Intraspecific Variation in Plant-Animal Interactions of the Brassicaceae Family Along a Steep Rainfall Gradient in the Eastern Mediterranean Basin

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Abstract

The future climate of dryland ecosystems is predicted to become increasingly arid as rainfall decreases and becomes more unpredictable and extreme. Consequently, dryland plant communities may risk extinction if species are unable to adapt to increasing aridity. A plethora of theoretical and empirical studies has investigated plant adaptations to arid environments, yet, adaptations in plant-animal interactions are often overlooked. Because the abiotic environment can determine the composition of pollinators and herbivores, which, in turn, can drive the coevolution and trade-offs in plant reproductive strategies and plant herbivore defences, it is critical that we investigate the dependencies of plants on the activities of their associated animals under different climatic conditions. Observing intraspecific variation in plant-animal interactions along environmental gradients provides compelling evidence of how the ecosystem may respond to the future climate. Whilst this approach has been exploited for latitudinal and elevation gradients, fewer studies have addressed intraspecific variation in plant-animal interactions along rainfall gradients. In addition, these studies often use a single plant species which reduces our ability to generalise.

This dissertation aims to provide much needed evidence for the effects of increasing aridity on plant performance, mediated through their interactions with pollinators and herbivores, as predicted by climate change. A steep rainfall gradient in the Eastern Mediterranean Basin was used to determine the effect of increasing aridity on pollinator services and herbivory pressure. Simultaneously, a pollinator-exclusion experiment in the field determined whether different plant species in the Brassicaceae family could secure their reproduction by self-fertilisation in the absence of pollinators. Furthermore, evidence of increasing self-compatibility as an adaptation to increasing aridity was ascertained by a pollination manipulation experiment. Finally, quantifying the concentration of glucosinolates in plant species highlighted the relationship between herbivory damage and plant chemical defence investment.

The abundance and diversity of pollinators decreased with increasing aridity, whilst self-fertilisation was variable across the plant species. This weak relationship suggests pollinator limitation plays a minor role in selecting for self-fertilisation in plants. Genetically, the plant species were more self-compatible in increasingly arid environments, suggesting that the mechanisms of prezygotic self-incompatibility weaken with increasing aridity. In congruence with decreasing pollinator activity, herbivore pressure also decreased with increasing aridity, yet, total leaf glucosinolate concentration in plants did not change. The lack of evidence for a trade-off in leaf damage by herbivory and total leaf glucosinolates concentration suggests herbivore pressure may not be the dominant factor selecting for plant chemical defence.

Taken together, the research of this thesis shows that plant performance is surprisingly robust to variations in the pollinator community and in herbivore pressure. I conclude that, whilst the diversity of the pollinator community and the resilience of herbivores may be under threat in future climates, the reproductive success and resource-allocation into constitutive herbivore defences in plants, at least in the Brassicaceae family, shall remain relatively unaffected by climate change.

Kurzfassung

Das künftige Klima von Trockengebieten wird voraussichtlich noch trockener durch weiter abnehmende Regenfälle deren Auftreten zudem immer unberechenbarer und extremer werden dürfte. Folglich können Pflanzengemeinschaften der Trockengebiete durch unzureichende Anpassung vom Aussterben bedroht sein. Eine Vielzahl von theoretischen und empirischen Studien hat die Adaption von Pflanzen an die abiotischen Bedingungen in Trockengebieten untersucht, jedoch sind in diesen Studien die Adaptionen der Pflanzen an die mit ihnen interagierenden Tieren weitgehend unbeachtet geblieben. Da die abiotische Umgebung die Zusammenstellung von Bestäubern und Herbivoren bestimmen kann, was wiederum die Koevolution und trade-offs zwischen Pflanzenvermehrungs-Strategien und Abwehrmechanismen gegen Herbivore antreiben kann, ist es erforderlich, die Abhängigkeiten von Pflanzen von den Aktivitäten ihrer assoziierten Tiere unter verschiedenen Klimabedingungen zu untersuchen. Die Analyse intraspezifischer Variation von Pflanzen-Tier-Interaktionen entlang von Umweltgradienten kann überzeugende Hinweise liefern, wie das Ökosystem auf das zukünftige Klima reagieren könnte. Diese intraspezifische Variationen in den Wechselwirkungen zwischen Pflanzen und Tieren wurden entlang von Nord-Süd- sowie Höhengradienten schon vielfach untersucht, doch nur wenige Studien haben sich mit den Pflanze-Tier- Interaktionen entlang von regionalen Niederschlagsgradienten auseinandergesetzt. Darüber hinaus basierten diese Studien meistens nur auf einer einzigen Pflanzenart, wodurch unsere Fähigkeit zur Verallgemeinerung eingeschränkt wird.

Diese Dissertation erarbeitet Hinweise auf die Auswirkungen der prognostizierten zunehmender Trockenheit auf die Performanz von Pflanzen in Abhängigkeit von ihren Wechselwirkungen mit Bestäubern und Pflanzenfressern. Die Effekte der zunehmenden Trockenheit auf Bestäuber-Dienste und Herbivoren-Exposition wurden in einem Gebiet mit steilem Niederschlagsgefälle im östlichen Mittelmeerbecken untersucht. Gleichzeitig stellte ein Bestäuber-Exklusionsexperiment im Freiland fest, ob verschiedene Pflanzenarten in der Brassicaceae-Familie ihre Reproduktion durch Selbstbefruchtung in Abwesenheit von Bestäubern sichern können. Darüber hinaus wurde durch eine

Manipulation der Bestäubung der Nachweis einer zunehmenden Eigenkompatibilität als Anpassung an zunehmende Trockenheit erbracht. Schließlich zeigten Konzentrations-Messungen von Glucosinolaten in den Pflanzenarten die Beziehung zwischen dem Schädigungsgrad durch Pflanzenfresser und der pflanzlichen Investition in die chemische Verteidigung.

Die Abundanz und Diversität von Bestäubern nahm mit zunehmender Trockenheit ab, während der Grad der Selbstbefruchtung der Pflanzenarten weitgehend unabhängig vom Trockenheits-Gradienten variierte. Dieser Befund deutet darauf hin, dass bei den untersuchten Pflanzen eine Beschränkung der Bestäuber keine entscheidende Rolle für die Ausprägung der Selbstbefruchtung spielt. Genetisch waren die Pflanzenarten in zunehmend trockener Umgebung mit sich selbst kompatibler, was darauf hindeutet, dass die Mechanismen der präzygotischen Selbst-Inkompatibilität mit zunehmender Trockenheit schwächer werden. In Übereinstimmung mit abnehmender Bestäubungsaktivität nahm auch die Belastung durch Pflanzenfresser mit zunehmender Trockenheit ab, jedoch änderte sich die Gesamtkonzentration von Blattglucosinolaten in Pflanzen nicht. Der Mangel an Beweisen für einen Trade-off zwischen Blattschädigungen durch Pflanzenfresser und der Gesamtkonzentration von Blattglucosinolaten legt nahe, dass der Pflanzenfresserdruck nicht der dominierende Faktor bei der Auswahl einer chemischen Verteidigung der Pflanze ist.

Zusammenfassend zeigen die Untersuchungen dieser Arbeit, dass die Pflanzen-Performanz gegenüber Veränderungen in der Bestäuber-Gemeinschaft und dem Fraßdruck durch Herbivore erstaunlich robust ist. Ich folgere daraus, dass die Vielfalt der Bestäuber-Gemeinschaften und die Widerstandsfähigkeit von Pflanzenfressern in künftig stärker ariden Klimaregimes durchaus gefährdet sein können, jedoch der Reproduktionserfolg und die Ressourcenverteilung in konstitutive Pflanzenfresser-Abwehr von Pflanzen, zumindest in der Familie der Brassicaceae, vom Klimawandel relativ unberührt bleiben.

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Contents

Declaration of my own working contribution to the present thesis

The present thesis is based on the work I carried out in the field in Israel, Palestine and Jordan and in the greenhouse in Germany during my PhD. Throughout the course of this research I was advised by my supervisor Prof. Dr Katja Tielbörger.

I designed each experiment together with my supervisor, Prof. Dr Katja Tielbörger, and I performed all practical work, data analysis, and writing, except for: Dr Merav Seifan helped with the experimental design and statistics in the second chapter; Viktoria Ferenc performed the hand-pollination experiment in the third chapter; Prof. Dr Caroline Müller and Karin Djendouci at the University of Bielefeld performed the glucosinolate analysis in the fourth chapter; and all commented on drafts of the chapters they were involved in, *i.e.*, Prof. Dr Katja Tielbörger commented on chapters 2, 3, and 4; Dr Merav Seifan commented on chapter 2; Viktoria Ferenc commented on chapter 3; Prof. Dr Caroline Müller commented on chapter 4.

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Chapter 1

Introduction

This thesis sets out to investigate the adaptations in plant mating systems and plant defence mechanisms in response to variation in pollinator activity and herbivory pressure along a rainfall gradient. In this introduction chapter, I provide the context for each of these topics (see contents) before ending the chapter by explaining the specific goals of my thesis research.

1.1. Plant-animal interactions

Most animals that directly interact with plants are insects, typically insect pollinators and herbivores (Herrera and Pellmyr, 2009). Consequently, both mutualist and antagonistic plant-animal interactions can occur (Travis, 1996; Thompson, 1988; Johnson et al., 2015). Plant-pollinator interactions provide a beneficial service to the plant because pollinators move pollen between plant individuals within a population and increase the rate of cross-fertilisation (Lloyd, 1992; Harder and Barrett, 1996). The diversity of plant-pollinator interactions provides numerous selection pressures that drive variation in plant mating systems, often in opposite directions (Barrett and Harder, 1996; Barrett, 2002; Barrett, 2010a; Barrett, 2010b; Barrett and Harder, 2017). Many angiosperm plant species have evolved self-incompatibility mechanisms to prevent inbreeding depression, whilst others have evolved self-compatibility to provide reproductive assurance when there is a risk of reproductive failure (Lloyd, 1979; Barrett, 1988; Lloyd, 1992; Pannell and Barrett, 1998; Barrett, 2003; Barrett et al., 2014).

Plant-herbivore interactions are typically disadvantageous to the plant because it can damage the plant by biomass loss. Plant-herbivore interactions perpetuate a so-called ‘arms-race’ between plant defence strategies and herbivore activity (Agrawal, 2011; Agrawal et al., 2012). Plant species have evolved a variety of defence strategies to protect themselves from herbivores, including chemical defences derived from secondary plant metabolites and mechanical defences, such as leaf toughness, thorns, and trichomes (Rhoades, 1979; Stamp, 2003; Rasmann et al., 2014; Agrawal et al., 2015; Agrawal and Weber, 2015).

Plants-animal interactions are influenced by local abiotic conditions (Thompson, 1988; Travis, 1996; Sotka and Hay, 2002; Menge, 2003; Sotka et al., 2003). This can select for clinal trends in plant-animal interactions over small spatial scales, and between populations of a single plant species, resulting in intraspecific variation in these interactions (Pratt and Mooney, 2013; Anderson et al., 2015). Geographic variation in plant-animal interactions within a species can result in adaptations in both plants and their associated animals to the local conditions (e.g. Thompson, 1988; Sotka and Hay, 2002; Sotka et al., 2003; Pennings et al., 2009). Whilst this may provide optimal fitness for plants under relatively constant conditions, plant populations may suffer as the climate changes (Sala et al., 2000). At present, it remains uncertain whether climate change will decrease the abundance of pollinators and herbivores and whether this will select for reproductive assurance (chapter 2), increasing self-compatibility (chapter 3), and the production of chemical defences (chapter 4) within populations of plant species (Barrett and Harder, 2017; Hargreaves and Eckert, 2014; Pennings et al., 2009; Hahn and Maron, 2016).

1.2. Plant mating systems

Many plant species are hermaphroditic, which means that both sex organs are housed within a single flower. Thus, hermaphroditic plants have the potential to fertilise their own ovules (selfing). Ubiquitous advantages of self-fertilisation include full gene transmission compared to cross-fertilisation (Nagylaki, 1976) and the ability to produce offspring should pollinators or mates be absent (Baker, 1955). Yet, offspring produced by self-fertilisation may experience lower fitness than offspring produced by cross-fertilisation and thus the advantage of self-fertilisation is traded-off against the negative effect of inbreeding depression (Jain, 1976; Lande and Schemske, 1985; Schemske and Lande, 1985; Charlesworth and Charlesworth, 1987). Furthermore, self-fertilisation over many generations can lead to loss of genetic variability at the population level (Charlesworth et al., 1993; Charlesworth, 2003).

The trade-off between self-fertilisation and the loss of genetic variability has led to the evolution of self-incompatibility mechanisms in up to 60% of hermaphroditic plant species (Bateman, 1955; Lloyd, 1965; Barrett, 1988; Hiscock and Kües, 1999; Hiscock and Tabah, 2003; Barrett, 2010a). Self-incompatibility mechanisms, both gametophytic and sporophytic self-incompatibility, are genetically controlled by the *S*-locus gene (Barrett, 1988). Gametophytic self-incompatibility is the most common mechanism in angiosperm plant species and sporophytic self-incompatibility is uncommon and known only in a few families, namely *Asteraceae*, *Brassicaceae* and *Compositae* (Bateman, 1955; Lloyd, 1965; Reinartz and Les, 1994). Genetically controlled self-incompatibility mechanisms prevent self-pollen from accessing the ovule because it is recognized as self-pollen on the stigma and its germination and pollen tube growth is inhibited (Bateman, 1955; Shivanna et al., 1978; Barrett, 1988; Hiscock and Tabah, 2003).

Loss-of-function mutations in the *S*-locus gene can result in the self-incompatibility mechanism becoming leaky, or being switched off completely which may result in viable offspring produced by self-fertilisation (Nasrallah et al., 2002; Levin, 1996). The break-down of self-incompatibility mechanisms and the subsequent increase in successful self-fertilisation can be selected for under conditions that limit cross-pollination (Lloyd, 1965; Reinartz and Les, 1994; Goodwillie, 2001). Securing reproduction by self-fertilisation in environments where cross-pollination is unlikely is called reproductive assurance (Lloyd, 1992). The selective pressures for the evolution of reproductive assurance include low mate abundance and low pollinator abundance within the local environment (Charlesworth and Charlesworth, 1979; Vallejo-Marín and Uyenoyama, 2004; Porcher and Lande, 2005).

Scarcity of mates can limit outcrossing success because low density plant patches attract fewer pollinators than high density patches (Fausto et al., 2001). Additionally, low mate density reduces the proportion of conspecific pollen relative to heterospecific pollen present on the stigma (Feinsinger, 1987; Ashman et al., 2004). Scarcity of pollinators can limit the transfer of pollen and can increase the rate of self-fertilisation within the plant (Ashman et al., 2004). Increased self-fertilisation as a result of pollinator foraging behaviour can result from geitonogamous selfing, where the pollinator visits numerous flowers on the same plant sequentially and transfers pollen between flowers on the same plant individual, or from facilitated selfing, where the pollinator transfers pollen within the same flower on the same individual (Lloyd, 1992; Eckert, 2000; Barrett, 2003). Consequently, self-fertilisation may be selected in plant populations even if the density of mates is high (Harder and Barrett, 1996; Wilcock and Neiland, 2002). Low abundances of pollinators and mates frequently occur in harsh environments, such as high altitude, high latitude and arid regions (Noy-Meir, 1973; Greenslade, 1983; Kearns, 1992; Ashman et al., 2004; Devoto et al., 2009). Furthermore, when plants colonise new environments either by invasion or by long-distance dispersal there may be low abundances of conspecific mates and their associated pollinators (Baker, 1955; Busch and Schoen, 2008; Petanidou et al., 2012; Razanajatovo et al., 2016).

Whilst reproductive assurance occurs only in self-compatible plant species, not all self-compatible plant species secure their reproduction by self-fertilisation. The classical theory predicts that self-compatible species reproduce either by outcrossing or by selfing (Lloyd, 1992). Many empirical studies have shown, however, that the bimodal distribution predicted by theory is rarely seen in the natural environment (reviewed in Goodwillie et al., 2005; Razanajatovo et al., 2016). Rather, self-compatibility is a quantitative trait that can be measured, for example, by calculating self-compatibility indices (Lloyd and Schoen, 1992). From this, self-compatible species then span a continuum of reproductive modes. Reproduction can occur by i) predominantly outcrossing because incompatibility is maintained by spatial and temporal separation of ripe anthers and stigmas, despite a breakdown in the self-incompatibility mechanism (reviewed in Barrett, 2010b), ii) a mixture of outcrossing and selfing (mixed mating), because selfing is delayed and occurs only when outcrossing is unlikely (reviewed in Goodwillie et al., 2005), or iii) predominantly selfing, because there is no structural or temporal separation within the floral unit and because selfing can occur before and during anthesis (Lloyd, 1979; 1992).

In summary, it remains uncertain whether selection for reproductive assurance is more common with increasing environmental harshness (Hargreaves and Eckert, 2014), whether stress induced by climate change can select for the evolution of selfing (Barrett and Harder, 2017), whether populations of a single species can increase their self-compatibility in response to increasing aridity and whether selfing to assure reproduction in such environments is advantageous (Cheptou and Massol, 2009; Barrett et al., 2014).

1.3. Plant defence systems

Plant populations exhibit large geographic variation in plant defences that affects palatability to herbivores. It remains unclear, however, whether this variation is caused in response to different environmental conditions or because herbivore pressure changes in the different locations (Pennings et al., 2009). On the one hand, plant metabolic activity is directly affected by environmental variation and thus resources available for herbivore defence are directly limited by the local environment (Rasmann et al., 2014). On the other hand, the abundance and activity of insect herbivores is directly affected by environmental variation because insect herbivores are ectotherms (Novotny et al., 2005; Rodríguez-Castañeda et al., 2010). Therefore, under varying environmental conditions, herbivore abundance and plant herbivore defences are expected to form clines (Rasmann et al., 2014). Consequently, a wealth of theories has been developed to explain the interspecific and intraspecific variation in plant herbivore defence.

Early theoretical studies concentrated on the physiological costs involved in the production of defences by correlating the costs and benefits of defence to plant fitness parameters such as growth or reproduction (van der Meijden, 1996). The resource allocation hypothesis (also known as the optimal defence theory) posits that the cost of producing defence chemicals in low-resource environments is less than the cost of replacing biomass lost to herbivory and so consequently plant fitness increases with increasing defence concentrations in low-resource environments and *vice versa* in high-resource environments (Coley et al., 1985). The plant stress hypothesis posits that plants with limited resources are more palatable to herbivores than plants with sufficient resources because plants under environmental stress increase the amount of nitrogen available in their tissues (White, 1984). The plant vigour hypothesis directly contradicts this suggestion by proposing that vigorous plants, by virtue of containing so many nutrients, are more palatable to herbivores and thus herbivore pressure is greater on vigorous rather than stressed plants (Price, 1991). The carbon-nutrient hypothesis posits that more resources are invested into defence when the environment limits growth more than it limits photosynthesis. This is because carbon is more readily available than nutrients, making it cheaper to invest in carbon-based secondary metabolites, such as chemical defences, than it is to invest in growth (Bryant et al., 1983). The growth-differentiation balance hypothesis develops this further by incorporating the trade-off between sufficient growth to compete with plant neighbours and sufficient defences to survive herbivore attack (Herms and Mattson, 1992). By incorporating other resource-based hypotheses on intraspecific variation in plant defences, the growth-differentiation balance hypothesis has been regarded as the most advanced hypothesis of its type (Stamp, 2003; Scogings, 2018).

Another explanation for the variation in plant defence concentration, independent of physiological costs to the plant, is the divergent selection pressure that generalist and specialist herbivores exert on the plant (van der Meijden, 1996). Plants are subject to a community of herbivores including both generalist and specialist herbivores. Generalist herbivores are deterred by high concentrations of defence chemicals, whilst specialist herbivores use these defence chemicals as cues to find and identify plants that provide ideal food and oviposition sites (van der Meijden, 1996). Consequently, the contrasting effects of generalist and specialist herbivores can explain the great interspecific and intraspecific variation in plant defence concentrations (van der Meijden, 1996).

Herbivore defence is predominantly a function of plant secondary metabolites (Bazzaz and Grace, 1997; Agrawal and Weber, 2015). Herbivore defences can be expressed constitutively, *i.e.*, permanently throughout a plant’s life, and this mode of defence is expected to be favoured in unpredictable environments and when damage is high (Karban and Baldwin, 1997). Conversely, herbivore defences can be synthesized upon herbivore attack and this temporary induced defence is expected to be favoured when herbivory is high but variable (Karban and Baldwin, 1997). Herbivory is typically measured as herbivore density and as leaf damage across multiple sites ranging along an environmental gradient (Pennings et al., 2009; Anstett et al., 2016). Whilst these methods do not measure natural selection directly, evidence of a strong geographic cline in herbivory can support the hypothesis that geographic variation in herbivory selects for geographic variation in plant defence traits (Pennings et al., 2009). Furthermore, using complementary common-garden environments determines whether plants express constitutive defences and whether this expression is under genetic control (Salgado and Pennings, 2005).

Plant secondary metabolites involved in herbivore defence include condensed tannins (Ward et al., 2011; Moreira et al., 2017a), monoterpenes (Pratt and Mooney, 2013), cardenolides (Agrawal et al., 2015), tropane alkaloids (Castillo et al., 2013), cyanogenic glucosides (Kooyers et al., 2014), phenolics (Abdala-Roberts et al., 2016), and glucosinolates (Wittstock and Halkier, 2002; Halkier and Gershenzon, 2006; Agerbirk and Olsen, 2012; Metz et al., 2014; Tomiolo et al., 2017). Glucosinolate chemicals and their hydrolysis products are detrimental to a wide variety of organisms and are found predominantly in the Brassicales order (Halkier and Gershenzon, 2006; Agerbirk and Olsen, 2012). Glucosinolates share a core structure of thiohydroximates with an *S*-linked β-glucopyranosyl residue and an *O*-linked sulphate residue and consist of a variable side chain derived from amino acids (Agerbirk and Olsen, 2012). Based on this amino acid precursor glucosinolates can be classified as methionine-derived (aliphatic glucosinolates), phenylalanine-derived (benzenic glucosinolates) or tryptophan-derived (indole glucosinolates) (Agerbirk and Olsen, 2012). Plants often produce a variety of glucosinolates to ensure survival against a range of herbivores (Müller et al., 2010). Glucosinolates can be constitutively expressed or can be inducible upon herbivore damage (Halkier and Gershenzon, 2006; Hopkins et al., 2009). Upon tissue damage, the enzyme myrosinase is activated and hydrolyses the glucosinolates into glucose and an unstable by-product, such as isothiocyanates and nitriles

(Halkier and Gershenzon, 2006; Agerbirk and Olsen, 2012; Wittstock and Halkier, 2002). These by-products are known to have anti-feeding, growth inhibition and toxic effects on many generalist herbivores yet can attract specialist herbivores (Halkier and Gershenzon, 2006; Agerbirk and Olsen, 2012).

In summary, because of the complexity of interactions between herbivores and the environment, between plants and the environment, and therefore between herbivores and plants it remains challenging to predict whether the concentration of plant chemical defences increases or decreases along an environmental gradient, especially within a species (Hahn and Maron, 2016).

