduces the ability to respond to selection, which decelerates arms races, cools down hotspots, and slows down differentiation.
- If the plant suffers from increased inbreeding and suboptimal trait values, increased herbivory further diminishes plant population size and/or drives the plant population to extinction. If the herbivore suffers from inbreeding, the disproportionately higher level of plant defences will drive the herbivore population to extinction. Therefore, inbreeding may lead to population extinctions and increase variation in the occurrence of hotspots and coldspots in the geographical mosaic.
- The degree of the negative effects of inbreeding depends on the genetic mechanism determining inbreeding depression. If heterosis determines inbreeding depression in the plant, and gene flow allows the inbred lines (from different populations) to cross, the resulting hybrids are highly resistant to herbivores ('hybrid vigor'). This increases selection for herbivore traits that enable continued use of the highly resistant plants, and may accelerate coevolution if herbivore population can respond to this selection. If inbreeding depression in the plant is due to partial dominance, reduction in population size has negative effects irrespective of gene flow, unless the population has been small and inbred for a long time. This has negative effects on coevolution as occurrence/appearance of new resistant or virulent genotypes would be prevented.

To what extent plant-herbivore coevolution is affected by habitat fragmentation as predicted depends on plant mating system, dispersal mode, and distance of both plants and herbivores, as well as on the life form and successional stage of the plant, and the level of specialization of the herbivore.

## Climate change and plant-herbivore coevolution

Studies on the effects of climate change on plant-herbivore interactions demonstrate direct and indirect effects of temperature and elevated $O_3$ and $CO_2$ on the life-history traits and abundance of insect herbivores, and on leaf chemistry and nutritive quality of host plants (Bale et al., 2002; Valkama et al., 2007; Lindroth, 2010). In their review on climate change effects on insect herbivores, Bale et al. (2002) reported direct effects of temperature on development, survival, range, and abundance of insect herbivores. In a meta-analysis Valkama et al. (2007) demonstrated, in turn, that elevated $O_3$ and $CO_2$ alone and in combination influenced the nutrient content and concentrations of carbohydrate and defensive chemicals in plants: nutrient content and defence chemicals were either increased or decreased, or not affected at all, depending on tree species, ontogeny, and type of chemical. In his recent review Lindroth (2010) also points out that the effects of elevated $O_3$ and $CO_2$ on plant defence chemistry and ecological interactions are highly context- and species-dependent, which makes it very difficult to identify and predict any general or global patterns. However, it is clear that these effects of climate change on vital life-history traits of herbivores and the effects on host-plant quality and defence will also influence plant-herbivore coevolution. In addition to the direct effects on plant and herbivore traits, elevated $O_3$ and $CO_2$

levels can affect plant-herbivore coevolution by altering the composition and dynamics of the communities the coevolving interaction is embedded in, or by altering the abundance and functional responses of natural enemies of the herbivores (reviewed in Lindroth, 2010). Finally, changes in $O_3$ and $CO_2$ levels can alter carbon sequestration and nutrient cycling in the ecosystem thereby indirectly affect plants and herbivores and their communities (reviewed in Lindroth, 2010).

Increased drought is another consequence of climate change, which is likely to substantially affect plant–insect interactions and coevolution. This is largely due to changes in plant primary and secondary metabolism. Concentrations of nitrogen-based compounds increase under moderate drought stress (Mattson & Haack, 1987; Gutbrodt et al., 2011). This provides herbivores with higher food quality, whereas secondary defence compounds are expected to increase under moderate drought stress and to decrease under strong drought stress (Herms & Mattson, 1992). In addition, drought increases leaf temperature, which is beneficial for insect growth (Mattson & Haack, 1987). The few studies that examined the effects of varying levels of water limitation on plant-herbivore interactions found that drought-stressed plants are more susceptible for specialist herbivores (Haugen et al., 2008; Gutbrodt et al., 2011) or they found no effect (Khan et al., 2010). The mixed impacts of drought stress on plant-herbivore interactions can increase variation in the G × G × E interactions altering the selection mosaics and thus plant-herbivore coevolution.

In addition to direct effects on traits central for an interaction, climate change can influence selection mosaics of plant-herbivore interactions by altering the environmental component (E) in the G × G × E interactions and by affecting genotype frequencies through its effects on selection and gene flow. Climate change increases the environmental stochasticity and variability, which can result in extinction of local populations (e.g., McLaughlin et al., 2002). McLaughlin et al. (2002) provided evidence for climate-change induced extinctions of a checkerspot butterfly (*Euphydryas* spec.) due to increased variability in precipitation. Local adaptation is more likely under conditions that persist relatively long and therefore increased temporal environmental variability may further influence coevolutionary dynamics by constraining local adaptation (Slatkin, 1983).

