The context dependence of the interaction between a semi-arid herb and its herbivores

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UNIVERSIDAD DE GRANADA

The context dependence of the interaction between a semi-arid herb and its herbivores

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The context dependence of the interaction between a semi-arid herb and its herbivores

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Facultad de
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Publications

This thesis is based on the following scientific articles:

Pre-dispersal seed predators boost seed production in a short-lived plant **(2021)**

M. Aguirrebengoa, C. Müller and A. González-Megías | Oecologia 194 (4), 971-982 (2021)

Ungulates enhance overcompensation for pre-dispersal seed predators

A. González-Megías, M. Aguirrebengoa, R. Menéndez | *In preparation*

Transgenerational effects of ungulates and pre-dispersal seed predators on offspring success and resistance to herbivory **(2018)**

M. Aguirrebengoa, M. García-Planas, C. Müller and A. González-Megías | PLoS ONE 13 (12), e0207553

Additiveness and density-dependence in simultaneous root and floral herbivory

M. Aguirrebengoa, C. Müller, P. Hambäck and A. González-Megías | *In preparation*

Plants facing floral herbivory while interacting with detritivores: a density-dependent perspective

M. Aguirrebengoa, C. Müller, P. Hambäck and A. González-Megías | *In preparation*

Density- and context-dependent transgenerational effects of detritivores, root herbivores and floral herbivores

M. Aguirrebengoa, C. Müller, and A. González-Megías | *In preparation*

Altered rainfall patterns reduce plant fitness and disrupt interactions between below-and aboveground insect herbivores **(2020)**

M. Aguirrebengoa, R. Menéndez, C. Müller, and A. González-Megías | Ecosphere 11 (5), e03127

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Main chapters

Resumen

La herbivoría es la tasa de consumo por parte de los animales de cualquier parte de la planta, incluidas las raíces, el follaje, los tallos o las estructuras reproductivas. La relación entre las plantas y sus herbívoros es uno de los aspectos cruciales de la ciencia biológica, ya que es una ubicua interacción biótica esencial para comprender la diversidad biológica actual, la distribución y abundancia de las diferentes especies de plantas y animales, los procesos biogeoquímicos, el funcionamiento de ecosistemas y sus servicios ecosistémicos.

Durante las últimas décadas se han realizado importantes avances en el estudio multifocal de esta interacción, aunque dada su complejidad intrínseca aún quedan muchas cuestiones por resolver. Aún hoy tenemos un conocimiento algo sesgado hacia sistemas más simples como los de las plantas cultivables, con pocos sistemas en los que se haya estudiado conjuntamente el conglomerado de herbívoros naturales de una determinada planta con tasas de consumo realistas, y menos aún que hayan considerado simultáneamente su resistencia y tolerancia frente a su comunidad de herbívoros. Esto es necesario tanto para el avance de este amplio campo y todas sus ramificaciones (por ejemplo, la dinámica de la comunidad), como para poder seguir desarrollando un control de plagas vegetales cada vez más respetuoso con el medio ambiente. En el presente trabajo hemos evaluado experimentalmente la interacción entre la herbácea silvestre *Moricandia moricandioides* (Brassicaceae) y diversas combinaciones de sus principales herbívoros, la respuesta defensiva de la planta y parte del contexto en el que tanto el daño infligido a la planta como la capacidad de la planta para hacer frente a sus herbívoros pueden variar. Además, profundizamos en aspectos novedosos como son los efectos transgeneracionales, la variación subindividual inducida por herbívoros y la potencialidad del cambio climático para modular las interacciones planta-herbívoro.

En el primer capítulo detallamos el experimento llevado a cabo para determinar el impacto de los depredadores de semillas pre-dispersivos sobre el éxito reproductivo de la planta (Capítulo 1, Fig. A1es). Este tipo de herbivoría se considera de las más perjudiciales para la planta pues reduce el número de potenciales embriones (semillas), aunque esta interacción ha sido casi exclusivamente estudiada de forma observacional. Al llevar a cabo

un experimento manipulativo de presencia/ausencia con este tipo de herbívoro comprobamos que su impacto sobre la planta fue ciertamente sorprendente; a través mecanismos de tolerancia la planta sobrecompensó el daño causado por los depredadores de semillas pre-dispersivos, produciendo más semillas que en ausencia de daño. En este mismo experimento también manipulamos a los detritívoros para determinar si la respuesta de la planta a estos herbívoros pudiera depender de los recursos adicionales que estos les proporcionan, pudiendo confirmar que los efectos derivados de la depredación pre-dispersiva de semillas ocurrieron independientemente de la presencia de detritívoros.

A lo largo de este trabajo continuamos el estudio de los depredadores de semillas pre-dispersivos, esta vez en combinación con otros herbívoros. Durante un periodo de diez años evaluamos el impacto de los depredadores de semillas conjuntamente con otros determinantes herbívoros como son los ungulados (ovejas en este caso; Capítulo 2, Fig. A1es). Observamos que los ungulados redujeron la incidencia e intensidad de los depredadores de semillas pre-dispersivos. Una intensidad moderada de los depredadores de semillas pre-dispersivos se correlacionó positivamente con la altura y el número de tallos reproductivos en la planta y, en consecuencia, con la producción de semillas, confirmando el efecto positivo sobre el éxito reproductivo de la planta previamente observado. Sin embargo, lo más evocador fue la interacción entre ambos herbívoros, ya que el efecto positivo de los depredadores de semillas pre-dispersivos en la planta se acentuó en las poblaciones expuestas a ungulados. A medida que avanzaba este estudio, y viendo la no-aditividad de los efectos de ambos herbívoros sobre la planta, nos preguntamos si estos podrían afectar también a la calidad de las semillas y la progenie a través de efectos transgeneracionales (Capítulo 3, Fig. A1es). Los resultados, como era de prever, fueron complejos. Solo los ungulados afectaron a la viabilidad de la descendencia, limitando su emergencia y supervivencia al reducir el contenido en carbono en las semillas. La interacción entre ambos herbívoros provocó que los efectos de los ungulados sobre la descendencia se viesen reforzados por el acaecimiento simultáneo de la depredación de semillas, aunque esa herbivoría dual materna también aumentó la resistencia de la descendencia frente a los insectos herbívoros. Esta resistencia pudo observarse en la descendencia derivada de plantas madres con depredación de semillas, pero a su vez, la descendencia derivada de frutos con depredación de semillas mostró aún más resistencia, por lo que observamos efectos transgeneracionales de ambos herbívoros a nivel de planta pero también a nivel de dentro de planta (descendencia de semillas en cuyo fruto de origen hubo depredación *vs.* descendencia de semillas en cuyas madres hubo depredación se semillas, pero no en su fruto de origen).

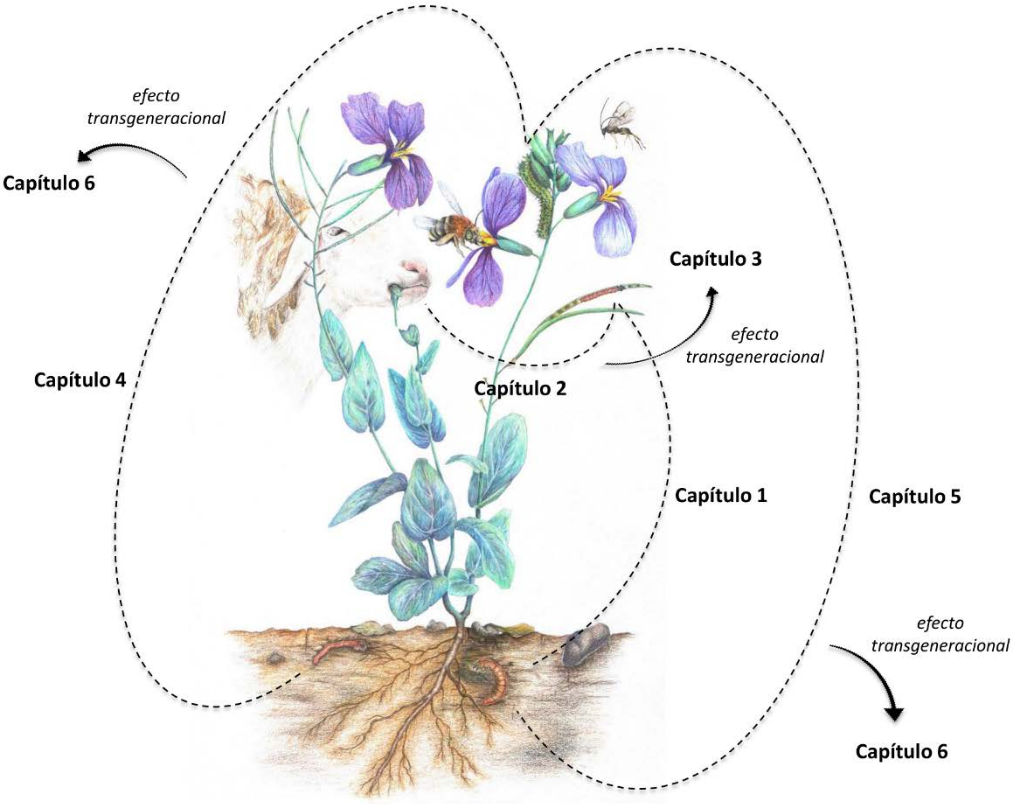


Figura A1es. *Moricandia moricandioides*, sus herbívoros y detritívoros, y los capítulos en los que se estudia cada interacción. Autor de la ilustración: Igor Vázquez.

Pasando a otras de interacciones de la planta, nuestro objetivo fue estudiar el efecto de otros tipos de herbívoros como son los florícolas en combinación con organismos subterráneos que interactúan con la planta. Con la intención de simular un mayor grado de realismo y, por lo tanto, de complejidad, manipulamos los diversos organismos de parte aérea y subterráneos en un gradiente de densidad. Cuando examinamos el efecto combinado de florícolas y radicícolas, encontramos que su impacto sobre el éxito reproductivo de la planta fue independiente, a pesar de que la alta densidad de ambos grupos de herbívoros conllevó un aumento no aditivo en la defensa química de la planta (concentración de glucosinolatos; Capítulo 4, Fig. A1es). El impacto moderadamente negativo de los radicícolas aumentó linealmente con su densidad, mientras que ese aumento no fue lineal para los herbívoros florales (la curva de daño se aplanó a densidades más altas), lo que es atribuible a una respuesta que combina tanto la resistencia como la tolerancia por parte de la planta. Al evaluar los efectos denso-dependientes de los florícolas en combinación con detritívoros, observamos que el impacto no lineal sobre el éxito reproductivo de la planta de estos herbívoros fue independiente de la presencia de detritívoros (Capítulo 5, Fig. A1es), en consonancia a lo observado para depredadores de semillas pre-dispersivos. Como habíamos hecho anteriormente para los ungulados y los depredadores de semillas pre-dispersivos, nos propusimos evaluar los posibles efectos transgeneracionales combinados e independientes que podrían tener las diferentes densidades de florícolas y organismos subterráneos (Capítulo 6, Fig. A1es). Encontramos que las tres interacciones (florícolas, radicícolas y detritívoros) tuvieron efectos transgeneracionales sobre la descendencia, aunque los efectos transgeneracionales dependieron tanto del contexto (otros interactuantes) como de la densidad con la que interactuaron con la planta madre. Entre otros resultados complejos, la emergencia de plántulas aumentó y disminuyó, respectivamente, con florivoría y alta densidad de detritívoros en las plantas madre, lo que podría relacionarse con el efecto de estos organismos sobre el provisionamiento de las semillas. En cambio, el éxito reproductivo de la descendencia aparentemente estuvo más condicionada por los fenotipos de defensa-crecimiento heredados y su interacción con los florícolas en esta segunda generación.

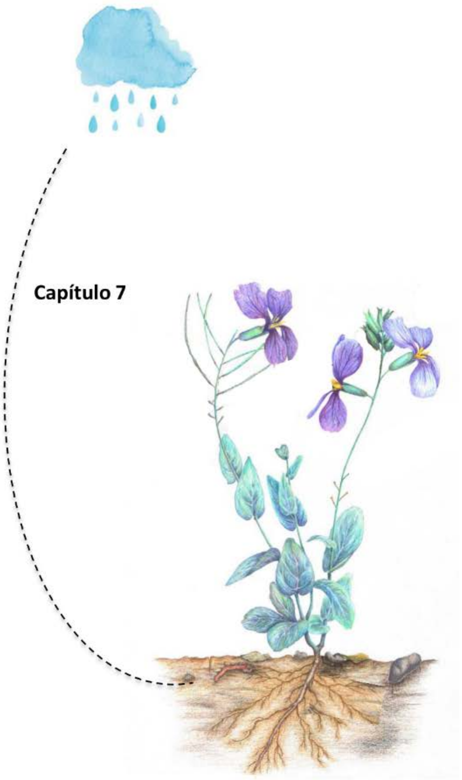


Figura A2es. Manipulación simultánea de un patrón de lluvia alterado y herbivoría de raíz en el sistema *Moricandia moricandioides* (Chapter 7).

En un capítulo final abordamos la posibilidad de que cierto aspecto del cambio climático pudiera modular la interacción entre la planta y sus herbívoros (Capítulo 7, Fig. A2es). Simulamos un potencial escenario futuro en el que variamos el momento y la intensidad de la precipitación, y como resultado observamos que la planta se vio muy afectada en su crecimiento y reproducción. Estas consecuencias para la planta conllevaron la disrupción del efecto positivo de los radicícolas mediante la prolongación del periodo de floración sobre los insectos masticadores de la parte aérea de la planta.

Finalmente, integramos los diferentes capítulos de este trabajo en una discusión más amplia, realizando una síntesis del conocimiento generado.

Abstract

Herbivory is the rate of consumption by animals of any plant parts, including roots, foliage, stems or reproductive structures. The relationship between plants and their herbivores is one of the crucial aspects of biological science, as it is an ubiquitous biotic interaction essential to understand the current biological diversity, the distribution and abundance of the different plant and animal species, biogeochemical processes, the functioning of ecosystems and their ecosystem services.

During the last decades, important advances have been made in the multifocal study of this interaction, although given its intrinsic complexity there are still many questions to be resolved. Even today we have a somewhat biased knowledge towards simpler systems such as like those of crop plants, with few systems in which the set of natural herbivores of any plant has been jointly studied with realistic rates of consumption, and even fewer that have considered simultaneously the plant resistance and tolerance towards its community of herbivores. This is necessary both for the advancement of this broad field and all its ramifications (e.g. community dynamics), as well as for being able to further develop an environmentally-friendly crop protection. In the present work we have experimentally evaluated the interaction between the wild herb *Moricandia moricandioides* (Brassicaceae) and various combinations of its main herbivores, the defensive response of the plant and part of the context in which both the damage inflicted on the plant and the ability of the plant to deal with its herbivores can vary. In addition, we delve into novel aspects such as transgenerational effects, herbivore-induced subindividual variation and the potentiality of climate change to modulate plant-herbivore interactions.

In the first chapter we detail the experiment carried out to determine the impact of pre-dispersal seed predators on plant fitness (Chapter 1, Fig. A1en). This type of herbivory is considered one of the most damaging for plants as it reduces the number of potential embryos (seeds), although this interaction has been almost exclusively studied observationally. When performing a manipulative presence/absence experiment with this type of herbivore, we found that its impact on the plant was certainly surprising; through tolerance mechanisms, the plant overcompensated for the damage caused by pre-dispersal

seed predators, producing more seeds than in the absence of damage. In this same experiment, we also manipulated detritivores to determine if the plant's response to these herbivores could depend on the additional resources detritivores provide, being able to confirm that the effects derived from pre-dispersal seed predation occurred independently of the presence of detritivores.

Throughout this work we continued the study of pre-dispersal seed predators, this time in combination with other herbivores. Over a period of ten years we evaluated the impact of seed predators together with other determinant herbivores such as ungulates (sheep in this case; Chapter 2, Fig. A1en). We observed that ungulates reduced the incidence and intensity of pre-dispersal seed predators. A moderate intensity of pre-dispersal seed predators was positively correlated with plant height and number of reproductive stalks and, consequently, with seed production, confirming the previously observed positive effect on plant fitness. However, the most evocative was interaction between both herbivores, since the positive effect of pre-dispersal seed predators on the plant was exacerbated in populations exposed to ungulates. As the study progressed, and seeing the non-additivity of the effects of both herbivores on the plant, we wondered if these dual herbivory could also affect seed quality and the progeny through transgenerational effects (Chapter 3, Fig. A1en). The results, as might be foreseen, were complex. Only the ungulates affected offspring recruitment, limiting their emergence and survival by reducing carbon content in seeds. The interaction between both herbivores led in ungulate effects on offspring being strengthened by pre-dispersal seed predation, but especially in that dual maternal herbivory increased offspring resistance against herbivorous insects. This resistance could be observed in the offspring derived from mother plants with seed predation, but in turn, the offspring derived from fruits with seed predation showed even more resistance, so joint transgenerational effects occurred at plant level but also at within-plant level (offspring from seeds in whose fruit of origin there were pre-dispersal seed predators *vs.* offspring from seeds whose mothers faced seed predation, but not in their fruit of origin).

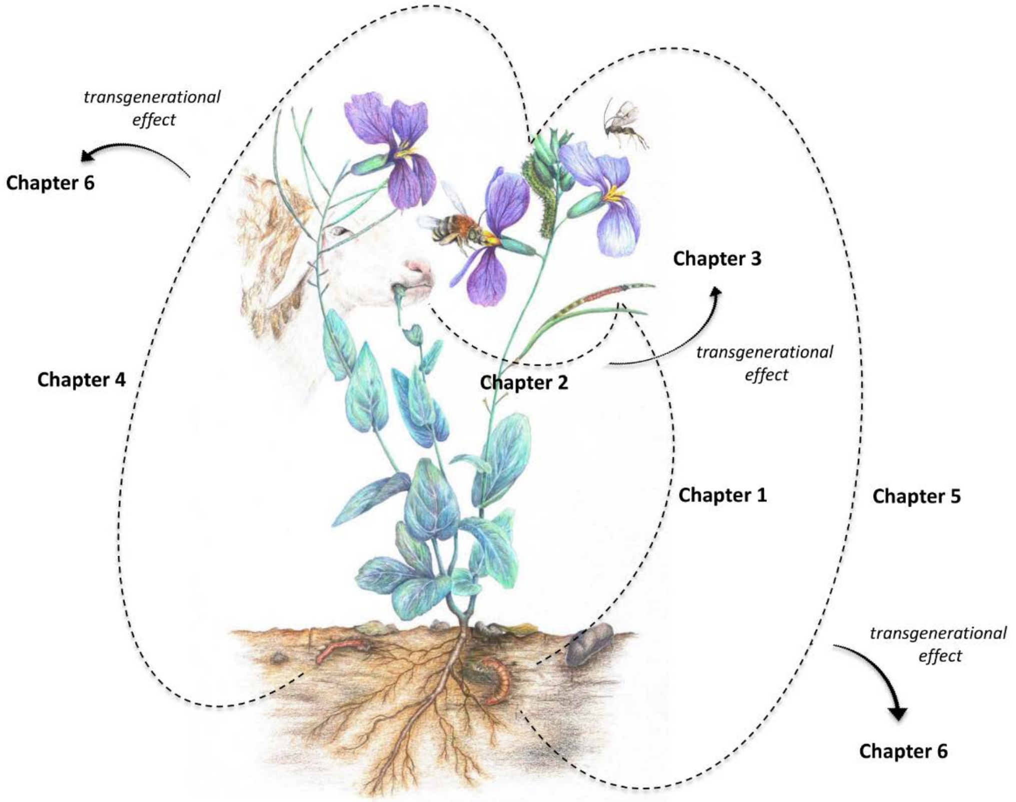


Figure A1es. *Moricandia moricandioides*, its herbivores and detritivores, and the chapters in which each interaction is studied. Author of the illustration: Igor Vázquez.

Moving on to other interactions, we aimed to study the effect of other types of herbivores such as florivores in combination with belowground organisms interacting with the plant. Intending a simulation with greater degree of realism and therefore complexity, we manipulated the various above- and belowground organisms in a density gradient. When we examined the combined effect of floral herbivores and root herbivores, we found that their fitness impact on plant was independent, despite high density of both herbivore groups entailed a non-additive increase in chemical defense (glucosinolate concentrations; Chapter 4, Fig. A1en). The moderately negative fitness impact of root herbivores increased linearly with their density, while that increase was non-linear for floral herbivores (the damage curve flattened at higher densities), which is attributable to the response that combined both resistance and tolerance on the part of the plant. When evaluating floral herbivore density-dependent effects in combination with detritivores, we observed that the non-linear fitness impact of these herbivores on the plant was independent of the presence of detritivores (Chapter 5, Fig. A1en), consistent with what was observed for pre-dispersal seed predators. As we had previously done for ungulates and pre-dispersal seed predators, we aimed to test the potential independent and combined transgenerational effects that the different densities of floral herbivores and belowground organisms could have (Chapter 6, Fig. A1en). We found that all three interactions (floral herbivores, root herbivores and detritivores) had transgenerational effects on offspring, although transgenerational effects depended both on the context (other interacting organisms) and on the density with which they interacted with the maternal plant. Between other complex results, seedling emergence increased and decreased, respectively, with florivory and high density of detritivores on the maternal plants, which could be related to the effect of these organisms on seed provisioning. Offspring reproductive output was apparently more conditioned by the inherited growth-defense phenotypes and their interplay with floral herbivores in this second generation.

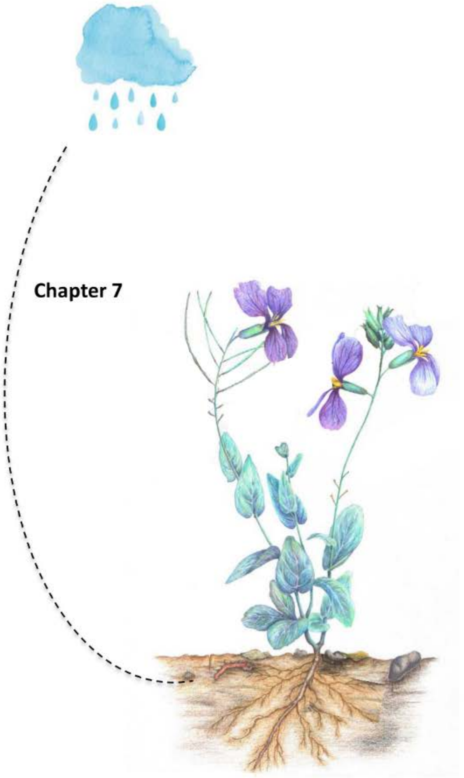


Figure A2en. Simultaneous manipulation of an altered rainfall pattern and root herbivory in the *Moricandia moricandioides* system (Chapter 7).

In a final chapter we addressed the possibility that a certain aspect of climate change could modulate the interaction between the plant and its herbivores (Chapter 7, Fig. A2en). We simulated a potential future scenario in which we altered the timing and intensity of precipitation, and we observed as a result that the plant was greatly affected in its growth and reproduction. These consequences for the plant led to the disruption of the positive effect of root herbivores by prolonging the flowering period on aboveground chewing insects.

Finally, we integrate the different chapters of this work into a broader discussion, carrying out a synthesis of the generated knowledge.

Introduction

Terrestrial biodiversity is dominated by plants and the herbivores that consume them. Herbivory has thoroughly determined ecosystem functioning and services, widely shaped biodiversity and favored complexification, since the actual diversity of life is the result not only from the diversification of species but also from the diversification of interactions among them (Huntly 1991, Stanley and Miikkulainen 2004, Thompson 2005, Futuyma and Agrawal 2009, Leimu et al. 2012, Valiente-Banuet et al. 2015, Guimarães et al. 2017, Levine et al. 2017). Insects have been recognized to be the most significant herbivores (Lawton 1983, Crawley 1989, 2009, Jaenike 1990), usually triggering an ongoing process of coevolution or reciprocal adaptations with plants (Ehrlich and Raven 1964, Janzen 1980, Gatehouse 2002, Strauss et al. 2004b, Futuyma and Agrawal 2009, Leimu et al. 2012). The great number of ecological niches afforded by the great diversity of plants has promoted insect diversification, since herbivorous insect clades are more species-rich than their non-herbivorous sister clades (Mitter et al. 1988, Jaenike 1990, Price 1991, Novotny et al. 2006, Winkler and Mitter 2008, Futuyma and Agrawal 2009, Barrett and Heil 2012). Herbivores, mainly insect herbivores, have evolved in terms of crypsis, phenological synchrony, life history and metabolic and behavioural adaptations towards the plants (Strong et al. 1984, Price 2002, West and Cunningham 2002, Clissold and Simpson 2015, Dussourd 2017, Endara et al. 2017). Along with insects, the importance of herbivorous mammals (particularly large ones) is also remarkable due to its acute impact on nutrient cycling, plant composition, cover and evolution, and its indirect effects on the herbivore community (Huntly 1991, Gómez and González-Megías 2007a, du Toit and Olff 2014, Takagi and Miyashita 2014, Gish et al. 2017). The wide range of herbivorous species with different guilds, feeding modes and specialization degrees has driven plant evolution and provoked differential plant responses and thus phenotypes, which in turn strongly influence the composition and diversity of the herbivore community in a feedback loop (Fritz and Simms 1992, Becerra 2007, Gripenberg et al. 2010, Utsumi et al. 2010, Ohgushi 2016).

Herbivory cause fitness losses in plants, either by consuming reproductive parts or by feeding on vegetative parts and thereby reducing the available resources (Marquis 1992). Thus from the plant perspective, herbivory is a fundamental type of interaction that presents substantial selection pressure to replace lost tissues and to prevent subsequent losses in fitness (Marquis 1992, Mauricio and Rausher 1997, Tiffin and Rausher 1999). Through adaptive evolution, plants have acquired the capacity to quickly and efficiently recognize the most relevant signals from their environment, which includes the challenge by herbivores (Cole et al. 2012, Mithöfer and Boland 2012, Karban 2015). The perception of species-specific herbivore-associated elicitors and vibrations may allow plants to distinguish the type of attacking herbivore (Peiffer et al. 2009, Poelman et al. 2011, Appel and Cocroft 2014, Hilker and Fatouros 2015), and plant inherent plasticity enables to present their most possible competent phenotype to defend against that particular herbivore (Karban et al. 1997, 2016, Ohgushi 2016). Plasticity is partially explained by their modular and unspecialized bodies in the case of vascular plants, whose metabolism is easily adjustable and whose meristems can give rise to almost any tissue at any ontogenetic time (Herrera 2009, 2017, Karban et al. 2016, Gómez et al. 2020). Likewise, plasticity permits plants to exert certain control over their phenology, and thus influence the timing of their interaction with both the physical and biotic environment (van der Putten et al. 2001, Yang and Rudolf 2010, Munguía-Rosas et al. 2011, Ehrlén 2015, Gómez et al. 2020).