1.4. Eastern Mediterranean Basin

The Eastern Mediterranean Basin, the landmass east of the Mediterranean Sea, is characterized by a rainfall gradient ranging from mesic-Mediterranean conditions to arid conditions (Black, 2009; Smiatek et al., 2011; Golodets et al., 2013). The region is considered a biodiversity hotspot because of its diverse flora and fauna (Myers et al., 2000). Many plant species are distributed across the rainfall gradient (Golan-Angelko and Bar-Or, 2008) and there is a high species turnover between the mesic-Mediterranean and arid environments (Holzapfel et al., 2006). However, some species, especially in the Brassicaceae family, are distributed across the length of the rainfall gradient, thus populations of these species inhabit a range of environments and experience different environmental selection pressures (Petrů et al., 2006). For example, plant populations inhabiting resource-rich, mesic-Mediterranean conditions are more likely limited by biotic competition whilst populations inhabiting resource-poor, arid conditions are most likely limited by rainfall (Golodets et al., 2013). Such divergent selection within a species may result in the different populations becoming locally adapted to their environment, including in their strategies to interact with their associated animals (Thompson, 1988; Travis, 1996; Galloway and Fenster, 2000). This may provide optimal fitness in the current climate, yet plant populations may suffer as the climate changes (Sala et al., 2000).

Climate change models predict increasing global temperatures and changing rainfall patterns globally (IPCC, 2014). Hydric environments are predicted to experience increasing mean annual rainfall, whilst xeric environments are predicted to experience decreasing mean annual rainfall. Furthermore, it is very likely that rain events will become more intense and that these extreme events will become more frequent as global mean surface temperature increases (IPCC, 2014). The Eastern Mediterranean Basin region is particularly vulnerable to climate change because mean annual rainfall in this area is expected to decrease in the order of 20% (Black, 2009; Evans, 2009; Smiatek et al., 2011; Golodets et al., 2013). Indeed, in 2017, unusually dry conditions were widespread in the Mediterranean region and the Eastern Mediterranean was badly affected by drought (WMO, 2018).

Investigating the adaptive potential of plant species to climate change can be achieved using natural rainfall gradients that allow a space-for-time approach (Sternberg et al., 2011). Populations currently inhabiting arid environments may represent the phenotype of populations inhabiting mesic environments in the future as these mesic environments become increasingly arid (Kigel et al., 2011). Any clines found in the traits of plant species distributed along the rainfall gradient can be used as proxies for future changes in plant fitness in response to climate change. Nevertheless, evidence of observed differences in populations currently inhabiting different environments suggests that these plant populations are locally adapted to the current climate (Galloway and Fenster, 2000). Local adaptation may render populations vulnerable to extinction if they are unable to rapidly adapt *in situ* or if they are unable to migrate to more favourable conditions as the climate changes (Peñuelas and Filella, 2001; Walther et al., 2002; Parmesan, 2006; Aitken et al., 2008). The Eastern Mediterranean Basin will likely experience large biodiversity loss should the climate change at a rate faster than species can adapt to increasing aridity (Sala et al., 2000).

1.5. Brassicaceae family

The Brassicaceae family is known globally for hosting a range of important crop species (e.g. *Brassica oleracea, Brassica napus*) and in ecology for hosting the most studied model species (*Arabidopsis thaliana*). The Brassicaceae family is one of the most diverse and abundant families in the Eastern

Mediterranean Basin where many species naturally grow together and many populations are distributed along the rainfall gradient (Boaz et al., 1990). These native species have a common phylogenetic history, a common local history, and are wild relatives of agricultural crops (Boaz et al., 1990; Gupta, 2016). Moreover, the Brassicaceae family contains a diverse range of self-incompatible and self-compatible species (Plitmann, 1993). Furthermore, Brassicaceae species predominantly use glucosinolates as herbivore defences (Halkier and Gershenzon, 2006; Agerbirk and Olsen, 2012). Maintaining these native species and understanding their natural ecology is highly relevant not only for biodiversity and conservation but also for maintaining genetic diversity that can be bred into crop varieties. Taken together, the characteristics of the Brassicaceae family make it a very convenient system for comparing to other studies and make the outcomes of this thesis highly general.

Altogether six species of the Brassicaceae family were used to for the research presented in this thesis: *Biscutella didyma* (L.), *Erucaria pinnata* (Viv.) Taeckh. & Boulos, *Hirschfeldia incana* (L.), *Isatis lusitanica* (L.), *Matthiola livida* (Delile) DC., and *Sinapis alba* (L.). Those species used for investigating plant-pollinator interactions include *B. didyma*, *I. lusitanica* and *S. alba*. *Biscutella didyma* is classified as self-compatible (Plitmann, 1993) and is distributed from mesic-Mediterranean to arid environments and has a Mediterranean-Irano-Turanian chorotype (Zohary, 1966). *Isatis lusitanica* is classified as self-incompatible (Plitmann, 1993) and is distributed from mesic-Mediterranean to semi-arid environments and has a Mediterranean-Irano-Turanian chorotype (Zohary, 1966). *Sinapis alba* is classified as self-incompatible (Plitmann, 1993), yet has been shown to successfully produce self-seeds in the absence of pollinators (E. Gibson-Forty, *personal observation*). *Sinapis alba* is distributed from mesic-Mediterranean to semi-arid environments and has a European-Siberian-Mediterranean-Irano-Turanian chorotype (Zohary, 1966). Those species used for investigating plant-herbivore interactions include *E. pinnata*, *H. incana*, *I. lusitanica*, *M. livida* and *S. alba*. *Erucaria pinnata* and *M. livida* are distributed from semi-arid to arid environments and have a Saharo-Arabian chorotype and *H. incana* is distributed from Mediterranean to arid environments and has a Mediterranean-Irano-Turanian chorotype (Zohary, 1966).

1.6. Thesis objective and outline

The main scope of this thesis was to investigate the intraspecific variation in plant-animal interactions in the Eastern Mediterranean Basin. Specifically, how plant fitness is affected by changes in interactions with pollinators and herbivores across a range of environments. Ultimately the goal of this thesis is to provide evidence on the potential effects of climate change on plant fitness through the interaction with pollinators and herbivores. Such scope was achieved through an integrated approach that investigated how changing climate (changes in rainfall amount along the gradient) influences biotic activity (pollinator activity and herbivore pressure) and how this in turn affects plant reproductive success and investment in chemical defences. The study was carried out during two subsequent years. Herbivore pressure was quantified in the first year in the field and pollinator activity and natural selfing rates of target species were measured in the second year in the field. The degree of target species' self-compatibility was measured in the first year in the greenhouse and the concentrations of glucosinolates in target species was measured in the second year in the greenhouse and in the laboratory.

First, I examined 1) whether pollinator abundance and diversity decreased with increasing aridity and 2) whether this selected for reproductive assurance in three Brassicaceae species: *B. didyma*, *I. lusitanica*, and *S. alba* (chapter 2). Second, I investigated whether populations of two Brassicaceae species (*B. didyma* and *S. alba*) have adapted to increasing arid environments by evolving greater self-compatibility (chapter 3). Third, I investigated the relationship between leaf damage by herbivory and investment into plant chemical defence along the rainfall gradient (chapter 4). Finally, I discuss the evidence from the three chapters together, draw conclusions from this research and consider the effects that climate change may have on plant performance in general in the Eastern Mediterranean Basin (chapter 5). For the purposes of independent reading of single topics, each chapter was written individually, consequently some background information is repeated for the readers' convenience. The results of this research are highly relevant to predicting the long-term consequences of climate change on plant fitness because I used a multispecies approach to increase taxonomic replication enabling more general and realistic conclusions to be made than from single species studies. Furthermore, the combined approach using controlled greenhouse conditions with field observations and experiments in the natural environment allows my experimental findings to be related to real-world patterns.

Chapter 2

Investigating the dependency of plant reproductive success on pollinator activity: a field approach

Self-fertilisation in plants is a common strategy that has evolved in response to lack of pollinators or mates. Because the abundance of mates and potential pollinators are often reduced under resource limitation, it is predicted that plants depend more on self-fertilisation as a mechanism of reproductive assurance under stressful environmental conditions. Compared with the wealth of theoretical studies on the topic, relatively few studies have tested this prediction in the field. Moreover, field studies were usually performed for a single species, which hampers our ability to generalize. Here, I used three species from the Brassicaceae family growing together along a steep rainfall gradient, ranging from mesic-Mediterranean (950mm mean annual rainfall) to arid (90mm mean annual rainfall) conditions. I hypothesised that pollinator abundance and diversity decrease with increasing aridity and, consequently, that plant reproduction would increasingly rely on selfing with increasing aridity. I first determined whether pollinator abundance and diversity decreased with decreasing rainfall. Second, I excluded pollinators in the field and evaluated the relative reproductive success from self-fertilisation *vs*. pollinator-mediated outcrossing. I showed that although abundance and diversity of the pollinator community decreased, and the composition of the pollinator community changed with increasing aridity, self-fertilisation decreased in one species, increased in a second species and did not change in a third one. Taken together, these results indicate that, even in very harsh environments, pollinator limitation may not play a dominant role in selecting for self-fertilisation.

2.1. Introduction

Many hermaphroditic flowers have the potential to produce offspring without external pollinating agents. Such autonomous self-pollination (selfing) may be highly advantageous because it promotes the transmission of twice as many gene copies as outcrossing processes (Nagylaki, 1976). However, selfing also increases the risk of inbreeding depression and the expression of deleterious alleles (Jain, 1976; Lande and Schemske, 1985; Schemske and Lande, 1985; Charlesworth and Charlesworth, 1987). Therefore, selfing should be favoured when there is a high probability of ovules remaining unfertilized (Lloyd, 1992) and when inbreeding depression is relatively low (Lande and Schemske, 1985; Schemske and Lande, 1985). The mechanisms that select for selfing are highly relevant for population dynamics not only because they ensure current survival, but because they may affect local adaptation and the capacity to buffer environmental changes (Charlesworth et al., 1993; Charlesworth, 2003; Barrett, 2010a; Levin, 2010; Barrett et al., 2014; Hargreaves and Eckert, 2014; Peterson and Kay, 2015) due to their long-term effect on population genetic diversity (Charlesworth et al., 1993; Charlesworth, 2003; Barrett, 2010a; Levin, 2010; Barrett et al., 2014).

Reproductive assurance through selfing can be promoted by plant population characteristics or by the pollinator community. On the one hand, individuals in a small population are expected to rely more on selfing compared to larger populations due to mate limitation (Charlesworth and Charlesworth, 1979; Griffin and Willi, 2014; Opedal et al., 2016). On the other hand, selfing may also be selected for in habitats with low pollinator abundance and low pollinator diversity because both may decrease the probability and efficiency of gamete transfer between plants (Feinsinger, 1987; Harder and Barrett, 1996; Fausto et al., 2001; Eckert, 2002; Ashman et al., 2004; Arceo-Gómez et al., 2016).

Compared to the wealth of theory around reproductive assurance via selfing (Charlesworth and Charlesworth, 1979; Lande and Schemske, 1985; Lloyd, 1992), sound empirical evidence is relatively rare. Namely, we do not know how common the phenomenon of reproductive assurance is in nature and under which specific ecological conditions reproductive assurance is observed (Hargreaves and

Eckert, 2014). For example, because both plant population size and density, as well as pollinator availability, are strongly correlated with the level of investment in selfing, it was suggested that reproductive assurance will be higher towards species' range margins where both pollinators and mates are rare (e.g. Busch, 2005; Griffin and Willi, 2014). However, the empirical evidence is mixed (Eckert, 2002; Herlihy and Eckert, 2005; Moeller et al., 2012; Koski et al., 2017).

Based on the central role of mate and pollinator limitation in the reproductive assurance hypothesis, environmental gradients provide an ideal system for testing these theoretical predictions. This is because with increasing environmental harshness, plant populations tend to decline in size and density and thus become mate-limited (Levin, 2012; Maron et al., 2014). Similarly, environmental harshness is often correlated with a decrease in pollinator abundance (Devoto et al., 2009; Quintero et al., 2010; Chalcoff and Aizen, 2016) and with limited pollinator services (Hegland and Boeke, 2006; Lázaro et al., 2009). However, here as well, the results are equivocal: some studies report increased selfing with increasing climatic extremes (Evans et al., 2011; Jones et al., 2013), while others show no evidence of increased selfing under environmental stress (Kay and Picklum, 2013). Unfortunately, these studies have also focused on a single species (but see Kay and Picklum, 2013), which furthermore limits our ability to draw general conclusions. Therefore, the question remains open whether selection for reproductive assurance is more common with increasing environmental harshness (Hargreaves and Eckert, 2014).

In order to test the generality of the reproductive assurance hypothesis, I took advantage of the highly diverse Eastern Mediterranean annual-plant communities distributed along a steep rainfall gradient (Tielbörger et al., 2014) and selected three coexisting annual species of the Brassicaceae family. Average density of plants and thus mates decreases towards more arid conditions (Tielbörger et al., 2014), but little is known about pollinator activity. By comparing the reproductive success of the species with and without pollinators, I assessed whether plants increasingly rely on selfing to produce seeds in harsher environmental conditions and whether this tendency is correlated with changes in the abundance and diversity of the local pollinator communities. Specifically, I tested two hypotheses: 1) pollinator abundance and diversity, which affect pollen transfer probability, will decrease with environmental harshness; and 2) in correlation with the change in pollinator activity, plant selfing rates will increase with environmental harshness.

2.2. Materials and Methods

Study sites

The study was carried out in spring 2017 at four sites located along a steep rainfall gradient in Israel (Table 2.1). These four sites differ approximately ten-fold in average annual rainfall. All sites are situated on the same calcareous bedrock with a south-facing aspect and have similar mean annual temperatures. The length of the growing season is driven predominantly by rainfall; germination of annual species typically begins in October-November, peak flowering is between February and April, and senescence of vegetative biomass occurs around April-May. The growing season is typically shorter in more arid sites (Tielbörger et al., 2014).

I focused on three common annual species from the Brassicaceae family because it is one of the most diverse families in the region with many very abundant species, increasing the generality of my conclusions (Boaz et al., 1990). The study species were selected based on the following criteria: i) they are abundant and occur in at least three of the four study sites; ii) they have hermaphroditic, showy flowers; iii) they are large enough to enable pollinator exclusion in the field; and iv) they all have an overlapping peak-flowering time between February and March. Furthermore, each of the three species have been described to differ in their ability to self: *Biscutella didyma* (L.) occurs from the mesic-Mediterranean to the arid region, is self-compatible, and is known to reproduce successfully by self-fertilisation in the absence of pollinators (Plitmann, 1993; Tielbörger et al., 2012); *Isatis lusitanica* (L.) and *Sinapis alba* (L.) occur from the mesic-Mediterranean to the semi-arid region. The two species are classified as self-incompatible (Plitmann, 1993), but both may exhibit a mixed strategy and are capable of selfing, albeit at a much lower rate than *B. didyma* (E. Gibson-Forty, *personal observation*).

Table 2.1: Location and environmental conditions of sites along the gradient (Holzapfel et al., 2006; Kurze et al., 2017).

	Arid	Semi-arid	Mediterranean	Mesic-Mediterranean
Site name	Sde Boker	Lahav	Matta	Harashim
Coordinates	N30°52’ E34°46’	N31°23’ E34°51’	N31°42’ E35°3’	N32°57’ E35°19’
Mean annual rainfall	90 mm	300 mm	540 mm	950 mm

Pollinator activity observations

I observed pollinator activity and approximated pollinator abundance as number of visits (Hegland and Boeke, 2006) during peak pollination between March and April 2017. All observations were conducted on days with similar weather conditions (dry and warm), resulting in two observation days per site occurring within 4 days of each other, except the Mediterranean site where access was not possible for a second day during peak pollinator activity.

On each observation day, six 50×50 cm quadrats were distributed across the site (approximately 100 m^2) in areas that had at least one target species. This quadrat size was selected because it was large enough to cover a representative sample of conspecific and heterospecific individuals, yet the whole quadrat could be observed continuously.

The following data were recorded in each quadrat: i) the number of pollinator visits to one randomly selected individual of each target species present; ii) the number of pollinator visits to the remaining conspecific individuals; iii) the number of pollinator visits to heterospecific individuals; iv) the total number of all flowers on each of the target species; v) the total number of flowers in the quadrat.

Pollinator activity was observed in each of the six quadrats for three 13 minute rounds in a random order, spread across the day between 09:00 and 16:30. The three sessions were summed to give 39-minutes observation per quadrat per day (method adapted from Seifan et al., 2014). Pollinator activity observations along the gradient followed the phenology, starting in the arid site and ending in the mesic-Mediterranean site.

During a pollinator activity session, a visit was defined successful when an insect contacted a flower in a manner which could lead to cross-pollination (see Seifan et al., 2014). Owing to the protected status of many pollinators and the nature of the observations, I was unable to identify all visitors to a species level. Instead, pollinator identity was assigned to six groups, representing different functional groups of pollinators: solitary wild bees, hoverflies (Syrphidae), other flies (Diptera), ants (Formicidae), beetles (Coleoptera), and others (see Hegland and Totland, 2012; Seifan et al., 2014). Solitary wild bees were further classified into small (3-7 mm body length), medium (10-25 mm body length) and large (>26 mm body length) because foraging behaviour (e.g. foraging duration and range) may be determined by body size (Gathmann and Tscharntke, 2002; Arianoutsou-Faraggitaki and Groves, 2012).

Autonomous selfing

To quantify self-compatibility and the level of selfing in the target species, I excluded pollinators from forty randomly selected plants for each species per site. A single branch (typically the main stem) was selected on each individual plant and any open flower was removed. The branch was enclosed in a fine-mesh bag that permitted light and airflow but prevented insects from approaching (see similar protocols in Kron et al., 1993; Jacobs et al., 2009; Hargreaves et al., 2015). To compare the success of seed production by autonomous selfing with seed production by pollinator-mediated outcrossing, I selected a second individual plant that grew in close physical vicinity to the pollinator-excluded plants (5-10 cm distance) and I chose a single branch that fitted the same developmental stage as its pollinator-excluded neighbour, marked it and removed all opened flowers (pollinator exposure treatment). After a month, when the flowering season was over, the branch in the pollinator exposure treatment was enclosed in a fine-mesh bag to minimize differences in developmental conditions between the pollinator exclusion and exposure treatments, and to facilitate fruit and seed collection.

At the end of the growing season (May 2017 at the arid site, June at the mesic-Mediterranean site) I collected the branches and counted the number of fruits that developed on each branch, distinguishing between fruits bearing seeds and empty fruits (*i.e.*, flowers that failed to produce a fruit). In addition, I harvested the entire plant and measured its dry-weight biomass after 24 h in an oven at 70 °C.

To estimate the potential effects of site and pollinators on plant reproductive success, I first estimated the number of seeds produced per branch for each plant individual per treatment and site. For *I. lusitanica,* that produces only one seed per fruit, this was estimated as the number of seed-bearing fruits. For *B. didyma*, that produces two seeds per fruit, I estimated seed production by multiplying the number of seed bearing fruits by two. For *S. alba*, that produces a variable number of seeds per fruit, I estimated the number of seeds produced by dividing the total number of seeds per branch by the number of seed-bearing fruits on this branch (brood size *sensu* Wiens, 1984). I then calculated, for each of the species, the relative seed production, as the proportion of seeds produced out of the number of flowers on the sampled branch (seed: ovule ratio; Wiens et al., 1987). Note, that while selfing can be directly derived from the reproductive success in the pollinator exclusion treatment, the pollinator exposure treatment represents reproductive success from both outcrossing and potential selfing. Nevertheless, I use the pollinator exposure treatment to provide indirect evidence of the reliance of plant reproductive success on pollinator-mediated outcrossing. That is, small differences in seed production between the two treatments suggests limited reliance on outcrossing, whilst large differences suggest substantial reliance on outcrossing.

I further tested for seed viability, because self-compatibility is not only expressed as the number of seeds but also in their quality, *i.e.*, the existence of a viable embryo (Metsare et al., 2015). All of the seeds produced were stored in a glasshouse at Tübingen, Germany, to over-summer. In October 2017, the seeds were sown on wet filter paper in petri dishes that were well irrigated before sealing and the number of germinated seeds was counted until no more seeds germinated. Because non-germinated seeds may be dormant, I pierced the seed coat of all non-germinated seeds with a needle to expose the embryo (Pake and Venable, 1996). The proportion of viable seeds out of the total number of seeds produced was calculated as the number of germinated and embryo-bearing seeds out of the total seeds.

Statistical analysis

I assessed the change in total number of pollinator visits per flower across sites using a generalised linear model (GLM) with gamma distribution (inverse link). The number of pollinator visits per flower was calculated as the number of pollinator visits to a target individual divided by the number of flowers on that target. Because I assumed that the number of neighbouring flowers affects pollinator activity (Hegland and Boeke, 2006), I tested for the additional potential effects of conspecific and total number of flowers per quadrat using model selection based on AICc values. I further calculated the number of pollinator visits per flower for each target species separately and tested, in a similar fashion, the effects of site and neighbouring flower numbers using GLM with gamma distribution (inverse link). I also calculated species richness and Shannon-Wiener diversity of the visiting pollinator community and analysed them as a function of site using GLM with normal distribution (identity link).

I further tested, for each species separately, whether the relative seed production and seed viability changed across sites using a normal distribution (log link) and binomial distribution (logit link), respectively. I used as explanatory factors the pollinator exclusion treatment (exclusion of *vs*. exposure to pollinators), the site and their interactions. I additionally checked for the contribution of the individual plant biomass as a covariate in these models based on AICc values, but as it did not contribute to the models, plant biomass was dropped from the presented results.

In cases of significant differences among sites, I applied Tukey post-hoc multiple comparisons. All statistics were carried out in R version 3.3.1 (R Core Team, 2016) with packages ‘Lsmeans’ (Lenth, 2016) and ‘Vegan’ (Oksanen, 2017).

2.3. Results

Pollinator activity

Overall, the number of pollinator visits to the observed quadrats decreased towards the arid site ($\chi_3^2 = 33.25; P < 0.001$; Fig. 2.1a). In addition, total visits to the quadrats were significantly affected by the number of overall flowers growing in them (Appendix Fig. A1, Tables A1-2).

Simultaneously, the pollinator community was significantly richer and more diverse towards the mesic-Mediterranean site (species richness: $F_{3,28} = 8.42; P < 0.001$; species diversity: $F_{3,28} = 7.19; P < 0.001$; Appendix Table A3). Specifically, beetles were the dominant floral visitors in the arid, semi-arid and Mediterranean sites, whilst in the mesic-Mediterranean site, medium and large wild bees were the dominant visitors (Fig. 2.1b).