Climate change may also influence plant-herbivore coevolution by altering the phenology of the interacting species, which can lead to phenological mismatches and even pull apart coevolving interactions (Bale et al., 2002; Boggs & Ehrlich, 2008). This threatens particularly highly specialized herbivores that require tight synchrony with their host plants to complete their life cycles (Bale et al., 2002). The inability of herbivores to track changes in host-plant phenology can force herbivores to feed on alternative food plants (Hodkinson, 1997), or to change the plant part/tissue they utilize (Hodkinson, 1997; Hill et al., 1998).

Plant and animal species can respond to climate change by shifting their ranges to track optimal climatic conditions (Parmesan, 1996; Parmesan et al., 1999; Thomas & Lennon, 1999; Warren et al., 2001). Altered migration patterns and changes in distribution patterns can further influence plant-herbivore coevolution. Herbivores are likely to be better able to track climate change than their sessile host plants. A more substantial increase in migration and gene flow of herbivores compared to those of the host plant will undoubtedly influence the coevolutionary dynamics (see above). It may lead to mismatches and maladaptation in coevolving populations, but also result in a higher rate of trait remixing. Range shifts may also affect coevolution by influencing levels of the distribution of genetic variation: genetic variation is often lower in populations at the front of range expansion. Moreover, after a range shift the component E of the G × G × E interaction inevitably changes as the coevolving interaction occurs in a new environment.

As mentioned above, it is difficult to provide general global predictions of the effects of climate change on plant-herbivore coevolution, because these effects are strongly context- and species-dependent. Yet, we hypothesize that:

- Increased $CO_2$ changes C/N ratio results in an increase in carbon-based secondary compounds. If these compounds are involved in defence against a coevolving herbivore, this gives the plant an advantage in the arms race. If nitrogen-based compounds are involved in defence against a coevolving herbivore, this gives the herbivores an advantage in the arms race, because increase in carbon-based compounds results in a decrease in nitrogen-based compounds.
- The predictions on the effects of elevated $CO_2$ level and increasing environmental stochasticity on plant-herbivore coevolution are contrasting. If the effects of elevated $CO_2$ are constant, they impose directional selection for plant herbivore defence and strengthen coevolution.
- Selection on defence compounds that have a lower metabolic cost increases under drought stress, because allocation of resources to growth is more constrained in plants growing under drought stress. This promotes and accelerates plant-herbivore coevolution.
- Increased environmental stochasticity (e.g., in temperature) can wipe out populations that can be either

coevolutionary hotspots or coldspots and, thus, modify the coevolutionary mosaic. Increased stochasticity causes variation in herbivore population density, and, consequently, reduces the strength and consistency of selection for plant herbivore resistance. The decreased strength of selection in turn slows down coevolution.

- Environmental stochasticity results in suboptimal environmental conditions and, thus, increases environmental stress. This can directly reduce population size and lead to negative genetic consequences (as those described above for habitat fragmentation) that may be further enhanced due to environmental stress.

These predicted effects of climate change on plant-herbivore coevolution depend on plant mating system and longevity, successional stage, the level of environmental specialization of the plant, food-plant specialization of the herbivore, and the type of defensive secondary compounds of the plant (e.g., carbon- vs. nitrogen-based).

## Species invasions and plant-herbivore coevolution

Attempts to understand why some species become invasive when introduced to new environments have largely focused on assessing life-history traits of invasive species and characteristics of the habitats invaded (e.g., Shea & Chesson, 2002; van Kleunen et al., 2010). According to two prevailing theories of plant invasions, the 'enemy release hypothesis' (Williamson, 1996; Keane & Crawley, 2002) and the 'novel weapons hypothesis' (Bais et al., 2003; Callaway & Ridenour, 2004), successful plant invasions can be explained by lack of natural enemies in the novel range or defence strategies that are novel in the new range thus providing escape from natural enemies respectively. More recently, plant invasion has been explained by dislocation from coevolved relationships (e.g., Hallett, 2006).

In a geographical mosaic that covers the species' distribution range, the dynamics of plant-herbivore coevolution can change if one of the species invades a new area. Disruption of coevolved interactions in an introduced range relaxes selection from the coevolving counterpart and can result in rapid radiation of the introduced species. As predicted by the 'enemy release' and 'novel weapons' hypotheses, plants may escape herbivory in the introduced range, which relaxes selection for defence and allows resources to be allocated to other functions such as growth and reproduction. Analysing herbarium specimens across a period of over 150 years, Zangerl & Berenbaum (2005) showed how the concentrations of defensive furanocoumarins in the wild parsnip, *P. sativa*, decreased dramatically after the species was introduced to North America where its coevolved specialist herbivore *D. pastinacella* was absent (Zangerl & Berenbaum, 2005). However, the wild parsnip rapidly re-evolved with high concentrations of furanocoumarins after an unintentional introduction of *D. pastinacella* to North America (Zangerl & Berenbaum, 2005). These findings demonstrate how introduction of coevolved herbivores to control the spread of toxic invasive weeds may result in even more noxious weeds.