Plant resistance and tolerance to herbivory

In response to herbivory, plants have developed various morphological, biochemical, and molecular level defense strategies, which imply reconfigurations of primary but also secondary metabolism (Howe and Jander 2008, Schwachtje and Baldwin 2008, War et al. 2012, Fürstenberg-Hägg et al. 2013). These diverse strategies are commonly grouped as tolerance and resistance, which can be simultaneously expressed (Mauricio et al. 1997, Strauss and Agrawal 1999, Pilson 2000, Rausher 2001, Kessler and Baldwin 2002, Siemens et al. 2003, Núñez-Farfán et al. 2007, Stevens et al. 2007, Muola et al. 2010, Peterson et al. 2017). Plants aim to reduce herbivore damage to minimize impact on fitness though resistance, while aim to maintain fitness despite of being damaged through tolerance. Resistance has two facets, physical and chemical resistance, while tolerance is presented in many ways: compensatory growth, increments in photosynthetic activity, meristem activation, phenological changes and modifications in resource allocation patterns, among others (Strauss and Agrawal 1999, Stowe et al. 2000, Tiffin 2000, Núñez-Farfán et al. 2007, Fornoni 2011, Orians et al. 2011). Despite their probable ubiquity in most plants, resistance and tolerance have been rarely simultaneously investigated within the same study system, partly because the study of plant defenses has been widely biased towards resistance, and tolerance has been steadily overlooked (Leimu and Koricheva 2006, Fornoni 2011, Lucas-Barbosa 2016, Peterson et al. 2017). Resistance and tolerance can be beneficial in terms of plant fitness, although they can also result costly if there is not an optimal balance between the need for defense, herbivore pressure itself and the need for growth and reproduction. Many times costs on fitness are inevitable due to herbivory, and plants are restricted to suboptimal phenotypes based on a single or a combination of defensive traits (Valladares et al. 2007, Orrock et al. 2015, Züst and Agrawal 2017). Both resistance and tolerance are thought to depend on plant life history, phylogenetic constraints, adaptation to abiotic stress or mating system, among other characteristics (Skogsmyr and Fagerström 1992, Grime 2001, Futuyma and Agrawal 2009, Těšitel et al. 2021; Fig. I1), and the costs associated with defense for both resistance and resistance would vary over time and plant ontogeny (Briggs and Schultz 1990, Boege and Marquis 2005, Boege et al. 2007, Barton and Koricheva 2010, Orians et al. 2011, Ochoa-López et al. 2020). Plant responses to herbivory would be contingent to plant ontogeny, the type and identity of the herbivore, the intensity of damage, the plant part under attack, and the environmental conditions (Marquis 1996, Maron 1998, Stowe et al. 2000, Boege and Marquis 2005, Barton and Koricheva 2010, Meldau et al. 2012, Carmona and Fornoni 2013, Massad 2013, Myers and Sarfraz 2017, Ochoa-López et al. 2020).

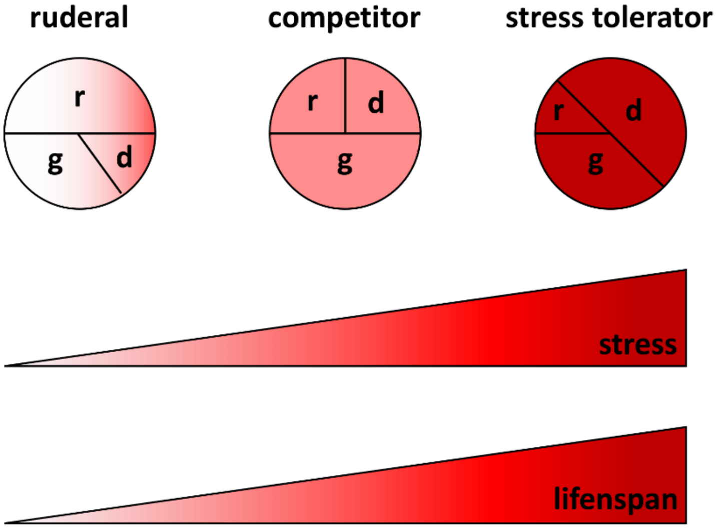


Figure I1. Conceptual model for the different plant strategies: ruderal, competitor or stress tolerator (sensu Grime 2001). Each plant strategy would vary on the relative investment in life history traits of growth (g), defense (d) and reproduction (r). Adapted from De Deyn (2017).

The fact that tolerance and resistance are many times simultaneously expressed would not mean that they are necessarily genetically correlated (Leimu and Koricheva 2006, Núñez-Farfán et al. 2007, Fornoni 2011, but see Pilson 2000, Huot et al. 2014). Many tolerance responses such as compensatory growth are considered generally extended ancestral characters among plants, which could be preadaptations to fire, trampling, desiccation, disease, wind and frost (Belsky et al. 1993, Rosenthal and Kotanen 1994, Tiffin 2000, Fornoni et al. 2003, Verdaguer and Ojeda 2005, Fornoni 2011). While herbivores impose selection on plant tolerance, whether the contrary occurs (plant imposing selection on herbivores through tolerance) is an ongoing debate, since it might not require specific counteradaptations beyond adapting to phenological plant changes (Garrido-Espinosa and Fornoni 2006, Agrawal and Fishbein 2008, Fornoni 2011, Peterson et al. 2017). What does actually seem plausible is that tolerance might slowdown herbivore adaptations to resistance (Jokela et al. 2000, Núñez-Farfán et al. 2007). Chemical defense, instead, would be a more novel response product of selection for increased fitness via coevolution with herbivores (Fraenkel 1959, Ehrlich and Raven 1964, Fritz and Simms 1992, Wheat et al. 2007, Winde and Wittstock 2011, Leimu et al. 2012, Mithöfer and Boland 2012, Speed et al. 2015, Hahn et al. 2019). Chemical defense, which is usually a faster response towards herbivores than physical resistance and tolerance, would probably be more suited to coevolve than tolerance as it would have less pleiotropic constrains (Renwick 2002, Futuyma and Agrawal 2009, Carmona et al. 2011, Moore et al. 2013, Speed et al. 2015, Züst et al. 2020, but see Rausher 1996, Purrington 2000, Strauss et al. 2002, Fornoni 2011, Huot et al. 2014). Chemical defense narrows the range of herbivore species each plant species face by affecting generalist herbivores and partially deterring specialists (Futuyma and Agrawal 2009, Ali and Agrawal 2012, Barrett and Heil 2012, Endara et al. 2017). Regarding chemical defenses, coevolutionary asymmetry between plants and herbivores may be common, as herbivores must adapt to various repellent, toxic or antinutritive compounds, and any novel compound could nullify herbivore adaptations (Futuyma and Agrawal 2009, Mithöfer and Boland 2012, Moore et al. 2013, Speed et al. 2015, Züst et al. 2020). Nevertheless, chemical defenses may also increase diversity and abundance of specialist herbivores on evolutionary time scale, due to the latter's adaptation and speciation, and promote genetic variation in both plants and herbivores (Ali and Agrawal 2012, Ohgushi and Hambäck 2015, Richards et al. 2015, Ohgushi 2016). As many times in the coevolutionary arms race the effectiveness of plant resistance is dampened due to herbivore counteradaptations, maintaining a mixed resistance-tolerance defense would be beneficial for plants (Jokela et al. 2000, Núñez-Farfán et al. 2007).

Resources can vary in space and time, as do the resource requirements by the plant (Burow and Halkier 2017). Both defense strategies require resources, but some tolerance responses such as an increment in photosynthetic activity or regrowth of below- and/or aboveground plant parts could also favor the acquisition of resources and minimize tolerance costs (Núñez-Farfán et al. 2007). The high resource requirement of chemical defenses could have been a limit for their own evolutionary escalation and would have favored mixed tolerance-resistance strategies (Coley et al. 1985, Simms and Rausher 1987, Herms and Mattson 1992, Simms 1992, Futuyma and Agrawal 2009, Bekaert et al. 2012, Gershenzon 2017). To reduce chemical defense costs, plants evolved inducibility in their chemical defense regulation. Like this, it has been predicted and generally demonstrated that constitutive chemical defenses prevail when the fitness losses of herbivory greatly outweigh production costs, and the probability of herbivory attack is not low (Zangerl and Rutledge 1996, Orrock et al. 2015, Karban et al. 2016). On the contrary, inducibility in chemical defenses should be more effective in environments with low and unpredictable rates of herbivory (Stamp 2003, Jung et al. 2012, Hahn and Maron 2016, Karban 2020).

Herbivory can influence plant chemistry, plant morphology, reproduction, and phenology. However, throughout their entire life cycle, plants are also challenged by many different abiotic and biotic stresses in addition to herbivory, such as temperature, light, water and nutrient availability, and other mutualistic, competitive and antagonistic biotic interactions (e.g. pollinators, concurrent plants and pathogens, Fig. I2). In the same way that the herbivore effect is thought to be dependent on their density and the damage intensity, the rest of plant stresses/interactions also exert selection, and their interaction strength with plants would be density/intensity-dependent as well (Thompson and Pellmyr 1992, Thompson 2005, Haloin and Strauss 2008, Gómez et al. 2009, Maron et al. 2014, 2019, Hahn and Maron 2016). Identifying which interactions are the main selective drivers for each plant on each environment is indeed one of the fundaments of ecological research (Strauss and Irwin 2004, Valiente-Banuet et al. 2015). These various stresses/interactions can even act as opposing selective pressures and impose allocation and ecological costs of defense (Mooney 1991, Agrawal and Karban 1999, Heil and Baldwin 2002, Strauss et al. 2002, Strauss and Irwin 2004, Fornoni 2011, Stowe et al. 2013). Within this contingency, plants have to optimally balance growth, resource acquisition, mating and defense, as trade-offs may arise between the different activities (Herms and Mattson 1992, Strauss and Agrawal 1999, Valladares et al. 2007, Karban et al. 2016, de Vries et al. 2017, Karasov et al. 2017, Züst and Agrawal 2017). Therefore, many plant traits related to growth, reproduction and defense would be under continuous conflicting selection, whose strength could even change throughout the life cycle (Gómez 2008, Campbell 2015, Johnson et al. 2015).

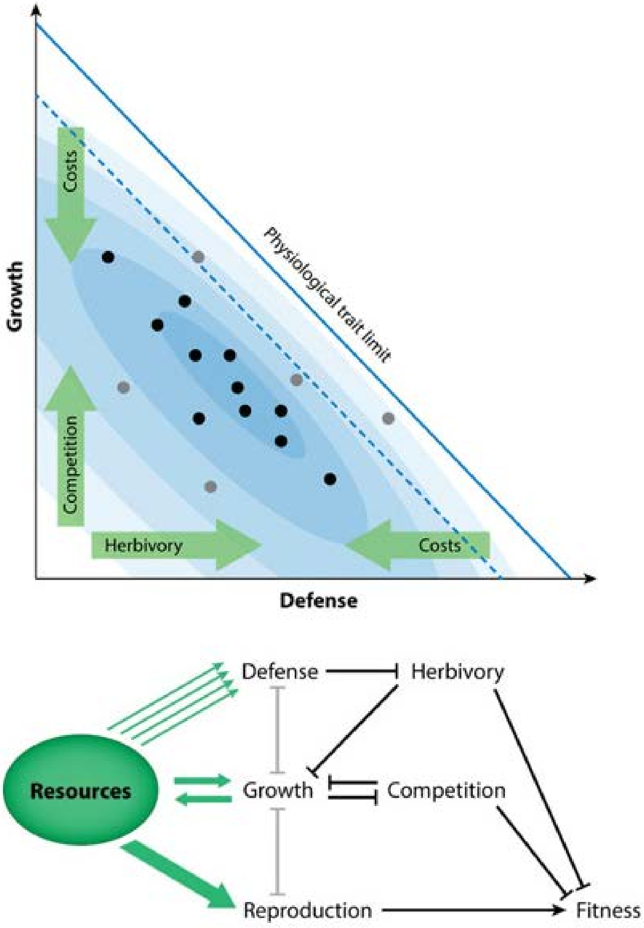


Figure I2. Relations between defense, growth, reproduction and fitness, and resource availability, herbivory and competition effects on them. Modified from Züst and Agrawal (2017).

Predominant plant defense hypotheses: resource availability and plant life history

Theoretical frameworks of plant defense center on the central dilemma of defense costs: resources are limiting, and defenses require resources that would otherwise be available for growth and reproduction. A secondary dilemma would be how plant life history conditions the relative effectiveness of the different defense strategies (see reviews Stamp 2003, Kant et al. 2015)**.** Environment plays a major role determining plant performance and the magnitude of defense costs, which are mainly paid in the form of energy, carbon and nitrogen (allocation and fitness costs; Koricheva 2002, Mithöfer and Boland 2012), being an essential factor in the ongoing discussion about plant defense hypotheses (Stamp 2003; Table I1). It has been proposed that high resource availability can positively covary with both growth and defense, reducing allocation costs (Leimu and Koricheva 2006, Hahn and Maron 2016). Stressful and stochastic environments with great inherent uncertainty/unpredictability (e.g. amount and timing of precipitation) can even generate maladaptation, or impose limits on the rate of adaptation to, for example, herbivores (Debeaujon et al. 2000, Valladares et al. 2007, Lenormand et al. 2009, Leimu et al. 2012, Sæther and Engen 2015, Lyberger et al. 2021). Other authors propose that plants somehow adapt to whatever their environment is, and develop high resilience (Miranda et al. 2009, Asbjornsen et al. 2011, Lloret et al. 2012, Escudero et al. 2015). The expanded growth-differentiation balance hypothesis points that resources do not abound in stressful environments, so defense (referred mainly to chemical resistance) would be costly, trading-off with plant growth over a resource availability gradient (Herms and Mattson 1992; Table I1). When resources are actually limiting, both growth and defense would be compromised, while when resources are available, growth at the expense of (chemical) resistance would be favored (Herms and Mattson 1992). The expanded growth-differentiation balance hypothesis takes into consideration plant life cycle, assuming that rapidly growing plants would have lower levels of secondary metabolites and vice versa (Table I1). Resource availability hypothesis, instead, predicts that high resource environments select for growth rather than for resistance, while low resource environments select for resistance rather than for growth, due to the low capacity of plants to compensate the damage caused by herbivores in these environments (Coley et al. 1985; Table I1). A minor hypothesis tied to resource availability hypothesis like the carbon-nutrient balance hypothesis postulates that the carbon-nutrient status of plants directly controls allocation to secondary metabolites (Bryant et al. 1983), although plant defense patterns are rarely predicted by this balance (Hamilton et al. 2001, Moore et al. 2013). The plant stress hypothesis, also linked to resource availability hypothesis, predicts that environmental stresses decrease plant capacity to resist herbivory by altering biochemical source-sink relationships (White 1984). On the contrary, Hahn and Maron (2016) argued that certain plant traits that are adaptive in stressful and stochastic environments, such as a more rapid phenology, might also facilitate greater tolerance to herbivory. Similarly, the growth rate model states that plants grow below their maximum growth rate under stressful conditions, so may have higher capacity for regrowth after receiving damage than those plants enduring benign conditions and thus growing near their maximum growth rate (Hilbert et al. 1981; Table I1). The compensatory continuum hypothesis mostly circumscribes its prediction to high resource environments: tolerance should be greater in high resource environments, since plants have abundant resources to replace lost tissues and recover from the damage (Maschinski and Whitham 1989; Table I1). Stevens et al. (2007; Table I1) also basically limited their prediction to high resource environments, pointing that in high resource environments resistance should be more costly, where allocation to resistance would hoard resources that could be invested in growth.

Defense hypotheses	**Reference**	**Main predictions**
Optimal defense theory	(McKey 1974, Rhoades 1979)	Resistance in specific plant parts would be favored when the benefits outweigh the costs, especially when probability of attack is high
Growth rate model	(Hilbert et al. 1981)	Plants grow below their maximum growth rate under stressful conditions, so may have a higher capacity for regrowth after receiving damage
Resource availability hypothesis	(Coley et al. 1985)	High resource environments select for growth rather than for resistance; low resource environments select for resistance rather than for growth
Compensatory continuum hypothesis	(Maschinski and Whitham 1989)	High resource environments allow a greater tolerance to herbivory
(Expanded) growth-differentiation balance hypothesis	(Herms and Mattson 1992)	Resistance more costly under stressful conditions
-	(Stevens et al. 2007)	Resistance more costly under high nutrient conditions
Limiting resource model	(Wise and Abrahamson 2005)	Focus on the resource that is limiting plant fitness, the resource affected by herbivory and how the acquisition of resources is affected by herbivory

Table I1. Current principal plant defense hypotheses.

The limiting resource model (Wise and Abrahamson 2005; Table I1), instead, focuses on the resource that is actually limiting plant fitness, which could be an abiotic factor, and how the acquisition of resources and resources themselves are affected by herbivory. The limiting resource model would give importance to the type and timing of damage with respect to plant ontological state, and thus encompasses different possible outcomes of defense (mostly different outcomes of tolerance) based on these aspects. Other minor hypothesis such as the defense-stress cost hypothesis focuses on biotic interactions such as competition, which would increase the costs of defenses since resources are primarily required for growth (Siemens et al. 2003). Finally, optimal defense theory does not take into account that much the environment but the costs in defense, together with the risk of attack and the value of the plant part attacked in terms of fitness (McKey 1974, Rhoades 1979; Table I1). This theory, despite being the oldest to be postulated, continues to have great empirical support, as its predictions fit for chemical defenses (Pichersky and Lewinsohn 2011), indirect defenses such as volatiles and extrafloral nectaries (Dicke and Baldwin 2010) and for fitness consequences of florivory *vs.* folivory (McCall and Fordyce 2010). Cost of defense would be linked to the risk of attack, as contemplated by the plant apparency hypothesis (Feeny 1976), a complement of optimal defense theory. It states that the likelihood of being attacked strongly determines plant investment in defense, thus only when herbivore pressure is high the benefits of defense would outweigh the costs. In addition, it predicts different outcomes for long-lived (apparent) and short-lived (unapparent) plants. Regarding plant parts' or tissues' value, plant capacity to survive and reproduce after removal of this parts is considered crucial in this theory. Reproductive organs such as flowers and developing seeds would be the most valuable tissues inherently defended with constitutive defenses, particularly defensive secondary metabolites (see also Karban and Baldwin 1997). That would be valid mostly for short-lived plants, although the fitness value of the different plant tissues would vary temporally, and even spatially (see also Meldau et al. 2012). Like this, the plants' developmental stage and the timing of herbivory would be also determinant in this theory, pointing that damage early on ontogeny and/or early at the season would be more detrimental (see also Crawley 1989, Maron 1998, Rusman et al. 2020, but see Boege and Marquis 2005).

Ecological realism in plant-herbivore studies

In the intricate subject of plant-herbivore interactions, awareness of the limitation of our studies and the conclusions that can be drawn from them is essential. A first point to take into account is that many plant-herbivore studies, mostly those focused on plant chemical defenses, do not estimate plant fitness. Fitness is a central concept in evolutionary biology, and its measurement is necessary to assess the selective potential of any phenotypic trait (Strauss et al. 2004b). Many studies use proximate measures such as plant growth, but such surrogate measures can be context-dependent and problematic. It is usually more convenient to estimate, at least in annual and short-lived plants, female fecundity by means of the total number of seeds (Herrera 1991, Züst and Agrawal 2017). In addition, the fitness of an individual would be influenced by all lifetime interactions with the abiotic and biotic environment, so that the fitness effects of plant interactions and defense phenotypes can only be optimally evaluated in the natural environment, the *field* (Tack and Roslin 2011, Ehrlén 2015, Kant et al. 2015, Laughlin et al. 2020). Given that plant interactions can have transgenerational consequences on progeny, biologists have realized in recent years that the estimation of total lifetime fitness should even contemplate offspring performance when possible (Fig I3).

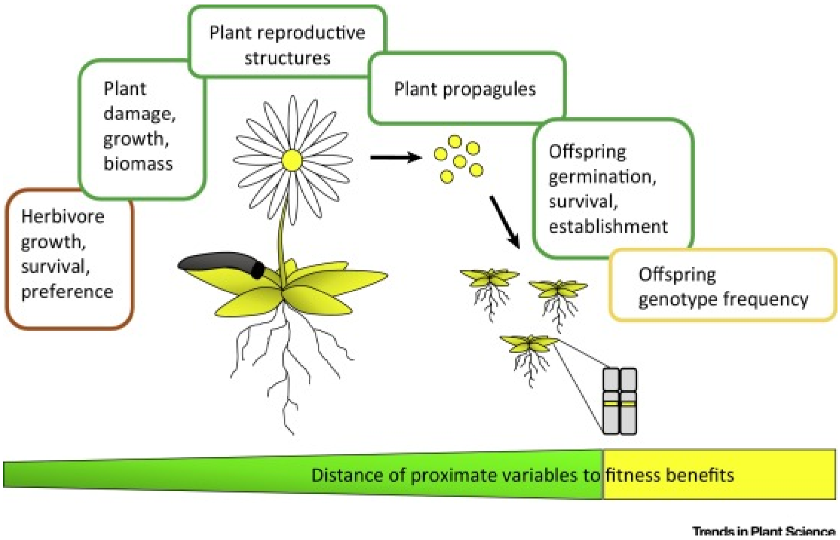


Figure I3. Representation of the suitability of the different fitness measures. The further to the right, the more representative of plant lifetime fitness. Extracted from Erb (2018).

Besides fitness, many other aspects should be taken into account for greater ecological realism in plant-herbivore studies, some of them well summarized in Harvey et al. (2015). First, it should be clear that the same conclusions could not be drawn from laboratory and field studies. Although many facets of plant-herbivore interactions can be elucidated in laboratory studies, the detection of trade-offs and costs of defenses can only be reflected in plants' natural environment (Agrawal 2011, Lucas-Barbosa 2016, Züst and Agrawal 2017). On the other hand, many studies to date have been based on crop plants, which have generally many domestication syndromes such as low genetic variability and thus high phenological uniformity, reduced physical and chemical defense, increased tissue palatability and enhanced apical dominance (Tscharntke et al. 2005, Gols et al. 2008, Chaudhary 2013, Chen et al. 2015, Gaillard et al. 2018, Fernandez et al. 2021). Crop plants also host a less diverse insect community and are often grown in chemically and structurally less complex systems than natural ones such as monocultures, in which agricultural practices can strongly determine herbivory rates (Chen et al. 2015, Harvey et al. 2015). Importantly, many studies principally with crop plants have used unnatural species combinations, such as herbivores with which the study plants have not coevolved. Between others, that is the case of most studies involving the principal plant model *Arabidopsis thaliana* (Harvey et al. 2015). Regarding artificial herbivory, in the last decades it has also been shown that artificial damage does not totally mimic herbivore damage, particularly insect herbivore damage, and some caution should be exercised about the conclusions drawn from these studies (Heil 2009, Fornoni 2011, Moreira et al. 2019), as well as from the rest of above mentioned cases. To conclude this section of common pitfalls and biased conclusions in plant-herbivore studies, it should be noted that experimental manipulation rather than observational data is the most appropriate way to determine strength and fitness impact of interactions under study (Züst and Agrawal 2017).

Plant-herbivore interactions in a semiarid environment

Drylands represent 41% of the terrestrial surface and account for more than 25% of global soil organic carbon (Safriel and Adeel 2005). These temperate ecosystems are characterized by unpredictable and extreme conditions, such as rainfall variability, nutrient poverty and many times hot summers with acute interannual temperature fluctuations (Reynolds et al. 2004, Morton et al. 2011). Because of that, abiotic factors are considered the key selective agents in these ecosystems, and several physiological, morphological and life history trait adaptations are required to survive and reproduce in such variable and limiting conditions (Noy-Meir 1985, Haloin and Strauss 2008, Balao et al. 2018). For plants, the most prominent are: dominance of annual and short-living cycles, low height and rosette morphology, shorter flowering duration, earlier onset of flowering and lower seed persistence (Nunes et al. 2017). Nonetheless, the hypothesized prevalence of abiotic factors determining adaptations in semiarid environments does not reduce the importance of biotic interactions structuring communities in these ecosystems. In fact, semiarid environments can be diverse systems in which ecological interactions are subjected to temporally variable resource-pulse dynamics (Polis 1991, Chesson et al. 2004, González-Megías et al. 2011, Doblas-Miranda et al. 2012, Nielsen and Ball 2015). Plant-herbivore interactions would be thus strongly conditioned by the temperature fluctuations and the timing and intensity of rainfall events that would determine plant and herbivore phenology and activity (Forrest and Miller-Rushing 2010, Yang and Rudolf 2010, Morton et al. 2011, González-Megías and Menéndez 2012, Johansson et al. 2013, Ehrlén 2015, Hänel and Tielbörger 2015).

Diverse plant defense theories predict that resource availability plays a crucial role in plant-herbivore interactions (Table I1). Plants in low-resource environments may support less herbivore pressure, particularly by generalists, because plants in this type of environments have lower tissue quality (Endara and Coley 2011, Hahn and Maron 2016). However, stressful environmental conditions and nutrient limitation could increase the manifestation of trade-offs among the essential plant functions of growth, defense and reproduction (Koricheva 2002, Züst and Agrawal 2017, Lyberger et al. 2021). Besides, plant defense could be directly compromised by less resources available due to the slowdown of photosynthesis caused by very high temperatures (Salisbury and Ross 1985, Grubb and Abel 2006, Jahangir et al. 2009). However, this very fact could free up resources such nitrogen-rich compounds, making these available for use in chemical defense (Schwachtje and Baldwin 2008). Overall nutrient poverty make litter decomposition and soil detrital-based food webs key factors in semiarid environments (Doblas-Miranda et al. 2009a, González-Megías et al. 2011, Morton et al. 2011, Escudero et al. 2015, Sagi et al. 2019, Sagi and Hawlena 2021). Detritivore activity would thus generate resource-rich spatial patches, in which plants could increase their performance and lessen the predicted trade-offs between their different functions (Wurst 2013, Züst and Agrawal 2017). Therefore, it might be highly advantageous for plants in this type of environment to send cues into the soil to attract specific decomposers (Rasmann and Turlings 2016).