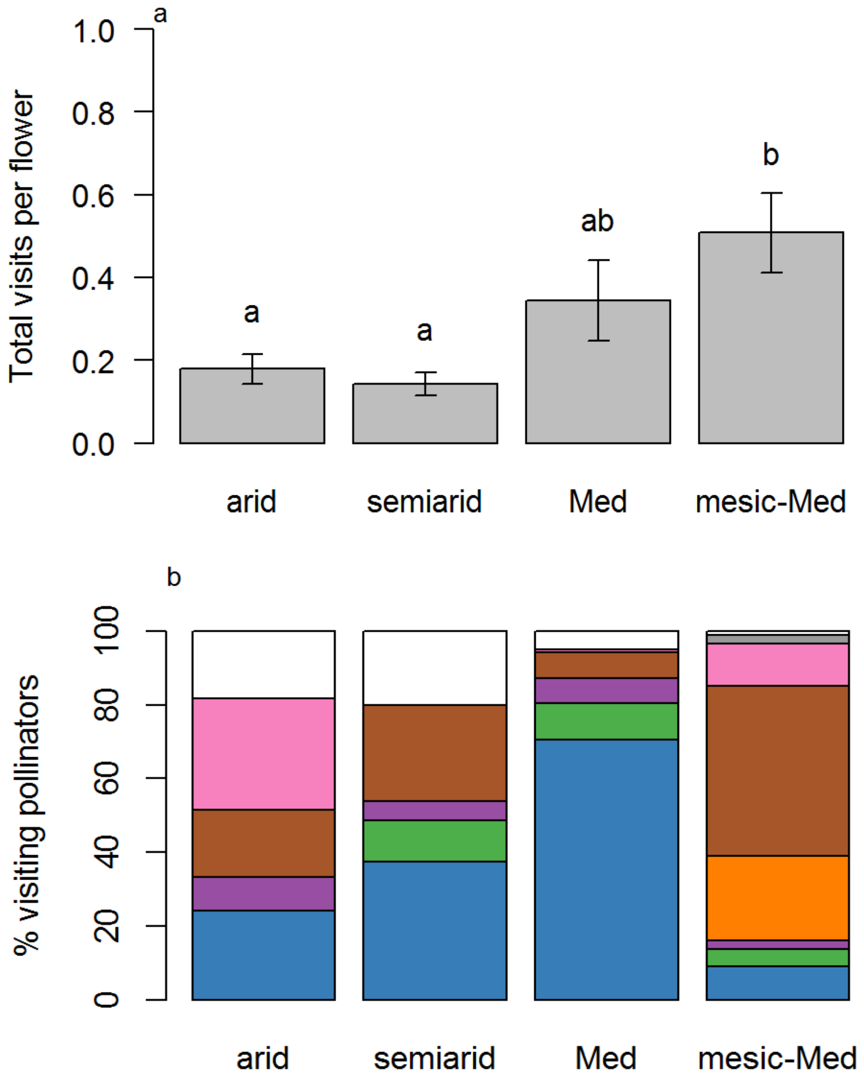

Figure 2.1: a) total number of pollinator visits to quadrats $(\bar{X} \pm SE)$ along the rainfall gradient. Different letters represent significant differences using Tukey multiple comparisons. b) morphospecies composition of pollinators visiting flowers of target species along the rainfall gradient. Within site, the different colours represent the relative percentage of visits by the different groups of pollinators. Colours represent the pollinator groups: pink = small wild bees; brown = medium wild bees; orange = large wild bees; grey = hoverflies; green = other flies; purple = ants; blue = beetles; white = other. Med = Mediterranean site; mesic-Med = mesic-Mediterranean site.

When analysing visitation rates for each of the target species separately the trends were less clear. In the case of *B. didyma*, whilst the factor site improved the statistical model (Appendix Table A1), it had no significant effect on pollinator visits to flowers ($\chi^2_3 = 7.08; P = 0.069$; Fig. 2.2). Instead, the number of *B. didyma* flowers in the quadrat had a significant effect on the number of pollinators visiting the plant ($\chi^2_1 = 10.57; P = 0.001$), but the effect was negative (Appendix Table A2).

The rate of pollinator visits to *I. lusitanica* was significantly different across sites ($\chi^2_2 = 8.14; P = 0.017$), with the most visits observed in the Mediterranean site (Fig. 2.2, Appendix Tables A1-2). Visits to *S. alba* were significantly affected by both site ($\chi^2_2 = 19.98; P < 0.001$) and the number of *S. alba* flowers in the quadrat ($\chi^2_1 = 9.00; P = 0.003$; Appendix Tables A1-2). Specifically, visits to *S. alba* decreased with aridity (Fig. 2.2) and with decreased flower number (Appendix Table A2).

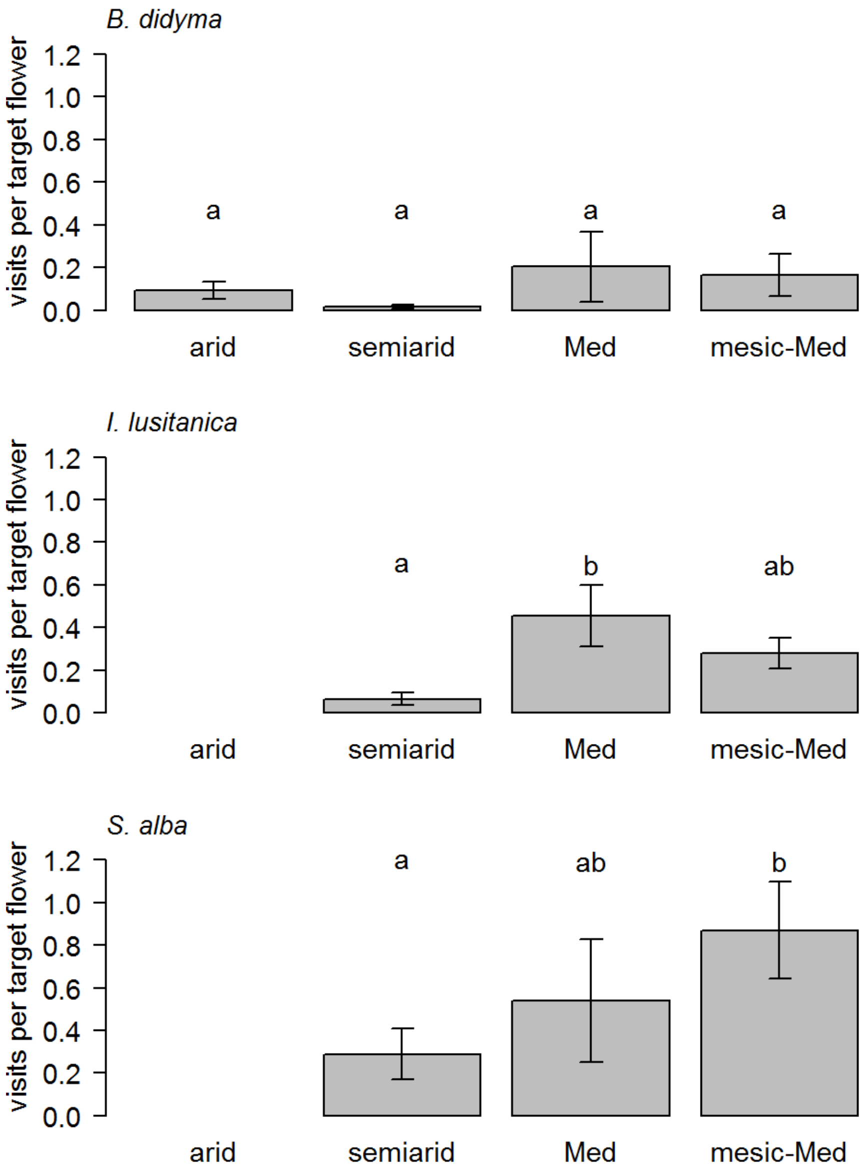

Figure 2.2: Number of pollinator visits to the target species ($\bar{X} \pm SE$) along the rainfall gradient. Different letters represent significant differences using Tukey multiple comparisons. Med = Mediterranean site; mesic-Med = mesic-Mediterranean site.

Autonomous selfing: Relative seed production per flower

Site had a significant effect on relative seed production of *B. didyma* (Table 2.2), so that, overall, seed production was higher at the mesic-Mediterranean site relative to the other sites (Fig. 2.3a). Additionally, a significant interaction between site and pollinator treatment was detected. Namely, seed production was significantly greater in the pollinator exclusion treatment than the exposure treatment in the arid site, while pollinator exclusion had no clear effect in the other sites.

In the case of *I. lusitanica*, only the pollinator exclusion treatment had a significant effect, decreasing the number of seeds produced (Fig. 2.3b, Table 2.2). Pollinator exclusion was also the only significant treatment in the case of *S. alba* (Table 2.2). However, in this case, the multiple comparison test revealed a significant trend of decreased seed production towards the mesic-Mediterranean site when flowers were exposed to pollinators (Fig. 2.3c). Additional information may be found in the Appendix Fig. A2, Tables A4-7.

Seed viability

Overall, all the produced seeds were viable in a similar, relatively high, proportion. The multiple comparison test revealed a significant reduction in viability in the case of *B. didyma* in the semi-arid site (Fig. 2.3d, Table 2.2). Exclusion of pollinators significantly reduced seed viability in *S. alba* (Fig. 2.3f, Table 2.2). Site and the interaction between site and treatment had no significant effect on the viability of the seeds produced by any of the target species (Fig. 2.3d-f, Table 2.2).

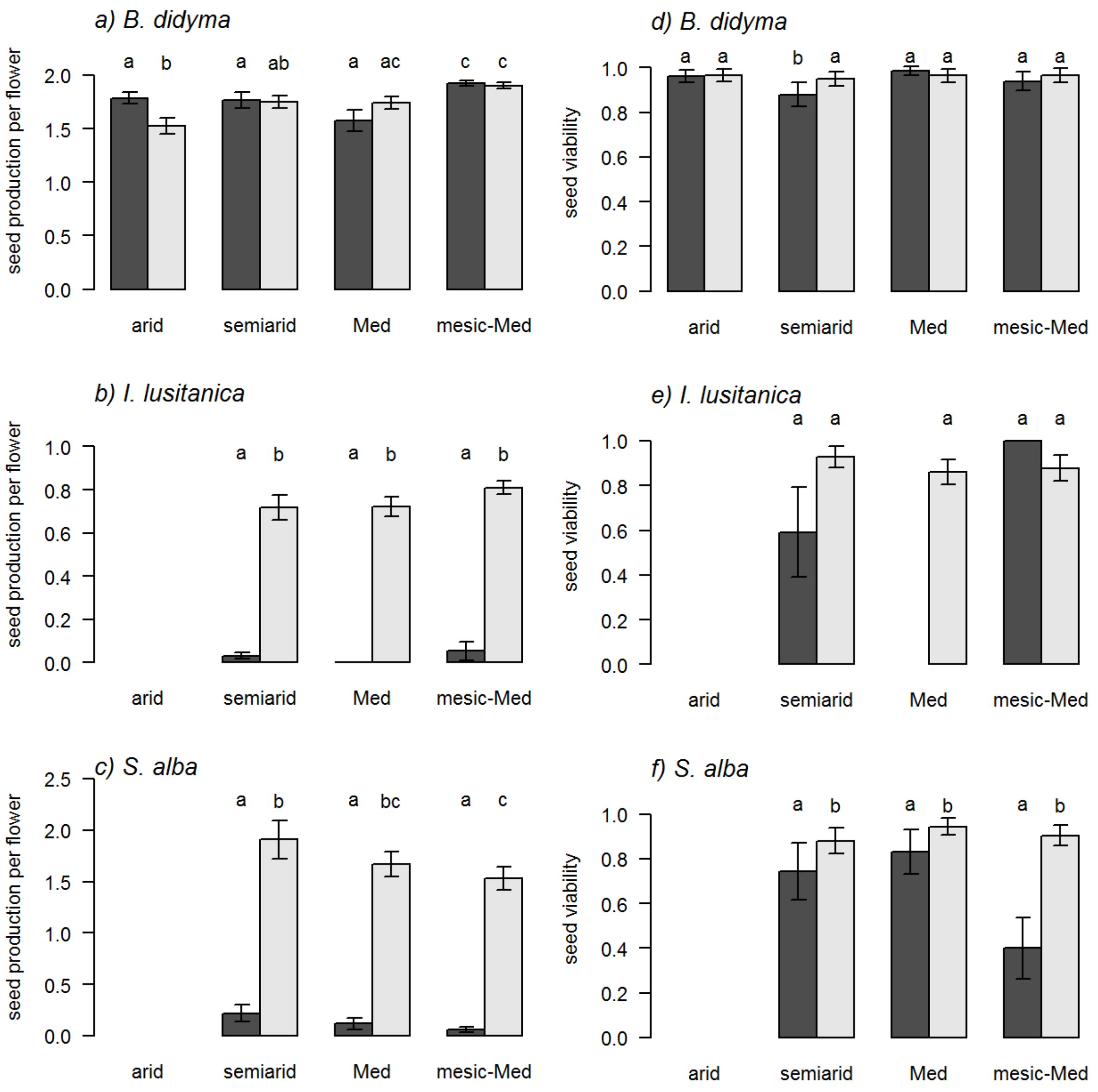

Figure 2.3: Relative seed production and seed viability of the produced seeds ($\bar{X} \pm SE$) per target species along rainfall gradient: a, d) relative seed production and seed viability for *B. didyma*; b, e) relative seed production and seed viability for *I. lusitanica*; c, f) relative seed production and seed viability for *S. alba*. Dark grey bars = pollinator exclusion treatment, light grey bars = pollinator exposure treatment. Different letters represent significant differences using Tukey multiple comparisons. Med = Mediterranean site; mesic-Med = mesic-Mediterranean site.

Table 2.2: Statistical results from the GLM models for each study species, testing for the effects of site, pollinator treatment (exclusion from *vs*. exposure to pollinators) and their interaction on relative seed production per flower and seed viability. Bold: P-values <0.001; **: 0.01>P-values >0.001; *: 0.05>P-values >0.01. The coefficient information for the models can be found in the Appendix (Tables A4-6).

		Site	Treatment	Site × Treatment
		Wald χ^2(df)	Wald χ^2(df)	Wald χ^2(df)
Relative seed production	*B. didyma*	**19.713(3)**	0.506(1)	12.066(3)**
	I. lusitanica	1.050(2)	**16.430(1)**	0.092(1)
	S. alba	0.943(2)	**14.899(1)**	0.435(2)
Seed viability	*B. didyma*	0.812(3)	0.704(1)	0.897(3)
	I. lusitanica	0.911(2)	0.392(1)	0.373(1)
	S. alba	0.656(2)	**0.048(1)**	0.171(2)

2.4. Discussion

Overall, this study shows that the abundance and diversity of the visiting pollinator community decreased along the rainfall gradient. However, these observations were not necessarily correlated with visitation rate to the target species and were not correlated with an increase in the plant species' reliance on selfing. Thus, our study does not unequivocally support the reproductive assurance hypothesis (Lloyd, 1992).

The overall decrease in pollinator abundance with increasing aridity followed our initial prediction and was accompanied by a decrease in pollinator diversity. This was mainly created by the unique presence of large solitary wild bees and hoverflies in the more mesic habitats, similar to observations in other Mediterranean regions (Moeller et al., 2012). These observed changes in the pollinator community may be caused by direct and indirect resource limitation. For example, water shortage may directly limit many stages of the insect life cycle, such as oviposition (Hegland and Boeke, 2006), feeding, mating, nesting, and pupation (Devoto et al., 2009). Furthermore, fewer and less predictable rain events in arid environments (Holzapfel et al., 2006) may indirectly limit pollinator abundance because extended time periods between rain events may reduce the quantity of nutritional resources for pollinators (Ghazoul, 2005). While I do not have direct evidence for a reduction in plant density along the studied gradient, previous studies in the region suggested such a trend (Holzapfel et al., 2006; Petrů et al., 2006; Tielbörger et al., 2014). Therefore, I suggest that not only the pollinator community changed along the gradient, but also the number of potential mates was reduced towards the arid edge of the target species' range.

Surprisingly, I found that neither pollinator abundance and diversity, nor the potential reduction in plant density, along the rainfall gradient were clearly correlated with a change in reproductive assurance. In particular, only in the case of *B. didyma* did I detect a reduction in overall seed production along the aridity gradient, in addition to indications for higher seed production by selfing in the most arid site. However, because there was no reduction in the pollinator visitation rate to *B. didyma* toward the arid region, these findings might not be a direct product of the shift in pollinator services, though they might indicate an effect of mate limitation. Similarly, in the case of *I. lusitanica,* a species that seems to rely almost exclusively on external pollination (Plitmann, 1993), no correlation between pollinator abundance and seed production was detected. Finally, in the case of *S. alba,* I found a negative correlation between the change in pollinator visitation rate and the relative seed production along the aridity gradient. My findings are in concordance with a case study from the Iberian Peninsula that showed a mismatch between pollinator services and investment in selfing along plant distribution ranges (Herrera et al., 2001). In particular, similar to my findings with *S. alba*, Herrera et al. (2001) found that in the case of *Helleborus foetidus*, the highest investment in self-pollination was found in the region in which pollinator services were the greatest.

The theoretical benefits of selfing in response to unpredictable pollinator environments and limited mate options have been intensively studied (Herlihy and Eckert, 2002; Barrett, 2003; Kalisz et al., 2004; Goodwillie et al., 2005; Eckert et al., 2006). However, the empirical evidence supporting the benefits of selfing in populations of species distributed along environmental gradients has been inconclusive. Recently, Koski et al. (2017) suggested three conditions that are required for reproductive assurance to occur: i) the reproductive system must allow within flower self-pollination; ii) pollen transfer between individuals must limit seed production in natural populations; and iii) selfing must increase seed production in the natural populations. Here, based on previous work on the species and from the present experiment, I could show that these conditions applied in only one of the three species (*B. didyma*) and it was in fact the only species exhibiting a trend towards reproductive assurance. In the case of the other two species, while the first two requirements were satisfied, selfing did not provide an advantage in the form of more seeds in two of the target species. On the contrary, selfing resulted in fewer seeds than outcrossing, and in the case of *S. alba*, seeds from autonomous selfing were also less viable, irrespective of the numbers of pollinators. Therefore, this study adds to the accumulating information that indicates that despite the theoretical appeal, the reproductive assurance hypothesis may not be supported in many plant species when put to test in the field (e.g. Busch, 2005; Herlihy and Eckert, 2005; Eckert et al., 2006; Mable and Adam, 2007; Koski et al., 2017).

Several reasons may explain my results, among them some methodological constraints. For example, plant-pollinator interactions and pollinator diversity and abundance can vary throughout the growing season (CaraDonna et al., 2017; Ogilvie et al., 2017). Therefore, it may be that the period of pollination observations in this study was too short and did not fully reflect the general patterns of pollinator activity. However, with four sites and a clear cline in abundance and diversity, I believe that this problem was minor. A potential stronger limitation to this study may be that I conducted the study in a single year in a region that experiences large inter-annual variation in rainfall amount (Holzapfel et al., 2006; Tielbörger et al., 2014). However, as the annual rainfall in the study year was below average and the annual temperature was above average (WMO, 2018), I believe that this year was representative of the conditions in which reproductive assurance is expected to be a significant process. Therefore, I suggest that the lack of pattern for two species in such a dry year is strong evidence against the existence of reproductive assurance in the system.

Other, more general, reasons may be at the heart of the discrepancy between my results and the theoretical predictions. First, Herrera et al. (2001) suggested that the reproductive assurance theory does not take into account a potential trade-off between the availability of pollinators and individual flower longevity. For example, plants may compensate for low visitation probabilities by increasing flowering duration (e.g. Ashman and Schoen, 1994; Herrera et al., 2001). However, this explanation seems unlikely in my study as the individual flowers of the target species do not survive more than 3 days (E. Gibson-Forty and V. Ferenc, *personal observations*). Second, it may be that the main determinant of seed production was not the level of selfing *per se* but a relative constant production of seeds by external pollination transfer. This may be related to the increase in pollinator diversity when the habitat becomes more benign, which may reflect less effective transfer of pollen between conspecifics (Eckert, 2002; Fenster et al., 2004; Benelli et al., 2017). In this system, long-tongued, large, solitary bees frequently visited the target species without landing on the flower (E. Gibson-Forty, *personal observation*). This behaviour may reduce pollen dispersal efficiency (Fenster et al., 2004). The higher pollinator abundance in the more benign habitat may also have contributed to excessive deposition of viable pollen that may clog the stigma and prevent pollen grain germination and pollen tube growth (Feinsinger, 1987; Plitmann, 1993; Feldman, 2008; Dauber et al., 2010). Finally, reproductive assurance may not necessarily be the main selective force shaping selfing evolution in the target-species. For example, it may be that selfing was developed as a mechanism to incur the costs of seed and pollen discounting, or that the mechanism was shaped by the presence of pathogens, or that selfing may be an unavoidable by-product of selection for outcrossing in species with multiple flower inflorescences (Goodwillie et al., 2005).

The mismatch between pollinator services and selfing ability observed in this study may not only question the reproductive assurance hypothesis but may also be related to the highly variable conditions that are a main feature of arid regions. Namely, many annual plants have adapted to the strong variability and unpredictability of resource availability in drylands by rapidly changing their growth and flowering activities (Crimmins et al., 2013). This may be supported by my finding that each target species could produce viable seeds via selfing (albeit in different numbers) even though the literature defined two of the species as incompatible (Plitmann, 1993). Moreover, it may be that, in this studied ecosystem, not only the plants, but also the pollinators are highly adapted to environmental cues and are therefore able to flexibly adjust their behaviour and activity to the local conditions (see Minckley et al., 2013). The rapid response of the pollinators may weaken the potential limitation on pollinator services and may allow plant species to maintain a relatively stable reproductive success even in the face of strongly limiting climatic conditions.

2.5. Conclusions

In conclusion, this study joins a body of work that attempts to understand the mechanisms regulating species performance along environmental gradients. Variability in a species' reproductive success is a significant determinant of genetic diversity and evolvability under global change (Pannell and Barrett, 2001; Cheptou and Massol, 2009; Hargreaves and Eckert, 2014). My overall findings suggest that despite decreasing pollinator abundance and diversity, and a change in composition of the pollinator community with increasing environmental harshness, the production of viable seeds from a range of annual plant species remained constant, or even increased. Such reliable seed production despite environmental uncertainties may be part of the mechanism by which plants in arid ecosystems buffer environmental changes.

Chapter 3

Self-compatibility as a plant adaptation to limited pollinator services: a mechanistic approach

Rainfall in dryland ecosystems, particularly the Mediterranean Basin, is predicted to decrease and occur in more unpredictable extreme events as the climate changes. Consequently, plant species must adapt to the increasingly arid environment or risk extinction. Different plant traits have been used to investigate whether dryland plant species can adapt to increasing aridity, ranging from seed dormancy, seed mass, and seed size, to flowering phenology and reproductive allocation. However, determining whether plant species adapt their reproductive strategy in response to increasing aridity, *i.e.*, by increasing their self-compatibility, has been overlooked. Here, we conducted a hand-pollination experiment using third generation inbred lines of populations originating from four sites along a natural aridity gradient in Israel. We tested whether two species inhabiting increasingly arid environments secure their reproduction by self-fertilisation. I predicted that populations originating from increasingly arid environments would produce more viable seeds through self-fertilisation than populations originating from benign environments. Based on this experimental approach, I could discern each species' natural selfing ability and could compare the success of selfing to outcrossing. Both species use a mixed-mating reproductive strategy, shown by viable seed set from both forced selfing and outcrossing, but with varying degrees of successful seed set by self-fertilisation. Both species produced more seeds by selfing with increasing aridity, suggesting that prezygotic self-incompatibility weakens with increasing aridity. Each species showed different trends in self-compatibility with respect to viability of the seeds produced. I speculate these differences arise from alternative selection pressures on the mechanism of selfing. This study demonstrates that increasing self-compatibility may help to maintain reproduction in harsh environments but is likely only one of numerous adaptations in reproductive strategies to increasingly arid conditions.