Even if introduced/invasive plants would eventually be attacked by native herbivores at the introduced range, these herbivores might cause very different selection pressures on defence compared with the coevolving herbivore at the native range. Likewise, if a coevolving herbivore is introduced to a novel range its success will depend on its ability to shift to a novel host plant. Host-plant shifts are more likely between related plant species and, thus, the success of an introduced herbivore in the novel range may depend on the occurrence of suitable plant species in the community. Introduction of a new herbivore into a community is likely to disrupt coevolution between native herbivores and the host plant(s) the novel herbivore feeds on. Moreover, coevolving plant-herbivore interactions can be indirectly influenced by an invasive species that occupies the same community and influences either the plant or the herbivore or both via its effects on competition, predation, or mutualistic interactions (Boggs & Ehrlich, 2008).

We often envisage long-distance introductions within or between continents in relation to introduction and invasion of species. However, introduction at a much more local scale can also influence evolutionary trajectories and coevolutionary dynamics (Benkman et al., 2008). Such introductions are likely to be more successful given the similarity of the biotic and abiotic conditions between the original and introduced sites. Local introductions can homogenize formerly distinct populations, which can restrict spatial variation in species interactions, therefore reducing the potential for speciation and levels of diversity (Benkman et al., 2008).

Sometimes coevolving interacting species both invade a new area, although the two species often invade or are introduced to a new area at different times. This is the case when specialized natural enemies are introduced to control an invasive host. The coevolutionary dynamics between a coevolving pair of introduced species may change in the novel abiotic and biotic conditions due to changes in the $G \times G \times E$ interactions. Moreover, introduced populations commonly have reduced genetic variation (e.g., Uller & Leimu, 2011) and restricted gene flow, which is likely to further affect the coevolutionary dynamics (see above). Controlling the spread of invasive plant species includes deliberate introduction of herbivores (i.e., biocontrol) that are often specialized to feed on the invasive plant species. Although the interactions

between invasive plants and potential biocontrol agents are commonly well studied before the release of, for example, a novel herbivore to control its invasive host plant, biocontrol projects could benefit from a better understanding of the coevolutionary dynamics of the species in question. In particular, biocontrol projects would benefit from a better understanding of interactions between particular genotypes (G × G) and how these interactions function in the native vs. introduced ranges and how they depend on the environmental conditions (G × G × E). For example, biocontrol is based on using species that show extreme host-plant specialization in their native range and the assumption that species show the same degree of specialization when introduced into new environments. However, specialists very often show flexibility in their reliance on particular host species and this flexibility can depend on the environment and the evolutionary history of host use, which explains failure of some of the biocontrol attempts (Thompson, 1999). For example, the introduction of a specialist insect herbivore to control its invasive host plant resulted in a rapid increase in the plant's defensive compounds, which consequently lead to an even more noxious weed that also causes human health problems (Zangerl & Berenbaum, 2005). In this case, neglecting the importance of the reciprocal selection plants and herbivores exert on each other contributed to the failure of biocontrol.

We hypothesize that species invasions can affect plant-herbivore coevolution in the following ways:

- Invasion of only the species involved in a coevolving interaction into a new area dislocates coevolution and creates new coldspots into a geographical mosaic where escape from the coevolving partner may lead to rapid radiation. For example, due to relaxed selection this may result in reduced plant defence or evolution of new defences.
- If introduced plants or herbivores interfere with existing coevolving interactions, this disrupts coevolutionary dynamics by altering selection on defence and counter defence. This may cool down hotspots and slow down the arms race depending on the relative strength of selection native and introduced interactors exert on each other.
- If both interacting species are introduced, changes in community structure, natural enemies, and the abiotic environment affect coevolutionary dynamics. This is because genotype × genotype × environment interactions change as the environmental component changes.
- Genetic variation is usually reduced in introduced populations and some resistant/virulent genotypes will be missing. This slows down local adaptation and coevolution in cases in which both interactors are introduced.

These predicted effects of species invasion on plant-herbivore coevolution depend on plant mating system, the generation times of plants and herbivores, and how specialized the herbivores are.

## Conclusions

It is clear that human activities are likely to dramatically alter evolutionary dynamics of interacting species thereby influencing biological diversity. In our attempts to conserve biological diversity, and the functioning of biological communities and ecosystems, we ought to focus more on conserving diversity of interactions, in particular that of highly specialized and tightly coevolving interactions. Many of the services that have been identified as indicators of ecosystem integrity are, in fact, the consequences of species interactions, not services provided by a single species acting on its own, for example, pollination, nutrient cycling, and decomposition. We should thoroughly utilize the current theoretical understanding of coevolutionary dynamics to construct a framework where we combine this understanding with that of conservation genetics. We also urgently need studies investigating the interactive effects of the main drivers of the current biodiversity loss, such as climate change and habitat fragmentation, on the evolution of interacting species. Finally, we should consider the coevolutionary history of interacting species when planning and carrying out biocontrol of invasive species.

## Acknowledgements

We thank Jess Metcalf for fruitful discussions and two anonymous reviewers for valuable comments.

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