Context-dependence in plant-herbivore interactions: a multiherbivore approach

Plants are simultaneously attacked below- and aboveground by a myriad of herbivores, being insects a prominent part of this community (Masters et al. 1993, Kaplan and Denno 2007, Morris et al. 2007, Stephens et al. 2013, Stam et al. 2014). Experimental studies have proven that multiherbivore effects on plant fitness, growth, and reproduction as well as plant responses to them are usually unique and could not be directly inferred from the response of plants to each individual attack in isolation (Strauss and Irwin 2004, Gómez and González-Megías 2007a, Pieterse and Dicke 2007, Utsumi et al. 2010, Rasmussen et al. 2013, Gols 2014, Stam et al. 2014). In fact, most plants coevolve in response to multiple herbivores in a diffuse rather than in a pairwise way, and the adaptive value of the different defense traits can only be comprehended when the entire gamut of herbivores is considered (Iwao and Rausher 1997, Futuyma 2000, Strauss and Irwin 2004, Ohgushi 2005, Haloin and Strauss 2008, Futuyma and Agrawal 2009, Strauss 2014, Salazar et al. 2018, Rusman et al. 2019). The fitness impact of each herbivore is likely to indicate the extent of its relative importance in terms of plant defense evolution (Ali and Agrawal 2012), and probably also the extent of its plant-mediated indirect effects on concurring herbivores (Strauss 1991, Ohgushi 2005, 2016, Gómez and González-Megías 2007a, Ohgushi et al. 2012, Barraclough 2015, terHorst et al. 2015, 2018). However, plants may also exhibit specialized traits for less damaging herbivores. The degree of these adaptations would depend on its costs in plant relationship with the most damaging herbivores (Hunter 1992, Strauss and Irwin 2004, Agrawal 2011). In this way, the plant's response at a specific moment will therefore be determined by its evolutionary background, herbivore species composition and density at that moment, and by herbivore relative importance in plant fitness and in canalizing or overriding the effect of other herbivores (Gómez and González-Megías 2007a, Utsumi et al. 2010, Erb et al. 2011, Stam et al. 2014). It could be thought that the responses of plants to different herbivores can compete for resources and conflict, and that this will be resolved in terms of the relative importance of each herbivore. Nonetheless, several studies show that resistance to multiple herbivores usually tends to be positively correlated (Leimu and Koricheva 2006), and tolerance to multiple herbivores could also be independent or positively correlated (Tiffin and Rausher 1999, Pilson 2000). The ubiquitous existence of multiple herbivores, plant diffuse coevolution with them and the positive correlation among plant defensive responses to the different herbivores would be another explanation of tolerance and resistance complementarity (Stinchcombe and Rausher 2001, Carmona and Fornoni 2013). This complementarity could evidence, as some authors suggest, that general mechanisms of defense rather than species-specific responses are likely to be favored because they lower the cost of defense (Núñez-Farfán et al. 2007).

Notably and as previously commented, we must not lose sight that in addition to the coevolution with herbivores, plants have evolutionarily differentiated along other axes in response to environmental variables such as climate, resource availability and interactions with their mutualists. These interactions interfere in plant defense, condition its costs and benefits and sometimes constrain its evolution (Strauss and Irwin 2004, Irwin et al. 2004, Denno and Kaplan 2007, Kaplan and Denno 2007, Núñez-Farfán et al. 2007, Parachnowitsch and Caruso 2008, Campbell 2015, Johnson et al. 2015, Lucas-Barbosa 2016, Pringle 2016, Jacobsen and Raguso 2018, Ramos and Schiestl 2019, 2020). All these facts highlight the need to progress from the study of pairs of interacting species to multispecies interaction studies in order to understand the role of ecological interactions configuring the actual natural complexity (Thompson 2005, 2013, Bascompte 2009, Stam et al. 2014, Levine et al. 2017). Indeed, plant-herbivore research is in the recent years moving towards multifactorial experimental studies (Agrawal 2011, Baldwin 2012, Stam et al. 2014, terHorst et al. 2015).

Novel aspects on plant-herbivore interaction: transgenerational effects and within-plant variation

In the last years, herbivore capacity to determine the progeny phenotypes through transgenerational effects has been acknowledged (Roach and Wulff 1987, Rossiter 1996, Agrawal 2001, 2002), complicating the estimates of herbivore impact on plants (Ehrlén 2003, Gómez 2008, Erb 2018; Fig. I3). Transgenerational effects can occur across a single generation, as a result of either parental (predominantly maternal) effects or induced genetic changes (Rossiter 1996). Maternal effects would refer to changes in resource allocation such as seed nutrient provisioning (also named as carry-over effects; Agrawal 2001, 2002, Steets and Ashman 2010, Zas et al. 2013), while induced genetic changes would refer to transgenerationally inherited modifications in gene expression regulation (e.g. epigenetic effects; Herman and Sultan 2011, Holeski et al. 2012, Herman et al. 2014, English et al. 2015, Karasov et al. 2017, Richards et al. 2017, Ashe et al. 2021, López Sánchez et al. 2021). In both cases, the environment experienced by the mother would condition offspring traits or performance (Roach and Wulff 1987). Nonetheless, only in the second case could one speak of adaptive plasticity if progeny is benefited by the transgenerationally induced modifications (Uller et al. 2013, Engqvist and Reinhold 2016).

The potential as a trigger for rapid evolution of transgenerational effects in plants and their ecological implications are still a great challenge (Thompson 2013, Auge et al. 2017, Richards et al. 2017, Ashe et al. 2021, López Sánchez et al. 2021). We still lack knowledge about their mechanisms, persistence and costs. Transgenerational inheritance may not be universal in plants, as it may depend upon the nature of the specific stress, and the selective advantages of transgenerational effects depending on habitat predictability and life history characteristics (Ezard et al. 2014, Herman et al. 2014, English et al. 2015, Auge et al. 2017, López Sánchez et al. 2021). Transgenerational effects are apparently stronger in short-lived plants, and occur in both stressful and benign environments (Yin et al. 2019, Zhang et al. 2020). When both parental and offspring environments are alike, transgenerational inheritance may attenuate any detrimental effect on fitness caused by the stressful conditions, as is the case of herbivores (Roach and Wulff 1987, Herman and Sultan 2011, Auge et al. 2017, Karasov et al. 2017). Like this, many times the progeny of attacked plants inherit constitutively expressed defense phenotypes, or higher inducibility upon herbivory (Agrawal 2001, Holeski 2007, terHorst and Lau 2012, Holeski et al. 2012, Rasmann et al. 2012, Ballhorn et al. 2016, Colicchio 2017, Karasov et al. 2017, Kellenberger et al. 2018, López Sánchez et al. 2021, Sobral et al. 2021). However, transgenerational effects may result from multiple abiotic and direct and indirect biotic interactions, and may be difficult to predict in species-rich, natural communities (Miller and Travis 1996, Irwin 2006, Novak et al. 2011, terHorst and Lau 2012, Walsh 2013, Lampei 2019). In this way, few advances have been made to date in the study of the transgenerational effects of multiple herbivores on plants (see Gómez 2008, González-Megías 2016).

In addition to transgenerational effects, there is an ample range of fronts on which the study of the interaction between plants and herbivores may be advanced. One of them is the ecological consequences of within-plant variation. Vascular plants are highly plastic, with a versatile metabolism and modular and unspecialized bodies, which give rise to large variations in the same tissues (i.e. leaves, flowers, seeds) within an individual plant (Herrera 2009, 2017, Karban et al. 2016). Within-plant heterogeneity also arises from genetic and epigenetic factors, which generate epigenetic mosaics among the same tissues (Herrera 2017, Alonso et al. 2018, Balao et al. 2018, Harder et al. 2019, Herrera et al. 2019). Like this, the same tissues can phenotypically vary within-plant in terms of morphology, structure, physiology, nutrient content and chemical defenses (Karban et al. 1997, Holeski 2007, Karban 2011, Holeski et al. 2012, Moore et al. 2013, Jakobs and Müller 2018, Chen and Giladi 2020, Gómez et al. 2020, Eisenring et al. 2021, Vescio et al. 2021). Within-plant variation enhances the exploitation of abiotically and biotically heterogeneous environment, and in turn modifies the outcome of plant interactions with their herbivores and mutualists (Herrera 2009, 2017, Siefert et al. 2015, Jakobs and Müller 2018, Wetzel and Meek 2019, Gómez et al. 2020). Regarding plant-herbivore interactions, plant plasticity would imply not only that herbivores select specific parts of the same tissues within-plant, but also that herbivores can affect plants at a subindividual level by differentially damaging within-plant parts (Jakobs et al. 2019, Wetzel and Meek 2019). In addition to the intragenerational consequences of subindividual herbivory for plants, herbivores could provoke plants to have a heterogeneous progeny. Heterogeneous progeny could derive from heritable within-individual effects such as differential epigenetic mosaics in seeds that could result in, for example, among-sibling variation in chemical defense (Moore et al. 2013, Herrera 2017, Harder et al. 2019). Among-sibling variation could also be the consequence of herbivore subindividual effects on seed provisioning, which is critical for seedling performance (Agrawal 2001, 2002).

Climate change on plant-herbivore interactions

Climate change is altering key agents of selection such as CO_2 concentrations, temperatures and precipitation patterns (Intergovernmental Panel on Climate Change (IPCC) 2014). These changes in the abiotic environment can influence trait expression in both plants and herbivores, and alter the long-standing interaction between them (Hamann et al. 2021). Plants, especially short-lived ones, can be highly sensitive to changing climatic conditions (Voigt et al. 2003, Morris et al. 2008, Jamieson et al. 2012, Siepielski et al. 2017). Regarding insects, warmer temperatures could accelerate their development, leading to earlier adult emergence, even to complete more generations per season (Jönsson et al. 2009, Altermatt 2010, Forrest 2016, Marshall et al. 2020, Rodrigues and Beldade 2020, Gutiérrez and Wilson 2021). This could lead to temporal and ecological mismatches between plants and herbivores. Nevertheless, how plant-herbivore interactions will respond to climate change still remains a great challenge (Tylianakis et al. 2008, Rasmann et al. 2014, Hamann et al. 2021). Warmer temperatures and increased CO_2 concentrations may induce greater food consumption by herbivores, and drought conditions can strengthen herbivore damage on plants (Zvereva and Kozlov 2012, Hamann et al. 2021). Less is known about how changes in precipitation regimes will influence plants, herbivores and their interaction. Changes in the timing and intensity of precipitations are expected to provoke shifts in the relationship between plants and the organisms with which they interact. Given that modifications in precipitation regimes due to climate change are predicted to be region specific, studies based on regional climate projections and expected seasonal changes can provide valuable insights into climate change effects on plant-herbivore interactions (Jamieson et al. 2012).

Objetivos del estudio

El objetivo de este estudio es determinar el efecto combinado de múltiples herbívoros sobre diferentes rasgos de las plantas, su capacidad de defensa y su éxito reproductivo usando como sistema modelo a *Moricandia moricandioides*. También estudiamos si el efecto de los herbívoros puede ser modulado por otros agentes bióticos y abióticos como los detritívoros y potenciales cambios en los patrones de precipitación.

Objetivos específicos de cada capítulo:

Capítulo 1

1) Discernir experimentalmente el efecto sobre el éxito reproductivo de la planta de los depredadores de semillas pre-dispersivos, la respuesta de la planta frente a este tipo de herbívoro y determinar si esta respuesta es modulada por los detritívoros.

Capítulo 2

2) Determinar si la respuesta a largo plazo (10 años) de 47 poblaciones de *M. moricandioides* a los depredadores de semillas pre-dispersivos depende tanto de la intensidad del daño como de la presencia de un herbívoro de gran tamaño, los ungulados.

Capítulo 3

3) Determinar los efectos transgeneracionales combinados de los depredadores de semillas pre-dispersivos y los ungulados sobre el reclutamiento de plántulas y su resistencia a la herbivoría.
4) Discernir si los potenciales efectos transgeneracionales de los depredadores de semillas pre-dispersivos varían del nivel de planta al nivel de dentro de la planta.

Capítulo 4

5) Analizar los efectos combinados de herbívoros que consumen partes aéreas (florícolas) y subterráneas (radicícolas) en el éxito reproductivo de la planta*,* así como si estos efectos son dependientes de la densidad de los herbívoros.

Capítulo 5

6) Estudiar los efectos dependientes de la densidad de herbívoros florícolas sobre el éxito reproductivo de la planta, y si la respuesta de la planta es modulada por detritívoros y/o la densidad de ambos interactuantes.

Capítulo 6

7) Determinar los efectos transgeneracionales dependientes de la densidad y del contexto biótico materno tanto de los detritívoros como de varios herbívoros (radicícolas y florícolas) sobre el destino de la F1 de la planta (tasa de emergencia, crecimiento y éxito reproductivo).

Capítulo 7

8) Finalmente, se pretende determinar si futuros escenarios de cambio climático van a afectar al éxito reproductivo de la planta y su interacción con herbívoros aéreos (florícolas) y subterráneos (radicícolas).

Aims of the study

The objective of this study is to determine the combined effect of multiple herbivores on different plant traits, plant defense capacity and plant fitness using *Moricandia moricandioides* as a model system. We also study whether the effect of herbivores can be modulated by biotic and abiotic agents such as detritivores and potential changes in precipitation patterns.

Specific objectives of each chapter:

Chapter 1

1) Experimentally discern pre-dispersal seed predator's effect on plant fitness, the response of the plant against this type of herbivore and determine if this response is modulated by detritivores.

Chapter 2

2) Determine if the long-term response (10 years) of 47 *M. moricandioides* populations to pre-dispersal seed predators depends both on the intensity of the damage and on the presence of a large herbivore, the ungulates.

Chapter 3

3) Determine the combined transgenerational effects of pre-dispersal seed predators and ungulates on seedling recruitment and resistance to herbivory.
4) Discern whether the potential transgenerational effects of pre-dispersal seed predators vary from the plant level to the within-plant level.

Chapter 4

5) Analyze the combined effects of herbivores that consume aboveground (floral herbivores) and belowground (root herbivores) parts on plant fitness, as well as discern whether these effects are dependent on the density of the herbivores.

Chapter 5

6) Study the density-dependent effects of floral herbivores on plant fitness, and if the plant response is modulated by detritivores and/or the density of both interacting organisms.

Chapter 6

7) Determine the density- and maternal biotic context-dependent transgenerational effects of both detritivores and various herbivores (root and floral herbivores) on the fate of F1 (emergence, growth and reproductive success).

Chapter 7

8) Finally, it is intended to determine if future climatic scenarios will affect plant fitness and its interaction with aboveground and belowground herbivores (floral and root herbivores).

General methodology

Study area: the semiarid Baza basin

All the field work associated with this thesis has been developed in Barranco del Espartal, a seasonal watercourse surrounded by steep hills located in the Baza basin (province of Granada, Andalusia, Fig. GM1). The climate at this area is strongly continental Mediterranean, with sharp temperature fluctuation across the year (ranging from -14ºC to up to 45ºC) and high seasonality (hot summers, cold winters). Due to isolation by a chain of mountains, precipitation in this harsh semiarid environment is low (rarely more than 300 mm), severely conditioning vegetation diversity and cover. The substrate is composed of silt with gypsum sediments, and the soil is characterized by a sandy-loam texture with low water retention capacity and high pH (Sánchez-Piñero 2007). The vegetation is an open shrub-steppe dominated by the perennials *Artemisia herba-alba* and *A. barrelieri* (Asteraceae), *Salsola oppositifolia* (Salsoloideae), *Stipa tenacissima* (Poaceae), *Retama sphaerocarpa* (Fabaceae), *Ononis tridentata L.* (Leguminosae) and *Lygeum spartum* (Poaceae) (Sánchez-Piñero 2007). Several perennial and short-lived herb species of the genera *Eruca*, *Lepidium*, *Sysimbrium*, *Mathiola*, and *Reseda* (Brassicales order, Brassicaceae and Resedaceae families) are also common, together with *M. moricandioides*. The anthropic influence in Barranco del Espartal includes the presence of roaming flocks of sheep in certain routes, as well as a gradual increase in the cultivation of cereals in the flat areas.

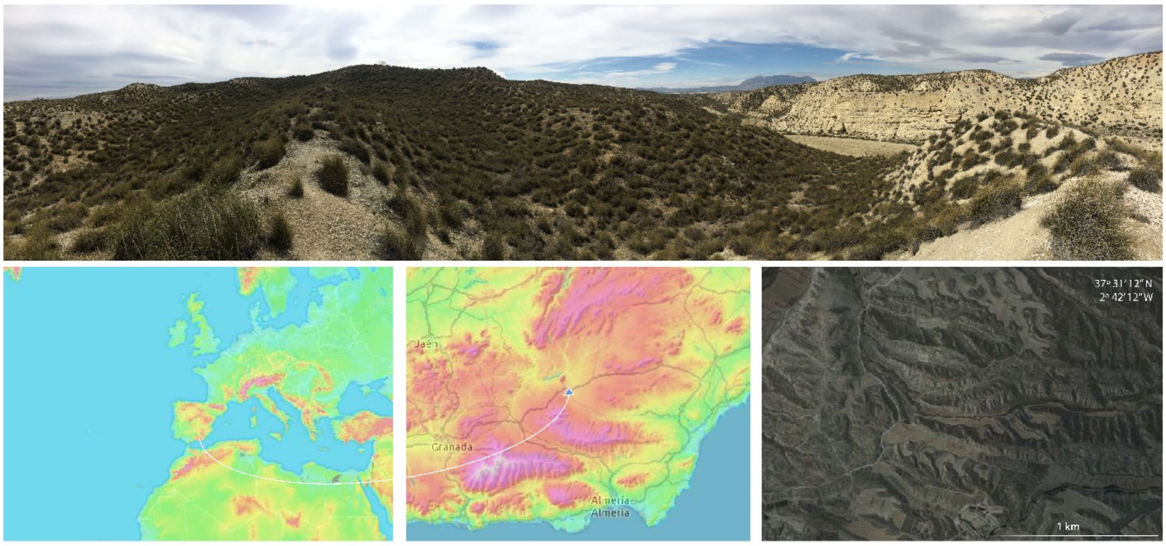


Figure GM1. The study area Barranco del Espartal in the Baza Basin (Granada, Andalusia; 37° 31′ 12″ N, 2° 42′ 11.99″ W).

As in our study area, a great part of Iberian Mediterranean basin is gypsic (Fig. GM2). Gypsum soils are characterized by gypsum contents over 15% and almost all are confined to arid and semiarid regions (Escudero et al. 2015). In such environment plants require a minimum adaptation to gypsum (the mineral rock calcium sulfate dihydrate), being either gypsophiles (plants specialized living on gypsum soils) or gypsovags (non-specialist plants that can live on gypsum soils). Gypsum does not significantly increase osmotic potential as saline soils do, but it increases the physical soil thickness and modulates the availability of water and nutrients (Herrero et al. 2009, Escudero et al. 2015). A limiting factor in these soils are nitrogen, phosphate and potassium deficiencies, while there is an excess of sulphur, calcium and magnesium (Herrero et al. 2009). Plant adaptations in these environments, which might be part of an adaptive syndrome of stress-tolerant species, include the ability to accumulate elements found in excess (the appearance of organic sulphur secondary metabolites such as glucosinolates could be originally a preadaptation to living on gypsum) together with macronutrients such as nitrogen and phosphate which are scarce in these soils, a mucilaginous seed coat, the physical ability to surpass the hard crust typically formed on the surface of gypsum soils, and a delayed reproductive phenological peak (Escudero et al. 2015). Most gypsum plants also maintain persistent soil seed banks, which are dense in the vicinity of mother plants due to the short-distance seed dispersal typically found in these species (Escudero et al. 2015).

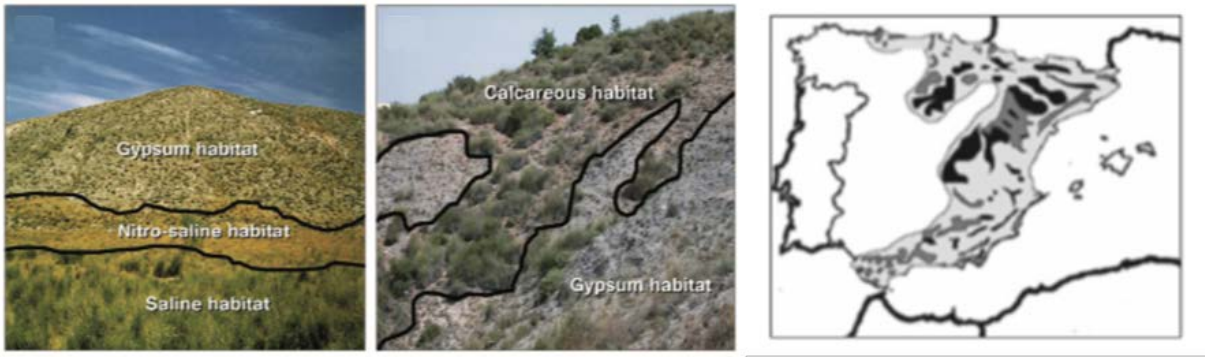


Figure GM2. On the left, example of a typical gypsum hill habitat. On the middle, example of the differential plant composition and cover between calcareous and gypsum soils. On the right, gypsum outcrops (in grey) and genuinely gypsum habitats (in black) in the Iberian Peninsula. All figures extracted from Escudero et al. (2015).

Study system: *Moricandia moricandioides* and its associated fauna

The Brassicaceae family, particularly the Brassiceae tribe, includes many economically relevant species broadly used as vegetables, edible oils, crop forages, condiments and fuel crops, having been the focus of a vast amount of genetic, agronomic, and ecological research (Gupta 2009, Schmidt and Bancroft 2011, Perfectti et al. 2017). The Brassiceae genus *Moricandia* represents a diverse group distributed across North Africa, Mediterranean basin, West Asia and South Asia (Tahir and Watts 2011, Perfectti et al. 2017). Our study plant *Moricandia moricandioides* (Boiss.) Heywood (Brassicaceae) is a herb inhabiting semiarid areas in the Iberian Peninsula (Sobrino Vesperinas 1993; Fig. GM3). This species normally lives at altitudes between 300 and 700 m a.s.l. and grow in patchy populations, with an individual size no greater than 80 cm. It is adapted to gypsum soils (classified as gypsovag by Escudero et al. 2015), where it predominates over the widely distributed *M. arvensis*, a ruderal species dominant in cultivated areas, roadsides and other human disturbed habitats. *Moricandia moricandioides* is diploid, with C3 photosynthetic metabolism, and has inferior growth rates although thicker leaves with more chlorophyll and slightly greater rates of photosynthesis per unit leaf area than several cultivated *Brassica* species (McVetty et al. 1989). The *baetica* subspecies used in this study is present in the southern provinces of Cadiz, Jaen, Malaga and Granada (Andalusia).

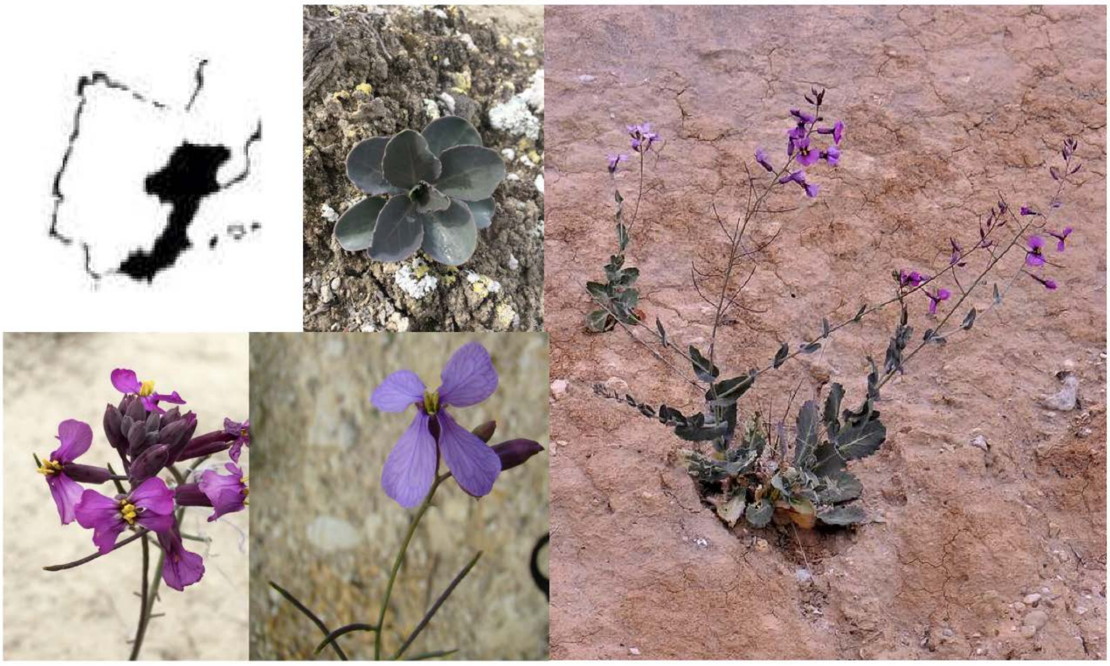


Figure GM3. Photos and distribution of *Moricandia moricandioides* in the Iberian Peninsula.

This herb grows as a vegetative rosette during winter and produces reproductive stalks during spring. The stalks are photosynthetically active during the entire season (González-Megías and Müller 2010). The inflorescences are racemes of commonly more than 20 flowers, which develop and open sequentially (Sobrino Vesperinas 1993, Gómez 1996, Torices et al. 2018). The flowering period is usually short and synchronic within populations due to the stochasticity of the environments in which this species live; occurs mostly in April and May, and last for 3-4 weeks. Flowers are hermaphrodite, with the stigma receptive for 3 to 5 days, and the mating is highly self-incompatible (what prevents defense costs derived from inbreeding depression; Núñez-Farfán et al. 2007, Bello-Bedoy et al. 2011, Campbell et al. 2013, Carr and Eubanks 2014, Johnson et al. 2015, Schrieber et al. 2019). Fruit production begins in the first few days of flowering. Fruits are dehiscent two-valve siliques with one seed series per valve, and usually 20-60 seeds develop in each fruit. The species is predominantly semelparous, since the vast majority of individuals die in August at the time of seed dispersal.

A characteristic feature of the Brassicaceae family is the production of specific secondary metabolites, the so-called glucosinolates (Fahey et al. 2001). Glucosinolates are non-volatile nitrogen- and sulphur-containing defensive metabolites, derived from amino acid biosynthesis (Halkier and Gershenzon 2006, Wink 2008, Mithöfer and Boland 2012, Burow and Halkier 2017). More than 140 aliphatic, indolic or aromatic compounds of this broad group of defenses have been identified, which differ among species and even among tissues of the same plant (Kliebenstein et al. 2001, Brown et al. 2003, Mithöfer and Boland 2012, Burow and Halkier 2017). The breakdown products resulting from glucosinolate hydrolysis represent the active toxic, repellent and/or antinutritive defensive components. The hydrolysis occurs when the compartmentalized glucosinolates and myrosinases come into contact upon tissue disruption (that is, upon herbivory; Figure GM4). Glucosinolates are present all over plant organs such as roots, stems, leaves, flowers and seeds. Glucosinolate profile and concentrations can vary within plant ontogeny and due to the intensity and duration of environmental conditions and biotic interactions such as herbivory, pathogen infection, competition, nutrient availability (most importantly nitrogen and sulphur), temperature and precipitation (Van Dam and Baldwin 1998, Heil and Baldwin 2002, Brown et al. 2003, Kliebenstein et al. 2005, Lankau and Strauss 2008, Ahuja et al. 2009, Jahangir et al. 2009, Moore et al. 2013, Metz et al. 2014, Burow and Halkier 2017). Glucosinolate transport across different tissues via the xylem and phloem is thought to be constant (Burow and Halkier 2017). Indeed, seeds are the organs that present higher concentrations of glucosinolates but lack *de novo* biosynthesis, so they depend on glucosinolate allocation from other tissues. As a wild Brassicaceae, *M. moricandioides* has lower amino acid content and higher glucosinolate concentrations that domesticated *Brassica* crops. Eleven glucosinolate compounds have been identified for our study species (González-Megías and Müller 2010, Chapters 1, 3, 4, 5, 6 and 7). Leaves have several aliphatic and indolic compounds that can be found at relatively high concentrations, while in seeds a single aliphatic compound is responsible almost entirely for the total concentration, 3-OH-butenyl (Fig. GM4).