3.1. Introduction

Drylands, such as the Eastern Mediterranean Basin, are considered biodiversity hotspots because they comprise many plant species that have evolved different strategies to cope with water limitations (Myers et al., 2000). However, climate change models predict that drylands will experience reduced rainfall and more unpredictable extreme rainfall events (Smiatek et al., 2011; IPCC, 2013). Consequently, these highly diverse drylands will likely become increasingly arid, rendering those plant species unable to adapt to aridity vulnerable to extinction (Sala et al., 2000).

Typically, studies investigating plant adaptations to arid environments have focused on traits determining plant fitness at different life stages. These traits include seed mass and seed size (e.g. Metz et al., 2015; Bergholz et al., 2017), seedling survival and recruitment (e.g. Volis et al., 2002a; Metz et al., 2010; Siewert and Tielbörger, 2010; Rysavy et al., 2016), flowering phenology, onset of reproduction and reproductive allocation (Aronson et al., 1990; Aronson et al., 1992; Aronson et al., 1993; Volis et al., 2002b; Volis et al., 2002c; Petrů et al., 2006; Liancourt and Tielbörger, 2009; Kigel et al., 2011; Hanel and Tielbörger, 2015), and combinations of these and other traits (e.g. Holzapfel et al., 2006; Schiffers and Tielbörger, 2006; Liancourt et al., 2009; Kurze et al., 2017). Nevertheless, there is a lack of studies considering the adaptive ability of plant species to change their reproductive strategies in response to environments becoming increasingly arid and to pollinators and mates becoming increasingly limited. Annual plants are ubiquitous in drylands (Tielbörger and Salguero-Gómez, 2014) and may contribute significantly to the ecosystem annual net primary productivity (Tielbörger et al., 2014). By virtue of the annual life-cycle strategy, securing reproduction in the face of reproductive uncertainty, *i.e.*, pollinator limitation, is critical to sustaining populations through time (Lloyd, 1992). Many studies have focused on bet-hedging strategies in response to potential reproductive failure, such as seed dormancy and the existence of seed banks (Pake and Venable, 1996; Venable, 2007; Petrů and Tielbörger, 2008; Lampei and Tielbörger, 2010; Tielbörger and Petrů, 2010; Salguero-Gomez et al., 2012; Tielbörger et al., 2012; Gremer et al., 2016; Lampei et al., 2017; Metz et al., 2018). Yet, no studies have investigated whether limited pollinator services in increasingly arid environments has selected for adaptations in the mode of reproduction in annual plant species.

Assuring reproduction by self-fertilisation when the fertilisation of ovules via outcrossing is uncertain is the basis of the reproductive assurance hypothesis (Lloyd, 1992). Typically, this condition arises when pollinators are limited (Feinsinger, 1987; Harder and Barrett, 1996; Fausto et al., 2001; Ashman et al., 2004) or when conspecific mates are limited (Charlesworth and Charlesworth, 1979; Griffin and Willi, 2014; Opedal et al., 2016). Increasing aridity is likely to limit pollinator abundance and services (Devoto et al., 2009; Hegland and Boeke, 2006; Lázaro et al., 2009; Quintero et al., 2010; Chalcoff and Aizen, 2016) and reduce population size (Levin, 2012; Maron et al., 2014), thereby leaving dryland plant species increasingly at risk of reproductive failure which may select for increasing reliance on self-fertilisation.

Reproduction by self-fertilisation is possible in hermaphroditic flowers but is typically inhibited by self-incompatibility mechanisms to prevent inbreeding depression within the population (Jain, 1976; Lande and Schemske, 1985; Schemske and Lande, 1985; Charlesworth and Charlesworth, 1987). Often, hermaphroditic plants use mixed-mating to assure reproduction, that is, they outcross when the environment is favourable and self when there is a risk of reproductive failure (Goodwillie et al., 2005). Recently some studies have evaluated the effect of climate on plant mating systems and whilst some report extremes in climate are associated with the evolution of self-compatibility (Evans et al., 2011; Jones et al., 2013), others show no evidence of increased selfing under environmental stress (Kay and Picklum, 2013). Therefore, the question of whether stress induced by climate change can select for the evolution of selfing remains open (Barrett and Harder, 2017).

To the best of my knowledge, no studies have investigated whether plant species exposed to increasing aridity in drylands increase their self-compatibility in response, and thus self-fertilise more in increasingly arid environments. On the one hand, increasing self-fertilisation within a population can reduce dispersal capacity, which may select for rapid adaptation to novel conditions *in situ* and thus populations may be able to buffer environmental change (Cheptou and Massol, 2009; Hargreaves and Eckert, 2014). Yet, on the other hand, increasing self-fertilisation can also reduce population genetic variation and thus limit potential adaptation to changing conditions (Charlesworth et al., 1993; Charlesworth, 2003; Barrett, 2010a; Levin, 2010; Barrett et al., 2014). In summary, it is uncertain whether populations of a single species can increase their self-compatibility in response to increasing aridity and whether selfing to assure reproduction in such environments is advantageous.

Our approach to test whether plant species can adapt to increasing aridity as predicted by climate change, and to the subsequent limited pollinator services, was to use a natural rainfall gradient that encompasses a range of environments from mesic-Mediterranean to arid. The phenotypes of populations inhabiting the present-day arid environment may represent the future phenotype of populations inhabiting mesic environments as these environments become increasingly arid following reduced rainfall (Kigel et al., 2011). By extension, therefore, any clines found in the self-compatibility of plant species distributed along the rainfall gradient can be used as a proxy for changes in reproductive strategies as a result of climate change. Here, we provide a systematic test for the reproductive strategies of two annual plant species distributed along a natural rainfall gradient in Israel. We used *Biscutella didyma* (L.) and *Sinapis alba* (L.) from the Brassicaceae family because i) they are present in multiple sites along the aridity gradient, ii) they can produce self-seed when pollination is not possible (E. Gibson-Forty, *personal observation*) and iii) the number of pollinators known to visit these species declines with increasing aridity (chapter 2), *i.e.*, a prerequisite for increasing selfing is fulfilled. By using third generation inbred lines of populations originating from four sites along the gradient I compared the success of self-fertilisation with increasing aridity. I hypothesised that both species are more self-compatible in increasingly arid environments, that is, the reproductive success of plants in populations originating from increasingly arid environments is less limited by selfing than in populations originating from benign environments.

3.2. Materials and methods

Study sites

The rainfall gradient in Israel ranges from an arid environment in the south to a mesic-Mediterranean environment in the north. Four sites were selected along this gradient that are characteristic of different rainfall regimes (Table 3.1), yet all sites are situated on the same calcareous bedrock, have the same south-facing exposure and they share similar annual temperatures (Holzapfel et al., 2006). The average annual rainfall amount differs approximately nine-fold between the arid and mesic-Mediterranean sites. Inter-annual rainfall variation is greatest and the growing season is shortest in the arid site. Germination of annual species follows the first rainfall, typically between October and November. Seed set and senescence of annual species typically occurs between April and May.

Table 3.1: Location and environmental conditions of sites along the gradient (modified from Holzapfel et al., 2006).

	Arid	Semi-arid	Mediterranean	Mesic-Mediterranean
Site name	Sde Boker	Lahav	Matta	Ein Ya’akov
Coordinates	N30°52’ E34°46’	N31°23’ E34°51’	N31°42’ E35°3’	N35°0’ E35°14’
Mean annual rainfall	90 mm/y	300 mm/y	540 mm/y	780 mm/y

Study species and seed collection

To study whether populations originating from increasingly arid environments have evolved greater self-compatibility than populations originating from benign environments I selected two annual Brassicaceae species, *Biscutella didyma* and *Sinapis alba* distributed along the rainfall gradient. *Biscutella didyma* has been classified as self-compatible (Plitmann, 1993) and is present in all four sites along the gradient. *Sinapis alba* has been classified as self-incompatible (Plitmann, 1993), yet is capable of successfully producing seeds in the absence of pollinators (E. Gibson-Forty, *personal observation*) and is present in the mesic-Mediterranean to the semi-arid sites.

The study material comprised third generation (F3) inbred lines. The F0 seeds of *B. didyma* were collected in 2012 in all four sites along the gradient and the F0 seeds of *S. alba* were collected in 2014 in the mesic-Mediterranean to the semi-arid sites. The subsequent F1 and F2 generations were grown in a greenhouse under standard conditions, optimal water (soil saturation three times per week) and optimal light (10 h light), and temperature regimes that reflected the growing season in Israel (daily temperature range: 18-25 °C, night temperature range: 15-18 °C). Pollinators were excluded by wrapping plants in organza mesh upon first flowering. Seeds from the F2 generation were collected and stored in dark, dry conditions until they were sown for the present study.

Hand pollination experimental design

In November 2016, three to five seeds from 13 individuals per site of *B. didyma* and *S. alba* were sown in 10 x 10 x 10 cm pots in a 1:1 mixture of topsoil and sand and placed randomly in a glasshouse in optimal conditions (see previous description). After successful germination, plants were thinned to one seedling per pot, resulting in a total of 91 plants.

Each plant received two pollination treatments (outcrossing and selfing) and two controls (forced selfing procedural control and spontaneous selfing control). All buds on the branches that received the two treatments were fully emasculated before anthesis by carefully removing all anthers using forceps. Ripe stigmas in the outcrossing treatment received pollen from a different individual of the same species from the same site (Fig. 3.1, blue arrows). Ripe stigmas in the selfing treatment received pollen from a different flower on the same individual (Fig. 3.1, red arrows). All buds used in the forced selfing procedural control had one anther removed that deliberately contacted the stigma before its removal and, therefore, ripe stigmas received pollen from the same flower on the same individual. No manipulation was applied to the spontaneous selfing control and, therefore, ripe stigmas received pollen from the same flower on the same individual. The spontaneous selfing control demonstrated the natural capacity of the plants to spontaneously self in the absence of conspecific pollen. Differences between the selfing treatment and the spontaneous selfing control may indicate the presence of prezygotic self-incompatibility (Barrett, 1988), thus providing additional information on the natural mating systems of these species.

The outcrossing and selfing treatments and the forced selfing procedural control were applied to ten buds on separate branches on each individual plant. If less than ten buds were produced on a single branch, then all buds produced were manipulated and the number of buds was recorded. No buds were manipulated in the spontaneous selfing control and this branch was wrapped in organza upon first bud formation and left untouched for the duration of the experiment. A fifth branch was left to develop and the flowers provided pollen to the outcrossing treatment on a different plant and to the selfing treatment on the same plant (Fig. 3.1, donor (d) flowers). Additional branches were removed by cutting the branch at its base with scissors. A pilot study showed that branch removal did not affect fruiting success and that hand-pollination was successful.

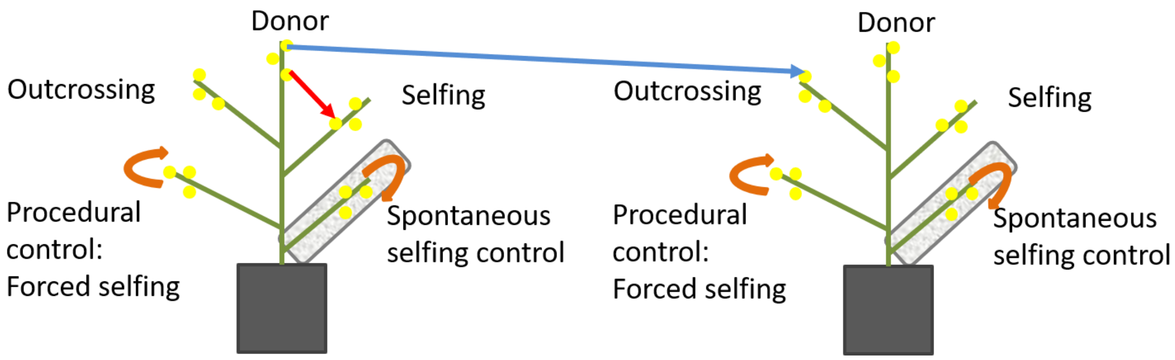

Figure 3.1: Schematic of experimental design per species per site. Each plant had 5 branches with a single treatment on each branch: donor flowers provided pollen for flowers in the selfing treatment (s) on the same individual (red arrow) and for flowers in the outcrossing treatment on a different individual (blue arrow), forced selfing procedural control and spontaneous selfing control received pollen from within the same flower (orange arrow) and thus did not receive pollen from a donor. Before flowers opened, all anthers were removed in the selfing treatment and outcrossing treatment and one anther was removed in the forced selfing procedural control. Flowers were not manipulated in the spontaneous selfing control and the branch was wrapped immediately in an organza mesh bag (grey rectangle). The spontaneous selfing control demonstrated the natural capacity for the plant to spontaneously self in the absence of conspecific pollen.

Testing viability

The seeds produced were tested for viability in October 2017 by recording germination. All seeds produced from the outcrossing and selfing treatments and the forced selfing procedural control were germinated and a subsample of 20 seeds produced from the spontaneous selfing control were germinated. A subsample of seeds was used for the spontaneous control to control for the accuracy of the viability ratio because the bud number in the spontaneous selfing control was not limited to ten and consequently more than ten times more seeds were produced compared to the other treatments. A maximum of 20 seeds were sown on wet filter paper in a single petri dish which was well irrigated before sealing. The petri dishes were placed in a glasshouse with a light regime reflecting winter conditions in the field in Israel (10 h) for 7 days. All germinated seeds were counted and recorded as viable seeds. The seed coat of all non-germinated seeds was pierced with a needle to expose the embryo in order to determine its viability. White fleshy embryos were counted as viable (method adapted from Pake and Venable, 1996).

Self-compatibility index

I used a self-compatibility index derived from Lloyd and Schoen (1992), calculated as the number of seeds produced per flower manipulated from the selfing treatment divided by the number of seeds produced per flower manipulated from the outcrossing treatment. I used this index first for the absolute number of seeds produced. Here, the index was calculated as the absolute number of seeds produced per flower manipulated from the selfing treatment divided by the absolute number of seeds produced per flower from the outcrossing treatment. I then used the index for the proportion of viable seeds out of total seeds produced. Here, the index was calculated as the number of viable seeds out of total seeds produced by selfing divided by the number of viable seeds out of total seeds produced by outcrossing. Finally, I used the index for the number of viable seeds produced. Here, the index was calculated as the number of viable seeds produced per flower manipulated from the selfing treatment divided by the number of viable seeds produced per flower from the outcrossing treatment. The index of the absolute number of seeds produced represents the potential reproductive success of the species, whilst the index of the number of viable seeds produced represents the actual reproductive success of the species.

Statistical analysis

To assess whether the manipulation treatments affected the absolute number of seeds produced per flower manipulated in each species I used an ANOVA with the absolute number of seeds produced per flower manipulated as the dependent variable and treatment, site, and their interaction as independent categorical variables. Conformation to the ANOVA assumptions was performed visually as proposed by Zuur et al. (2009). Post-hoc pairwise comparisons were performed for meaningful interactions (*i.e.*, between sites, between treatments within each site, and between the spontaneous control across all sites) and the *P*-values were adjusted using the Tukey method where necessary.

To assess whether populations of *B. didyma* and *S. alba* originating from increasingly arid environments were significantly more self-compatible than populations originating from benign environments, I compared the three indices of self-compatibility as dependent variables against the continuous independent variable rainfall using linear regression models. Conformation to the linear regression assumptions was performed visually as proposed by Zuur et al. (2009). Linear regression models were used first for the absolute number of seeds produced per flower manipulated, second for the proportion of viable seeds out of total seeds produced, and third for the number of viable seeds produced per flower manipulated. All statistical analyses and graphics were performed in R 3.3.1 (R Core Team, 2016) with the package 'multcomp' (Hothorn et al., 2008).

3.3. Results

The population of *B. didyma* originating from the arid site produced significantly fewer seeds overall than populations originating from the semi-arid and mesic-Mediterranean sites (Fig. 3.2, Table 3.2). There was no effect of the manipulation treatment on the number of seeds produced within populations (Fig. 3.2, Table 3.2). Significantly fewer seeds were produced by spontaneous selfing in the population of *B. didyma* originating from the mesic-Mediterranean site than from the populations originating from the semi-arid and arid sites (Fig. 3.2, Table 3.2).

The site of origin had no significant effect on the total number of seeds produced in *S. alba* (Fig. 3.2, Table 3.2). Populations originating from the semi-arid and Mediterranean sites produced significantly more seeds in the manipulated outcrossing treatment compared to the forced selfing procedural control and spontaneous selfing control (Fig. 3.2, Table 3.2). There was no significant effect of the manipulation treatment in the population originating from the mesic-Mediterranean site. There was no significant effect of site of origin on the production of seeds in the spontaneous selfing control (Fig. 3.2, Table 3.2).

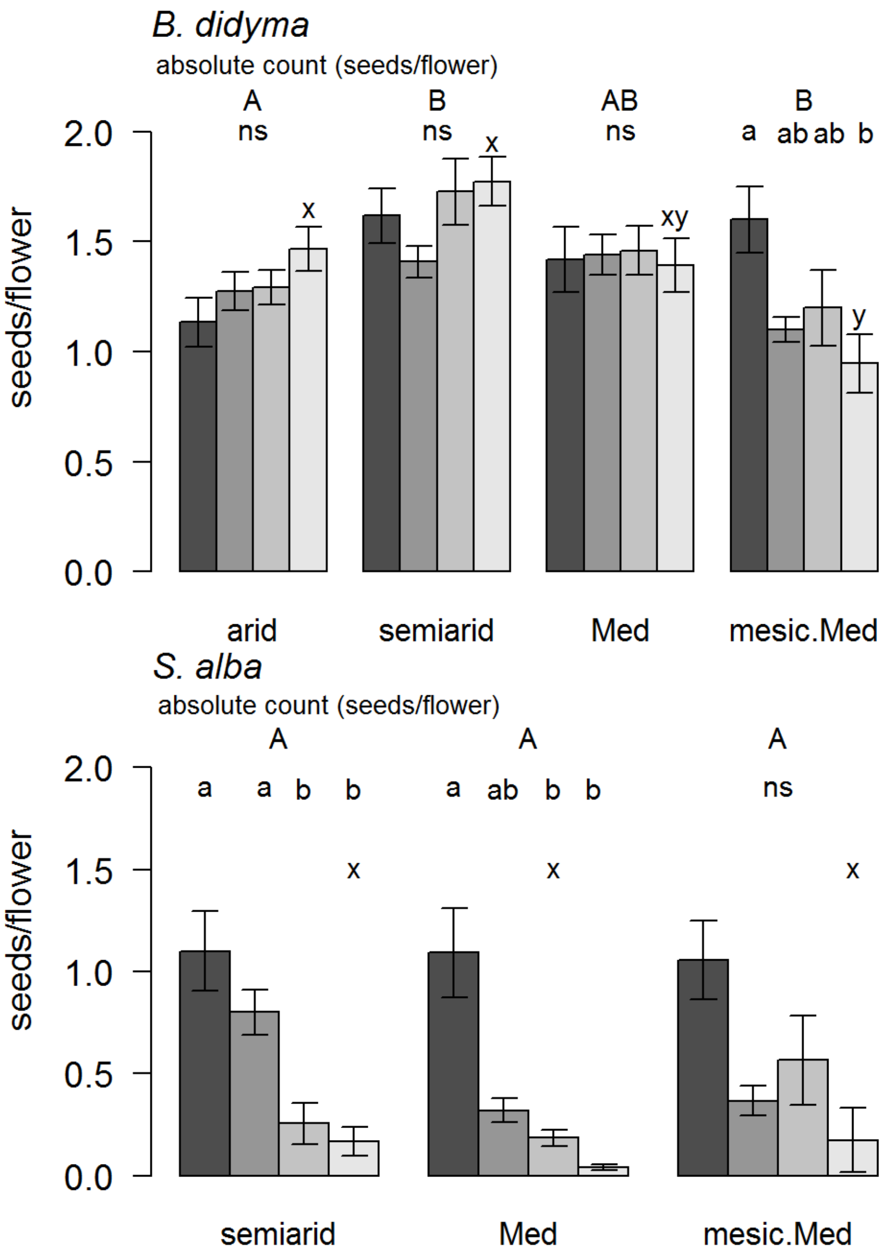

Figure 3.2: the absolute number of seeds produced per flower in *B. didyma* and *S. alba* for each treatment in each site of population origin. Black bars = outcrossing treatment, dark grey bars = selfing treatment, grey bars = forced selfing procedural control, light grey bars = spontaneous selfing control. Capital letters indicate the significant differences in absolute number of seeds per flower produced across sites as a main factor, lower case a/b indicate the significant differences in absolute number of seeds per flower produced between treatments within the same site, lower case x/y indicate the significant differences in absolute number of seeds per flower produced in the spontaneous control across sites, ns indicates no significant difference. Pairwise comparisons were performed using a Tukey post-hoc test. Med= Mediterranean site, mesic.Med= mesic-Mediterranean site.

Table 3.2: Coefficients ± SE for the analysis of the effects of site (arid, semi-arid, Mediterranean and mesic-Mediterranean), treatment (outcrossing, selfing, forced selfing procedural control and spontaneous selfing control) and their interactions on the seed production per flower for *B. didyma* and *S. alba*.

		B. didyma		*S. alba*	
		β_1 ±SE	*P*-value	β_1 ±SE	*P*-value
	Intercept	**1.133±0.113**	**<0.001**	**1.100±0.143**	**<0.001**
Site	Semi-arid	**0.485±0.163**	**0.003**	N.A.	N.A.
	Med	0.287±0.167	0.088	-0.008±0.198	0.969
	mesic-Med	**0.467±0.163**	**0.005**	-0.045±0.207	0.827
Treatment	Selfing	0.142±0.159	0.375	-0.300±0.212	0.161
	Procedural control	0.158±0.159	0.322	**-0.845±0.264**	**0.002**
	Spontaneous control	**0.334±0.163**	**0.042**	**-0.933±0.212**	**<0.001**
Site x Treatment	Semi-arid x selfing	-0.351±0.230	0.130	N.A.	N.A.
	Semi-arid x Procedural control	-0.049±0.231	0.831	N.A.	N.A.
	Semi-arid x Spontaneous control	-0.178±0.236	0.451	N.A.	N.A.
	Med x selfing	-0.122±0.236	0.608	-0.472±0.336	0.164
	Med x Procedural control	-0.118±0.236	0.617	-0.062±0.351	0.861
	Med x Spontaneous control	-0.361±0.239	0.133	-0.120±0.336	0.723
	Mesic-Med x selfing	**-0.642±0.230**	**0.006**	-0.388±0.329	0.242
	Mesic-Med x Procedural control	**-0.558±0.230**	**0.017**	0.357±0.417	0.394
	Mesic-Med x Spontaneous control	**-0.987±0.233**	**<0.001**	0.052±0.386	0.893

The self-compatibility index for the absolute number of seeds produced per flower manipulated in *B. didyma* and *S. alba* increased with the cline of increasing aridity (Fig. 3.3, Table 3.3). There was no cline in the self-compatibility index for the proportion of viable seeds out of total seeds produced along the gradient in either species (Fig. 3.4, Table 3.3), although there was a trend towards decreasing viable seeds with increasing aridity in *S. alba* (Fig. 3.4, Table 3.3). Similarly, there was no cline in the self-compatibility index for the number of viable seeds produced per flower manipulated in either species (Fig. 3.5, Table 3.3), although there was a trend towards increasing self-compatibility of viable seeds per flower in *B. didyma* with increasing aridity (Fig. 3.5, Table 3.3).