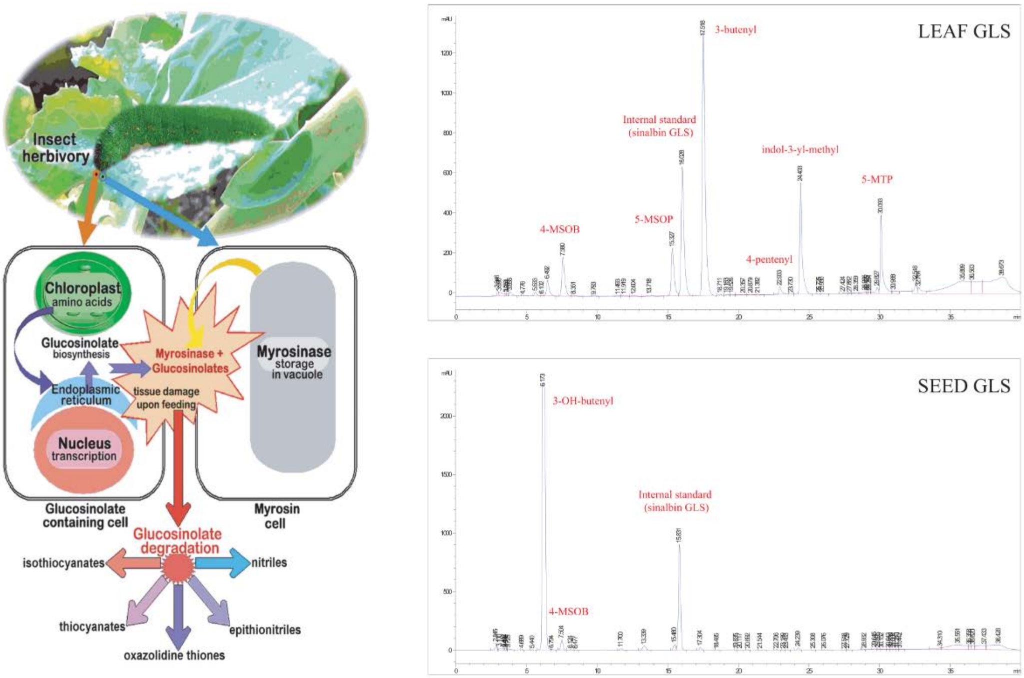


Figure GM4. On the left, insect herbivory brings glucosinolates and myrosinase together and facilitates the hydrolysis of glucosinolates. Extracted from Ahuja et al. (2009). On the right, examples of leaf and seed glucosinolate (GLS) profiles in *Moricandia moricandioides* obtained by high-performance liquid chromatography (HPLC).

The glucosinolate-myrosinase defense system is a highly dynamic constitutive as well as inducible type of defense, and plays an important role in plant-herbivore (mostly insect) interactions (Müller and Sieling 2006, Travers-Martin and Müller 2008, Ahuja et al. 2009, Agrawal and Weber 2015, Wagner and Mitchell-Olds 2018). Glucosinolates and their hydrolysis products negatively affect a wide range of herbivores such nematodes, mollusks and insects, and can also be detrimental for mammals and birds (Hopkins et al. 2009, Textor and Gershenzon 2009, Machado et al. 2016). In the case of insects, glucosinolates may stimulate oviposition and feeding by specialist herbivore species, which have developed mechanisms to detoxify, sequester, excrete, or selectively bind these defensive compounds (Opitz and Müller 2009, Winde and Wittstock 2011). Nevertheless, and although to a lesser extent than for generalists, glucosinolate ingestion may have detrimental consequences for the survival, growth, and fecundity of many of these specialist species (Ali and Agrawal 2012, Moore et al. 2013, Kant et al. 2015). The advantage that glucosinolates provide as defense against antagonists is counterbalanced by the metabolic costs associated with their high requirement for photosynthates (Bekaert et al. 2012). Nevertheless, under certain circumstances plants may entirely switch off the synthesis of glucosinolates, or even up-regulate resource acquisition via increased photosynthetic capacity or nutrient uptake to minimize potential trade-offs between glucosinolate synthesis and other plant functions (Moore et al. 2013)

Moricandia moricandioides interacts with a wide range of animal species in the study area. As for herbivores, it is remarkable the strong grazing pressure exerted by ungulates such as domestic sheep, which intensely condition the plant population dynamics in their areas of passage (Gómez 1996). These ungulates could even cancel the potential selection mediated by other herbivores such as insects. To our knowledge, the rest of most important herbivores for its potential impact on the plant's fitness are the following insect species: the pre-dispersal seed predator *Crossobela (Mesolephs) trinotella* (Lepidoptera, Gelechiidae), the floral herbivores *Euchloe crameri* and *Pontia daplidice* (Lepidoptera, Pieridae) and the root herbivore *Cebrio gypsicola* (Coleoptera, Cebrionidae). We have worked with all these herbivore guilds throughout this study, as well as with the detritivore *Tentyria incerta* (Coleoptera, Tenebrionidae; Fig. GM5). *Tentyria incerta* and other tenebrionid species such as *Morica hybrida* and *Alphasida clementei* play a determinant bottom-up role as is litter decomposition in semiarid environments such as ours (Doblas-Miranda et al. 2007, 2009b, 2009a, 2009c, 2012, González-Megías et al. 2011). The plant is thought to be highly plastic in its response to these herbivores. Root and floral herbivores, as well as detritivores, modulate the defensive response of the plant (González-Megías and Müller 2010, González-Megías and Menéndez 2012), and their impact on the plant can even transcend intragenerational effects, affecting the performance of offspring through transgenerational effects (González-Megías 2016). The underlying adaptive mechanisms towards glucosinolates of pre-dispersal seed predator and floral herbivore species, all lepidopterans and Brassicaceae specialists, are unknown. In the case of the pierid species, their diversification indeed occurred through adaptation to glucosinolate metabolization (Wheat et al. 2007, Winde and Wittstock 2011, Edger et al. 2015). Less is known yet about the possible adaptions of cebrionid root herbivores towards glucosinolates. As far as we know, none of these herbivores is a plant reprogrammer, in the sense of manipulating their host via changes in resource allocation or downregulation/upregulation of plant defenses (Kyndt et al. 2012, Savchenko et al. 2013, Giron et al. 2016, Guiguet et al. 2016, Zhang et al. 2017, Arena et al. 2018, Oates et al. 2021). On the contrary, less is known about the plant's tolerance mechanisms towards herbivores.

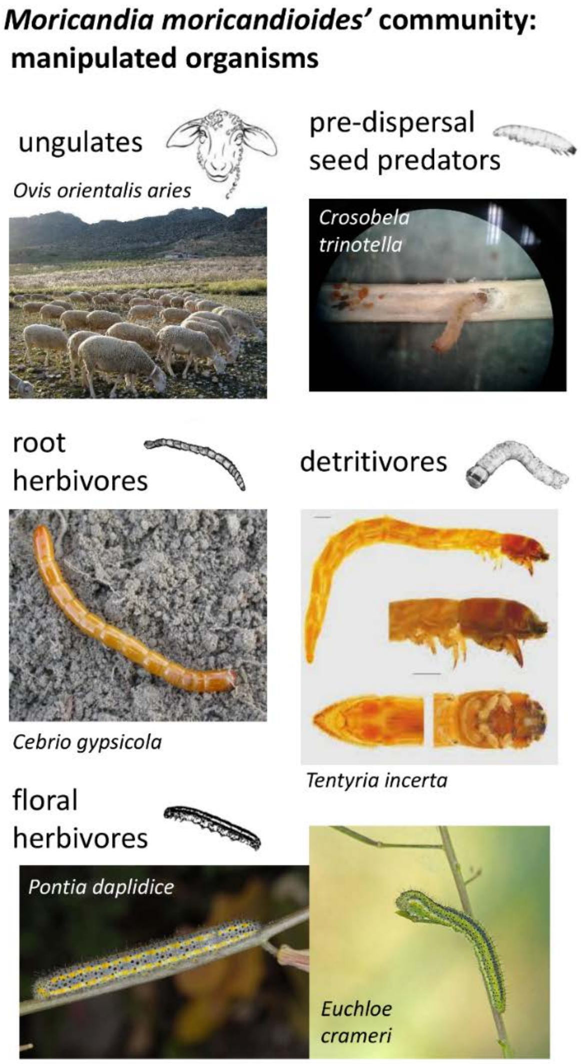


Figure GM5. Fauna associated to *Moricandia moricandioides* manipulated in this study.

It is worth mentioning that among the main herbivores, folivore and sucking insect guilds do not stand out. These herbivore guilds are the main herbivores in most cultivated herbs, being actual research on plant-herbivorous insect interactions somewhat biased towards them. Important *Brassica* crop pests such as the diamondback moth *Plutella xylostella* (Lepidoptera, Plutellidae), the small cabbage white butterfly *Pieris rapae* (Lepidoptera, Pieridae), the flea beetles *Phyllotreta* spp. (Coleoptera, Chrysomelidae) and the mustard and cabbage aphids *Lypaphis erysimi* and *Brevicoryne brassicae* (Hemiptera, Aphididae) are among *M. moricandioides'* herbivores, but they are not the most determinant (Fig. GM6). All these species are Brassicaceae specialists, therefore adapted to deal with glucosinolates

(Bridges et al. 2002, Ratzka et al. 2002, Wittstock et al. 2004, Kazana et al. 2007, Stauber et al. 2012, Beran et al. 2014, Rahfeld et al. 2014). A wide range of insect species complete the *M. moricandioides'* herbivore community: leaf mining dipterans, chewing species such as grasshoppers, *Galeruca angosta* (Coleoptera, Chrysomelidae), *Mylabris quadripunctata* and *M. hieracii* (Coleoptera, Meloidae), *Hellura* spp. (Lepidoptera, Cambridae) and *Tenthredo sebastiani* (Hymenoptera, Tenthredinidae), and the suckers *Agalmatium bilobum* (Hemiptera, Issidae), *Myzus persicae* (Hemiptera, Aphididae), *Aphis fabae* (Hemiptera, Aphididae) and several mirid, pentatomid and cicadellid species (Fig. GM6). Pathogen infestation in *M. moricandioides* is rare at the study area, and only occasionally fungal colonization in senescent leaves has been observed (personal observation). As for mutualists, the nearly zygomorphic flowers with dark pink-purple petals of this herb are commonly visited by an assembly of insects, although mostly composed of long-tongued Antophoridae bees, (Gómez 1996, Gómez et al. 2016, Torices et al. 2018; Fig GM6). With regard to the third trophic level, the parasitoid species *Cotesia kazak* (Hymenoptera, Braconidae), koinobiont endoparasitoid of the floral herbivore pierid caterpillars, is also a non-negligible top-down agent in the system due to its high abundance (Fig. GM6).

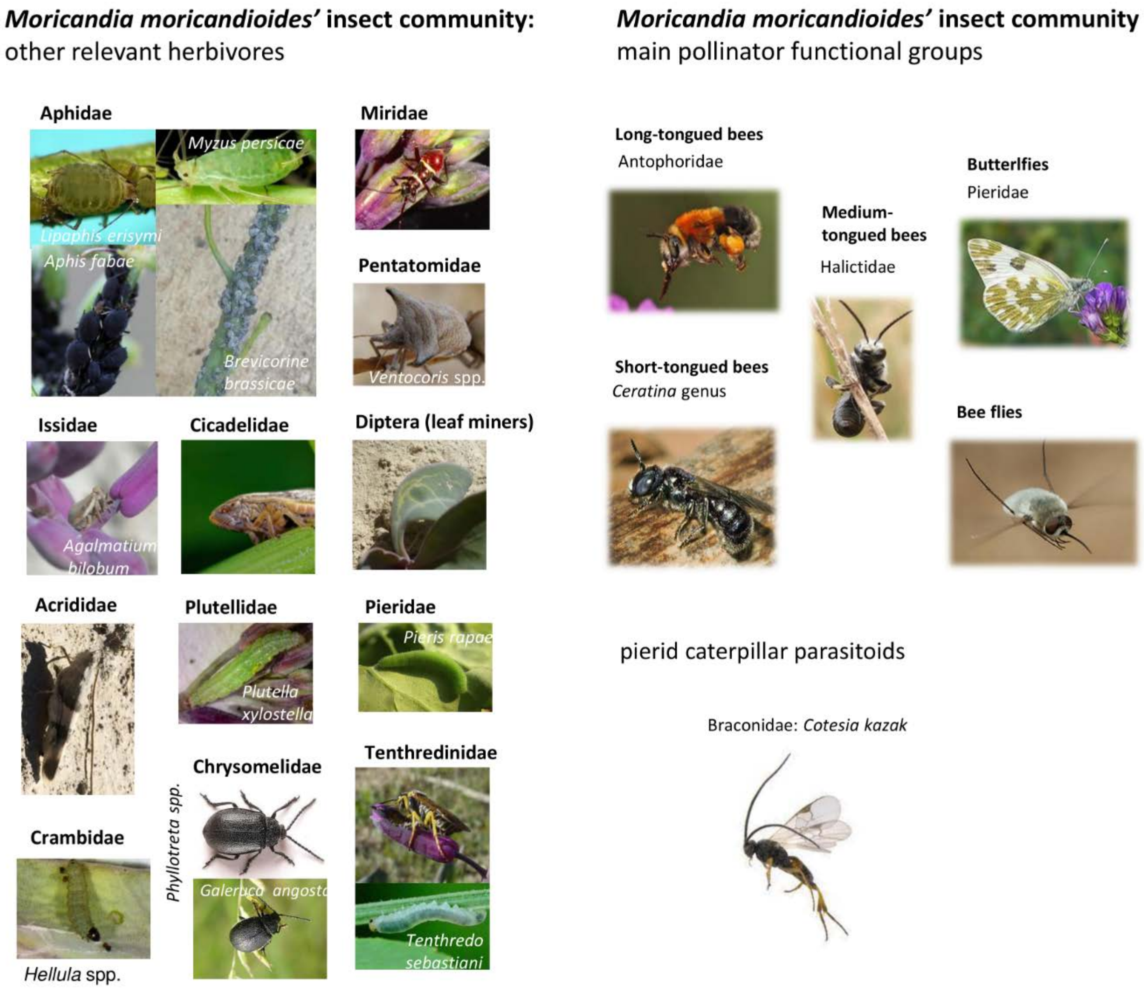


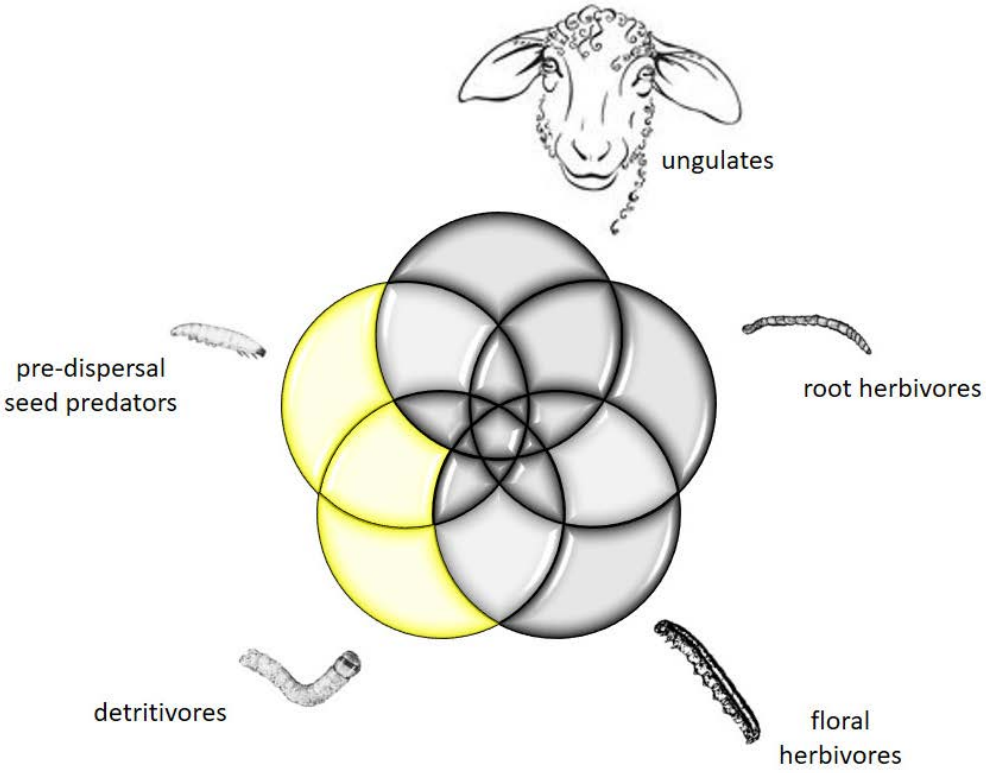
Figure GM6. On the left, a detailed list of *Moricandia moricandioides'* herbivore species not manipulated in this study. On the right, mutualists associated with *M. moricandioides*: the main pollinator functional groups and the main pierid caterpillar parasitoid.

General experimental design

Getting as close as possible to ecological reality has been the cornerstone in the experimental development of this study. Our approach has been always experimental rather than observational, so that the strength and fitness impact of the interactions under study could be more accurately quantified. In all experiments we have tested elaborate hypotheses based on the implementation of two realistic treatments in a full factorial design to determine the possible interactions. In these experiments we have always quantified the total number of seeds per individual plant as fitness measure. In addition, we measured defensive traits such as glucosinolate concentrations, and other traits that could indicate that a tolerance response had been given (e.g. plant height, aboveground biomass, reproductive stalks and flowers, fruit set, seed set), so that plant fitness could be quantified as a function of defensive traits (Züst and Agrawal 2017, Erb 2018). In each case we have worked with robust sample sizes, as large as our logistics allowed. In addition, we have always worked in the natural environment (except for seedling emergence tests) of the wild species *M. moricandioides* and the associated entomofauna, so that the fitness consequences of these interactions and subsequent defense phenotypes could be optimally evaluated. Finally, it should be noted that we have tried not to completely overlook certain marginally significant effects, because when dealing with field trials, with all the noise that this implies, such effects without robust statistical significance can undoubtedly have some biological relevance (Harvey et al. 2015, Amrhein et al. 2017).

Chapter 1

Pre-dispersal seed predators boost seed production in a short-lived plant



1.1 Abstract

Pre-dispersal seed predation diminishes fitness and population growth rate of many plant species. Therefore, plants have developed multiple strategies to reduce the harmful effects of this type of herbivory. The present study aims to determine the effect of pre-dispersal seed predators (PSPs) on the fitness of a short-lived herb, and to discern the mechanisms allowing the plants to reduce the impact of pre-dispersal seed predation. Knowing that the interplay between pre-dispersal seed predators and plants is strongly shaped by the presence of other co-occurring organisms, we tested whether detritivores modulate plant responses towards pre-dispersal seed predators. To do so, we experimentally manipulated in the field pre-dispersal seed predators and detritivores interacting with the short-lived herb *Moricandia moricandioides*. We found that detritivores did not alter the response of plants to PSPs. Strikingly, the plant overcompensated for pre-dispersal seed predation, almost doubling the number of seeds produced. Plant response to PSPs led to substantial changes in shoot architecture, reproductive traits, chemical defenses in leaves and seeds and in seed nutrient content. The overcompensating mechanism seems to be meristem activation, which allowed plants to produce more reproductive tissue, and increasing the proportion of ovules that became seeds, a response which specifically compensates for pre-dispersal seed predation. As far as we know, this is the first experimental evidence of a positive effect of PSPs on plant lifetime fitness as a consequence of plant overcompensation.

Keywords

Brassicaceae · Insect herbivory · Overcompensation ·
Plant resistance · Plant tolerance · Seed predation

1.2 Introduction

Pre-dispersal seed predators influence the abundance and distribution of many plant species by consuming seeds and affecting germination and seedling survival (Janzen 1971, Moles et al. 2003, Schelin et al. 2004, Kolb et al. 2007). The impacts of pre-dispersal seed predators on plant fitness may range from negligible to highly severe, with consumption of the entire seed yield (see reviews Preisser and Bastow 2005, Kolb et al. 2007). However, there are also some examples suggesting a positive relationship between pre-dispersal seed predator (PSP) attack rates and plant seed production (Lortie and Aarssen 2000, Gagic et al. 2016). The impact of PSPs on plant fitness is a consequence of the interplay between the capacity of plants to defend from the attack and the capacity of the PSPs to circumvent plant defenses (Ramírez and Traveset 2010).

Plants respond to PSPs using different tolerant strategies that enable them to reduce the detrimental effect on fitness (Pilson 2000, Herrera et al. 2002, Ehrlén 2003, Sakai and Harada 2007). Plants could produce a surplus of flowers, fruits or seeds to compensate for a future random or selective abortion of some of them (Ehrlén 1993, Östergård et al. 2007, Sakai and Harada 2007, Ghazoul and Satake 2009, Meyer et al. 2014). Plants could also increase the number of ovules per fruit to compensate for future potential losses (Sakai and Harada 2007), or even reduce seed quality by decreasing nitrogen content (Herrera et al. 2002, Östergård et al. 2007). Alternatively, plants could invest in resistance strategies against PSPs such as the investment in deterrent chemicals in seeds (Herrera et al. 2002, Muñoz et al. 2014). How plants respond to PSPs can also be related to the cues used by PSPs to select individual plants. To maximize their success some PSP species select plants according to flowering time, flower number, plant size, the color or odor, or a combination of several of these stimuli, with generally a strong preference for early flowering plants and/or plants with a large flower display (Herrera et al. 2002, Elzinga et al. 2007, Kolb et al. 2007, Östergård et al. 2007).

Curiously, strategies proposed for plants to cope with PSPs have mainly been explored in long-lived plants, but less in short-lived ones, which cannot defend themselves against PSPs with inter-annual strategies such as seed masting (Kolb et al. 2007). However, studies with short-lived plants can give a more realistic picture of the consequences of PSP attack for plants due to the possibility to quantify lifetime seed production, and hence better estimate the interaction strength (Louda and Potvin 1995, Ehrlén 2002, 2003). Furthermore, there are some more fronts on which the study of the interaction between plants and PSPs may be advanced, since most studies to date have been fundamentally observational, and very few studies have addressed more than one plant strategy for dealing with PSPs (Elzinga et al. 2007, Kolb et al. 2007). Consequently, there is a need for experimental studies not only to discern potential causal effects of PSPs on plant performance and fitness but also to focus on the mechanisms involved (DeSoto et al. 2016).

PSP effects on plants can also depend on the presence of various other organisms interacting with the plant (Strauss and Irwin 2004). Other herbivores could affect pre-dispersal seed predation by altering flowering time, floral display size or plant and fruit chemistry (Strauss and Irwin 2004). Pre-dispersal seed predation also depends on the impact of PSP predators, parasitoids, and other natural enemies (Preisser and Bastow 2005, von Zeipel et al. 2006). Several mutualistic endophytes have been also shown to diminish pre-dispersal seed predation (Saari et al. 2010, Zhang et al. 2012). Interestingly, some belowground organisms, such as detritivores, can also diminish PSP attack rates on plants (González-Megías and Müller 2010, González-Megías and Menéndez 2012). Detritivore activity enhances microbial turnover, nutrient recycling and the breakdown of organic matter, favoring plants in many ways (De Deyn and Van der Putten 2005). These organisms frequently increase tissue quality and plant performance, and even in some cases enhance plant fitness (Bonkowski et al. 2001, Haase 2001, Poveda et al. 2005, Laossi et al. 2009, González-Megías 2016). Therefore, a detritivore-mediated increase in plant resources may enhance plant attractiveness to PSPs if it results in, for example, greater flower production. On the contrary, this same detritivore-mediated extra provision of resources may allow plants to reduce plant attractiveness to PSPs through altering chemical defenses and/or diminish PSP impact on plants through the investment in tolerance mechanisms (Bardgett and Wardle 2003, Schröter et al. 2004, Poveda et al. 2005, González-Megías and Müller 2010).

The present study aims to experimentally explore the effect of PSPs on the lifetime fitness of a short-lived plant. Specifically, we aimed to discern the strategies used by plants to reduce PSP impact on fitness and the mechanism involved. For this purpose, we used the predominantly semelparous herb *Moricandia moricandioides* (Boiss.) Heywood (Brassicaceae) as a model system. As we knew that detritivores diminish PSP attack in this system (González-Megías and Müller 2010, González-Megías and Menéndez 2012), we tested if detritivores can also shape plant responses towards PSPs. We manipulated the presence of PSPs and detritivores under field conditions and analyzed several plant traits related to growth, phenology, reproduction and tissue quality. We hypothesized that: (i) pre-dispersal seed predation reduces the reproductive success of *M. moricandioides*, (ii) detritivores diminish the negative impact of PSPs on the plant by increasing the capacity to tolerate and/or resist PSP damage, (iii) plants may produce an excess of flowers/seeds to increase fruit and/or seed abortion, increase the number of ovules per fruit, decrease seed quality and/or increase chemical defenses in seeds to cope with PSPs, and (iv) taller early flowering plants or taller plants with more flowers are more susceptible to attack by PSPs (see detailed hypotheses in Table 1.1).

Hypotheses (H) tested	Expected results
Effect on plant reproductive traits	
H1: PSPs reduce the reproductive success of the plant	Plants not attacked by PSPs (PSP− bagged and PSP− unbagged plants) will have higher seed yield than attacked plants (PSP+)
H2: Detritivores diminish the negative impact of PSPs on the plant, via promoting tolerance and/or resistance	The subset of plants attacked by PSPs with detritivores (D+ PSP+) will have higher seed yield than plants without detritivores (D−PSP+)
Plant tolerance/resistance mechanisms towards PSPs	
H3: Plants produce a surplus of flowers or fruits to compensate for a future random or selective abortion of some of them	Plants attacked by PSPs (PSP+) will produce more flowers and have lower fruit set than plants without PSPs (PSP− bagged and PSP− unbagged plants)
H4: Plants produce a surplus of seeds to compensate for increased seed abortion	Plants attacked by PSPs (PSP+) will have lower seed set than plants without PSPs (PSP− bagged and PSP− unbagged plants)
H5: Plants produce more ovules per fruit to compensate for future potential losses	Plants attacked by PSPs (PSP+) will produce more ovules than plants without PSPs (PSP− bagged and PSP− unbagged plants)
H6: Plants reduce seed quality by decreasing nitrogen content	Plants attacked by PSPs (PSP+) will have higher C/N ratio in seeds than plants without PSPs (PSP− bagged and PSP− unbagged plants)
H7: Plants invest in defensive chemicals in seeds	Plants attacked by PSPs (PSP+) will have a different glucosinolate profile than plants without PSPs (PSP− bagged and PSP− unbagged plants)
Plant trait selection by PSPs	
H8: PSPs will select early flowering plants, which are generally taller	Plants attacked by PSPs (PSP+) will start flowering earlier and will be taller than those not selected by the PSPs (PSP− unbagged plants). No differences will be found between PSP+ and PSP− bagged treatments
H9: PSPs will select taller plants with more flowers	Plants attacked by PSPs (PSP+) will be taller with more flowers than those not selected by the PSPs (PSP− unbagged plants). No differences will be found between PSP+ and PSP− bagged plants

Table 1.1. Hypotheses tested and the expected results of the study regarding: the effect of pre-dispersal seed predators (PSPs) and detritivores (D) on *M. moricandioides'* reproductive traits, the potential tolerance and resistance mechanisms used by the plant in response to PSPs, and the potential plant traits selected by PSPs. PSP+ = plants with PSPs, PSP− = plants without PSPs; PSP− unbagged = unbagged plants not selected by the PSPs, PSP− bagged = bagged plants to elude the attack of PSPs.

1.3 Materials and methods

Study system

The study was conducted in 2015 at Barranco del Espartal, a seasonal watercourse located in the semiarid Guadix-Baza Basin (Granada Province, south-eastern Spain). The climate at the study area is continental Mediterranean with strong temperature fluctuations (ranging from − 14 °C to up to 45 °C) and high seasonality (hot summers, cold winters). Annual precipitation rarely exceeds 300 mm.

The short-lived Brassicaceae species *M. moricandioides* is highly abundant in monospecific stands in this habitat and no litter accumulates underneath the plants. The vast majority of individuals live and reproduce within a single year. This species germinates in autumn, grows as a vegetative rosette during winter, and produces reproductive stalks during spring. The stalks remain photosynthetically active during the entire season (González-Megías and Müller 2010).

Flowers are hermaphrodites and the mating is highly self-incompatible. Pollination primarily occurs not only via highly efficient solitary bees, but also by bee flies and pierid butterflies (Gómez 1996). The plants produce glucosinolates, which are the characteristic defense compounds occurring in the order Brassicales (Fahey et al. 2001).

Crossobela trinotella Herrich-Schäffer (Lepidoptera, Gelechiidae) is usually the main PSP on *M. moricandioides* in the study area (Chapter 3). The nocturnal adults of this Brassicaceae specialist species emerge in spring and oviposit on the flowers or early fruits of the host plant (Li and Sattler 2012). Caterpillars hatch in a few days' time and develop inside the fruits, feeding on seeds. There can be more than one caterpillar per fruit, each of them eating 8–12 seeds (*M. moricandioides* fruits have usually 20–60 seeds), which can be counted since the PSPs consume the embryo and leave coat remains.

Belowground organisms such as *Morica hybrida* Charpentier, *Tentyria incerta* Solier and *Alphasida clementei* Pérez (all of them Coleoptera, Tenebrionidae) are among the most abundant detritivores in the study area and encompass ~ 32% of belowground macroinvertebrate biomass (Doblas-Miranda et al. 2007).

Experimental set-up

During the autumn of 2014, seeds of *M. moricandioides* collected in the study area were germinated in pots and grown in a common garden. Plants were moved to the field in mid-March (10 and 11-March-2015). In the field, plants were re-potted using mixed soil (free of macroarthropods) from the study site. The pots consisted of fiberglass-mesh cylinders (15 Ø × 20 width cm) of 1 mm mesh size to inhibit the entrance or escape of belowground macroinvertebrates. These pots were then buried with the upper surface level with the ground. Plants were set up in 8 blocks with 18 plants per block. Within each block, plants were located in three lines (6 × 3 plants per block), and at least 30 cm apart from each other (144 plants in total).

The experiment consisted of a full factorial design with two factors. One factor was the presence (D +) and absence (D−) of a detritivore in the plant rhizosphere, and the second factor was the presence (PSP +) and absence (PSP−) of PSPs in the fruits. For the detritivore factor, a single larva of *T. incerta* collected in the study area was added to the enclosure soil of D + treatment plants two weeks after the plants were moved to the field. On average, a single tenebrionid larva per *M. moricandioides* plant is the most realistic density in the study area (Doblas-Miranda et al. 2007). The addition of tenebrionid detritivore larvae in fiberglass-mesh pots has been satisfactorily used before in this system (González-Megías and Müller 2010, González-Megías and Menéndez 2012).

The PSP treatment was not an addition but an exclusion. Reproductive tissues were bagged with 500 µm mesh size rearing bags in the case of PSP-plants to impede oviposition by PSPs. These tissues were bagged when the first floral bud was produced. Once the flowers started to produce fruits, we used larger bags to avoid any potential negative effect of constriction of the fruits on their development. The bags were removed as soon as the fruits were mature enough. We decided to use this method to avoid the use of larger bags that could potentially break the stalks on windy days because of their weight. When plants began to flower, rearing bags were opened every other day (at midday, for several hours) to allow pollination. Opening the bags every two days was sufficient to ensure pollination since the stigma remained receptive for 3 to 5 days. Additionally, when pollinators were scarce due to weather conditions, all open flowers from bagged and unbagged plants were hand-pollinated with pollen from at least two natural *M. moricandioides* individuals (in only 3 of the 36 times when bags were opened to allow pollination, hand pollination was required). We actively removed florivore eggs and caterpillars by hand every other day from all experimental plants to avoid florivory-derived effects. The removal of florivore caterpillars has been successfully tested before in this system (see González-Megías and Müller 2010, González-Megías 2016 for details). The rest of free-living herbivores were not manipulated, but the abundance of all herbivore species and guilds was quantified (Suppl. 1.1, Table 1.S1, Figs. 1.S1-1.S2).

To test for the potential effect of bags on plant reproductive traits we set up a prior experiment in spring 2014. For this experiment, we randomly selected plants growing naturally in the field, without controlling for plant age, microhabitat or previous herbivory. We found that PSP presence but not the bagging significantly affected plant reproductive traits, indicating that the methodology used in this study is suitable. This experiment is described in detail in Suppl. 1.2 (including methods, statistical analyses, and results; Suppl. 1.2, Tables 1.S2-1.S3 and Figure 1.S3).

The experimental design was unbalanced, with twice as many unbagged plants as bagged ones, in expectation that not all unbagged plants would have PSPs (24 D−PSP−, 24 D+PSP−, 48 D−PSP+ and 48 D+PSP+plants). Each block had randomly assigned 3 D−PSP−, 3 D+PSP−, 6 D−PSP+ and 6 D+PSP+ plants. None of the plants had a reproductive stem when moved to the field. During the first week in the field, and in the absence of rain, all plants were watered and net-covered to ensure their establishment.

We excluded from the analyses plants that did not produce either flowers or fruits, as well as the three bagged plants that had PSPs. From the remaining plants, one-third of the unbagged plants (25) were not attacked by PSPs and were classified as PSP− unbagged plants. Incorporating this last set of plants (unbagged plants without PSPs) as a treatment in the analyses allowed us to identify plant traits influenced by PSP predation (bagged PSP− plants vs. unbagged PSP+ plants; Hypothesis 1, Table 1.1) and those plant traits selected for by the PSPs (unbagged PSP− plants vs. unbagged PSP+ plants; Hypotheses 8 and 9, Table 1.1). The final sample size for each treatment was D−PSP− bagged plants n = 14, D+PSP− bagged plants n = 17, D−PSP− unbagged plants n = 12, D+PSP− unbagged plants n = 13, D−PSP+ unbagged plants n = 25 and D+PSP+ unbagged plants n = 25.

Data collection from the experiment

We recorded flowering onset and end of flowering for each individual plant during the experiment (flowering occurred between 03-April and 26-June). At the end of the experiment (01-July, 114 days after the plants were moved to the field), we measured plant height, counted the number of reproductive stalks and the total number of flowers and fruits produced by each plant. Fruit set was calculated as the proportion of flowers that became fruits.

Fruits were collected after complete maturation of seeds but before seed dispersal. All fruits were brought to the laboratory and checked for PSPs. Fruit attack rate was calculated as the proportion of fruits with PSPs. The numbers of ovules, predated seeds, aborted seeds, and intact healthy seeds were counted. We calculated seed set as the proportion of ovules per plant that produced seeds, including both predated and intact healthy seeds. We also calculated the number of seeds produced by the plant by summing the number of intact healthy seeds in each fruit in the plant (number of seeds hereafter).

Leaves were harvested and dried at 40 °C for 48 h to determine C and N content in leaf tissue using a CHN Elemental Analyser (CIC, University of Granada, Granada, Spain). C and N content was also determined in seeds. To quantify glucosinolate (GLS) concentrations in leaves, the youngest leaf of one stem of each of the experimental plants was collected before leaf senescence. Leaves were immediately freeze-dried, and the dried material was ground and extracted three times in 80% methanol after the addition of *p*-hydroxybenzyl GLS (sinalbin) used as an internal standard. GLS extraction and conversion to desulfoGLSs was done following previously established methodology using high-performance liquid chromatography (HPLC; Agilent Technologies, Santa Clara, California, USA) (González-Megías and Müller 2010, Chapter 3). DesulfoGLSs were identified by comparison of UV-spectra and retention times to those identified in earlier studies (González-Megías and Müller 2010, Chapters 3 and 7). GLS concentrations in seeds were also quantified, using a mix of seeds from different fruits of each plant, following the same methodology.

Statistical analyses

We analyzed whether detritivores affected the probability of PSP attack (proportion of plants with PSPs) and fruit attack rate (proportion of fruits with PSPs) on unbagged plants with a linear model, with detritivore presence/absence as predictor (D− n = 37, D+ n = 38).

We performed general and generalized linear mixed models (GLMMs) to test the effects of each factor, detritivores (with two levels) and PSPs (with three levels; PSP+ unbagged plants, PSP− unbagged plants, PSP− bagged plants) and their interaction on all measured reproductive, phenological, morphological and quality plant traits. Block was included in all analyses as a random factor. Variables were transformed when necessary (number of flowers and seeds; log-transformed). All variables except one were analyzed with Gaussian distribution according to their error distributions and their homoscedasticity compliance. Number of stalks was analyzed with Poisson distribution, log link function and additional observation-level random effect to correct overdispersion. We used Tukey's HSD pairwise post hoc comparisons to determine significant differences between levels of PSP factor for the different variables.

Last, we performed two structural equation models (SEMs). Structural equation models are suitable to evaluate direct and indirect effects, where all parameters could act as both predictor and response variables (Shipley 2016). PiecewiseSEM enables the inclusion of random effects on component models (Lefcheck 2016, Shipley 2016). GLMMs conformed the set of component models in the piecewiseSEM, using standardized variables (mean = 0, SD = 1) and including block as random factor for all component models. We started with an initial full model where the hypothesized pathways were based on the prior results (Suppl. 1.3, Fig. 1.S4; Suppl. 1.4, Fig. 1.S6) and we used Shipley's test of d-separation to select the final model (Shipley 2013, 2016; Suppl. 1.3, Table 1.S4; Suppl. 1.4, Table 1.S6). The d-separation approach is based on the removal of irrelevant paths and the inclusion of identified missing paths. It generates a Fisher's C test statistic, which can be used to assess overall fit of the SEM and to calculate Akaike's information criterion corrected for small sample sizes (AICc) for model selection (Shipley 2013, 2016).

We evaluated in the first SEM the potential bagging manipulation effect on plant fitness using the information obtained from the previous analyses. For that purpose, we analyzed the effects of the bagging manipulation to see whether plant bagging negatively impacted plant fitness (Suppl. 1.3, Table 1.S5). The SEM showed that the bagging manipulation did not negatively influence plant fitness. Furthermore, the bagging positively affected plant fitness through its direct and indirect effects on various fitness components when compared to unbagged plants without PSPs (Suppl. 1.3, Table 1.S5, Fig. 1.S5). Having verified the optimal functioning of the bagging manipulation, another SEM was performed to evaluate the direct and indirect effects of PSPs on plant fitness. This time, we only used plants from PSP- bagged and PSP + unbagged treatments to investigate the effects provoked by PSPs on the plant (Suppl. 1.4, Tables 1.S6-1.S7).

All analyses were performed with R (R Core Team 2017), using packages *lme4* (Bates et al. 2015) and *emmeans* (Lenth 2018) for GLMMS and package *piecewiseSEM* (Lefcheck 2016) for SEM. Mean ± standard errors (SE) are shown through the manuscript.

1.4 Results

Probability of attack, fruit attack rate and seed predation rate by PSPs

Detritivores had no effect on the probability of a plant to be attacked by PSPs on unbagged plants (D− 0.67 ± 0.07, D+ 0.65 ± 0.07, $F_{1,73} = 0.13$, $P = 0.71$), nor on fruit attack rate (D− 0.21 ± 0.04, D+ 0.23 ± 0.04, $F_{1,73} = 0.08$, $P = 0.77$). Seeds were predated by the moth *C. trinotella* (42% of plants), an unidentified gelechiid species (20% of the plants) or by both species (38% of plants). Average seed predation by PSPs was 80.38 ± 10.01 seeds per plant, representing 31.51 ± 4.39% of the total number of seeds per plant.

Effects on plant reproduction

Plants attacked by PSPs produced 60% more (intact healthy) seeds than PSP- unbagged plants and 40% more than PSP- bagged plants (Table 1.2, Fig. 1.1a). Detritivores had no effect on seed production. PSP treatments, but not detritivores, also affected both seed and fruit set (Table 1.2), although the fruit set differed only between unbagged and bagged plants that had no PSPs (Table 1.2, Fig. 1.1b). On the contrary, the seed set was significantly higher in plants with PSPs compared to plants without PSPs (Table 1.2, Fig. 1.1c).

	D			PSP			D x PSP		
	F/χ^2	*P*	Df	*F/χ^2*	*P*	df	*F/χ^2*	*P*	df
Plant reproduction									
Number of seeds	0.17	0.68	1,93	**8.20**	**0.0005**	2,93	0.63	0.53	2,93
Fruit set	1.15	0.28	1,93	**4.28**	**0.016**	2,93	0.03	0.97	2,93
Seed set	1.83	0.17	1,93	**19.97**	**< 0.0001**	2,93	0.32	0.84	2,93
Flowering phenology									
Flowering onset	0.15	0.69	1,92	**9.40**	**0.0001**	2,92	1.19	0.30	2,92
Flowering end	2.66	0.10	1,92	1.38	0.25	2,92	0.77	0.46	2,92
Other plant traits									
Height	**4.42**	**0.038**	1,93	15.19	< 0.0001	2,93	2.36	0.10	2,93
Number of stalks	**6.54**	**0.010**	1,93	11.63	0.002	2,93	0.63	0.72	2,93
Number of flowers	**5.30**	**0.021**	1,93	12.85	0.001	2,93	3.47	0.17	2,93
Number of ovules/fruit	2.93	0.09	1,93	2.29	0.10	2,93	0.85	0.43	2,93

Table 1.2. Results of general and generalized linear mixed models showing the effects of detritivore (D) and pre-dispersal seed predator treatments (PSP) on variables related to plant reproduction, phenology and morphology. χ^2 value is shown for number of stalks, *F* value is shown for the rest. Significant results ($P < 0.05$) are indicated in bold.

Effects on plant traits

Plants with detritivores were taller (D+ = 38.60 ± 1.47 cm, D− = 35.33 ± 1.58 cm; Table 1.2) and produced more stalks (D+ = 3.40 ± 0.22, D− = 2.57 ± 0.23; Table 1.2) and more flowers (D+ = 51.33 ± 4.37, D− = 42.33 ± 4.21; Table 1.2) than plants without detritivores. In a similar way, plants attacked by PSPs were also taller (Table 1.2, Fig. 1.1d) and produced more stalks and flowers than plants without PSPs, although only significantly for PSP− unbagged plants (Table 1.2, Fig. 1.1e-f). There was no difference among PSP treatments in the number of ovules per fruit (Table 1.2).

There was a significant effect of PSP treatment on flowering onset (Table 1.2). Both plants with PSPs and PSP− bagged plants began to flower earlier than PSP− unbagged plants, with no significant difference among them (PSP+ = 117.84 ± 1.09, PSP−

bagged = 121.06 ± 1.45, PSP− unbagged = 127.376 ± 2.07 Julian day of flowering onset; Table 1.2). Neither PSP treatment nor detritivores influenced flowering end (Table 1.2).

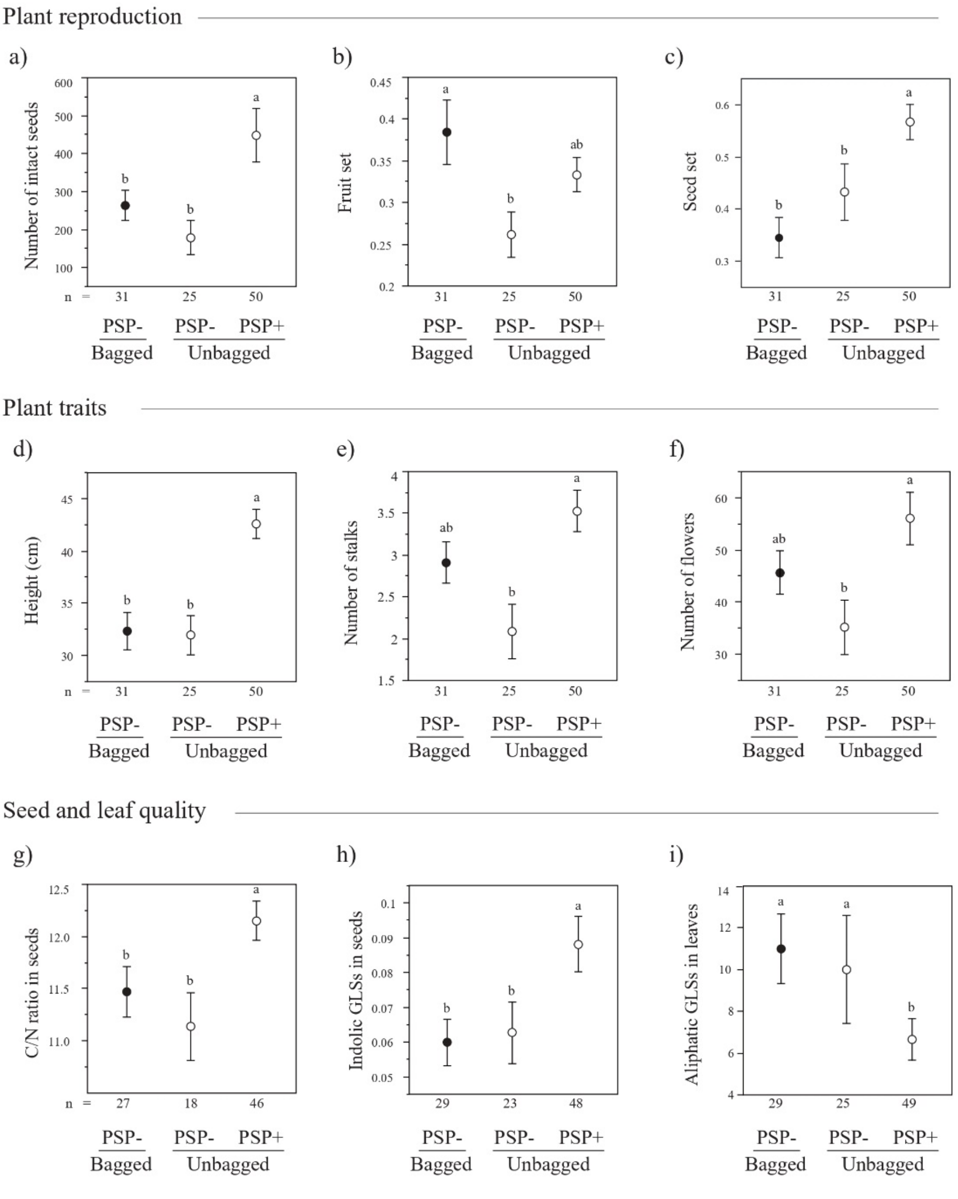


Figure 1.1. Pre-dispersal seed predator (PSP) treatment effects on plant reproduction (a–c: number of intact seeds, fruit set and seed set), plant traits (d–f: height, number of stalks and number of flowers) and seed and leaf quality (g–i: aliphatic GLS concentrations in leaves, C/N ratio in seeds and indole GLS concentrations in seeds; both GLS concentrations as μmol g^{-1} dry weight). Means ± SE are shown. Different upper-case letters indicate significant differences ($P < 0.05$), tested using Tukey's HSD pairwise post hoc comparisons. Numbers below each chart denote sample size per treatment.

Effects on plant quality

C/N ratio in leaves was lower in plants with detritivores (D+ = 52.16 ± 1.88, D− = 57.36 ± 1.76; Table 1.3) but this ratio was not affected by PSP treatment. Detritivores did not affect GLS concentrations in leaves, but total and aliphatic GLS concentrations in leaves were lower in plants with PSPs compared to plants without PSPs (Table 1.3, Fig. 1.1g).

C/N ratio in seeds was higher in plants with PSPs than in plants without PSPs (Table 1.3, Fig. 1.1h). Indolic GLS concentration in seeds was also higher in plants with PSPs than in plants without PSPs (Table 1.3, Fig. 1.1i). However, aliphatic and total GLS concentrations in seeds were interactively affected by detritivores and PSP treatment (Table 1.3). In the presence of detritivores, plants attacked by PSPs produced seeds with higher total GLS concentrations than plants without PSPs, whereas in the absence of detritivores, there was no significant effect of PSPs on seed GLS concentration. However, in the absence of detritivores and PSPs, bagging of plants had a significant effect on seed GLS concentration, with seeds from PSP− unbagged plants having higher total GLS concentrations than PSP− bagged plants (Suppl. 1.5, Fig. 1.S7).

	D			**PSP**			**D x PSP**		
	F	*P*	df	*F*	*P*	df	*F*	*P*	df
Leaf quality									
C/N ratio	**4.05**	**0.047**	1,88	0.70	0.49	2,88	0.45	0.63	2,88
Indolic GLSs	0.05	0.82	1,90	0.24	0.78	2,90	0.21	0.81	2,90
Aliphatic GLSs	0.07	0.79	1,90	**3.97**	**0.022**	2,90	0.10	0.90	2,90
Total GLSs	0.06	0.79	1,90	**7.97**	**0.018**	2,90	0.19	0.90	2,90
Seed quality									
C/N ratio	0.25	0.61	1,78	**3.32**	**0.041**	2,78	0.93	0.39	2,78
Indolic GLSs	0.36	0.55	1,87	**3.35**	**0.039**	2,87	0.72	0.48	2,87
Aliphatic GLSs	0.10	0.75	1,87	0.43	0.65	2,87	**4.27**	**0.017**	2,87
Total GLSs	0.10	0.75	1,87	0.32	0.72	2,87	**3.93**	**0.023**	2,87

Table 1.3. Results of general linear mixed models showing the effects of detritivore (D) and pre-dispersal seed predator treatments (PSP) on leaf and seed tissue quality (C/N ratio and glucosinolate (GLS) concentrations). Significant results ($P < 0.05$) are indicated in bold.

SEM: direct and indirect effects of PSPs

The SEM shows that herbivory by PSPs had a positive effect on seed production through the indirect and positive effects on seed set and plant height (Fig. 1.2). Plants with PSPs grew taller, which was associated with an increase in the number of reproductive stalks and flowers, and in the fruit set, which led to an increase in seed numbers (Fig. 1.2; Suppl. 1.4, Table 1.S7). Additionally, plants with PSPs increased their seed set, positively affecting seed numbers (Fig. 1.2; Suppl. 1.4, Table 1.S7). Despite the direct negative effect of PSP on the fruit set the net effect of PSPs on *M. moricandioides* fitness was positive (net effect = 0.65; Suppl. 1.4, Table 1.S7).

1.5 Discussion

Detritivore-PSP interaction

Detritivores are considered beneficial for the plants because they increase mineralization, alter soil properties and generally increase the dispersal and establishment of seeds (De Deyn and Van der Putten 2005). By altering plant chemical defenses, detritivores can also benefit plants by affecting above- and belowground herbivores (Newington et al. 2004, Poveda et al. 2005, González-Megías and Müller 2010). Based on this empirical evidence, we predicted that detritivores should provide *M. moricandioides* plants with resources to mitigate or compensate for PSP damage (Hypotheses 1–2, Table 1.1). Our results only partially supported our prediction, with detritivores positively affecting some plant traits but not altering the interaction between *M. moricandioides* and PSPs. *Moricandia moricandioides* plants with detritivores were taller, produced more flowers and leaves of higher quality than plants without detritivores, as generally occurs in this system (González-Megías and Müller 2010, González-Megías and Menéndez 2012) and in other Brassicaceae species (Newington et al. 2004, Poveda et al. 2005). Detritivores in our system also affected seed GLS concentrations although this effect was apparently influenced by the bagging manipulation with no clear effect of PSPs. Thus, our results indicate that *M. moricandioides* benefited from the presence of detritivores, but detritivores did not reduce the number of seeds consumed by PSPs nor alter plant responses to pre-dispersal seed predation. This may be because the PSPs benefited more than harmed the plant, and this occurred irrespective of resource availability.

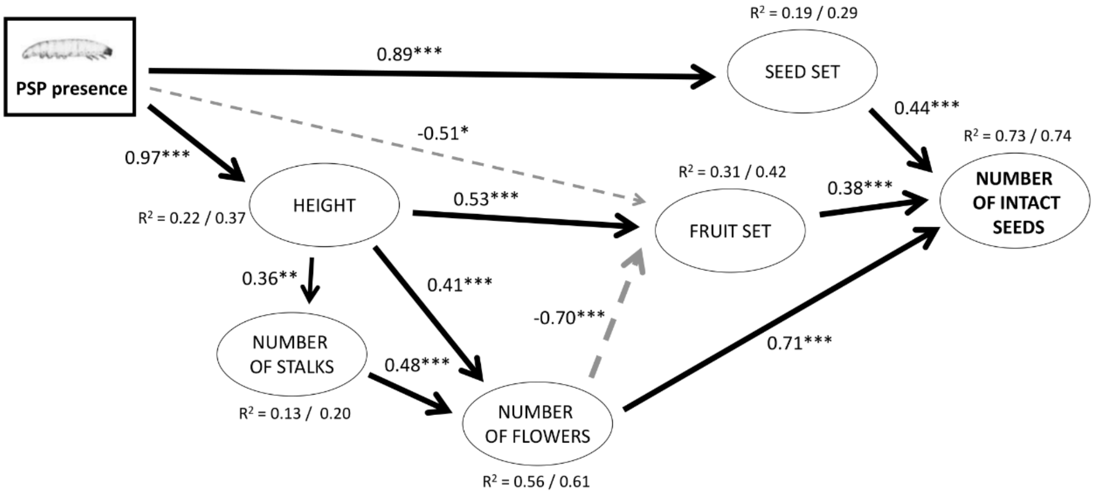


Figure 1.2. Final SEM showing the effects of PSPs on plant fitness. Standardized path coefficients are shown next to each path, and their significance level is shown as $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Solid lines denote positive and dashed lines negative relationships, and their thickness is scaled to the magnitude of the path strength. Variance explained by the component models (R^2) is reported as marginal/conditional.