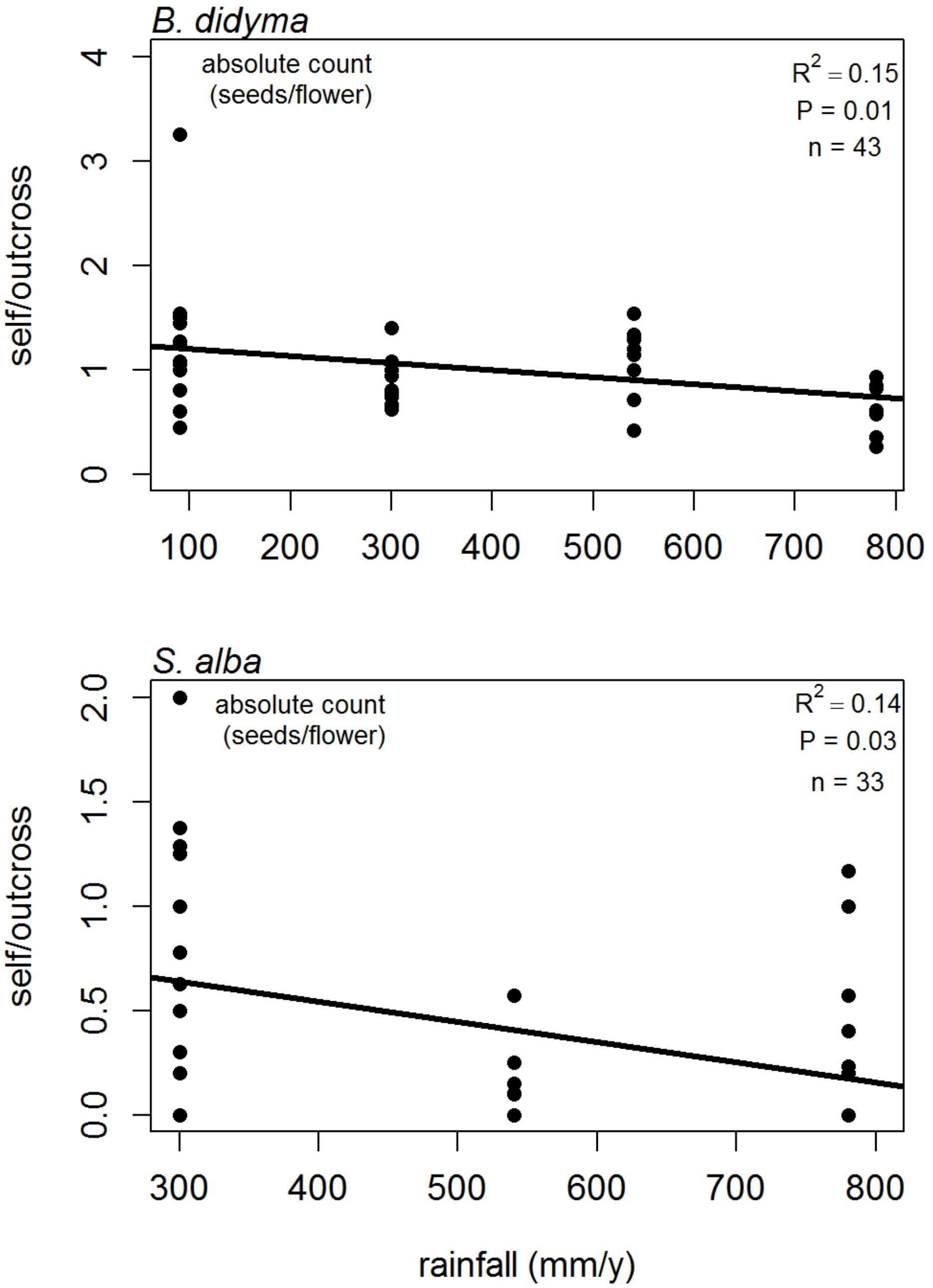

Figure 3.3: Self-compatibility index of *B. didyma* and *S. alba* as a cline along the rainfall gradient for absolute number of seeds produced per flower manipulated. The index is calculated as the absolute number of seeds produced per flower manipulated by selfing divided by the absolute number of seeds produced per flower by outcrossing. The mean annual rainfall ranges from 90 mm/y (*B. didyma*: N=12), 300 mm/y (*B. didyma*: N=11, *S. alba*: N=12), 540 mm/y (*B. didyma*: N=10, *S. alba*: N=11) to 780 mm/y (*B. didyma*: N=11, *S. alba*: N=11). The black lines indicate the significant cline in self-compatibility with increasing aridity.

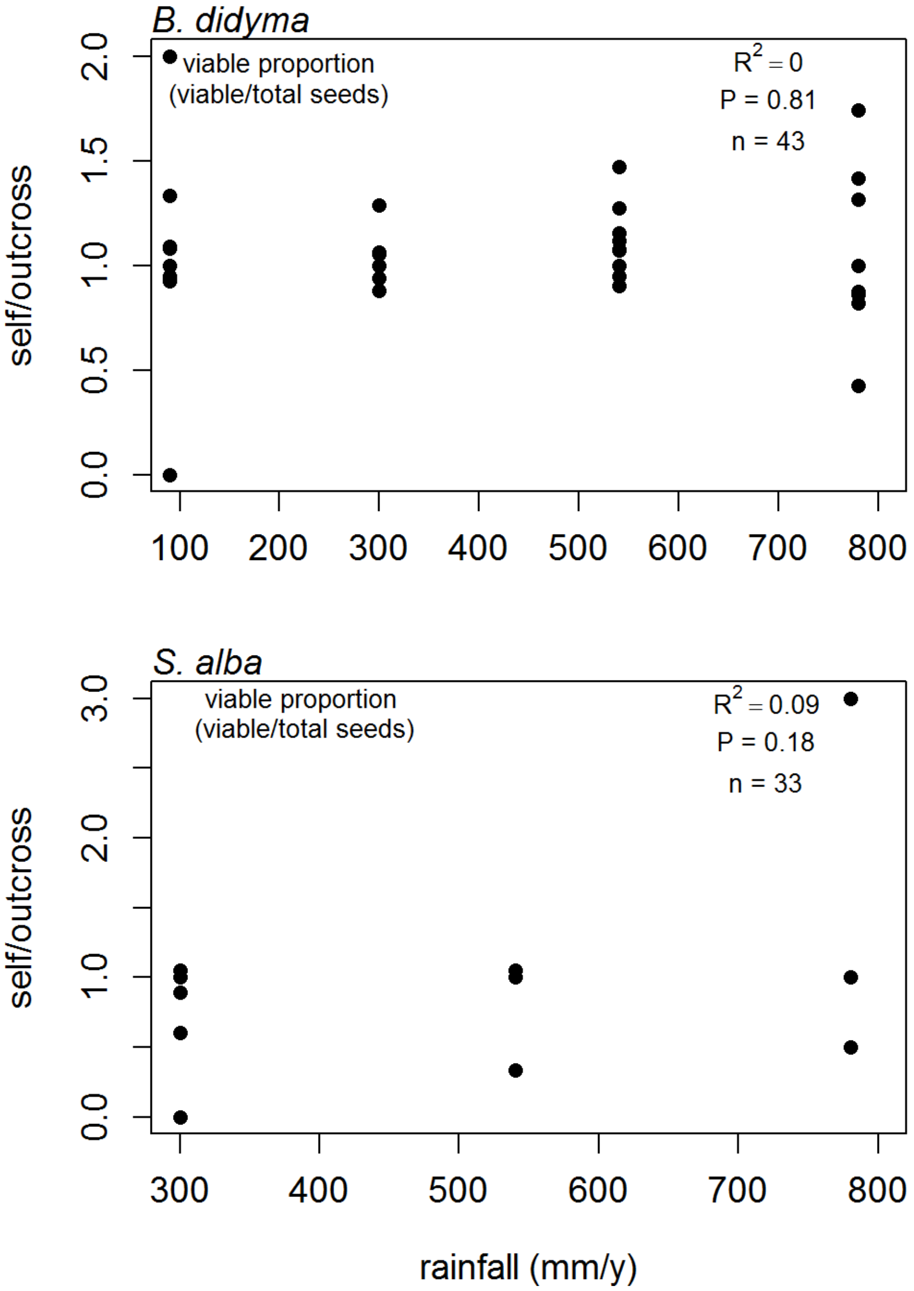

Figure 3.4: Self-compatibility index of *B. didyma* and *S. alba* as a cline along the rainfall gradient for the proportion of viable seeds out of total seeds produced per flower manipulated. The index is calculated as the proportion of viable seeds per total seeds produced by selfing divided by the proportion of viable seeds per total seeds produced by outcrossing. The mean annual rainfall ranges from 90 mm/y (*B. didyma*: N=12), 300 mm/y (*B. didyma*: N=11, *S. alba*: N=5), 540 mm/y (*B. didyma*: N=10, *S. alba*: N=10) to 780 mm/y (*B. didyma*: N=11, *S. alba*: N=6).

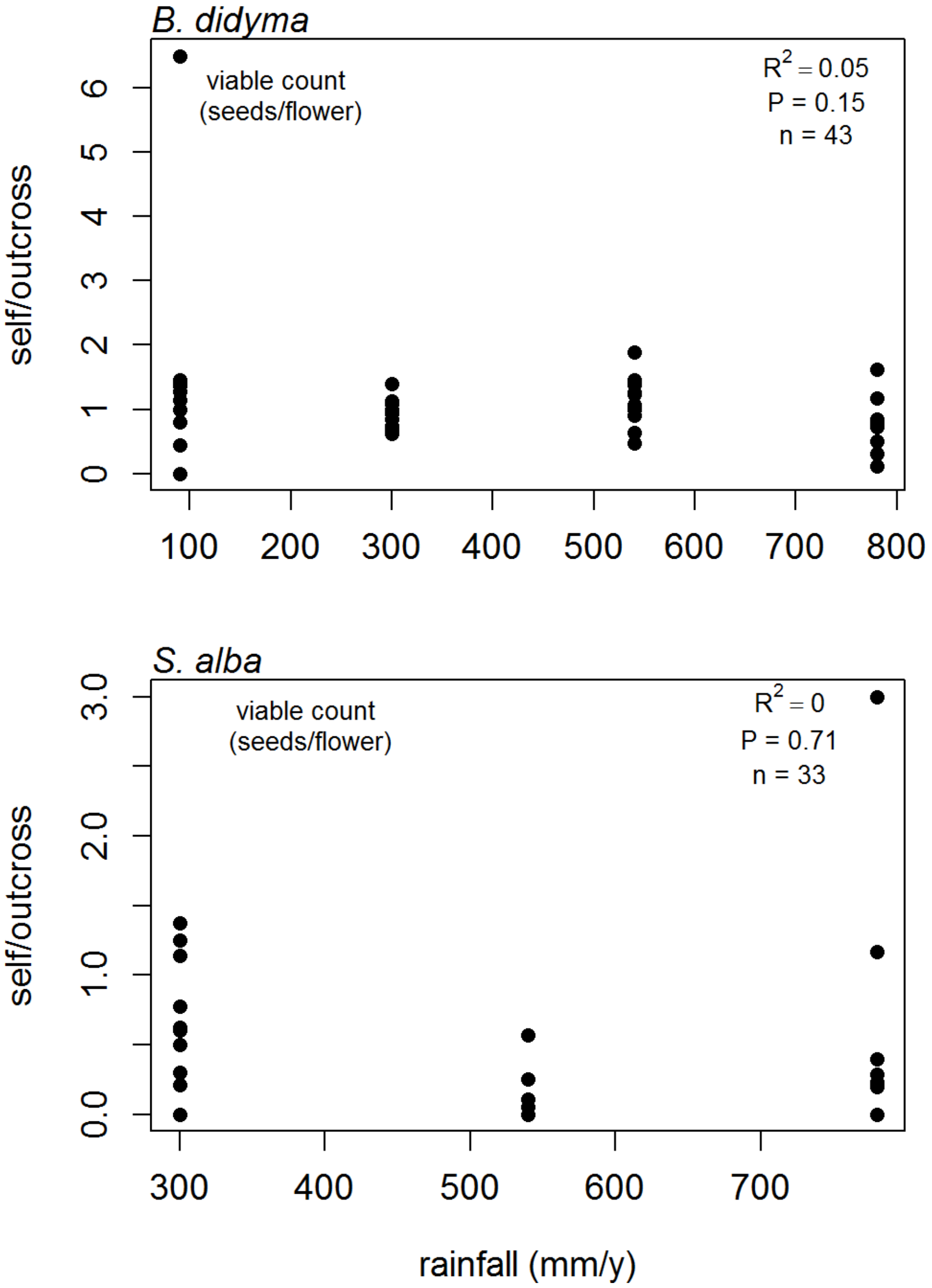

Figure 3.5: Self-compatibility index of *B. didyma* and *S. alba* as a cline along the rainfall gradient for the number of viable seeds produced per flower manipulated. The index is calculated as the number of viable seeds produced per flower manipulated by selfing divided by the number of viable seeds produced per flower by outcrossing. The mean annual rainfall ranges from 90 mm/y (*B. didyma*: N=12), 300 mm/y (*B. didyma*: N=11, *S. alba*: N=12), 540 mm/y (*B. didyma*: N=10, *S. alba*: N=10) to 780 mm/y (*B. didyma*: N=11, *S. alba*: N=11).

Table 3.3: Test statistics for the effect of mean annual rainfall at the site of origin on the self-compatibility indices of absolute seed count, proportion of viable seeds, and viable seed count for *B. didyma* and *S. alba*. Separate linear regression models were performed for each species. Increasing self-compatibility with increasing aridity (+), no change in self-compatibility with increasing aridity (0). Coefficients ± SE presented for the intercept (β_0) and rainfall (β_1). The sample size was not the same for each species, resulting in different degrees of freedom in each model. Values in bold highlight the significant results ($P < 0.05$).

	Self-compatibility indices for					
	Absolute seed count		Proportion of viable seeds		Viable seed count	
	B. didyma	*S. alba*	*B. didyma*	*S. alba*	*B. didyma*	*S. alba*
R^2	**0.146**	**0.137**	0.001	0.091	0.049	0.004
With increasing aridity	+	+	0	0	0	0
β_0 ±SE	**1.266±0.125**	**0.929±0.243**	1.030±-0.008	0.588±0.302	1.393±0.257	0.494±0.308
β_1 ±SE	**0.000±0.000**	**0.000±0.000**	0.000±0.000	0.001±0.001	0.000±0.000	0.000±0.001
F-value	7.194	5.073	0.057	1.902	2.161	0.138
DF	43	33	43	20	43	33
P-value	**0.010**	**0.031**	0.813	0.184	0.149	0.713

3.4. Discussion

This study shows that both Brassicaceae species were more self-compatible in populations originating from increasingly arid environments. The clear increase in the number of seeds produced by selfing relative to outcrossing with increasing aridity suggests that prezygotic self-incompatibility reduces seed production by selfing in mesic environments. *Biscutella didyma* produced as many viable seeds from selfing as from outcrossing consistently across the gradient, yet there was a trend in *S. alba* to produce fewer viable seeds from selfing with increasing aridity. This study shows that both species can secure their reproduction by self-fertilisation with increasing aridity, even though selfing in *S. alba* is not as successful as selfing in *B. didyma*.

The spontaneous selfing control represents the natural selfing success. Differences in the number of seeds produced in the spontaneous selfing control between the extreme environments would suggest that *B. didyma* maintains prezygotic self-incompatibility in mesic environments and that this mechanism becomes weaker with increasing aridity. Here, I provide evidence to support this, as spontaneous selfing was less successful in the mesic-Mediterranean environment than in the arid environment. The successful production of seeds by self-fertilisation in *B. didyma* indicates that the genetic sporophytic self-incompatibility mechanism is not active. Rather, prezygotic self-incompatibility in populations of *B. didyma* in the mesic-Mediterranean environment is most likely maintained by spatial or temporal partitioning of the sex organs. That is, the anthers and the stigma may be separated in space by floral morphology, or in time by the anthers ripening before the stigma, or *vice versa* (Barrett, 1988; Hiscock and Tabah, 2003). Moreover, these results corroborate previous observations of *B. didyma* grown in controlled greenhouse conditions in the absence of pollinators, which showed lower seed production by selfing from populations originating from the mesic-Mediterranean site than populations originating from the arid site (K. Tielbörger, *personal observation*).

In contrast to *B. didyma*, *S. alba* produced the greatest number of seeds in all populations by outcrossing, showing clearly that self-fertilisation is relatively unsuccessful in this species regardless of environmental conditions. Moreover, fewer seeds were produced in the spontaneous selfing control compared to the selfing manipulation treatment, particularly in the semi-arid site. Temporal or spatial prezygotic self-incompatibility might remain active in all populations of *S. alba*. Alternatively, the higher seed production as a result of pollination manipulation could indicate that *S. alba* is naturally pollen limited. The stigma in the selfing manipulation treatment actively received pollen whilst the stigma in the spontaneous selfing control was untouched. The manipulation treatments presumably received more pollen than the flower is naturally able to provide to its own stigma, thus increasing the number of seeds produced in the manipulated selfing treatments relative to the spontaneous selfing control. Reproductive success has been recorded before to be greater in pollen-supplemented flowers (*i.e.*, hand-pollinated) than in flowers which are naturally pollinated (Kron et al., 1993; Goodwillie, 2001; Johnson et al., 2009; Petanidou et al., 2012). Furthermore, the number of seeds produced per flower in the spontaneous selfing control did not increase with decreasing rainfall which suggests that this species does not naturally rely more on self-fertilisation with increasing aridity.

Despite species-specific responses in seed production by spontaneous selfing, both species showed a clear cline of increasing self-compatibility with increasing aridity in the total number of seeds produced. This supports the reproductive assurance hypothesis which posits that plants in harsher environments may be under selection to secure their reproduction by self-fertilisation (Lloyd, 1992). The results show that selfing is as successful as outcrossing in *B. didyma* and that self-pollen may compete with outcross pollen for ovule fertilisation (E. Gibson-Forty and V. Ferenc *personal observations*). It is unsurprising, therefore, that *B. didyma* can compensate its seed production by selfing in arid environments which are predicted to have reduced outcrossing opportunities (Lloyd, 1992). Conversely, these results show that *S. alba* produces only few seeds by self-fertilisation when the risk of complete reproductive failure is high, lending support towards a delayed-selfing mechanism (Lloyd, 1992; Goodwillie et al., 2005).

Seed production by self-fertilisation produced as many viable seeds as seed production by outcrossing in *B. didyma* (viable proportion self-compatibility index) and consequently there was a trend towards increasing self-compatibility in the number of viable seeds per flower in *B. didyma* with increasing aridity. This suggests that postzygotic barriers to self-fertilisation are weak in *B. didyma* as self-embryos developed normally and were viable. Furthermore, this suggests that *B. didyma* populations along the rainfall gradient do not experience strong inbreeding depression as a result of self-fertilisation, as inbreeding depression can manifest as the failure of fertilized embryos to develop (Seavey and Bawa, 1986). In contrasting, there was a trend towards decreasing viability of self-seeds relative to outcross-seeds in *S. alba* with increasing aridity. Ovule abortion and the failure of self-embryos to develop is likely the results of postzygotic self-incompatibility or inbreeding depression (Seavey and Bawa, 1986) and these results suggest that these processes were stronger in populations originating from increasingly arid environments. Populations of *S. alba* originating from mesic environments may produce more viable seeds by selfing than populations originating from arid environments because of continued selection from pollinator foraging behaviours (Eckert, 2002; Fenster et al., 2004; Benelli et al., 2017). The systematic foraging behaviour, particularly of bees, is known to increase the chance of self-fertilisation events (Lloyd, 1992; Eckert, 2000; Barrett, 2003). As pollinators are typically more abundant in mesic environments than in arid environments (Hegland and Boeke, 2006; Devoto et al., 2009; E. Gibson-Forty, *pers. obvs.*), *S. alba* in mesic environments may self-fertilise more, may have purged deleterious alleles from the population and, thus, suffer less from inbreeding depression than populations of *S. alba* in arid environments.

As some of the trends I have explained for each species are not supported statistically, I briefly provide alternative explanations, both methodological and ecological, for the results of the self-compatibility indices of viable seeds. Methodologically, the lack of significance in the self-compatibility index of the proportion of viable seeds out of total seeds produced in *S. alba* is likely a result of reduced sample size (Table 3.3). Meaningful information was lost because of mathematical reasons to calculate the viable proportion self-compatibility index. Many individuals of *S. alba* did not produce any self-seeds so were removed from the proportion analysis, and consequently, self-compatibility in this species may be overestimated. This is corroborated by the lack of reproductive assurance observed in the spontaneous selfing control of *S. alba*. Similarly, calculating the self-compatibility index of the number of viable seeds out of flowers manipulated exacerbates the variation in the data and it is likely that with a larger sample size, the trend of increasing self-compatibility in the number of viable seeds produced per flower manipulated for *B. didyma* would become significantly different from random variation within the population. Ecologically, the lack of increasing self-compatibility in the number of viable seeds produced in both species weakens the support for the reproductive assurance hypothesis and therefore requires the consideration of other environmental pressures selecting for reproductive strategies. Arid environments are challenging to plant populations in multiple related ways and thus the reproductive success of plant populations inhabiting arid environments is under multiple selection pressures and different adaptations to secure reproduction may manifest (Noy-Meir, 1973; Aronson et al., 1990; Kigel et al., 2011; Aronson et al., 1993). Previous studies comparing reproductive strategies of annual plants distributed along a Mediterranean-desert transect showed no consistent strategy of securing reproduction (Aronson et al., 1990; Aronson et al., 1993), highlighting that reproductive assurance may not be the dominant strategy to avoid reproductive failure. Furthermore, particularly *B. didyma* uses extensive seed banks and seed dormancy as bet-hedging techniques to buffer environmental variation in reproductive success (Lampei and Tielbörger, 2010; Petrů and Tielbörger, 2008; Tielbörger and Petrů, 2010; Tielbörger et al., 2012; Lampei et al., 2017). By using seed banks to avoid reproductive failure, the selection for reproductive assurance by selfing should be reduced and therefore should reduce the probability of self-compatibility (Evans et al., 2011).

3.5. Conclusions

In conclusion, this work supports the growing body of literature showing that strategies to adapt to arid environments are highly diverse amongst plant species. These results show that both species were able to increase seed production by self-fertilisation with increasing aridity, suggesting that prezygotic self-incompatibility mechanisms may reduce seed production by self-fertilisation in these species in mesic environments. Self-fertilisation was as successful as outcrossing in *B. didyma* across the gradient highlighting that this species reproduces by self-fertilisation frequently regardless of environmental conditions. Conversely, there was a trend towards fewer viable seeds produced by self-fertilisation in *S. alba* with increasing aridity indicating that successful self-fertilisation has been selected for in populations from mesic environments, presumably by pollinator foraging behaviour. These results strongly support the predictions of the reproductive assurance hypothesis in *B. didyma* and lend support towards reproductive assurance in *S. alba*. In light of the costs of self-fertilisation, it is likely that both species have evolved other adaptations to assure reproduction in the face of outcrossing failure, such as by using seed banks and seed dormancy. Adaptations to increasing aridity in traits other than increasing self-fertilisation allow for the maintenance of population genetic variation and therefore the potential for annual plant species to continually adapt to future dryland environments.