Overcompensation for PSP damage

The most striking result by far of this study is that total fitness was higher in plants attacked by PSPs, contradicting our initial hypothesis (Hypothesis 1, Table 1.1). Indeed, plants with PSPs were taller and had a higher seed set than plants without PSPs. Although overcompensation to PSPs has been suggested by observational studies (Lortie and Aarssen 2000, Pilson 2000, Gagic et al. 2016), as far as we know, our study is the first one demonstrating experimentally the occurrence of overcompensation to PSPs at the level of lifetime total fitness (see Garcia and Eubanks 2018 for a recent meta-analysis of overcompensation for insect herbivory). Overcompensation has been observed in a variety of systems in response to grazing/browsing (Paige and Whitham 1987, Turner et al. 1993), simulated defoliation (Paige 1999, Tito et al. 2016) and bud or meristem removal (Naber and Aarssen 1998, Huhta et al. 2000, Pilson and Decker 2002, Piippo et al. 2009, Olejniczak 2011, Thomsen and Sargent 2017). There is also an increasing body of evidence of plant overcompensation for insect herbivory, such as in response to folivores (Agrawal et al. 1999, Schat and Blossey 2005, Lu et al. 2010), gall makers (Fay et al. 1996, Omoloye et al. 2002) and stem borers (Rosenthal and Welter 1995, Utsumi and Ohgushi 2007, Gerber et al. 2008). Overcompensation has been prominently associated with the activation of dormant meristems and the production of new lateral branches, which is triggered by the loss of apical dominance (Argall and Stewart 1984, Aarssen 1995, Strauss and Agrawal 1999, Agrawal 2000, Rautio et al. 2005). However, as highlighted by Garcia and Eubanks (2018) and Paige (2018) in their recent reviews, the genetic, developmental and physiological bases by which plants overcompensate for insect damage and their cascading consequences on the other traits have only begun to be elucidated.

In the case of *M. moricandioides* upon damage by PSPs, overcompensation resulted in considerable changes in shoot architecture, reproductive traits, chemical defenses in leaves and seeds and in seed nutrient content. The timing of PSP attack in relation to the plant reproductive stage may be relevant to properly understand how overcompensation takes place. In *Verbascum thapsus* (Scrophulariaceae), the proportion of fruits damaged by PSPs was significantly lower at the top of the main stalk than at the bottom, indicating that PSPs attack fruits that are produced early in the season (Lortie and Aarssen 2000). An early attack by PSPs can act as a cue to induce lateral branching through meristem activation, boosting the growth of their host plants and potentially causing overcompensation (Lortie and Aarssen 2000). A similar pattern occurs in *M. moricandioides*, in which the majority of damaged fruits were located at the base of the stalks (Suppl. 1.6, Fig. 1.S8), suggesting a similar mechanism and response as in *V. thapsus*. Early PSP attack and sufficient remaining growing and reproductive season are ideal conditions for overcompensation to take place (Fig. 3).

Plant overcompensation may have further consequences, such as the negative crosstalk between the hormones auxins and gibberellins, which regulate cell elongation, meristem activity and aboveground architecture, and the jasmonate-mediated signaling pathway, responsible for the induction of GLSs (Meldau et al. 2012, Huot et al. 2014). On the contrary, Mesa et al. (Mesa et al. 2017) demonstrated a positive relation between tolerance (overcompensation) and induced chemical resistance in overcompensating ecotypes of *Arabidopsis thaliana* (Brassicaceae), a close relative of *M. moricandioides*. Increased tolerance and resistance in *A. thaliana* is caused by lower auxin concentrations due to herbivore damage, which gives rise to upregulating of the jasmonate pathway and to activating dormant meristems via the induction of endoreduplication (Scholes and Paige 2014, Mesa et al. 2017, Paige 2018). The ultimate mechanism responsible for changes associated with overcompensation in *M. moricandioides* needs to be explored.

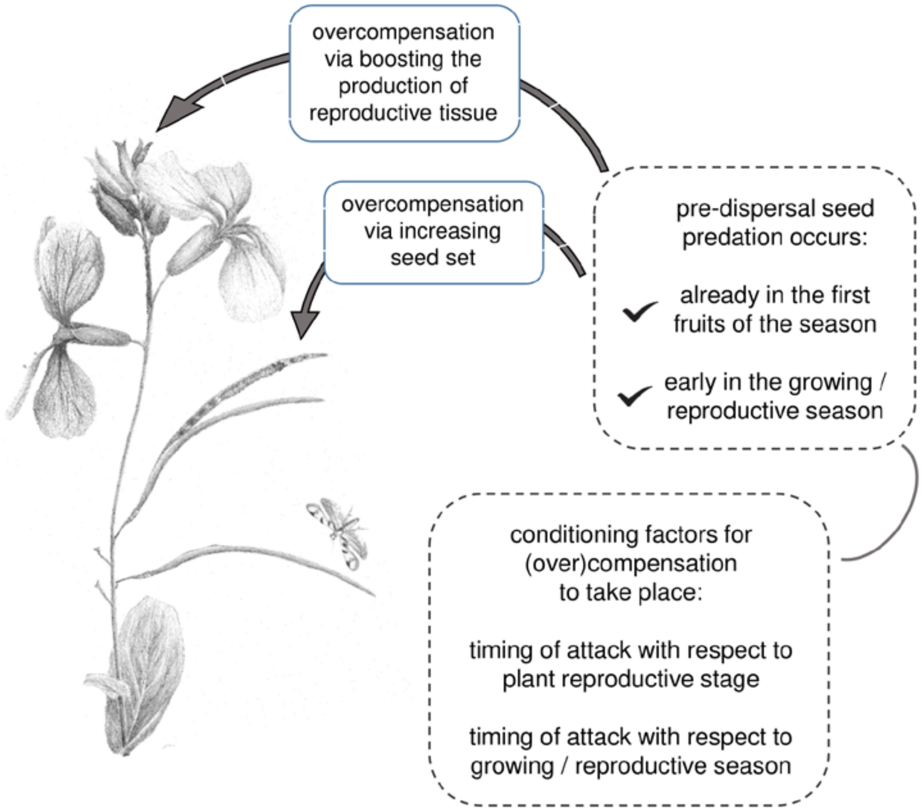


Figure 1.3. Illustration of the temporal factors that can trigger or constrain the plant's capacity to overcompensate for pre-dispersal seed predation.

Plant tolerance and resistance mechanisms towards PSPs

Contrary to our initial prediction, almost none of the more commonly described plant tolerance mechanisms for coping with PSP damage fitted for our study system (Hypotheses 3–5, Table 1.1). The first hypothesis stated that plants could reduce the harmful effects of PSPs by random or selected fruit abortion (Hypothesis 3, Table 1.1). Our results did not convincingly support this hypothesis because, although the experimental plants with PSPs produced more flowers, the fruit set did not differ from plants without PSPs. An alternative strategy against PSPs is the selective abortion of seeds inside attacked fruits (Hypothesis 4, Table 1.1). This strategy is more efficient when PSPs lay eggs in young fruits rather than on flowers (Sakai and Harada 2007, Meyer et al. 2014). However, it seems that the attack by PSPs enhanced seed set in *M. moricandioides* plants. Future works are needed to disentangle whether this outcome is the consequence of the plant manipulation by PSPs to guarantee a minimum amount of seeds, or on the contrary, is a tolerance mechanism exhibited by the plant to safeguard its fitness. Either way, because both partners seem to benefit from this interaction it may be considered mutualistic. Theoretically, when the plant produces surplus seeds, there is a limit to the number of seeds that develop successfully, and therefore, uneaten seeds will not be aborted if their number is below that limit (Sakai and Harada 2007). On the contrary, without surplus seeds, all uneaten seeds will develop irrespective of the number of predated seeds (Sakai and Harada 2007). Another hypothesis predicts that plants attacked by PSPs could also invest in more ovules within fruits rather than investing in more flowers (Hypothesis 5, Table 1.1). We found no evidence to support this; in fact, the number of ovules per fruit remained similar in plants with and without PSPs, despite this trait being variable within and between individuals. It seems, therefore, that *M. moricandioides* does not rely on any of the above-mentioned strategies to cope with PSP damage.

Our results fitted with those hypotheses proposing tolerance and resistance responses that entail changes in seed quality (Hypotheses 6–7, Table 1.1), such as poorer nutrient content and an increase in chemical defenses (Herrera et al. 2002). Seeds in plants attacked by PSPs were of lower quality than those escaping PSPs (higher C/N ratio), and had higher concentrations of indolic GLSs in seeds. It is noteworthy that this decrease in seed quality due to the attack of the PSPs does not lead to a reduction in *M. moricandioides* seedling recruitment (Chapter 3). It remains to be seen if the observed increase in C/N ratio is a strategy to impede the successful development of PSP caterpillars or if it is a by-product of overcompensation. Although specialist herbivores are more tolerant than generalists to chemical plant defenses, they can also be deterred and their performance be affected by these metabolites (Hopkins et al. 2009, Ali and Agrawal 2012). In particular, indole GLSs and their breakdown products play a significant role in plant defense (Agerbirk et al. 2009). Changes in seed quality can affect the survival, performance and lifetime fecundity of several seed predators (Muñoz et al. 2014), and further studies are needed to clarify the resource efficiency of this potential strategy towards PSPs.

Plant trait selection by PSPs

In many species, PSPs select early flowering plants, which are generally taller and have a greater reproductive success than late flowering ones (Campbell 1991, Elzinga et al. 2007, Kolb et al. 2007, Brody and Irwin 2012). Many PSPs also use the plant size and the flower display as cues for oviposition (Kolb et al. 2007, Östergård et al. 2007). Hence, tall-growing early-flowering plants could be more susceptible to the attack by PSPs (Hypotheses 8–9, Table 1.1). Supporting these predictions, *M. moricandioides* plants attacked by PSPs started flowering earlier, were taller and displayed more flowers than those not selected by the PSPs (the PSP− unbagged plants). However, plants attacked by PSPs were also significantly taller and displayed more flowers than those excluded from PSPs in our experiment (PSP− bagged plants), and there were no differences in the flowering onset with these plants. So, without denying that the PSPs may somehow select plants offering higher resources, our results undoubtedly point out that the greatest fitness observed in plants with PSPs is not only due to the individual selection of plants but also to an overcompensation effect, enhancing their reproductive effort when detecting the presence of PSPs.

1.6 Conclusions

It has been previously shown that PSPs can transgenerationally induce resistance to herbivory on *M. moricandioides* offspring (Chapter 3). Here, our experimental study made it possible to disentangle the within-generational pre-dispersal seed predation effects on *M. moricandioides*. We provide evidence that a short-lived herb overcompensated for the damage caused by PSPs, almost doubling the lifetime number of seeds produced. Overcompensation can be triggered by one or more mechanisms and can lead to important changes in various plant traits. The study also shows that overcompensation occurred irrespective of the presence of detritivores. Consequently, *M. moricandioides* seems to benefit more from a herbivore (PSP) than from a detritivore that has a potential positive indirect effect on plant fitness. More experimental studies manipulating PSPs are necessary to demonstrate whether this result is an exception rather than the norm.

1. X Supplementary material

Supplementary material 1.1. Detritivore and pre-dispersal seed predator treatment effects on free-living herbivorous insect abundance.

Data collection

During the study period, several species of herbivorous insects visited and fed on the experimental plants. To score the abundance of free-living herbivorous insects, the number of naturally occurring individuals was recorded on each experimental plant 3 times per week after the set-up of the experiment, giving a total of 44 surveys. In the case of aphids, we subtracted at each census the number of individuals of the same instar/type (winged versus not winged) counted in the previous census to avoid problems of summing individuals twice. Total abundance of each herbivore species or family was calculated by summing the number of individuals recorded during all the surveys. Herbivores were in adittion assigned to their corresponding guild.

When considering the experimental plants, 13.75 ± 1.25 suckers per plant were observed, which belonged to 5 different taxonomic families: 5.77 ± 0.59 Cicadellidae, 3.99 ± 0.53 Miridae (mainly *Hadrodemus* spp.), 1.25 ± 0.53 Aphidae (over 75% of them belonging to the specialist species *Brevicoryne brassicae* and *Lipaphis erysimi*), 1.76 ± 0.19 Issidae (mainly *Agalmatium bilobum*) and 0.52 ± 0.19 Pentatomidae. Chewers per plant were also noted (2.74 ± 0.30), and more than 87% of them belonged to 4 taxonomic groups that were abundant enough to be separatelly analyzed: 1.07 ± 0.18 *Plutella xylostella*, 0.43 ± 0.07 florivore pierid caterpillars (*Euchloe crameri* and *Pontia daplidice*), 0.42 ± 0.10 spp. flea beetles and 0.47 ± 0.08 *Galeruca angosta* leaf beetles. It must be noted that all hatched *Plutella xylostella* and florivore pierid caterpillars were immediately removed from plants. We also found 0.43 ± 0.15 leaf miners per plant.

Statistical analyses

We performed generalized linear mixed models (GLMMs) to test the effects of each factor, detritivores (with two levels) and PSPs (with three levels) and their interaction on herbivorous insect abundance on the experimental plants at both taxonomic and guild level (see Table 1.S1). We fitted the most appropriate distribution according to the errors (Poisson, Negative Binomial), and block was included in all analyses as a random factor. When overdispersion was observed, GLMMs with observation-level random effects were run, which allowed for variation at plant level (Table 1.S1). Zero-inflation was also modelled when necessary. GLMMs were performed with R package *glmmADMB*. When necessary, post hoc tests were done to determine significant differences between levels of PSP factor with R package *emmeans*, with Tukey's HSD pairwise comparisons test.

Additionally, we aimed to determine the influence of herbivorous guilds compared to the one of PSPs on key plant traits such as height and seed set (due to their importance as overcompensation mechanisms), seed traits affected by PSPs (C/N ratio and indolic glucosinolate concentration) and healthy intact seeds (measure of fitness). We also tested the influence of Miridae, the only taxonomic group for which we found significant post hoc differences. For that, we applied the Boruta algorithm (with the R package *Boruta*; Kursa and Rudnicki 2010). The Boruta algorithm is primarily designed to automatically perform feature selection, but it is also used to determine variable (feature) importance. In Boruta, features do not compete among themselves but with a randomized version of them based on multiple iterations.

Results

GLMMs

We found no experimental effect on the abundance of most herbivores associated with the plant (Table 1.S1). PSP treatment had a significant effect on the abundance of sap suckers (Table 1.S1), with more sap suckers on PSP+ plants than on PSP- unbagged plants (17.14 ± 2.15 individuals per plant on PSP+ plants, 14.52 ± 1.72 on PSP- unbagged plants and 7.33 ± 1.05 PSP- bagged plants). A similar results was obtained for Miridae (5.76 ± 0.93 individuals per plant on PSP+ plants, 3.92 ± 0.85 on PSP- unbagged plants and 1.19 ± 1.19 on PSP- bagged plants; Table 1.S1). PSP treatment had also a significant effect on the abundance of Cicadellidae, although we observed no post hoc differences among the different levels (Table 1.S1). Similarly, the abundance of Pentatomidae was interactively affected by detritivores and PSPs, although we observed no post hoc differences among the different levels (Table 1.S1). We observed no effects for the abundance of Issidae and Aphidae (Table 1.S1). We neither observed any effect for the abundance of chewers and leaf miners (Table 1.S1).

	Fitted distribution	Random effects	D		PSP		D x PSP	
			χ^2	*P*	χ^2	*P*	χ^2	*P*
Total sap suckers	Negative binomial	Block	1.82	0.17	**27.30**	**<0.0001**	1.18	0.55
Cicadellidae	Negative binomial	Block	1.02	0.31	**12.88**	**0.001**	2.19	0.33
Issidae	Negative binomial	Block	0.38	0.53	1.63	0.44	0.22	0.89
Miridae	Negative binomial	Block, Plant	2.50	0.11	**22.63**	**<0.0001**	2.63	0.26
Pentatomidae	Zero-inflated Poisson	Block	1.56	0.21	0.92	0.63	**9.72**	**0.007**
Aphidae	Zero-inflated Poisson	Block, Plant	0.21	0.63	0.76	0.68	5.47	0.064
Total chewers	Negative binomial	Block	0.09	0.75	1.84	0.39	1.36	0.50
P. xylostella	Zero-inflated Negative binomial	Block	1.04	0.30	1.87	0.39	5.10	0.078
Floral pierids	Zero-inflated Negative binomial	Block	0.89	0.34	0.68	0.70	3.36	0.18
Phyllotreta spp.	Negative binomial	Block, Plant	0.44	0.50	0.16	0.91	0.25	0.88
G. angosta	Zero-inflated Poisson	Block	0.60	0.43	0.00	0.99	1.85	0.39
Leaf miners	Zero-inflated Poisson	Block	0.01	0.88	5.82	0.054	0.06	0.96

Table 1.S1. Results of selected generalized linear mixed models showing the effects of detritivore (D) and pre-dispersal seed predator treatment (PSP; with three levels, see methods) effects on herbivorous insect abundance. Significant results ($P < 0.05$) are indicated in bold.

Boruta

The permutational analyses with the Boruta algorithm showed that PSP presence was the main feature determining plant height, seed set, C/N ratio in seeds, indolic glucosinolate (GLS) concentration in seeds and number of seeds, both when compared to guilds (Fig. 1.S1) and relevant taxonomic groups (Fig. 1.S2).

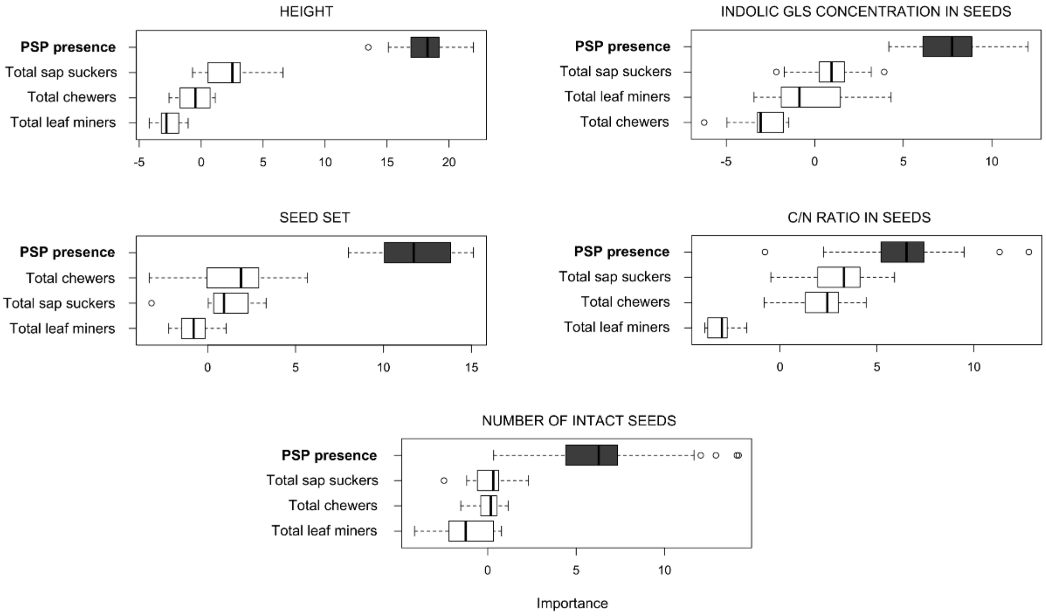


Figure 1.S1. Boruta plots showing the importance (achievable at random, estimated using their permuted copies) of the included features (PSP presence and the other herbivorous insect guild's abundance) on each of analyzed plant traits. Dark grey boxplots denote important features and white boxplots denote unimportant features.

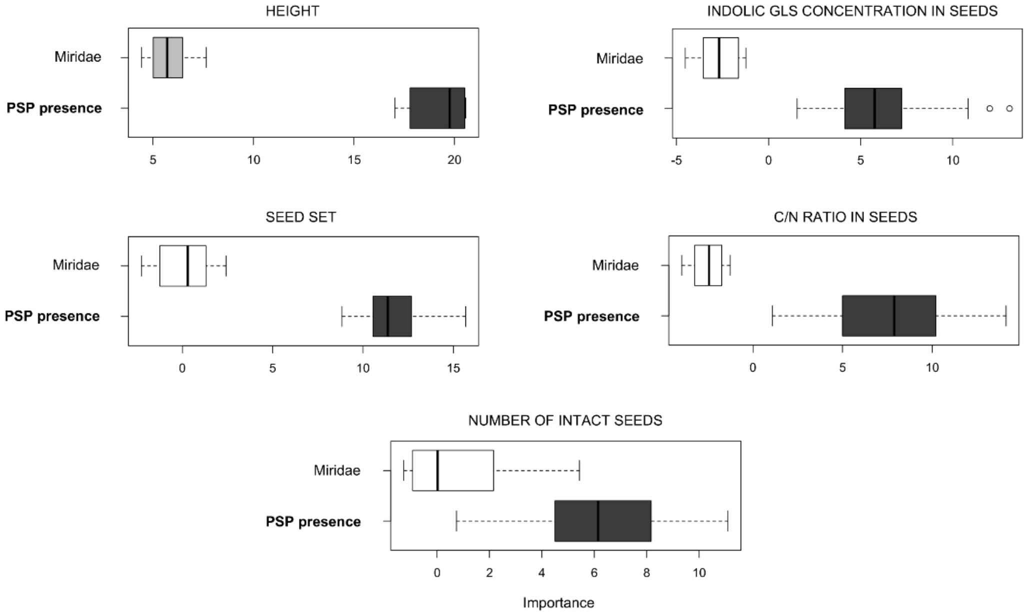


Figure 1.S2. Boruta plots showing the importance (achievable at random, estimated using their permuted copies) of the included features (PSP presence and mirid abundance) on each of analyzed plant traits. Dark grey boxplots denote important features, light grey boxplots denote tentative features, and white boxplots denote unimportant features.

Supplementary material 1.2. Bagging effect experiment: Detailed description of the experiment set up in 2014 to determine the potential effect of the manipulation of the plants to exclude pre-dispersal seed predators (PSPs).

Experimental set up

To achieve our goals it was necessary to manipulate plants to exclude PSPs. The use of bags to exclude insects from flowers is a common method for research on plant reproductive biology and pollination. Bags sometimes negatively compromise fruit development (usually by reducing fruit number, seed production, etc.), and therefore affect the reliability of the results obtained from the experiments. Therefore, to test for the potential effect of bags on plant reproductive traits we set up an experiment in spring 2014.

We carried out an experiment with natural *M. moricandioides* plants at the study area to test if bagging reproductive tissues would affect the development of plant reproduction. We selected 90 random plants from a population and randomly assigned them 3 treatments: no manipulation (unbagged plants, n=30), reproductive tissue bagging (bagged plants, n=30) and a procedural control treatment with open bags at the top that allowed pollinator and herbivorous insect visitation to reproductive tissues (open bagged plants, n=30). This last treatment was used as an indicator of possible constriction-derived effects of bags.

Reproductive tissues were bagged with 500 µm mesh size rearing bags when the first floral bud was produced (beginning of April 2014) until the end of the experiment (end of May 2014). Each reproductive stalk was bagged separately, and the size of the rearing bag was always adapted to the stalk. When plants began to flower (plants flowered from 09-April to 12-May), open flowers from bagged plants were hand-pollinated with pollen from two natural *M. moricandioides* individuals collected at the study area. To avoid hand-pollination derived effects, open flowers from unbagged plants were also hand-pollinated with the same pollen donors. While florivore chewers were unable to feed on bagged plants, we actively removed them by hand from all treatments to avoid florivory-derived effects. All eggs and caterpillars of species with florivory habits (the diamondback moth *Plutella xylostella* and the pierids *Euchloe crameri* and *Pontia daplidice*) were removed from all experimental plants. Once the flowers started to produce fruits, we used larger bags to avoid any potential negative effect of constriction of the fruits on their development.

Data collection

At the end of the experiment (end of May 2014) we counted the total number of flowers and fruits produced by each plant. Meanwhile, fruits were collected after complete maturation of seeds but before seed dispersal. All fruits were carried to the laboratory where the presence or absence of PSPs in the fruits was noted: 21 out of 30 unbagged treatment plants (70%), 8 out of 29 open bagged treatment plants (27%, a plant was dismissed as it produced no fruits) and 2 out of 30 bagged treatment plants (6%) had PSPs in their fruits. We also counted the total number of healthy intact seeds per fruit and per plant. We calculated the fruit set as the proportion of flowers that passed to fruits, and the seed set as the proportion of ovules per plant that produced intact healthy seeds.

Statistical analyses

Analyses were performed at two levels: 1) we tested whether treatments (unbagged, open bagged and bagged) influenced plant reproductive traits, and 2) due to PSP effects on plant reproduction, we tested treatment, PSP presence and their interaction effects on plant reproduction. This last analysis allowed to parse the potential bagging manipulation effects from those provoked by PSP activity, and to elucidate whether PSP effect depended on the bagging treatment applied. Plant reproductive traits were analyzed according to their error distributions. Fruit set and seed set were analyzed with linear models, and flower and healthy intact seed number with generalized linear models (Poisson distribution and additional observation-level random effect to correct overdispersion). When necessary, we performed Tukey's HSD pairwise post hoc comparisons to determine significant differences between levels of bagging treatments.

Results

Bagging treatment effect

We found that the bagging treatment did not influence the number of flowers, the fruit set and the number of healthy intact seeds (Table 1.S2, Fig. 1.S3). However, the bagging treatment had a significant effect on the seed set (Table 1.S2). Open bagged plants had lower seed set than unbagged plants (t ratio = -2.99, P = 0.01, Fig. 1.S3), while there were no significant differences between bagged and open bagged plants (t ratio = 0.72, P = 0.75, Fig. 1.S3). Additionally, there were no significant differences on the seed set between bagged and unbagged plants (t ratio = -2.29, P = 0.06, Fig. 1.S3), indicating that hand pollination was as efficient as open pollination.

	Bagging treatment		
	F/χ^2	P	df
Number of flowers	1.23	0.27	2,85
Fruit set	2.58	0.08	2,86
Seed set	**4.91**	**0.01**	2,86
Number of intact seeds	4.17	0.12	2,85

Table 1.S2. Linear and generalized linear models for testing bagging treatment effects on *M. moricandioides*' reproductive traits. χ^2 value is shown for number of flowers and number of seeds, F value is shown for fruit set and seed set. Significant results ($P < 0.05$) are indicated in bold.

Bagging treatment x PSP presence effect

At this level, we found that the bagging treatment had no significant effect on any reproductive trait (Table 1.S3, Fig. 1.S3). On the contrary, reproductive traits seemed to be more influenced by PSP presence (Table 1.S3, Fig. 1.S3), and previous bagging treatment effect on seed set waned when PSP presence was considered in the analyses. Indeed, PSP presence had a significant effect on healthy intact seed number (Table 1.S3, Fig. 1.S3): plants with PSPs produced 66% more seeds than plant without PSPs (503.32 ± 53.09 vs. 332.79 ± 40.90, mean ± SE). We found no significant interactive effect of bagging manipulation and PSP presence on any reproductive trait (Table 1.S3, Fig. 1.S3).

	Bagging treatment			PSP			Bagging treatment x PSP		
	F/χ^2	P	df	F/χ^2	P	df	F/χ^2	P	df
Number of flowers	1.33	0.51	1,82	3.82	0.05	2,82	1.42	0.15	2,82
Fruit set	0.81	0.44	1,83	3.07	0.08	2,83	1.03	0.36	2,83
Seed set	1.78	0.18	1,83	2.47	0.08	2,83	0.87	0.42	2,83
Number of intact seeds	0.21	0.89	1,82	**5.59**	**0.017**	2,82	1.11	0.57	2,82

Table 1.S3. Linear and generalized linear models for testing bagging treatment, pre-dispersal seed predator (PSP) presence and their interaction effects on *M. moricandioides'* reproductive traits. χ^2 value is shown for number of flowers and number of seeds, F value is shown for fruit set and seed set. Significant results ($P < 0.05$) are indicated in bold.

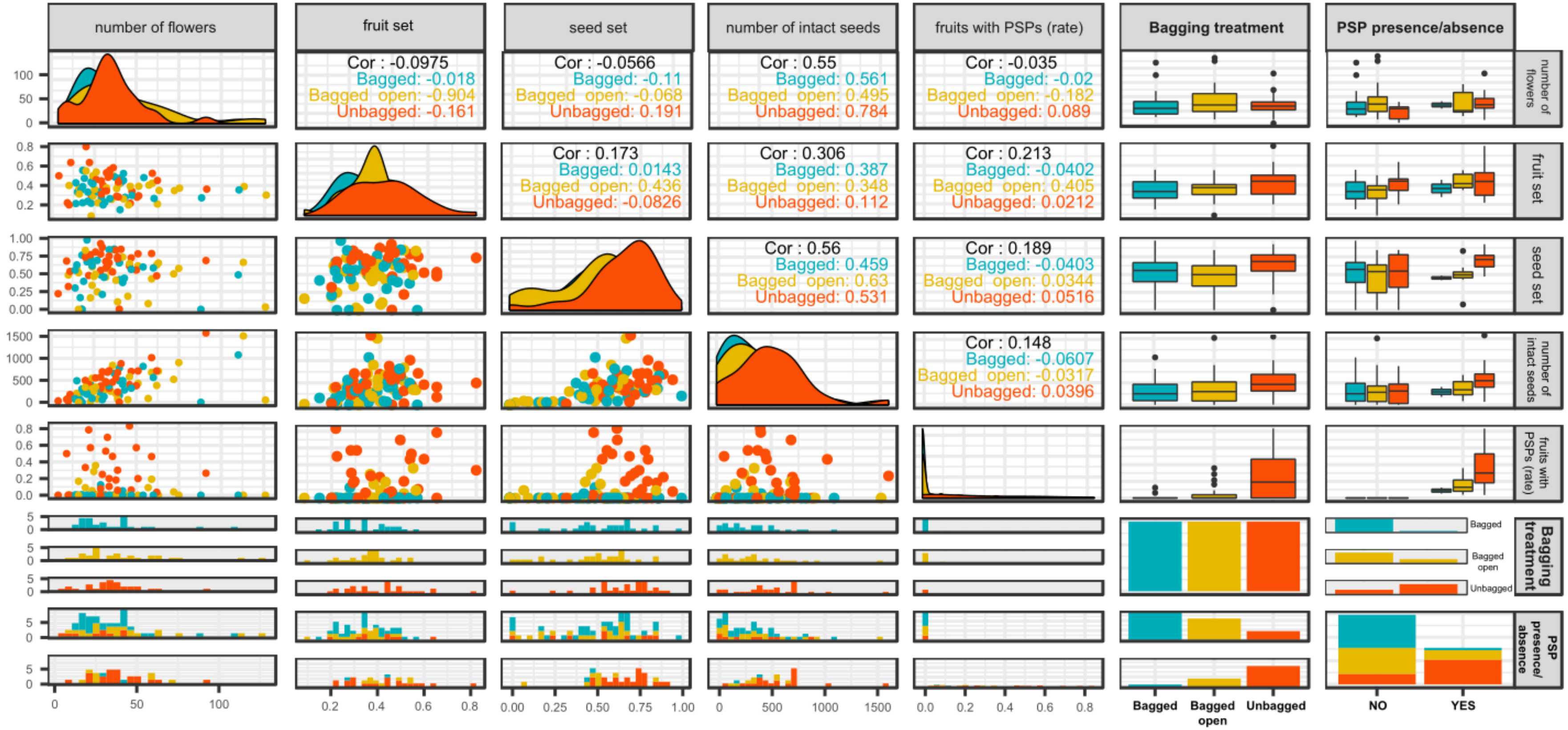


Figure 1.S3. Boxplot and scatterplot matrix of bagging treatment and pre-dispersal seed predator (PSP) effects on *M. moricandioides'* reproductive traits.

Supplementary material 1.3. Piecewise structural equation model (SEM) carried out to evaluate the direct and indirect effects of the bagging manipulation and pre-dispersal seed predator (PSP) presence on the number of healthy intact seeds of *Moricandia moricandioides*.

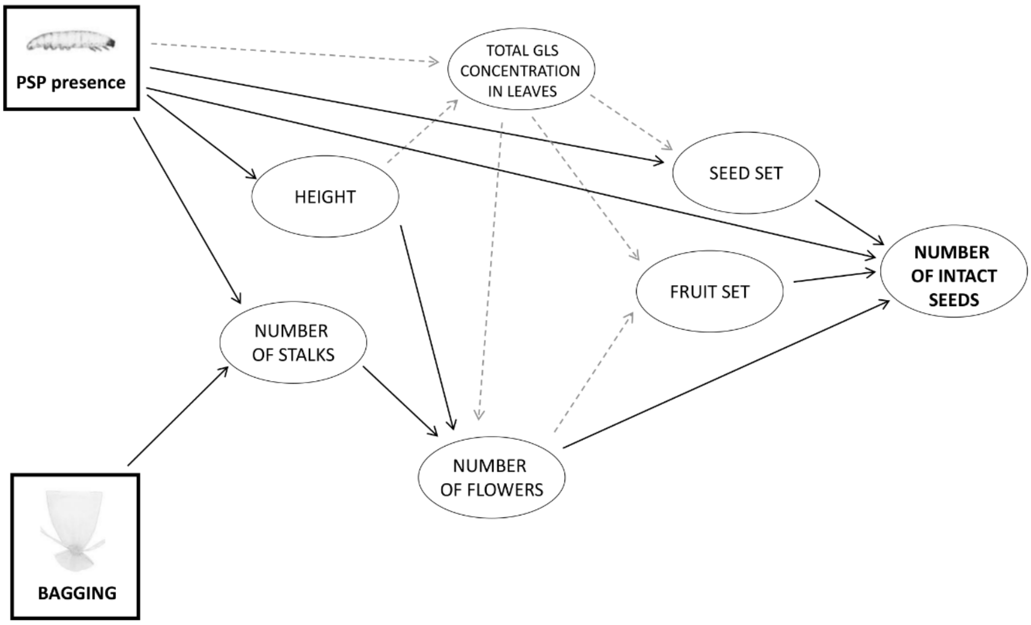


Figure 1.S4. Initially hypothesized SEM for the direct and indirect effects of the bagging manipulation (bagged PSP- plants) and PSP presence (unbagged PSP+ plants) on the number of seeds regarding unbagged PSP- plants (bagged PSP- plants, unbagged PSP- plants and unbagged PSP+ plants as the three options of a discrete variable). Solid lines denote positive and dashed lines negative relationships.

Model	Removed paths	Added paths	AICc	ΔAICc	df	Fischer’s *C*	*P*
Full initial model	-	-	261.886	96.701	28	77.64	0
model 2	Total GLSs in leaves → Number of flowers Total GLSs in leaves → Seed set PSP treatments → Seed set	-	269.273	104.088	34	100.544	0
model 3	-	PSP treatments → Fruit set Height → Total GLSs in leaves Height → Number of stalks Height → Seed set Number of stalks → Fruit set Number of stalks → Seed set	209.401	44.216	22	33.948	0.09
model 4	Height → Total GLSs in leaves Height → Seed set Number of stalks → Fruit set	-	240.857	75.672	28	64.982	0
model 5	Number of stalks → Seed set	-	240.160	74.975	30	69.894	0
model 6	-	Height → Total GLSs in leaves	227.356	62.171	28	56.855	0.001

model 7	**PSP treatments → Total GLSs in leaves** **Height → Total GLSs in leaves** **Total GLSs in leaves → Fruit set**	-	165.185	-	20	43.912	0.12

Table 1.S4. Stepwise SEM selection procedure for bagging manipulation effect on plant fitness.

Cause	Effects on	Direct	Indirect	Total
Height	Number of stalks	0.20	-	0.20
	Number of flowers	0.33	0.10	0.43
	Fruit set	-	-0.16	-0.16
	Number of seeds	-	0.24	0.24
Number of stalks	Number of flowers	0.54	-	0.54
	Fruit set	-	-0.20	-0.20
	Number of seeds	-	0.29	0.29
Number of flowers	Fruit set	-0.38	-	-0.38
	Number of seeds	0.68	-0.13	0.55
Fruit set	Number of seeds	0.35	-	0.35
Seed set	Number of seeds	0.42	-	0.42
Bagging	Height	0.09	-	0.09
	Number of stalks	0.48	0.01	0.49
	Number of flowers	-	0.29	0.29
	Fruit set	0.69	-0.11	0.58
	Seed set	-0.14	-	-0.14
	Number of seeds	-	0.35	0.35
PSP presence	Height	0.90	-	0.90
	Number of stalks	0.62	0.18	0.80
	Number of flowers	-	0.73	0.73
	Fruit set	0.71	-0.27	0.44
	Seed set	0.58	-	0.58
	Number of seeds	-	0.88	0.88

Table 1.S5. Standardized total, indirect and direct size effects of the bagging manipulation, PSPs and the component plant traits in the final SEM.

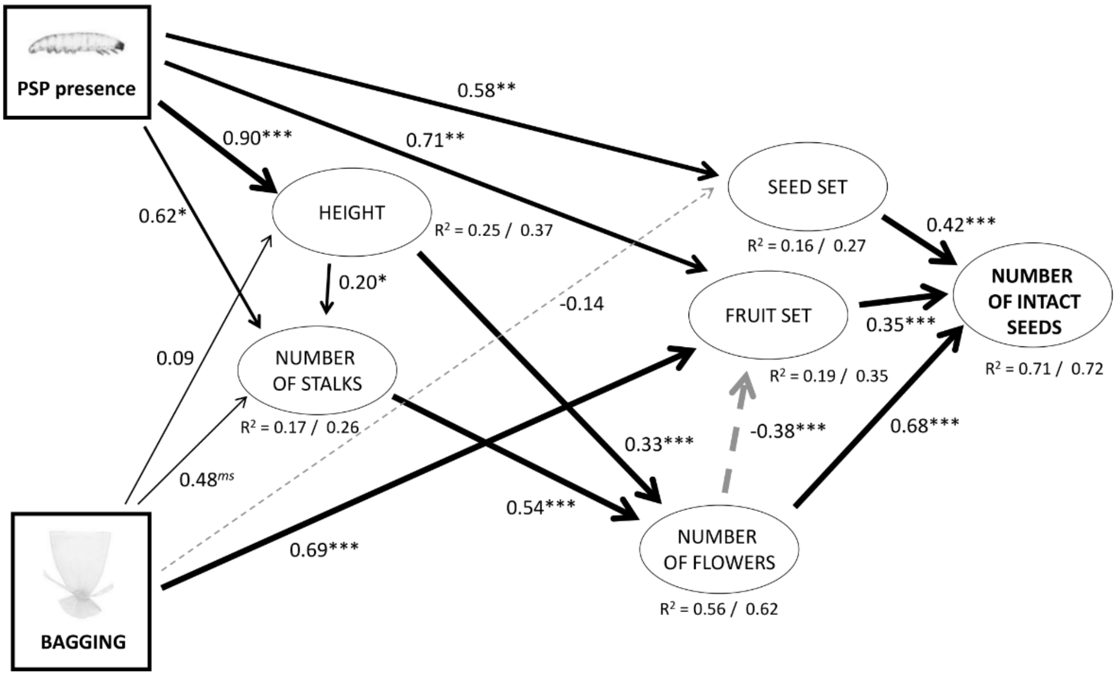


Figure 1.S5. Final SEM showing the effects of the bagging manipulation (bagged PSP- plants) and PSP presence (unbagged PSP+ plants) on plant fitness regarding unbagged PSP- plants (bagged PSP- plants, unbagged PSP- plants and unbagged PSP+ plants as the three options of a discrete variable). Standardized path coefficients are shown next to each path, and their significance level is shown as ^{ms} P <0.06, * P <0.05, ** P <0.01, *** P <0.001. Solid lines denote positive and dashed lines negative relationships, and their thickness is scaled to the magnitude of the path strength. Variance explained by the component models (R^2) is reported as marginal / conditional.

Supplementary material 1.4. Supplementary information of the piecewise structural equation model (SEM) carried out to evaluate the direct and indirect effect of pre-dispersal seed predators (PSPs) on the number of healthy intact seeds of *Moricandia moricandioides*.

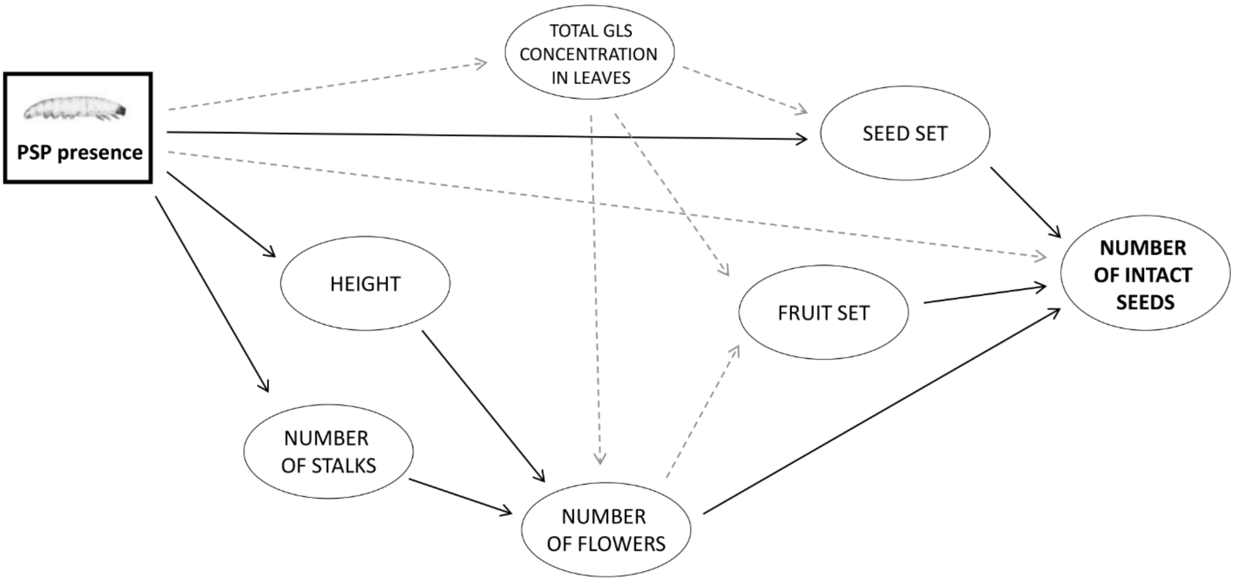


Figure 1.S6. Initially hypothesized SEM for the direct and indirect PSP effect on the number of seeds. Solid lines denote positive and dashed lines negative relationships.

Model	**Removed paths**	**Added paths**	**AICc**	**ΔAICc**	**df**	**Fischer's *C***	***P***
Full initial model	-	-	215.40	94.28	28	46.00	0.01
model 2	Total GLSs in leaves → Number of flowers Total GLSs in leaves → Fruit set Total GLSs in leaves → Seed set PSP presence → Number of seeds	-	193.04	71.92	36	51.84	0.04
model 3	-	Height → Fruit set	182.64	61.52	34	41.37	0.18
model 4	-	PSP presence → Fruit set	176.00	54.88	32	33.28	0.40
model 5	-	Height → Number of stalks	165.60	44.48	30	23.29	0.80
model 6	PSP presence → Number of stalks	-	159.82	38.70	32	24.15	0.83
model 7	PSP presence → Total GLSs in leaves	-	**121.12**	**-**	**20**	**18.26**	**0.57**

Table 1.S6. Stepwise SEM selection procedure for PSP effect on plant fitness.

Cause	Effects on	Direct	Indirect	Total
Seed set	Number of seeds	0.44	-	0.44
Height	Number of stalks	0.36	-	0.36
	Fruit set	0.53	-0.41	0.12
	Number of flowers	0.41	0.17	0.58
	Number of seeds	-	0.47	0.47
Number of stalks	Number of flowers	0.48	-	0.48
	Fruit set	-	-0.33	-0.33
	Number of seeds	-	0.21	0.21
Number of flowers	Fruit set	-0.70	-	-0.70
	Number of seeds	0.71	-0.26	0.44
Fruit set	Number of seeds	0.38	-	0.38
PSP presence	Seed set	0.89	-	0.89
	Height	0.97	-	0.97
	Fruit set	-0.51	0.11	-0.40
	Number of stalks	-	0.35	0.35
	Number of flowers	-	0.56	0.56
	Number of seeds	-	0.65	0.65

Table 1.S7. Standardized total, indirect and direct size effects of PSPs and component plant traits in the final SEM.

Supplementary material 1.5. Interactive effect on total glucosinolate concentrations in seeds.

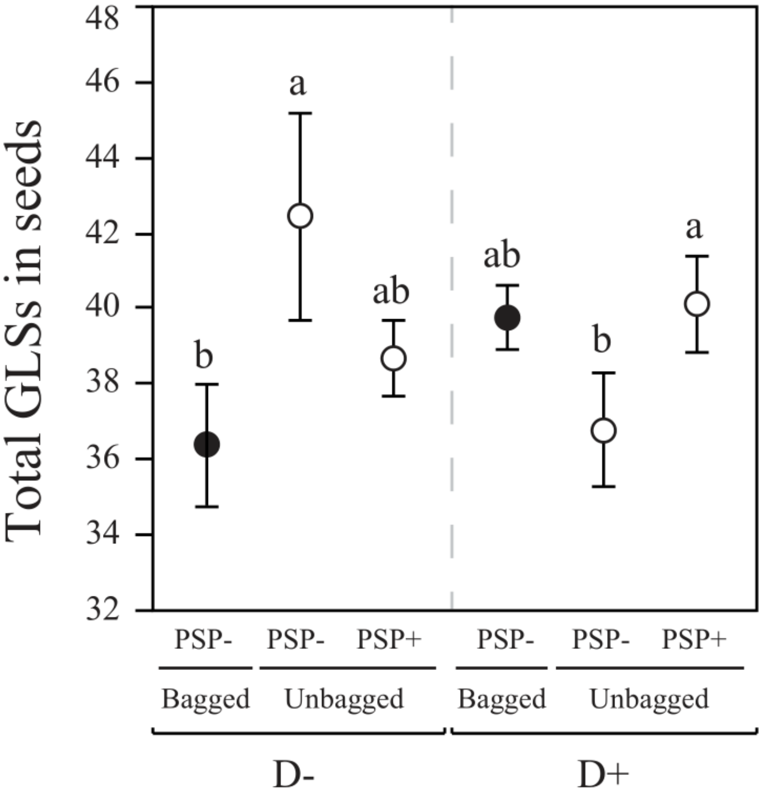


Figure 1.S7. Interactive effect of detritivores (D) and pre-dispersal seed predator (PSP) treatment on total seed glucosinolate (GLS) concentrations (μmol g^{-1} dry weight). Means ± SE are shown. Different upper-case letters indicate significant differences (*P* <0.05), tested using Tukey's HSD pairwise post hoc comparisons.

Supplementary material 1.6. Pre-dispersal seed predator (PSP) attack within plant's reproductive lifetime.

Data collection

The spur to carry out the main text experiment was partly encouraged by the results obtained from previously collected *M. moricandioides* fruits in the study area in 2012. A total of 1490 fruits from 303 plants from 28 natural *M. moricandioides* populations were collected (mean ± SE; 4.92 ± 0.02 random fruits per plant, 10.82 ± 0.83 random plants per population). Total number of fruits for each plant was counted, and the relative position of the collected fruits on reproductive stalks in relation to the number of fruits was estimated. At the lab, PSP presence or absence in the collected fruits was noted (568 fruits had PSPs).

Statistical analysis

We performed a linear mixed model with PSP presence/absence in each fruit as predictor variable, fruit relative position as response variable and plant identity as random factor to avoid pseudoreplication. We used the R package *lme4* for the analysis (see main text).

Results

There was a significant effect of PSP presence on the relative position of fruits on natural *M. moricandioides* plants at the study area, with fruits with PSPs being at a lower relative position than those without PSPs ($F_{1,1487}$ = 4.08, P = 0.043; Fig. 1.S8).

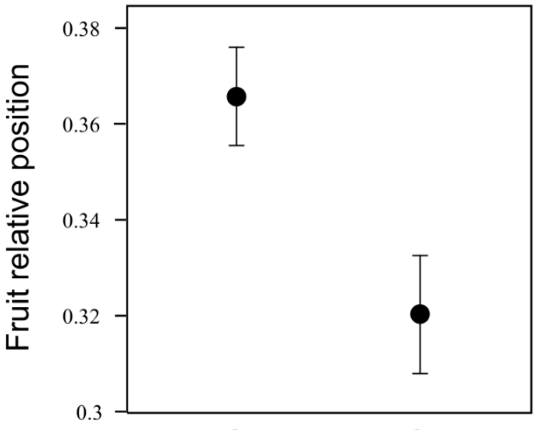
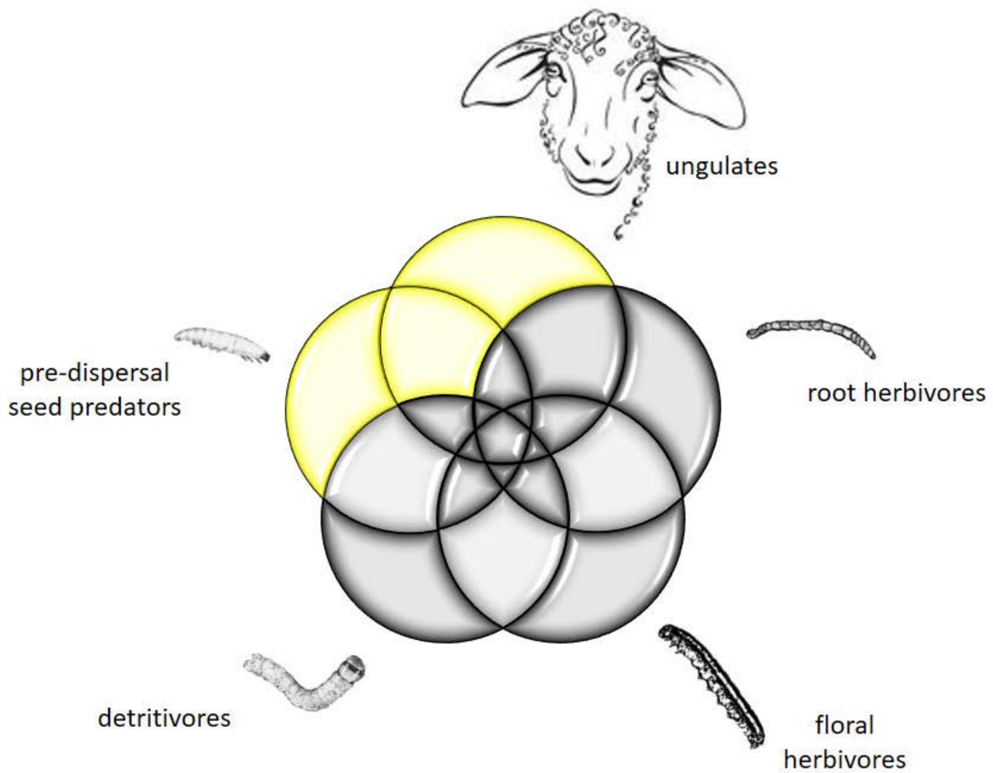


Figure 1.S8. Mean ± SE relative position of fruits on reproductive stalks according to PSP absence (PSP-) and presence (PSP+).

Chapter 2

Ungulates enhance overcompensation for pre-dispersal seed predators



2.1 Abstract

Overcompensation for insect damage seems to be a common phenomenon that has been neglected by the scientific community until recently. The ability of plants to deal with herbivore damage by compensating (tolerance mechanism) depends among others factors on the herbivore identity, the plant part under attack and the intensity and frequency of damage. Moreover, the interacting effect of multiple herbivores on the plant capacity to compensate for their combined damage has been seldom explored. In this study, we focus on *Moricandia moricandioides*, a short-lived plant with the ability to overcompensate for pre-dispersal seed predators (PSPs). We experimentally explored the effect of pre-dispersal seed predators on the total seed yield of *M. moricandioides* on 47 natural populations over 10 years. We used structural equation models to discern the direct and indirect effects of PSPs on plant fitness, the potential effect of damage intensity in the net effect on plant fitness and whether ungulates alter somehow the interaction between PSPs and the plant. Although the plant was subjected to high PSP incidence, in average plants overcompensated for the herbivore damage. Nonetheless, the ability of the plant to compensate for PSPs was reduced as the intensity of the damage increased. Remarkably, the ability of the plant to compensate for PSP damage increased in those populations with ungulate pressure. In summary, our study points out overcompensation for PSPs as a common strategy in multiple populations during a decade, with ungulates exacerbating the ability of the plant to overcompensate for ungulate damage.

Keywords

Brassicaceae · Grazer · Herbivorous mammal · Insect herbivory · Plant tolerance · Seed predation

1.2 Introduction

One of the main challenges faced by plants is to defense themselves from herbivores feeding on reproductive tissues (e.g. flowers and seeds; McCall and Irwin, 2006, Kolb et al., 2007). To defense from herbivores, plants have developed several strategies that go from resistance to tolerance mechanisms (Strauss and Agrawal 1999, Núñez-Farfán et al. 2007, Agrawal et al. 2012, Myers and Sarfraz 2017). Most plant species usually relay in a combination of resistance and tolerance mechanisms (mixed defense strategy; Leimu and Koricheva 2006, Núñez-Farfán et al., 2007), although the contribution of each mechanism may vary according to the part of the plant attacked, the type of herbivore, or even the environmental conditions (Carmona and Fornoni 2013, Myers and Sarfraz 2017). In particular, plants seem to favor growth and reallocation strategies (tolerance mechanisms) to compensate, or even overcompensate, for herbivore damage in reproductive tissues (Agrawal et al. 2012).