Chapter 4

Increasing rainfall selects for greater herbivore pressure and investment into specific plant defence compounds

The abiotic environment can determine the composition and abundance of herbivores which, in turn, can drive the coevolution of plant herbivore defences. Numerous distinct hypotheses have been developed to explain the cause of variation in plant defences, particularly intraspecific variation. Environmental gradients provide a unique opportunity to determine how intraspecific variation in herbivore pressure and plant defences changes in response to environmental variation and to predict changes in plant-herbivore interactions in the face of climate change. Whilst this has been exploited for latitudinal and elevation gradients, fewer studies have addressed intraspecific variation in plant-herbivore interactions along rainfall gradients. As yet, the results are equivocal, confounded by the diversity of defence compounds analysed, inconsistency of species used and the differences arising between natural and controlled conditions. Therefore, there is a need for intraspecific studies of herbivore pressure and plant defence investment replicated across numerous related species that combine observational studies of natural herbivore pressure with controlled greenhouse studies. Here, I observed natural leaf damage by herbivory on five species of the Brassicaceae family distributed along a steep rainfall gradient in the Eastern Mediterranean. Moreover, the glucosinolate profiles were determined of the same species grown in controlled conditions from seeds collected in the field. I investigated the alternative hypotheses that either leaf damage by herbivory increases with increasing rainfall whilst total leaf glucosinolate concentrations decreases with increasing rainfall, or *vice versa*, and predict that this relationship is driven by a trade-off in leaf damage by herbivory and plant defence production. My results showed overall increasing herbivore pressure with increasing rainfall. However, total leaf glucosinolate concentration did not change with increasing rainfall. Nevertheless, I found that the concentrations of the specific classes of glucosinolates (aliphatic, benzenic, indole) were affected by rainfall in different ways and that this was consistent between and

within plant species. Surprisingly, I found no evidence for a trade-off in leaf damage by herbivory and total leaf glucosinolate concentration. Overall, my results suggest that even though investment into total leaf glucosinolates appears to be independent of herbivore pressure, with increasing aridity herbivore pressure will likely decrease whilst plants will likely increase the production of aliphatic glucosinolates.

4.1. Introduction

Patterns of plant herbivore defence can be determined directly by abiotic conditions and indirectly by biotic interactions (Callis-Duehl et al., 2017). The abiotic environment can directly influence the herbivore community present and this can select for variation in plant herbivore defences (Coley and Aide, 1991; van der Meijden, 1996; Fornoni et al., 2004; Pennings et al., 2009; Schemske et al., 2009; Agrawal, 2011; Ali and Agrawal, 2012). The diversity of plant defences is seen both between plant species and within plant species. Theories developed to account for intraspecific patterns in plant defences often build on the abiotic environment as driving defence differentiation, such as the growth-differentiation hypothesis (Herms and Mattson, 1992). The resource allocation hypothesis (Coley et al., 1985), which was originally developed as a framework for explaining interspecific variation in plant defences (Endara and Coley, 2011), has recently been applied to explain intraspecific variation (Hahn and Maron, 2016). Typically, empirical tests of the abiotic environment altering plant defences use a single target species, unfortunately, this approach often overlooks natural herbivore pressure and limits our ability to draw general conclusions.

Environmental gradients provide a natural experimental design to test how plant-herbivore interactions are affected by different abiotic conditions and can be used to predict how these interactions may change in response to climate change (Kigel et al., 2011; Sternberg et al., 2011). Herbivore damage has been found repeatedly to be greater at lower latitudes compared to high latitudes (Coley and Barone, 1996; Pennings et al., 2009; Więski and Pennings, 2014; Moreira et al., 2017a) and greater at lower elevations compared to high elevations (Mitton and Ferrenberg, 2012;

Rasmann et al., 2014; Moreira et al., 2017b). Typically, the drier conditions in higher latitudes and the colder conditions at higher elevations, together with the shorter growing seasons and typically sparse plant cover, limit the size of herbivore populations compared to herbivore populations at lower latitudes and elevations (Coley and Barone, 1996; Pennings et al., 2009; Mitton and Ferrenberg, 2012). Surprisingly, measuring herbivore pressure along rainfall gradients without latitudinal and elevational variation has not been extensively studied. The few studies on herbivore pressure along rainfall gradients report conflicting results from greater herbivore pressure in wetter sites (Brenes-Arguedas et al., 2009) to no relationship between herbivore pressure and rainfall (Sinclair and Hughes, 2008). Consequently, further studies to quantify herbivore pressure along rainfall gradients without covariation in latitude and elevation are urgently needed.

Local abiotic conditions can directly influence plant allocation to defence compounds (Menge, 2003; Woods et al., 2012; Moreira et al., 2017a). Therefore, variation in the abiotic environment over small spatial scales can drive clinal trends of intraspecific variation in plant defences (Gols et al., 2008; Harvey et al., 2011; Pratt and Mooney, 2013; Anderson et al., 2015). In addition to herbivore pressure studies, intraspecific variation in plant defence investment has been measured extensively along latitudinal gradients (e.g. Moreira et al., 2017a; Baskett Carina et al., 2018; and reviewed in Moles et al., 2011; and Anstett et al., 2016) and elevational gradients (e.g. Galen et al., 1991; Pratt and Mooney, 2013; Rasmann et al., 2014; Anderson et al., 2015; Ferrenberg et al., 2017). Fewer studies have focused on evaluating the intraspecific variation in plant defences along rainfall gradients, and the results are contrasting. For example, some studies found enhanced plant defence levels with increasing rainfall (Ward et al., 2011; Castillo et al., 2013; Metz et al., 2014; Agrawal et al., 2015; Tomiolo et al., 2017), whilst others showed increasing plant defences with decreasing rainfall (Pratt and Mooney, 2013; Kooyers et al., 2014; Abdala-Roberts et al., 2016; Moreira et al., 2017a), or that the investment in plant defences was not affected by rainfall (Katz et al., 2013).

The variation in results on the relationships between plant defences and rainfall are likely explainable by the numerous defence compounds analysed and the different species used, making comparisons between studies difficult. For example, plant defence metabolites measured along rainfall gradients comprise condensed tannins (Ward et al., 2011; Moreira et al., 2017a), monoterpenes (Pratt and Mooney, 2013), cardenolides (Agrawal et al., 2015), tropane alkaloids (Castillo et al., 2013), cyanogenic glucosides (Kooyers et al., 2014), phenolics (Abdala-Roberts et al., 2016), and glucosinolates (Metz et al., 2014; Tomiolo et al., 2017). Moreover, plant secondary metabolites are often species-specific and can perform other functions in addition to herbivore defence (Anstett et al., 2016; Scogings, 2018). The physiological variation in plant defences, together with the methodological variation in experimental studies makes it difficult to determine the direction and strength of relationships between the climate, herbivory and plant defences. The conflicting results from studies of herbivore damage and plant defence investment along rainfall gradients, together with the lack of taxonomic replication, leaves the question open of how changing environmental conditions effect plant-herbivore interactions within species. One approach to answer this question and to draw more reliable conclusions is to incorporate both measurements of herbivore damage and plant defence investment in numerous related species along many sites of a steep rainfall gradient in a single study. Ultimately these studies should be corroborated by manipulative experiments.

Here, I combine observations of leaf damage by herbivory on five Brassicaceae species along twelve sites of a steep rainfall gradient in the Eastern Mediterranean Basin with glucosinolate concentrations of the same species grown in controlled conditions from seeds collected along the gradient. The hydrolysis products of glucosinolates are detrimental to a wide range of herbivores and are used extensively in the Brassicaceae family (Halkier and Gershenzon, 2006; Agerbirk and Olsen, 2012). These properties of the glucosinolates, together with the wide distribution of the Brassicaceae family (Boaz et al., 1990; Lysak and Koch, 2011), offer a convenient system to study intraspecific variation in plant defences along a rainfall gradient. I studied multiple species in many sites along the gradient to test whether overall patterns in herbivore pressure and plant defence investment is corroborated between and within species distributed along the rainfall gradient. I used the rainfall gradient as a proxy for climate change, as the Eastern Mediterranean Basin is expected to become increasingly arid (Black, 2009; Evans, 2009; Smiatek et al., 2011).

The relationships between leaf damage by herbivory and the concentration of total leaf glucosinolates may be positive or negative with increasing rainfall and thus both alternative hypotheses are presented. On the one hand, leaf damage by herbivory may increase with increasing rainfall and plant defence concentration may decrease because more resources in mesic environments enable plants to invest in compensatory growth and biomass production (Hilbert et al., 1981; Maschinski and Whitham, 1989; Siemens et al., 2003). On the other hand, leaf damage by herbivory may decrease with increasing rainfall and plant defence concentration may increase because more resources in mesic environments enable plants to invest in both growth and defence (Herms and Mattson, 1992). I predicted that these patterns were driven by a trade-off between leaf damage by herbivory and plant investment into constitutive defence, *i.e.*, the greater the plant defence, the lower the leaf damage by herbivory. Here, I first tested for the independent relationships of leaf damage by herbivory with increasing rainfall and the concentration of total leaf glucosinolates with increasing rainfall, and then I tested for the trade-off in leaf damage by herbivory and glucosinolate concentration.

4.2. Materials and Methods

Study sites and species

A field study was carried out in spring 2016 at twelve sites along a steep rainfall gradient, spanning the political borders of Israel, Palestine and Jordan (Table 4.1). The mean annual rainfall amount differs approximately tenfold between the arid (approx. 90 mm mean annual rainfall) and mesic-Mediterranean (approx. 950 mm mean annual rainfall) sites of the rainfall gradient, with rainfall variability being highest at the arid sites. The development of natural vegetation is determined chiefly by rainfall amount. The broadly Mediterranean climate generates warm, rainy winters and hot, rainless summers, with germination of annual species - the dominant life form - typically following the first rains of the season in October-November and seed set occurring during April and May. Five annual species from the Brassicaceae family were selected for the study: *Erucaria pinnata* (Viv.) Taeckh. & Boulos, *Hirschfeldia incana* (L.), *Isatis lusitanica* (L.), *Matthiola livida* (Delile) DC., and *Sinapis alba* (L.) (Table 4.1). Species were selected for the study if they occurred in at least four sites (Table 4.1). *Erucaria pinnata* and *M. livida* have a Saharo-Arabian chorotype, *H. incana* and *I. lusitanica* have a Mediterranean-Irano-Turanian chorotype, and *S. alba* has a European-Siberian-Mediterranean-Irano-Turanian chorotype (Zohary, 1966).

Table 4.1: The broad environmental conditions, location, and species present for each of the 12 sites along the rainfall gradient. The asterisk in site 5 denotes that two populations of *E. pinnata* and *M. livida* were sampled at 200 and 300 mm/y rainfall. Mean annual rainfall (mm/y) values were obtained from Holzapfel et al. (2006); Liancourt and Tielbörger (2009); Liancourt et al. (2009); Kurze et al. (2017) and C. Holzapfel (*unpublished*).

Site	Mean annual rainfall (mm/y)	Broad environmental conditions	Coordinates	Species present
1	90	Arid	N30°52’ E34°46’	*E. pinnata,* *M. livida*
2	90	Arid	N31°41’ E35°26’	*E. pinnata,* *M. livida*
3	190	Arid	N31°56’ E35°49’	*H. incana,* *M. livida*
4	300	Semi-arid	N31°23’ E34°51’	*H. incana,* *I. lusitanica,* *S. alba*
5	300*	Semi-arid	N31°48’ E35°19’	*E. pinnata,* *H. incana,* *M. livida*
6	330	Semi-arid	N31°58’ E35°44’	*E. pinnata,* *H. incana,* *M. livida*
7	370	Dry Mediterranean	N32°0’ E35°44’	*H. incana,* *I. lusitanica*
8	415	Mediterranean	N32°01’ E35°43’	*H. incana,* *M. livida*
9	500	Mediterranean	N31°45’E35°07’	*H. incana,* *M. livida,* *S. alba*
10	540	Mediterranean	N31°42’ E35°3’	*H. incana,* *I. lusitanica,* *S. alba*
11	780	Mesic-Mediterranean	N35°0’ E35°14’	*S. alba*
12	950	Mesic-Mediterranean	N32°57’ E35°19’	*I. lusitanica,* *S. alba*

Herbivore damage observations

Between February and March 2016, *i.e.*, at peak season, 50 individuals per species per site (Table 4.1) were randomly selected to be included for herbivory observations. Of these 50 individuals, a single, fully expanded green leaf was randomly selected and leaf damage as a result of herbivory was estimated as a percentage of total leaf area. As individuals were selected at random, some individuals were vegetative whilst others were already flowering. I used leaf damage as a proxy for herbivore pressure (Andrew et al., 2012).

Plant material

Seeds from 15 individuals of each species were collected from approximately two sites in each broad environment: arid (sites 2 and 3), semi-arid (sites 5 and 6), Mediterranean (sites 7, 8 and 9), and mesic-Mediterranean (site 12, Table 4.1) and were germinated in a greenhouse. In November 2016, three to five seeds per individual were sown in 10 x 10 x 10 cm pots with a 1:1 mixture of topsoil and sand. Plants were grown in a greenhouse under standard conditions: optimal water (soil saturation three times per week), optimal light (10 h per day), and temperature regimes that reflected the growing season in Israel (daily temperature range: 18-25 °C, night temperature range: 15-18 °C). Each pot was thinned to one individual after successful seedling establishment. All plants were sprayed with insecticide three times between seedling establishment and leaf harvesting to avoid induction of defence compounds in the greenhouse. Within 65 d of germination and before anthesis, the youngest fully expanded green leaf was removed from the plant, flash frozen in liquid nitrogen, lyophilised and analysed for the total concentration and composition of glucosinolates. In cases where the fresh leaf weighed less than 10 mg an additional young leaf was collected and pooled, typically in *M. livida* and *E. pinnata*. Six *I. lusitanica* individuals from site 7 did not germinate, reducing the replicate number from 15 to nine in this site. Aphids present in the greenhouse infested three individuals of *M. livida*, four individuals of *E. pinnata*, four individuals of *I. lusitanica*, 18 individuals of *H. incana*, and nine individuals of *S. alba*. As aphids may affect defence compound production (Mewis et al., 2005; Kim and Jander, 2007; Kuśnierczyk et al., 2007; Kuśnierczyk et al., 2008; Barah et al., 2013; Stolpe et al., 2017), I took this into account in the data analyses (see below).

Glucosinolate analysis

Dry material was weighed, homogenised and extracted threefold in 80 % methanol. For samples of *M. livida*, benzylglucosinolate (Phytoplan Diehm & Neuberger, Heidelberg, Germany) was added as internal standard at the first extraction, whereas for the other species, 2-propenylglucosinolate (Sigma, Taufkirchen, Germany) was used. Supernatants were applied on anion exchange columns, treated overnight with sulfatase [E.C. 3.1.6.1, type H-1, from *Helix pomatia*, Sigma; purified following Graser et al. (2001)] and desulfoglucosinolates eluted with water. Samples were analysed on a high-performance liquid chromatograph (1200 Series, Agilent Technologies, Inc., Santa Clara, CA, USA) coupled to a diode array detector. Separation was performed on a reversed phase column (Supelcosil LC 18, 150 mm × 3 mm, 3 µm, Supelco, Bellefonte, PA, USA) using the gradient as described in Metz et al. (2014). Peaks were integrated at 229 nm and concentrations calculated in relation to the dry sample weight and the internal standard, considering response factors as in Buchner (1987) and using 1.0 for unknown putative aliphatic glucosinolates.

Statistical analysis

Leaf damage by herbivory

To determine whether leaf damage by herbivory increased with the cline of rainfall, I first averaged the leaf damage by herbivory per species per site along the rainfall gradient. Then, I log-transformed the averaged percentage leaf damage by herbivory across all species to conform to the assumptions of linear regression. I used a linear regression with the overall averaged log-transformed leaf damage as the dependent variable and rainfall as the independent continuous variable. I also used separate linear regression models for each species. Again, I log-transformed percentage leaf damage by herbivory and used this as the dependent variable and rainfall as the independent continuous variable for each species model.

Glucosinolate analysis

To determine whether glucosinolate concentrations increased with the cline of rainfall, I pooled all species together and summed the total glucosinolate concentrations for all individuals. Then I averaged the total glucosinolate concentration per species per site. I used a linear regression with the overall average glucosinolate concentration as the dependent variable and rainfall as the independent continuous variable.

The total leaf glucosinolate concentrations were also analysed separately for each species. I tested whether aphid infestation had an effect on glucosinolate concentration and found that there was no effect, so, consequently, I included all plant individuals in my analyses. I used separate linear regression models for each species with total leaf glucosinolates (and total aliphatic, benzenic and indole glucosinolates separately, where appropriate) concentrations as the dependent variable with rainfall as the continuous independent variable for each species model.

Leaf damage by herbivory and glucosinolate concentration trade-off

I determined whether there was a trade-off in leaf damage by herbivory and the concentration of total leaf glucosinolates. I used a linear regression with the overall averaged log-transformed leaf damage as the dependent variable and the average total leaf glucosinolate concentration as the independent variable. All statistical analyses and graphics were carried out in R version 3.4.3 (R Core Team, 2017).

4.3. Results

Leaf damage in the field

Across all species, the overall averaged leaf damage in the field significantly increased with the cline in rainfall (Fig. 4.1a, Table 4.2), even though the leaf damage on these species was generally very low in the field; leaf damage increased from 0.5% at the arid extreme to 2% at the mesic-Mediterranean extreme. At the intraspecific level, the percentage of leaf damage increased with increasing rainfall in three of the five species (*E. pinnata*, *H. incana*, and *S. alba*, Fig. 4.1b-d, Table 4.2), yet did not change with increasing rainfall in two of the five species (*M. livida*, *I. lusitanica*, Fig. 4.1e-f, Table 4.2).

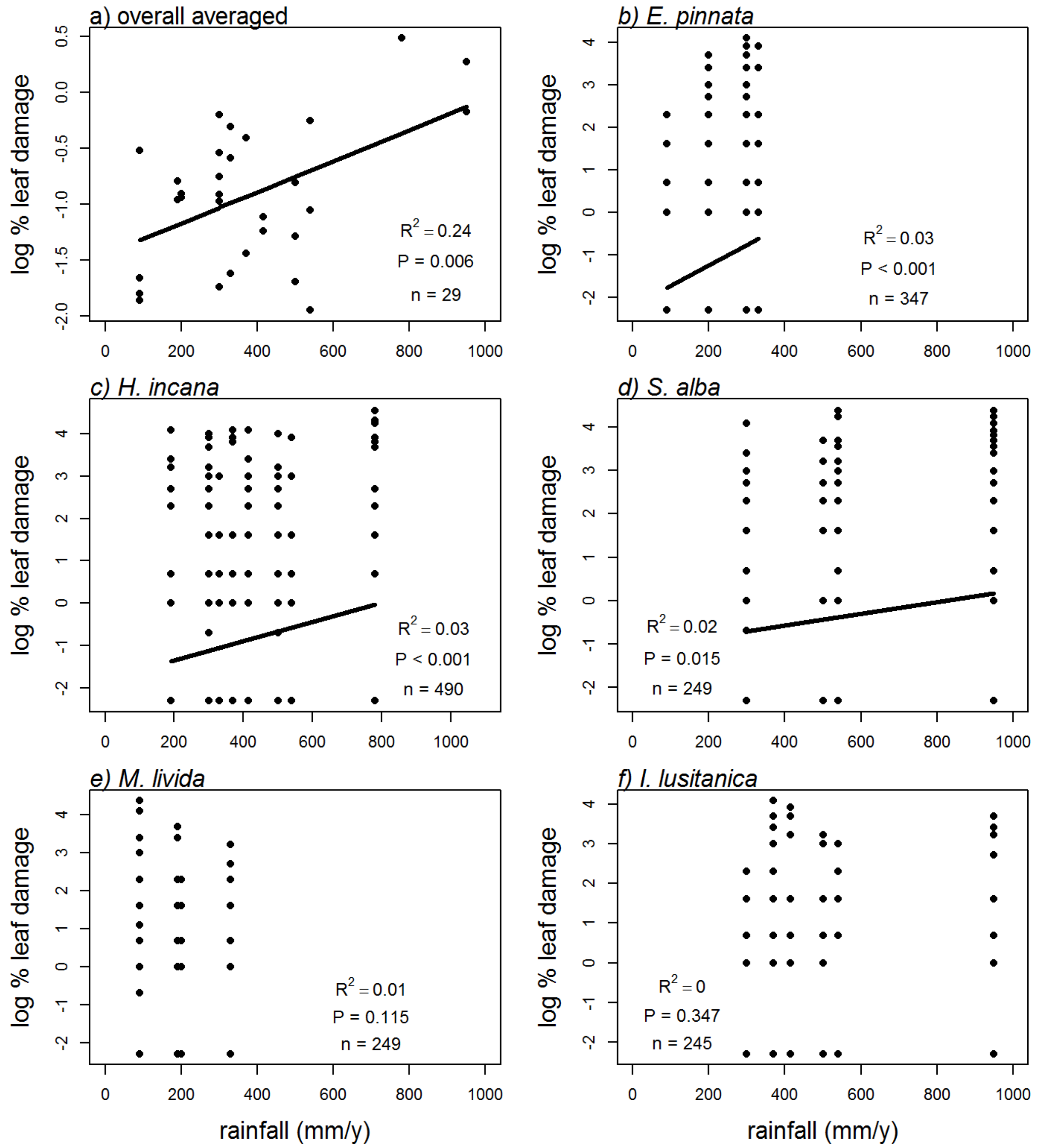

Figure 4.1: Log-transformed percentage leaf damage by herbivory observed a) overall averaged per species per site along the rainfall gradient, b) in *E. pinnata*, c) in *H. incana*, d) in *S. alba*, e) in *M. livida*, f) in *I. lusitanica* species along the rainfall gradient. The black lines represent the significant cline in percentage leaf damage with increasing rainfall (mm/y). The coefficient of determination (R^2), *P*-values and number of observations (n) are presented for each relationship. Rainfall (mm/y) on the x-axis represents the mean annual rainfall at the sites where leaf damage was measured.

Table 4.2: Test statistics for the effect of mean annual rainfall on overall percentage leaf damage by herbivory averaged per species per site along the rainfall gradient and for each plant species along the rainfall gradient. Separate linear regression models were performed for each species. Increasing leaf damage with increasing rainfall (+), no change in leaf damage with increasing rainfall (0). Coefficients ± SE are presented for the intercept (β_0) and rainfall (β_1). The sample size was not the same for each species resulting in different degrees of freedom in each model. Values in bold highlight the significant results ($P < 0.05$).

Leaf damage	R^2	Relationship	β_0 ±SE	β_1 ±SE	F-value	DF	*P*-value
Overall averaged	0.241	+	-1.446 ± 0.203	0.001 ± 0.000	8.896	28	**0.006**
E. pinnata	0.064	+	-2.211 ± 0.247	0.004 ± 0.000	23.57	346	**<0.001**
H. incana	0.032	+	-1.804 ± 0.249	0.002 ± 0.001	16.17	489	**<0.001**
S. alba	0.023	+	-1.103 ± 0.383	0.001 ± 0.001	5.947	248	**0.015**
M. livida	0.010	0	-1.294 ± 0.259	0.002 ± 0.001	2.503	248	0.115
I. lusitanica	0.004	0	-1.466 ± 0.355	0.001 ± 0.001	0.889	244	0.347

Glucosinolate concentrations of plants grown in controlled conditions

Across all species, the average total glucosinolate concentration of plants grown in controlled conditions did not change with increasing rainfall at the site of origin (Fig. 4.2a, Table 4.3). The total leaf glucosinolate concentration increased with increasing rainfall at the site of origin in one of the five species (*S. alba*, Fig. 4.2b, Table 4.3), decreased with increasing rainfall at the site of origin in two of the five species (*E. pinnata*, *M. livida*, Fig. 4.2c-d, Table 4.3), and was not affected by mean annual rainfall at the site of origin in two of the five species (*I. lusitanica*, *H. incana*, Fig. 4.2e-f, Table 4.3).

In total, four glucosinolates were found in the two populations of *S. alba*, namely two benzenic glucosinolates (benzyl glucosinolate and *p*-hydroxybenzyl glucosinolate) and two indole glucosinolates (indol-3-ylmethyl glucosinolate (I3M) and 4-methoxy-indol-3-ylmethyl glucosinolate (4OHI3M)). The total benzenic glucosinolates concentration accounted for >99% of glucosinolates and thus followed the same trend as total leaf glucosinolate concentration (Fig. 4.2b, Table 4.3). In total, nine glucosinolates were found in *E. pinnata*, namely seven aliphatic glucosinolates (1-methylethyl glucosinolate, 3-butenyl glucosinolate, 4-pentenyl glucosinolate, 6-methylsulfinylhexyl, three unidentified aliphatic glucosinolates), and two indole glucosinolates (I3M and 4OHI3M). The total aliphatic glucosinolate concentration accounted for >99% of glucosinolates and thus followed the same trend as total leaf glucosinolate concentration (Fig. 4.2c, Table 4.3). In total, ten glucosinolates were found in *M. livida*, namely nine aliphatic glucosinolates and one indole glucosinolate (4OHI3M). The total aliphatic concentration accounted for >99% of glucosinolates and thus followed the same trend as total leaf glucosinolate concentration (Fig. 4.2d, Table 4.3). One indole glucosinolate (I3M) was found in *I. lusitanica*. The total indole concentration accounted for 100% of glucosinolates and was not affected by rainfall at the site of origin (Fig. 4.2e, Table 4.3). In total, seven glucosinolates were found in the six populations of *H. incana*, namely four aliphatic glucosinolates (2-hydroxy-3-butenyl glucosinolate, 3-butenyl glucosinolate, 4-pentenyl glucosinolate, 5-methylsulfinylpentyl), one benzenic glucosinolate (2-phenylethyl glucosinolate), and two indole glucosinolates (I3M and

4OHI3M). The total aliphatic glucosinolate concentration accounted for 91% of the total glucosinolates and significantly decreased with increasing rainfall at the site of origin (Fig. 4.3, Table 4.3). The total benzenic glucosinolate concentration accounted for 5% of the total glucosinolates and significantly increased with increasing rainfall at the site of origin (Fig. 4.3, Table 4.3). The total indole glucosinolate concentration accounted for 4% of the total glucosinolates and significantly increased with increasing rainfall at the site of origin (Fig. 4.3, Table 4.3).

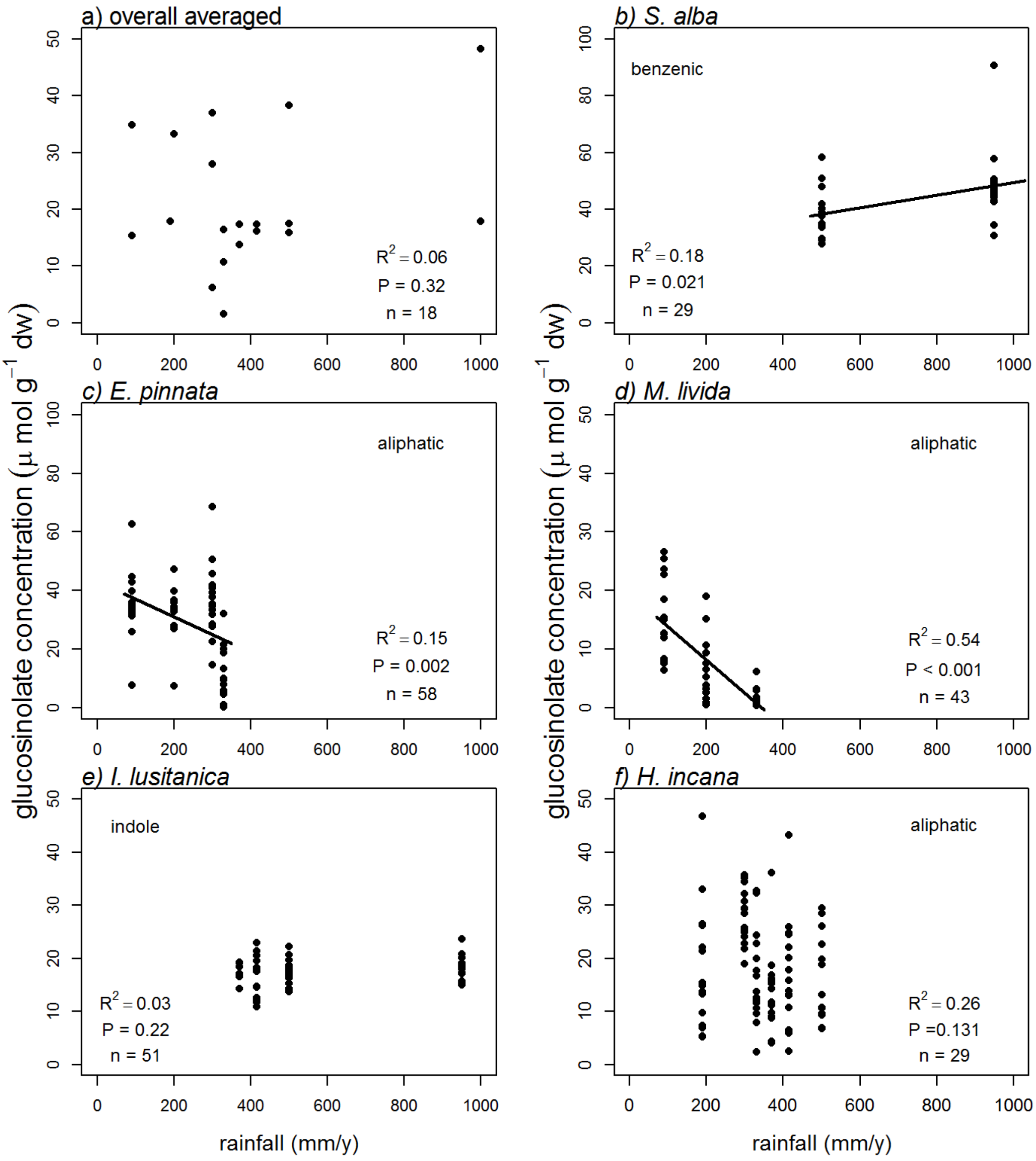

Figure 4.2: Glucosinolate concentrations a) overall averaged per species per origin site, b) in *S. alba*, c) in *E. pinnata*, d) in *M. livida*, e) in *I. lusitanica*, f) in *H. incana* species grown under controlled conditions in relation to the rainfall at the site of origin. The black dots represent the total leaf glucosinolate concentrations. The lines represent the significant cline in total leaf glucosinolate concentrations with increasing rainfall (mm/y). The dominant glucosinolate class, the coefficient of determination (R^2), *P*-values and number of observations (n) are presented for each relationship. Rainfall (mm/y) on the x-axis represents the mean annual rainfall at the sites of origin.

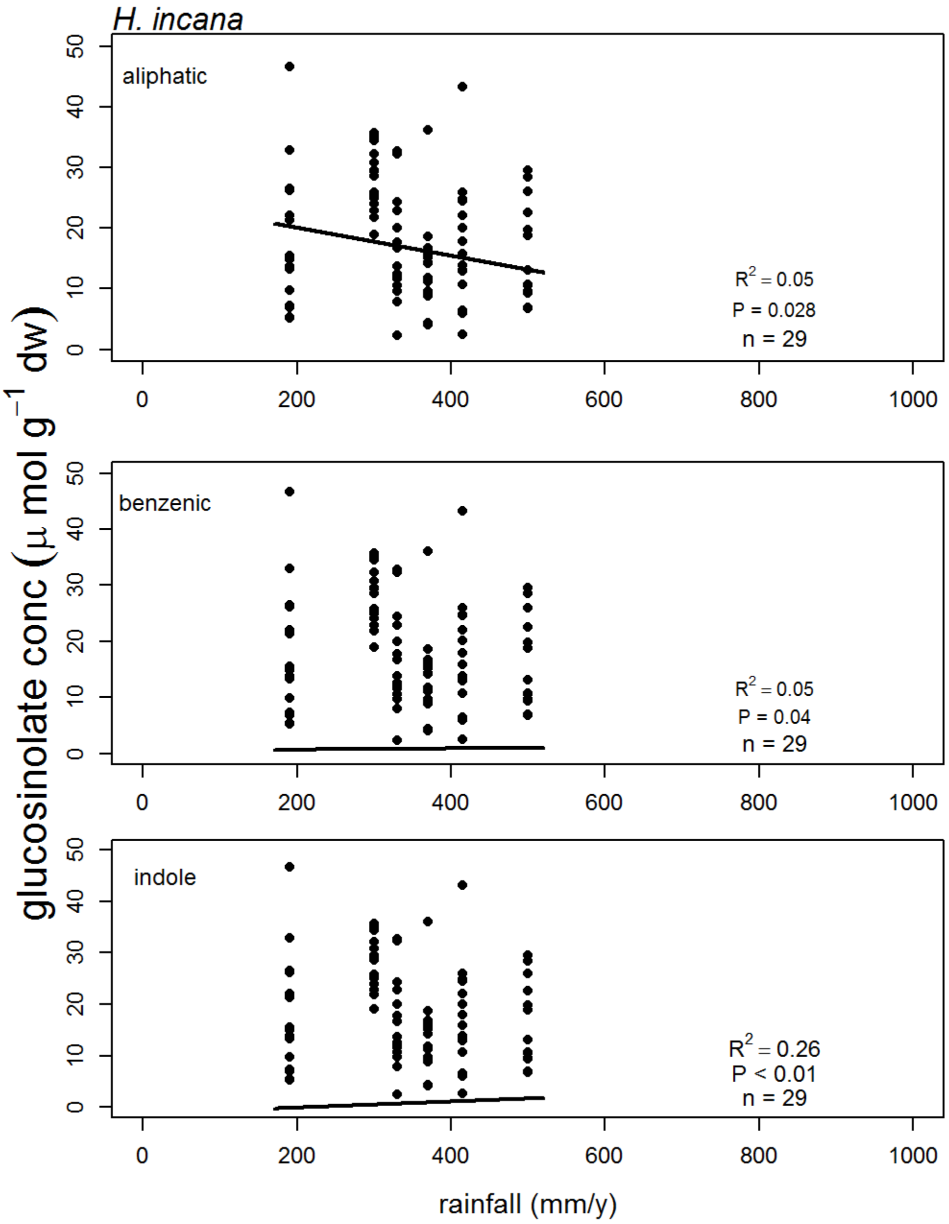

Figure 4.3: The concentrations of each class of glucosinolates present in *H. incana* species grown under controlled conditions in relation to the rainfall at the site of origin. The black dots represent the total leaf glucosinolate concentrations. The lines represent the significant cline in the concentration of each class of glucosinolate with increasing rainfall (mm/y). Each glucosinolate class, the coefficient of determination (R^2), *P*-values and number of observations (n) are presented. Rainfall (mm/y) on the x-axis represents the average annual rainfall at the sites of origin.

Table 4.3: Test statistics for the effect of mean annual rainfall at the site of origin on the total leaf glucosinolate concentration overall averaged per species per site, the total leaf glucosinolate concentration for each plant species, and the concentration of the dominant glucosinolate class for each species. Separate linear regression models were performed for each species. Increasing concentrations of glucosinolates with increasing rainfall (+), no change in concentration of glucosinolates with increasing rainfall (0), decreasing concentrations of glucosinolates with increasing rainfall (-). Coefficients ± SE are presented for the intercept (β_0) and rainfall (β_1). The sample size was not the same for each species, resulting in different degrees of freedom in each model. Values in bold highlight the significant results ($P < 0.05$).

Total glucosinolates	R^2	Relationship	β_0 ±SE	β_1 ±SE	F-value	DF	*P*-value
Overall averaged	0.059	0	16.514 ± 5.351	0.012 ± 0.012	1.062	17	0.317
S. alba	0.175	+	27.161 ± 6.907	0.022 ± 0.009	5.953	28	**0.021**
E. pinnata	0.151	-	43.086 ± 4.690	-0.061 ± 0.019	10.16	57	**0.002**
M. livida	0.544	-	19.470 ± 1.836	-0.057 ± 0.008	50.09	42	**<0.001**
I. lusitanica	0.030	0	15.971 ± 1.063	0.002 ± 0.002	1.534	50	0.221
H. incana	0.026	0	23.849 ± 3.851	-0.016 ± 0.011	2.319	88	0.131
Glucosinolate class							
S. alba benzenic	0.176	+	26.958 ± 6.878	0.022 ± 0.009	5.973	28	**0.021**
E. pinnata aliphatic	0.155	-	43.109 ± 4.690	-0.061 ± 0.019	10.45	57	**0.002**
M. livida aliphatic	0.544	-	19.459 ± 1.835	-0.057 ± 0.008	50.15	42	**<0.001**
I. lusitanica indole	0.030	0	15.971 ± 1.063	0.002 ± 0.002	1.534	50	0.221
H. incana aliphatic	0.054	-	24.666 ± 3.734	-0.023 ± 0.010	5.008	88	**0.028**
H. incana benzenic	0.045	+	0.468 ± 0.180	0.001 ± 0.000	4.159	88	**0.044**
H. incana indole	0.286	+	-1.285 ± 0.358	0.006 ± 0.000	35.24	88	**<0.001**

Leaf damage by herbivory and glucosinolate concentration trade-off

There was no relationship between the amount of leaf damage by herbivory in the field and the total leaf glucosinolate concentration of species grown in controlled conditions (R^2=0.011, $F_{1,17}$=0.194, P=0.665, Fig. 4.4).

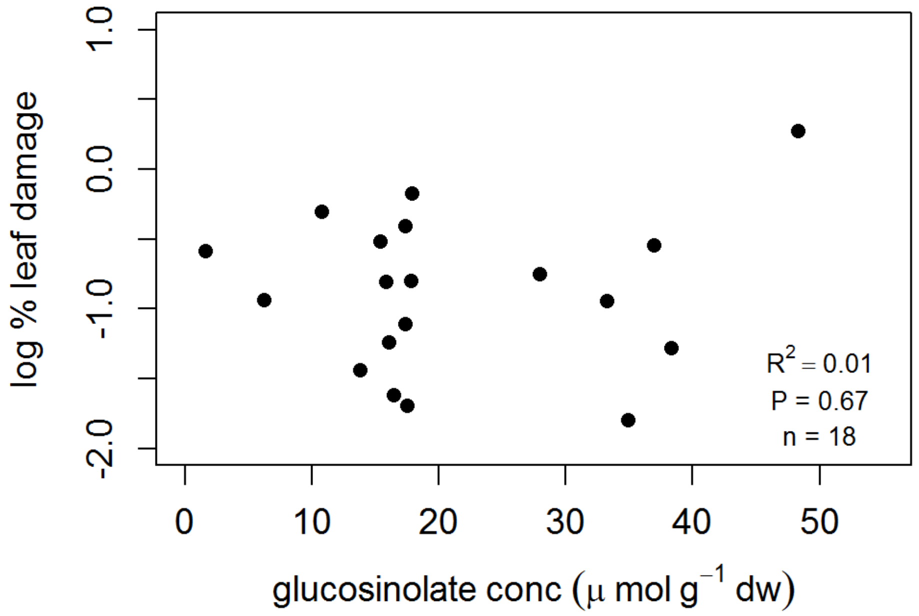

Figure 4.4: The average log of percentage leaf damage as observed in the field as a function of the average total leaf glucosinolate concentration of all plant species grown under controlled conditions. The coefficient of determination (R^2), *P*-value and number of observations (n) are presented.

4.4. Discussion

Here, I observed leaf damage by herbivory on five species of the Brassicaceae family along a steep rainfall gradient in the Eastern Mediterranean Basin. I showed that overall leaf damage, and therefore, herbivory pressure, increased with the cline of increasing rainfall. Furthermore, this trend was corroborated in three of the five target species which had the greatest leaf damage by herbivory in mesic environments. In addition, my results showed that total leaf glucosinolate concentration of the target species grown in controlled conditions did not change with increasing rainfall at the site of origin. Nevertheless, I found that the concentration of each class of glucosinolates was affected by rainfall. Moreover, each species tended to invest in one dominant class of glucosinolates. In contrast to my prediction, I found no trade-off in leaf damage by herbivory and total leaf glucosinolate concentration. The high taxonomic replication of this study enables me to identify patterns of herbivory and plant defence investment at different levels. That is, overall along the rainfall gradient, between species distributed along the gradient, and, of particular importance for predicting the effects of climate change, between populations within species distributed along the rainfall gradient.

Variation in herbivore damage

Overall, leaf damage increased along the entire length of the rainfall gradient from the arid to the mesic-Mediterranean environments. I used leaf damage as a proxy for herbivory pressure (Andrew et al., 2012), and accordingly support the first of the two alternative hypotheses, that there is a cline of increasing herbivory pressure with increasing rainfall. These results corroborate previous studies that showed increasing herbivory pressure in wetter conditions (Coley and Barone, 1996; Wright, 1999; Brenes-Arguedas et al., 2009). Favourable environments, such as the mesic-Mediterranean environment of the present study, can support more herbivores because the abiotic conditions promote greater ectothermic activity than in arid environments (Novotny et al., 2005; Rodríguez-Castañeda et al., 2010). In addition, herbivore abundance may be greater in favourable environments because plant biomass is typically greater and thus provides more resources for insect herbivores such as food, oviposition and nesting sites than in arid conditions (Price, 1991; Hegland and Boeke, 2006; Devoto et al., 2009; Louthan et al., 2015; Hahn and Maron, 2016).

Acknowledging that herbivore damage increases with increasing rainfall without taking plant species identity into account provides limited evidence for the effects of climate change on plant damage by herbivory. Rather, stronger evidence is provided by observing trends in herbivore damage within a plant species. Nevertheless, using only a single species to compare differences between populations provides limited support for general changes in plant-herbivore interactions (Anstett et al., 2016). Therefore, identifying a consistent pattern between plant populations across numerous species provides robust evidence towards a general trend in plant-herbivore interactions. These study results showed that across three species each population inhabiting the most mesic environment of their distribution received the greatest leaf damage. This may be driven directly by abiotic conditions or indirectly by plant growth strategies. First, plants may experience greater leaf damage by herbivory in mesic environments because the growing season lengthens with more mesic conditions and thus plants are exposed to herbivory pressure for longer (Strong, 1977). Second, resources are more abundant in mesic environments and therefore plants may invest resources into biomass production and compensatory growth after herbivory damage thus leaving many leaves highly damaged by herbivory (Hilbert et al., 1981; Maschinski and Whitham, 1989; Siemens et al., 2003). Taken together, my results from the herbivory observations imply that increasing aridity in the Eastern Mediterranean Basin is likely to reduce herbivore damage within and between plant species.

Variation in plant defence production

Overall, the total leaf glucosinolate concentration of plants grown in controlled conditions was not affected by increasing rainfall at the sites of origin, and consequently does not support either hypothesis. The direction of change in plant defence investment is difficult to predict along resource gradients because of the opposing selection pressures from the abiotic environment and from herbivores. Plants inhabiting arid environments may produce greater concentrations of chemical defences than plants inhabiting more mesic environments because biomass is less easily replaceable in low-resource environments (Coley et al., 1985). Conversely, plants inhabiting mesic environments may produce greater concentrations of chemical defences because resources are more abundant and herbivory pressure may be greater, therefore plants may allocate resources into both growth and defence production (Herms and Mattson, 1992). The lack of evidence for a trend in overall glucosinolates production in the present study may suggest species-specific responses in defence production. Alternatively, it may suggest that calculating the total leaf glucosinolate concentration masks differences in the concentration of glucosinolate classes to increasing rainfall at the sites of origin, although this may be unlikely as each species tended to invest in one dominant class of glucosinolate. Accounting for changes in chemical composition has been advised in previous studies because herbivore insensitivity to certain chemicals can select for a range of defence compounds to be produced within a single plant (Siemens and Mitchell-Olds, 1996; Müller et al., 2010).

All five target plant species produced one dominant class of glucosinolates (>90% of the total leaf glucosinolate production) and the identity of this dominant class varied between the species. Moreover, each class of glucosinolates (aliphatic, benzenic and indole) consistently responded to rainfall in different ways. Benzenic glucosinolates, which were dominant in *S. alba* and minor in *H. incana*, increased in concentration in populations originating from mesic sites. Increasing production of constitutive glucosinolates not only provides immediate defence against herbivory, but may also attract natural enemies of herbivores through the increased release of nitrile emissions during glucosinolate hydrolysis (Agerbirk and Olsen, 2012). Using natural enemies of herbivores could be particularly advantageous to plants inhabiting mesic environments that typically support greater insect abundance and diversity (Hegland and Boeke, 2006; Devoto et al., 2009). I am cautious in my conclusions, however, as samples for glucosinolate analysis in *S. alba* were obtained from only two sites and benzenic glucosinolates in *H. incana* were minor (5% total leaf glucosinolate concentration).

Aliphatic glucosinolates were the dominant class in three of the five target species and the concentration of this glucosinolate class consistently decreased in populations originating from mesic sites along the gradient. A similar pattern was reported in *A. thaliana* plants grown under drought conditions, in that the concentration of aliphatic glucosinolates increased with decreasing rainfall (Mewis et al., 2012). Similarly, a co-occurring annual species in the Eastern Mediterranean Basin, *Biscutella didyma*, showed decreasing concentrations in three out of five aliphatic glucosinolate compounds with increasing rainfall (Metz et al., 2014). Plants have been found to produce glucosinolates in drought tissue at low turgor, particularly short-chain constitutive glucosinolates (Mewis et al., 2012). Thus, plants that have evolved in water-limited environments may increase their production of short-chain constitutive glucosinolates precursors for later use in herbivory defence. Aliphatic glucosinolates are short-chained methionine derivatives (Mewis et al., 2012), hence potentially explaining the increase in aliphatic glucosinolate concentrations in populations that originate from water-limited sites in the present study.

Indole glucosinolates were the dominant class in only one of the five target species. This is most likely because my experiment was designed to quantify the concentration of constitutive glucosinolates. Aliphatic glucosinolates are commonly the dominant constitutively expressed glucosinolates whilst indole glucosinolates are commonly the dominant glucosinolates induced upon tissue damage (Harvey et al., 2011). Expressing indole glucosinolates after tissue damage occurs because they are more readily hydrolysed into their toxic by-products than aliphatic glucosinolates (Agerbirk et al., 2008).

Taken together, my results from the glucosinolate analysis imply that increasing aridity in the Eastern Mediterranean Basin is likely to select for the increasing production of aliphatic glucosinolates in species which dominantly express this class, whilst simultaneously reducing the concentration of benzenic glucosinolates and potentially indole glucosinolates. Consequently, increasing aridity may not select for greater chemical defence overall, but may act as a divergent selection pressure on the glucosinolate composition in Brassicaceae species.

4.5. Conclusions: Linking herbivore pressure and defence production

Disentangling the direct effects of the abiotic environment on plant defence concentration and the indirect effects mediated by herbivore pressure remains difficult. Evidence of clines in both herbivore pressure and glucosinolate concentrations along the rainfall gradient may support the hypothesis that herbivore pressure selects for geographic variation in plant defences (Pennings et al., 2009). However, the assumption that plants have higher defences in regions of higher herbivory has recently been argued as problematic in the latitudinal herbivory defence hypothesis (Anstett et al., 2015). Anstett et al. (2015) argue that plants which are more heavily defended will likely suffer from less, rather than more herbivore damage. In the present study, I show that not only was there no trade-off in leaf damage by herbivory and total leaf glucosinolate concentration, but that there was no relationship between the two. This could be because overall leaf damage by herbivory was very low in the field and thus does not act as a selection pressure on glucosinolate production. Alternatively, it could be because these species use other forms of defence, such as tolerance that do not influence the amount of plant damage (Anstett et al., 2015). Consequently, herbivore observations, chemical analysis and manipulative experiments are required together to ascertain the mechanisms driving plant chemical defence evolution. The present study has provided the first steps towards understanding this complex relationship in the Eastern Mediterranean Basin. I provide here compelling evidence that increasing aridity reduces herbivory damage to plants between and within species and that whilst overall defence concentration may not increase *per se*, increasing aridity seemingly selects for the specific production of aliphatic glucosinolates. Manipulative experiments are now required to rigorously test for the trade-off in leaf damage and defence production particularly in induced defences to separate and quantify the relative effects of climate and herbivory in selecting for variation in plant herbivore defences.

Chapter 5

Discussions and Conclusions

Changes in the climate will not only affect plant species directly, but they will also affect the interactions between plants and their associated animals (Hegland et al., 2009; Hargreaves and Eckert, 2014). Because of the direct and indirect effects that the abiotic environment has on plant fitness, it remains a challenge to predict how plants may respond to climate change (Franks et al., 2014; Merila and Hendry, 2014; Hahn and Maron, 2016). The importance of understanding the influence of the abiotic and biotic environment on plant fitness is especially evident in the increasing number of field experiments related to testing plant-animal interactions along environmental gradients (e.g. Devoto et al., 2005; Devoto et al., 2009; Moles et al., 2011; Sternberg et al., 2011; Hahn and Maron, 2016). However, as outlined in chapter 1, the selection pressures, mechanisms, and consequences of securing reproduction and investment into chemical defence in an increasingly arid climate remains uncertain. This is regrettable as, particularly in the Eastern Mediterranean Basin, rainfall amounts are predicted to decrease by 20% with rain events becoming less frequent and more extreme (Black, 2009; Evans, 2009; Smiatek et al., 2011; Golodets et al., 2013). This thesis resolves some of the aforementioned uncertainty by addressing the research goals put forward in chapter 1 in the form of hypothesis testing through observations and experiments as reported in chapters 2, 3, and 4.

I hypothesised that populations of plant species inhabiting increasingly arid environments will secure their reproduction by self-fertilisation and thus I predicted that populations from increasingly arid environments will produce more viable seeds by self-fertilisation in the natural environment (chapter 2). Moreover, I predicted that plant populations have adapted to increasing aridity by evolving greater self-compatibility (chapter 3). The pollinator observations (chapter 2) clearly showed a decline in pollinator visits with increasing aridity confirming that pollinators become increasing limited with decreasing rainfall. This fulfils one of the two prerequisites for reproductive assurance by self-fertilisation to evolve (Lloyd, 1992) in annual plant species in the Eastern Mediterranean Basin. The second prerequisite to select for the evolution of reproductive assurance is mate limitation (Lloyd, 1992) and previous research along this rainfall gradient showed that the average density of conspecific mates decreases with increasing aridity (Tielbörger et al., 2014). Taken together, this research provides compelling evidence that the conditions in the Eastern Mediterranean Basin will likely select for reproductive assurance in plant species distributed along the rainfall gradient.

Despite a clear decrease in pollinator abundance with increasing aridity, the pollinator visits to the three-target species showed less clear trends, thus potentially reducing the clear selection pressure for reproductive assurance. Pollinator visits to *Isatis lusitanica* were lower in the semi-arid site than the Mediterranean site, yet this had no effect on the number of seeds produced by self-fertilisation. These findings corroborate previous research classifying *I. lusitanica* as essentially self-incompatible (Plitmann, 1993), thus rendering reproduction by self-fertilisation nearly impossible. Nevertheless, *I. lusitanica* maintained a constant seed production by outcrossing across the rainfall gradient despite decreasing pollinator activity. I conclude from these combined results that *I. lusitanica* is completely reliant on pollinator visits to ensure successful fertilisation, yet, despite significantly fewer pollinator visits in the arid environment, the reproductive success of *I. lusitanica* remains secure. This resilience of plant reproductive success in arid environments may arise because the associated pollinator community may be highly efficient at cross-pollination and may be highly adapted to harsh environments by rapidly adjusting their activity and foraging behaviour in accordance with local climatic conditions (Minckley et al., 2013). Such flexibility in the plant-pollinator interactions of *I. lusitanica* implies that this species, and potentially other outcrossing annual species, may be able to maintain reproductive success as the environment becomes increasingly arid as predicted by climate change (Smiatek et al., 2011).

Pollinator visits to *Biscutella didyma* remained constant across the rainfall gradient, therefore reducing the selection pressure for populations in increasingly arid environments to secure their reproduction by self-fertilisation (Lloyd, 1992). Nevertheless, the results from the pollinator exclusion experiment in chapter 2 provide compelling evidence that *B. didyma* increases seed production by self-fertilisation in arid environments. This evidence for reproductive assurance in *B. didyma* in the natural environment was corroborated by the controlled manipulation experiment reported in chapter 3. Here, *B. didyma* exhibited greater self-compatibility with the cline of increasing aridity. Moreover, these results were confirmed by the spontaneous selfing control in the manipulation experiment which showed unequivocally that populations of *B. didyma* originating from the arid environment are genetically able to produce significantly more seeds by spontaneous self-fertilisation than populations originating from the mesic-Mediterranean environment. Based on the constant pollinator visitation rate, I conclude that the selection of reproductive assurance in *B. didyma* is most likely driven by mate limitation which has weakened prezygotic barriers of self-incompatibility (Barrett, 1988; Barrett, 2010b). Additional pollinator observations would be necessary to confirm whether the trend observed in the consistent pollinator visits to *B. didyma* across the rainfall gradient represents the long-term patterns in the plant-pollinator relationship, or whether the pattern identified during this study year was abnormal.

The number of pollinator visits to *Sinapis alba* reflected the general pattern of pollinator visits along the rainfall gradient, *i.e.*, decreasing visits with increasing aridity. Therefore, I expected to find strong evidence for reproductive assurance in populations of *S. alba* inhabiting the arid environment. However, the results from the field experiment provided no evidence that *S. alba* can increase the number of seeds produced by self-fertilisation in harsher environments (chapter 2). The results from the controlled manipulation experiment do not, on first sight, support the field results (chapter 3). The controlled manipulation experiment showed a clear cline in self-compatibility in *S. alba* with increasing aridity. I am cautious in concluding that *S. alba* has evolved to assure its reproduction by self-fertilisation, however, because there was no evidence of reproductive assurance in the spontaneous selfing control. I conclude therefore, that the self-compatibility index, by virtue of the mathematics involved in its calculation, overestimates the capacity of *S. alba* to successfully self-fertilise. The combined evidence from the natural environment (chapter 2) and the spontaneous control from the controlled manipulation experiment (chapter 3) showed that whilst *S. alba* may exhibit trends towards assuring its reproduction by self-fertilisation, this may not be its main strategy to secure reproduction in arid environments. Other strategies that this species, and potentially other annual plant species using mixed mating, use to secure reproduction without increasing self-fertilisation include seed dormancy and seed banks which prevent reproductive failure in unfavourable years (e.g. Lampei and Tielbörger, 2010; Tielbörger et al., 2012; Evans et al., 2011).

The combined evidence accumulated from field observations of natural pollinator activity, field manipulation experiments, and controlled manipulation experiments on populations of numerous plant species distributed along a steep rainfall gradient permits some predictions of how plant-pollinator interactions may change in response to future climate change. The research assimilated in chapters 2 and 3 of this thesis suggests that pollinator activity and diversity will decline with increasing aridity, yet this seems to have little effect on the efficiency of cross-pollination. Moreover, my research implies that the number of large solitary wild bees and hoverflies may decline with increasing aridity. This is in line with recent research that shows that bees may be vulnerable to climate change, particularly to increasing temperatures and decreasing rainfall (Hegland et al., 2009; Benelli et al., 2017). The robustness of pollinators to provide sufficient cross-pollen to annual plant species regardless of environmental conditions implies that, despite the negative impacts of climate change on the pollinator community, annual plant communities will continue to reproduce by outcrossing. Reproduction by outcrossing will maintain genetic variation within the gene pool, thus providing the opportunity for adaptation to novel climatic conditions (Charlesworth et al., 1993; Charlesworth, 2003; Barrett, 2010a; Levin, 2010; Barrett et al., 2014). The evidence showing that assuring reproduction by self-fertilisation was species-specific limits my ability to generalise and predict how increasing aridity will influence reproductive strategies overall in annual plant communities in the Eastern Mediterranean Basin.

In chapter 4, I put forward two alternative hypotheses to describe the relationship between leaf damage by herbivory and the concentration of plant chemical defences. This relationship may be positively or negatively affected by increasing aridity (see also Hilbert et al., 1981; Maschinski and Whitham, 1989; Herms and Mattson, 1992; Siemens et al., 2003). In both cases, I predicted that this relationship was driven by a trade-off between leaf damage by herbivory and the concentration of plant chemical defence. The results of the herbivore pressure observations corroborate the results of the pollinator observations, that is, decreasing herbivore pressure (and decreasing pollinator activity) with increasing aridity. The general reduction in leaf damage by herbivory with increasing aridity implies that many species of the annual plant community will benefit from reduced herbivore pressure with climate change. Plant investment into the total concentration of glucosinolate chemical defence will likely not change with climate change, yet the composition of glucosinolate defence is sensitive to increasing aridity. Surprisingly, there was no evidence of a relationship between leaf damage by herbivory and glucosinolate concentration suggesting that interactions between plants and their associated herbivores may provide weaker selective pressures for the evolution of chemical defence than the pressure from the abiotic environment.

Overall, the research of this thesis shows that large intraspecific variation exists in plant-pollinator and plant-herbivore interactions in populations of annual Brassicaceae species distributed along a steep rainfall gradient. Moreover, this thesis provides evidence that variation in the pollinator community and herbivore pressure has limited consequences for the reproductive success and defence mechanisms of annual Brassicaceae species. Each of the experiments and the observations of pollinator activity and herbivore pressure was conducted in a single growing season in a region that experiences large inter-annual variation in rainfall amount (Holzapfel et al., 2006), thus there is a limitation to the generality of these results. However, the annual rainfall in the second study year (chapter 2) was below average for the region whilst the temperature was above average (WMO, 2018) thus providing realistic conditions for which reproductive assurance is expected to occur. Naturally, long term observations might provide more exhaustive results, and there is scope to further investigate the guilds of herbivores present along the rainfall gradient and the specific effects of specialists *vs*. generalist herbivores on the constitutive and induced chemical defences of the annual Brassicaceae community. Nevertheless, in light of the combined research of this thesis, I conclude that the fitness of plants, at least in the Brassicaceae family, will remain relatively unaffected by climate change and that the continuation of outcrossing will maintain genetic diversity in a region that harbours great biodiversity (Myers et al., 2000; Sala et al., 2000; Golan-Angelko and Bar-Or, 2008).

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Appendix

Pollinator activity

I compared AICc values for models composed of the factor site and all combination of the covariates representing the number of flowers on the specific target species and the total number of flowers (regardless of species) present in the quadrat (Table A1).

Table A1: AICc values for potential models explaining pollinator visits to the whole quadrat, to *Biscutella didyma*, to *Isatis lusitanica,* and to *Sinapis alba*. The potential explanatory variables were site (arid, semi-arid, Mediterranean and mesic-Mediterranean), total number of flowers in the quadrat, and the number of flowers on the specific target plant. The selected models are indicated in bold.

	Visits to			
	quadrat	*B. didyma*	*I. lusitanica*	*S. alba*
Site	-34.04	5.72	**5.82**	30.82
Site, # target flowers	-31.64	**-58.88**	9.20	**22.66**
Site, # total flowers	**-44.62**	1.45	7.31	30.64
Site, # target flowers, # total flowers	-42.58	-55.69	11.50	27.74

Table A2: Coefficients (SE) for the selected models for pollinator visits to the quadrat and visits to each of the target species separately (Table A1). The potential explanatory variables were site (arid, semi-arid, Mediterranean and mesic-Mediterranean), total number of flowers in the quadrat, and the number of flowers on the specific target plant.

Bold: P-values <0.001; **: 0.01>P-values >0.001; *: 0.05>P-values >0.01.

	Visits to			
	quadrat	*B. didyma*	*I. lusitanica*	*S. alba*
Intercept	-0.78(0.218)	2.134(0.751)**	**-1.270(0.285)**	2.704(0.842)
Arid	**-1.225(0.240)**	-1.679(6.731)		
Semi-arid	**-1.128(0.246)**	-8.061(9.682)	-1.440(1.332)	**-2.733(0.618)**
Med.	-0.664(0.296)*	-1.480(0.557)**	0.485(0.343)	-2.033(0.731)**
# target flowers		-0.327(0.100)**		0.040(0.013)**
# total flowers	**0.005(0.001)**			

In addition, I tested for the potential correlation between visits to the target species and the general level of visits to the quadrat. I could show (Fig. A1) that overall, the species of the Brassicaceae family were an integral part of the flower community in my study and the visits to their flowers were strongly correlated (r=0.928; P <0.001) with the overall visits to the quadrats.

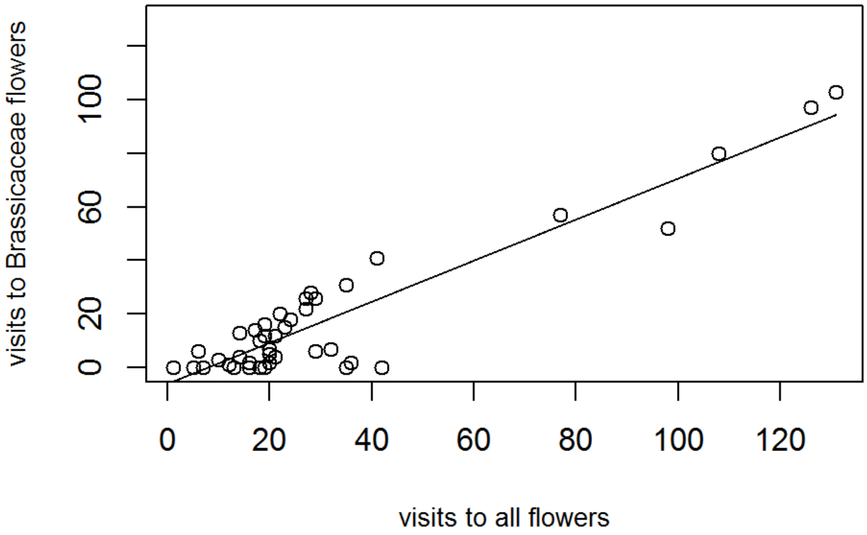

Figure A1: Correlation between the number of pollinator visits to the target species and the number of pollinator visits to all flowers in the quadrat.

Pollinator diversity

Table A3: Coefficients (SE) for the analysis of the effect of site (arid, semi-arid, Mediterranean and mesic-Mediterranean) on pollinator diversity (species richness and Shannon-Wiener diversity index) in the observed quadrats. Bold: P-values <0.001; **: 0.01>P-values >0.001; *: 0.05>P-values >0.01

	Richness	S-W diversity
Intercept	4.714(7.793)	0.384(0.145)*
Semi-arid	5.286(10.671)	0.298(0.199)
Med.	12.286(11.471)	0.131(0.213)
Mesic-Med.	**43.195(9.968)**	**0.778(0.185)**

Autonomous selfing

Tables A4-S6 present model coefficients (SE) for the analysis of the effect of site, treatment (exclusion of *vs*. exposure to pollinators) and their interactions on the seed production per flower and seed viability for each target species.

Table A4: Model coefficients (SE) for the relative seed production and seed viability for *B. didyma*. Bold: P-values <0.001; **: 0.01>P-values >0.001; *: 0.05>P-values >0.01*.*

Biscutella didyma		Seed production	Seed viability
Intercept		**0581(0.038)**	-0.041(0.045)
Site	Semi-arid	-0.013.054)	-0.087(0.077)
	Med.	-0.127(0.052)	0.027(0.088)
	Mesic-Med.	**-0.072(0.004)**	-0.023(0.080)
Treatment	Exposure	-0.160(0.054)	0.006(0.077)
Site × Treatment	Semi-arid × Exposure	0.151(0.074)	0.070(0.118)
	Med × Exposure	0.262(0.074)	-0.029(0.130)
	Mesic-Med × Exposure	0.150(0.079)	0.022(0.124)

Table A5: Model coefficients (SE) for the relative seed production and seed viability for *I. lusitanica*.

Bold: P-values <0.001; **: 0.01>P-values >0.001; *: 0.05>P-values >0.01

Isatis lusitanica		Seed production	Seed viability
Intercept		**-3.476(0.048)**	-0.526(0.350)
Site	Med.	-14.030(0.710)	-0.076(0.144)
	Mesic-Med.	-0.557(0.068)	0.526(0.639)
Treatment	Exposure	**3.146(0.054)**	0.452(0.365)
Site × Treatment	Med × Exposure	14.039(0.079)	N.A.
	Mesic-Med × Exposure	-0.438(0.074)	-0.582(0.653)

Table A6: Model coefficients (SE) for the relative seed production and seed viability for *S. alba*.

Bold: P-values <0.001; **: 0.01>P-values >0.001; *: 0.05>P-values >0.01.

Sinapis alba		Seed production	Seed viability
Intercept		**0.424(0.068)**	-0.295(0.233)
Site	Med.	-0.222(0.090)*	0.113(0.327)
	Mesic-Med.	-0.335(0.093)	-0.621(0.393)
Treatment	Exposure	-2.169(1.769)	0.169(0.242)
Site × Treatment	Med × Exposure	-0.486(1.863)	-0.044(0.343)
	Mesic-Med × Exposure	-1.034(1.996)	0.647(0.403)

Although I did not have a direct way to connect pollinator observations to the relative seed production, I wanted to estimate the potential effect of pollinator abundance on the relative seed production. To do that, I analysed, for each species separately, the effect of the average number of visiting pollinators per site as a covariate in addition to the effects of treatment and their interaction (GLM normal distribution and log link).

None of the factors had an effect on relative seed production in *B. didyma* and *I. lusitanica*, whilst treatment had a significant effect in *S. alba* ($\chi_1^2 = 4.98; P = 0.026$; Fig. A2, Table A7).

Table A7: Model coefficients (SE) for the relative seed production as a function of pollination exclusion treatment and the average number of visiting pollinators to the quadrats. Bold: P-values <0.001; **: 0.01>P-values >0.001; *: 0.05>P-values >0.01

	Relative seed production		
	B. didyma	*I. lusitanica*	*S. alba*
Intercept	**0.601(0.038)**	**-0.372(0.053)**	**0.626(0.056)**
Treatment: Exclusion	-0.036(0.057)	-4.021(2.227)	-2.089(0.935)*
# visits to quadrat	-0.026(0.013)	0.007(0.004)	-0.003(0.001)*
Treatment × # visits	0.025(0.020)	0.054(0.107)	-0.019(0.048)

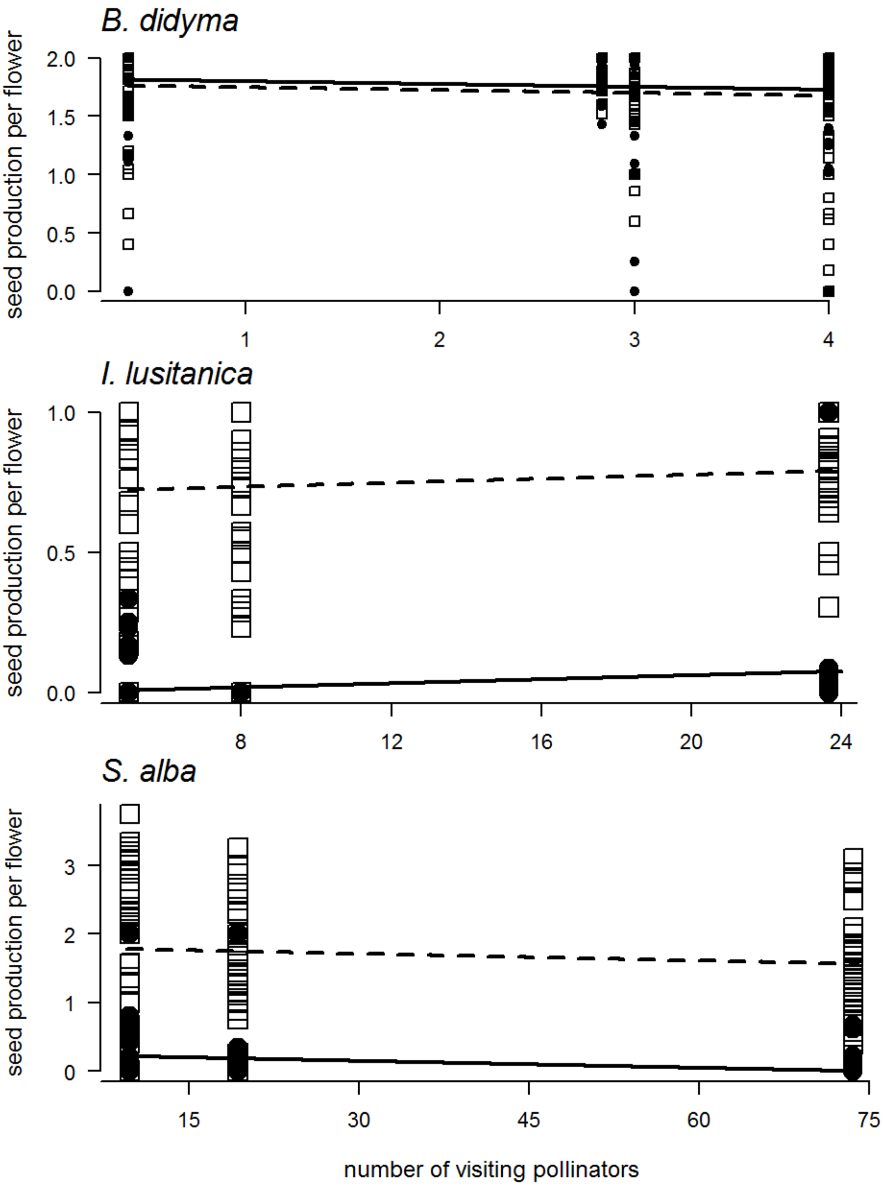

Figure A2: Relationship between the average number of visiting pollinators to a quadrat and relative seed production for the three-target species. Black symbols and dotted lines = pollinator exclusion treatment, white symbols and solid lines = pollinator exposure treatment.