Overcompensation is a common and broadly demonstrated tolerance mechanism in plants when dealing with ungulates (Paige and Whitham 1987, Turner et al. 1993, Piippo et al. 2009, Thomsen and Sargent 2017). On the contrary, the real extent and importance of overcompensation for insect damage has been only recently brought to attention (Garcia and Eubanks 2018). Insect damage has been shown to stimulate vegetative and reproductive overcompensation in plants belonging to 26 families (Garcia and Eubanks 2018). Overcompensation for insect herbivory has been to date observed for feeding guilds such as chewers (Gagic et al. 2016), gallers (Fay et al. 1996, Omoloye et al. 2002) and stem borers (Gerber et al. 2008). Overcompensation for pre-dispersal seed predators (PSPs) has being suggested to occur in a few studies (Lortie and Aarssen 2000, Pilson 2000, Gagic et al. 2016), and experimentally proved in a wild Brassicaceae species (Chapter 1). PSPs are generally small and specialized insects, which have the ability to circumvent resistance traits of the plant (Hulme and Benkman 2002, Muola et al. 2010). Consequently, plants attacked by PSPs usually rely on tolerant mechanisms that go from the production of a surplus of flowers, fruits or seeds, to compensate for random or selective abortion, to reducing seed quality (Ehrlén 1993, Östergård et al. 2007, Sakai and Harada 2007, Ghazoul and Satake 2009, Meyer et al. 2014). Attack by PSPs induce also apical growth and branching by meristem activation ("the reserve meristem hypothesis", Aarssen, 1995), boosting plant growth in some plant species (Lortie and Aarssen 2000, Chapter 1).

Discerning PSP effects on plant can be difficult since the intensity and even the sign of plant-PSP interaction can vary as a function of other interacting herbivores (Gómez and González-Megías 2007b, Takagi and Miyashita 2014). Recent studies, such as the meta-analysis performed by Stephens et al. (2013), found that the effects of multiple herbivores on plant performance were independent in the three-quarters of case studies. Nevertheless, PSP were underrepresented in that study since few attempts to date have been carried out to test PSP effect on plants in combination with other herbivores. In one of these few, Gagic et al. (2016) surprisingly found that reproductive overcompensation for PSPs only occurred when combined by the attack of other herbivore. It is well known that large herbivores such as ungulates have profound effects on plants by affecting their reproduction, abundance and distribution (Crawley 1983, Cyr and Face 1993), and consequently affecting insect herbivores (Hunter 1992, Foster et al. 2014, Filazzola et al. 2020). Their impact may be stronger on endophytic insects that cannot move to another plant, such as PSPs, making them more vulnerable to incidental predation by grazers (Gómez and González-Megías 2007b, Takagi and Miyashita 2014, Gish et al. 2017). Incidental predation by consuming flowers and fruits would not only reduce the resources for the PSPs, but also accidentally consume eggs and larvae of PSPs (Gish et al. 2017). Ungulates can also affect PSPs indirectly by reducing plant height and the number of flowers, what would diminish plant attractiveness to PSPs, since they usually select bigger plants with large floral displays (Herrera et al. 2002, Kolb et al. 2007, Brody and Irwin 2012). A reduction in flower number caused by ungulates could also diminish plant attractiveness to pollinators (Strauss et al. 1996, Mothershead and Marquis 2000, Ramos and Schiestl 2019). A decline in pollinator visitation rate would directly decrease fruit and seed production, reducing the resources for PSP development (Xi et al. 2018). Consequently, ungulates would also lessen PSP effect on plants. Due to the huge differences in size, the interaction between grazers and PSPs is expected to be asymmetrical, with ungulates affecting PSPs and their effects on plants, but not the other way around (Gómez and González-Megías 2002, 2007a).

The degree of compensation in an overcompensating population varies according to herbivore damage frequency and intensity (Levine and Paige 2004). In fact, overcompensation seems to be a common strategy in plants when herbivore damage is severe and predictable (Paige and Whitham 1987, Lennartsson et al. 1997). In this paper, we experimentally assessed how long-term ungulate pressure affected the interaction between PSPs and its host plant, and the ability of the plant to overcompensate for PSPs under ungulate pressure in several natural plant populations. As a model system, we used *Moricandia moricandioides* (Boiss.) Heywood (Brassicaceae), a predominantly semelparous species capable to overcompensate for PSP damage (Chapter 1). In this species, early attack by PSPs at the beginning of the reproductive season provoke an increase in plant height and the in number of reproductive stalks, resulting in increased seed yield (Chapter 1). On the contrary, *M. moricandioides* does not have the ability to compensate for ungulate damage (Gómez 1996) or even for other insects feeding on the reproductive tissues such as florivore caterpillars (González-Megías 2016). Here, by means of a long-term manipulation of ungulate herbivory (>25-year exclusion), we explored the relationship between PSPs and *M. moricandioides,* and the potential modulatory effect of ungulates in this interaction, in 47 populations. We studied the interaction over 10 years, taking into account that the attack of PSPs usually has important interannual oscillations (Kolb et al. 2007). First, we explored the net effect of PSPs on plant fitness. Then, we used structural equation models to discern the direct and indirect effect of PSPs on plant fitness. Next, we explored whether the intensity of PSP attack alters the plant capacity to compensate for PSP damage. Finally, we assessed the effect of ungulates on PSPs and their interaction with *M. moricandioides*. We hypothesized that although PSP incidence and intensity will be elevated in in *M. moricandioides* populations, the plant will be able to compensate for PSP damage. We also hypothesized that the ability of the plant to compensate for PSP damage will be reduced when in combination with ungulate herbivory.

2.3 Material and methods

Study system

The study site is a seasonal watercourse located in the semiarid Guadix-Baza Basin (Granada, south-eastern Spain). The climate is continental Mediterranean with sharp temperature fluctuations (ranging from -14ºC to up to 45°C) and high seasonality (hot summers, cold winters). Plant cover is scarce, dominated by drought-tolerant perennial shrubs (Sánchez-Piñero 2007, Chapter 3). The area comprises private and governmental lands and is exposed different anthropic pressures: some of the land is used exclusively for hunting (mainly rabbits, hares and partridges), and domestic ungulates have been excluded from it for at least the last 25 years. The rest is open access land used by domestic ungulates (mainly sheep). The pressure of ungulates during the study years changed from the historical numbers of 1.4 to 1.6 sheep ha^{-1} (since at least 50 years ago) to 0.6-0.8 sheep ha^{-1} by 2019. The shepherd moves sheep around the open area everyday all year around.

The short-lived Brassicaceae species *M. moricandioides* grows here in mono-specific stands. This species grows as a vegetative rosette over winter and produces reproductive stalks in spring that remain photosynthetically active during the reproductive period of the plant. Most individuals (~90%) are annuals, although some individuals can live up to three years. The most abundant PSP of *M. moricandioides* in the study area is the specialist moth *Crossobela trinotella* (Herrich-Schaffer, 1856, Gelechiidae) (González-Megías and Müller 2010, Chapters 1 and 3). This moth species lays the eggs on flowers and immature fruits, and develops inside the fruits, feeding on seeds (Li and Sattler 2012).

Data collection

In 2009, we selected 50 patches (populations hereafter) of *M. moricandioides* in the study area which were at least 20 m apart from each other (Suppl. 2.1, Fig. 2.S1). *Moricandia moricandioides* populations were assigned to two categories: 24 populations with ungulate pressure, and 26 populations without ungulate pressure (Suppl. 2.1, Fig. 2.S1).

Every year from 2009 to 2019 (except for 2015, when most populations did not have reproductive plants due to drought), all populations were sampled from the end of June to the beginning of July to collect mature fruits before seed dispersal. Each of the years, we randomly selected 15 reproductive plants in each population from which we measured height, number of reproductive stalks, number of fruits, and the distance to the nearest conspecific neighbor. We collected five random fruits per plant located at different position within the plant's reproductive stalks. Fruits were kept in the lab until winter to allow PSPs to develop. We then noted PSP presence in the fruits and quantified the number of ovules, aborted seeds, healthy intact seeds (seeds hereafter) and the number of predated seeds per fruit and per plant. Total number of seeds per plant was calculated by multiplying the mean number of seeds in the five collected fruits x total number of fruits per plant. Individual plant fitness was estimated as total number of fruits and seeds per plant. We estimated PSP incidence as a binary variable, classifying plants as ones with or without PSPs. PSP intensity was calculated as the rate of fruits per plant attacked by PSPs. Only the subset of plants attacked by PSPs were included in the analyses using this variable.

Statistical analyses

Relationship between ungulates, PSP and plant over the study years

We used linear mixed models (LMMs) to evaluate the effect of PSPs, year, and their interaction on plant fitness, and to test the effect of ungulates, year and their interaction on PSP intensity. A generalized linear mixed model (GLMM) with PSP incidence as the response variable and ungulates, year and their interaction as the predictor variables was also performed with PSP incidence as binomial distribution and logit link function. Population was used as a random factor in all the analyses. Mixed models were also used to evaluate the relationship between the distance to the nearest conspecific plant with PSP incidence (GLMM) and intensity (LMM), in which year and population were included as random factors in the models.

Direct and indirect effects of both herbivores on M. moricandioides

We fitted structural equation models (SEMs) to evaluate the direct and indirect effects of PSPs on plant fitness, and ungulate effects on PSP-plant interaction, using a piecewise approach (Lefcheck 2016). While the classical methods solve the models using a global estimation from a variance-covariance matrix, piecewise SEM solves each component model (each linear equation) separately (Lefcheck 2016). Therefore, this method allows models with random factors, non-normal distribution, and small sample sizes (Lefcheck 2016). In piecewise SEM, model fit is evaluated using Shipley´s test of *d*-separation through Fisher´s *C* statistic (Shipley 2009). This method also provides an estimate of AICc (Akaike information criteria corrected for small sample size) and BIC (Bayesian information criteria) for each model. In our performed SEMs (see below), we reduced the number of variables from initial full models using AICc and BIC until the lowest value of both criterions were procured. We selected the final models among the subset of best nested models with a difference in AICc (ΔAICc) lower than two, which are considered as equivalent (Shipley 2013, 2016). We standardized all variables to have a mean of zero and a standard deviation equal to one prior running the models. The standardized coefficients of each path and its *p*-values are shown for the selected models. Standardized coefficients indicate the direct effects, and allow estimating the indirect effects though coefficient multiplication. In each model, net effects were thus calculated by adding up all the direct and indirect effects obtained by all paths. The conditional R^2, which is the variance explained by both fixed and random effects, is shown for the response variables in all models.

General and generalized linear mixed models (LMM and GLMMs) conformed the set of component models in all SEMs to discern the direct and indirect effect of one (PSPs) or both herbivores (ungulates and PSPs) on plant fitness. All models included year and population as random factors. Some few populations were excluded for the SEM analyses, because they had no reproductive individuals in two consecutive years and/or in at least three of the study years.

Effect of PSP incidence on plant fitness

We build a full model conformed by a set of four component linear mixed models: 1) a LMM with the total number of seeds per plant as the response variable and plant height, the number of fruits and stalks and PSP incidence as predictor variables; 2) a LMM with number of fruits as the response variable, and number of stalks, plant height, PSP incidence and the distance to the nearest plant as predictor variables; 3) a LMM with number of stalks as the response variable, and plant height, PSP incidence and the distance to the nearest plant as the predictor variables; and 4) a LMM with plant height as the response variable, and PSP incidence and the distance to the nearest plant as the predictor variables. Given the relationship between plant density and plant performance and compensative ability (Postma et al. 2021), controlling for the distance between plants (a proxy of plant density) allowed a more precise assessment of plant responses to herbivores.

Effect of PSP intensity on plant fitness

To test the effect of PSP intensity in plant fitness a SEM similar to the previous one was built, but replacing in the component models (LMMs) PSP incidence for PSP intensity. Only the subset of plants attacked by PSPs were included in the analysis.

Effect of ungulates in the interaction PSPs-plant interaction

To determine whether ungulates altered the interaction between PSPs and *M. moricandioides*, we fitted a multigroup SEM (Shipley 2016) using ungulates as the group variable (see full model in Suppl. 2.2, Fig. 2.S2). This type of SEM statistically compares between groups, and determines with parts of the models are the same or differ in each group by testing the differences in the covariance structure between groups (Shipley 2016).

All analyses were performed in R 3.3.2 (R Core Team 2017) using libraries *lme4* (Bates et al. 2015), *emmeans* (Lenth 2018) and *piecewiseSEM* (Lefcheck 2016). Mean ± SE are shown through the manuscript.

2.4 Results

Relationship between ungulates, PSP and *M. moricandioides* over the study years

Overall incidence by PSPs per plant was 0.52 ± 0.007, and seed predation intensity was 0.42 ± 0.051 in the study area among the study years. The number of seeds consumed by PSPs per fruit was 7.32 ± 0.14 (24.16 ± 0.44 % of seed/fruit). Average PSP incidence varied among years from more than 80% in 2011 to less than 30% of the plants attacked by PSPs in 2009 (Suppl. 2.3, Fig. 2.S3). PSP intensity also varied among years in a similar pattern than PSP incidence (Suppl. 2.3, Fig. 2.S3).

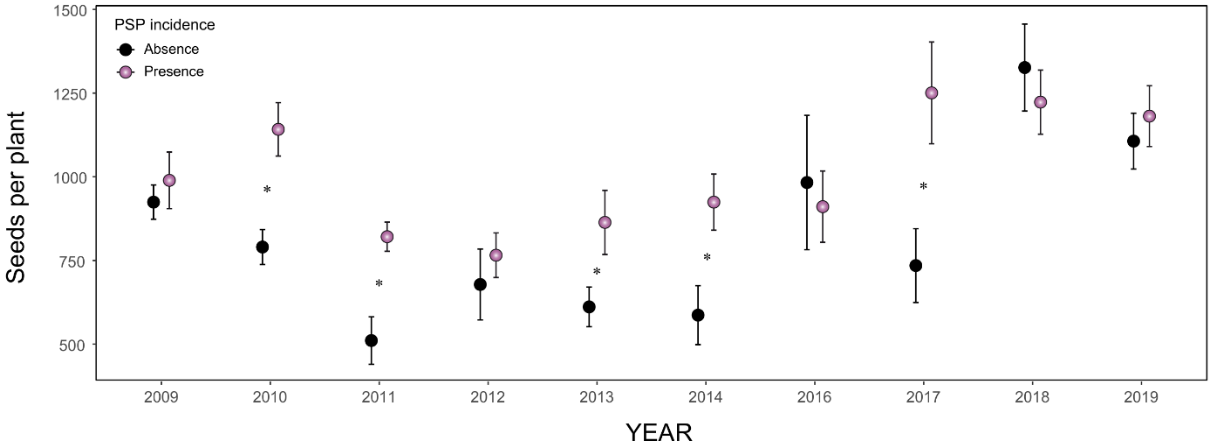


Figure 2.1. Average seeds per plant produced by *Moricandia moricandioides* with and without pre-dispersal seed predators (PSPs) each study year. Asterisks reveal years with significant Tukey's HSD post hoc differences between plants with and without PSPs. Mean ± SE.

Ungulates significantly affected PSP incidence and intensity, although the effect varied among year (Table 2.1). In general, ungulates had a negative effect on both PSP incidence and intensity (Table 2.1; Suppl. 2.3, Fig. 2.S3). PSP intensity and incidence were not significantly affected by the distance among conspecific *M. moricandioides* plants ($P > 0.05$ in both cases).

Both PSP incidence and intensity affected plant fitness (Table 2.2). Plants with PSPs produced 16% more seeds than those without PSPs (PSP+= 1014.43 ± 30.01 and PSP-= 874.56 ± 28.64 seeds per plant; Fig. 2.1), although only in five of the study years this difference in seed production was statistically significant (Fig. 2.1). On the contrary, seed yield was negatively related to PSP intensity (slope = -0.10, Table 2.2). Models for incidence and intensity with the random factor (population) were better than models without the random factor, indicating population variability (see PSP incidence effects on plant fitness in the studied populations in Suppl. 2.3, Fig. 2.S3). Ungulate effect on seed yield was less consistent, being either negative, positive or neutral depending on the year (Table 2.2, Suppl. 2.4, Fig. 2.S4). Distance between *M. moricandioides* plants was not affected by ungulates (LMM: F = 2.42, P = 0.12, df = 1).

	Ungulates			**Year**			**Ungulate x Year**		
	F/χ^2	P	df	F/χ^2	P	df	F/χ^2	P	df
PSP incidence	1.71	0.19	1,45	**491.13**	**< 0.0001**	9,5281	**77.10**	**< 0.0001**	9,5281
PSP intensity	3.88	0.055	1,45	**30.33**	**< 0.0001**	9,2672	**3.72**	**0.0001**	9,2672

Table 2.1. LMM and GLMM results for the effects of year and ungulates on pre-dispersal seed predator (PSP) incidence and intensity. χ^2 is shown for PSP incidence, F is shown for PSP intensity. Significant results ($P < 0.05$) are indicated in bold.

Direct and indirect effects of PSPs on *M. moricandioides*.

Effect of PSP incidence on plant fitness

The hypothesized SEM for PSP incidence (Ficher's C = 4.32, P =0.34, AICc = 60.32, BIC = 241.09, k = 28) explained a high proportion of the variation for seed production (Fig. 2.2A). In the model, plant fitness (in terms of both fruits and number of seeds) was directly affected by plant height and by the number of stalks (Fig. 2.2A). Taller plants produced more stalks, fruits and seeds. Plants with more stalks also produced more fruits, which indirectly increased the number of seeds produced by the plant, although the number of stalks also had a small negative direct effect on the number of seeds. PSP positively affected plant performance (height and number of stalks). Plants attacked by PSPs grew taller and produced more stalks, resulting on an indirectly positive effect of PSPs on the number of fruits. PSP incidence also had a direct negative effect on the number of seeds produced by the plants, although the net effect of PSPs on seed yield was positive (net effect=0.13; Fig. 2.2A). Finally, the distance to the nearest conspecific plant was also positively related to the number of stalks and plant height.

	Plant fitness		
	F	*P*	df
PSP incidence	**10.39**	**0.001**	1,5281
Year	**10.54**	**< 0.0001**	9,5281
PSP incidence x Year	**2.29**	**0.014**	9,5281
PSP intensity	**21.25**	**< 0.0001**	1,2671
Year	**3.76**	**0.0001**	9,2671
PSP intensity x Year	1.38	0.19	9,2671
Ungulates	2.32	0.13	1,45
Year	**9.70**	**< 0.0001**	9,5281
Ungulates x Year	**3.59**	**0.0001**	9,5281

Table 2.2. LMM results for the effects of pre-dispersal seed predator (PSP) incidence x year, PSP intensity x year and ungulates x year on plant fitness (number of seeds per plant). Significant results ($P < 0.05$) are indicated in bold.

Effect of PSP intensity on plant fitness

The SEM for PSP intensity also showed that PSP intensity had a direct negative effect on the number of seeds produced by the plant (Ficher's $C = 4.10$, $P = 0.58$, AICc = 58.69, BIC = 218.39, k = 27). In this model PSP intensity had only a slight non-significant effect on plant height and the number of stalks (Fig. 2.3A). The net effect of PSP intensity on the number of seeds produced by the plant was negative (net effect= -0.07).

Effect of ungulates on the interaction between PSPs and M. moricandioides.

The effect of PSP incidence on plant fitness differed between populations with and without ungulates, being the net positive effect of PSP incidence on the number of seeds produced by M. *moricandioides* higher in populations with ungulates (Fig. 2.2B). The difference between both types of populations was explained by the effect of ungulates strengthening the relationship between PSP incidence and the number of stalks (F = 903.2, P = 0.01, df = 1). Ungulates also affected the strength of the relationship between the number of stalks (F = 36.7, P = 0.001, df = 1), plant height (F = 36.7, P = 0.03, df = 1) and the number of fruits (F = 36.7, P = 0.001, df = 1) with seed yield (Fig. 2.2B). The strength of the interaction between the number of stalks with the number of fruits (F = 2663.9, P = 0.001, df = 1), and the relationship between the number of stalks and the distance to the nearest *M. moricandioides* plant (F = 903.1, P = 0.03, df= 1; Fig. 2.2B) also differed between population with and without ungulates.

Ungulates had no significant effect on the interactions between PSP intensity and plant fitness (Fig. 2.3B). However, ungulates affected the strength of the relationship between plant height (F = 34.9, P = 0.001, df = 1) and the number of fruits (F = 34.9, P = 0.04, df = 1) with seed yield (Fig. 2.3B). Ungulates also affected the strength of the relationship between plant height and the number of fruits (F = 1290.7, P = 0.04, df = 1). Finally, the strength of the interaction between the distance to the nearest *M. moricandioides* plant (F = 436.5, P = 0.01, df = 1) and plant height (F = 436.5, P = 0.01, df = 1) with the number of stalks also differed between populations with and without ungulates (Fig. 2.3B).

2.5 Discussion

PSP effects on plant fitness

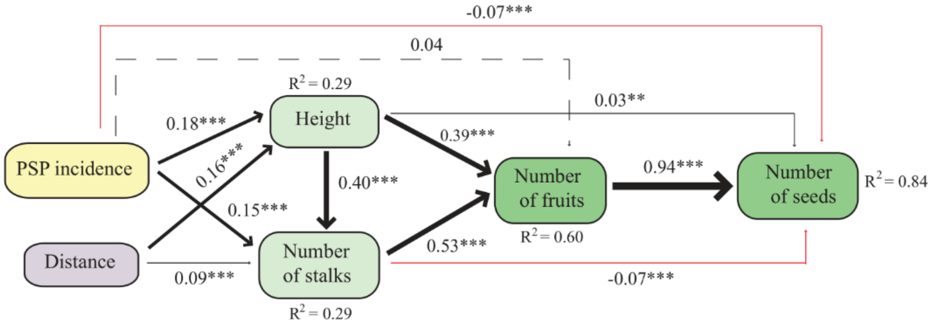
We found a general pattern over the years in the studied *M. moricandioides* populations indicating that plants produced more seeds when attacked by PSPs. Interestingly, the SEM showed that the effect of PSPs on plant fitness go mostly through the positive indirect effect of PSPs on plant height and number of reproductive stalks. This finding agrees with and extends our previous experimental results obtained in an experimental population (Chapter 1). The current study suggests that overcompensation for PSPs, rather than being circumscribed to a few localities, is a common strategy in *M. moricandioides*. Reproductive overcompensation induced by herbivores feeding on reproductive tissue seems to be frequent and common in plants (Garcia and Eubanks 2018). It remains to be seen whether in our case overcompensation is consequence of local adaptation or a general phenomenon occurring in other populations of this species. We presume the latter to be true in our system. In fact, overcompensation is predicted to occur in populations subjected to high risk of herbivory (Tuomi et al. 1994, Nilsson et al. 1996, Boalt et al. 2010). About 50% of the *M.*

moricandioides plants were attacked by PSPs during our study, and just nine out of the 47 populations were free of PSPs at least one of the 10 study years.

Interestingly, our results suggest that the capacity of the plant to overcompensate for PSP damage depends on the intensity of the interaction. An increase in PSP attack rate was associated with a reduction in seed yield. This result supports the idea that the degree of compensation depends on damage levels (Huhta et al. 2003, Zvereva et al. 2010, Poveda et al. 2018, Ramula et al. 2019), and contradicts previous findings reporting vegetative and reproductive overcompensation for insect damage to occur irrespective of the intensity of the interaction (Garcia and Eubanks 2018). Plants ability to overcompensate is related to physiological and genetic processes triggered in the plants when herbivores harm their tissue (Paige and Whitham 1987, Scholes et al. 2013). In *M. moricandioides*, the capacity of the plant to overcompensate for PSPs is related to the activation of meristems, leading to taller plants with more stalks, once the first fruits produced are attacked by PSPs (Chapter 1). However, an increase in PSP intensity was not associated with a positive effect on plant performance. This means that plants respond quickly to PSP presence but the capacity of the plant to compensate for seed consumption decreases with increasing attack intensities. Experimental evidence would help to determine whether the reduction in seed production observed in highly damaged *M. moricandioides* plants leads to under-compensation, equal-compensation or even slight overcompensation.

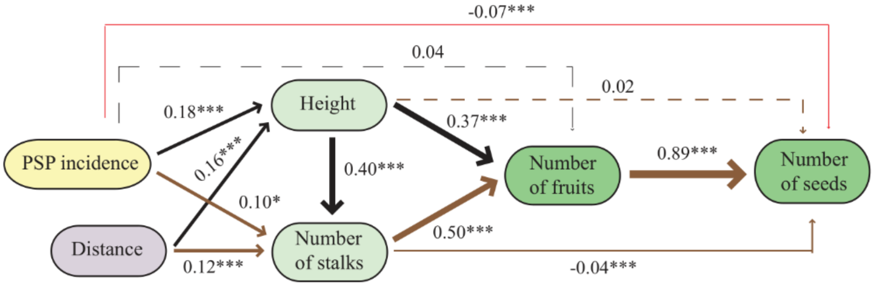
Despite finding variability among populations, most populations showed over- and equal-compensatory responses for PSPs, and only a few populations undercompensated for PSPs (for example, populations 13 and 47; Suppl. 2.4, Fig. 2.S4). A similar result was also found among years, in which the plant's response varied from compensation to overcompensation. Spatial and temporal variation in PSP attack is usually common (Kolb et al. 2007); our results indicate that this variability may also occur in the plant's ability to compensate for PSP damage. We presume that some ecological factors acting at local level may mediate the ability of plants to respond to PSP damage, as it is known that plants' ability to compensate by herbivore damage depends on several biotic and abiotic factors (Ramula et al. 2019). It remains to be explored which environmental conditions may or not favor *M. moricandioides'* ability to compensate for PSP attack.

A) PSP-*Moricandia moricandioides* interaction model



B) Ungulates effect on PSP-*Moricandia moricandioides* interaction

No ungulates: PSP net effect on the number of seeds = 0.10



Ungulates: PSP net effect on the number of seeds = 0.21

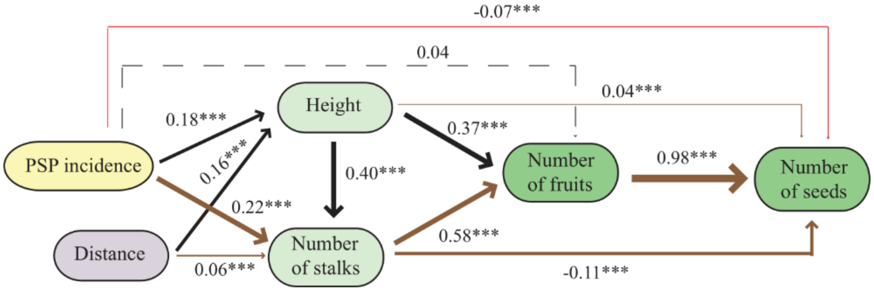
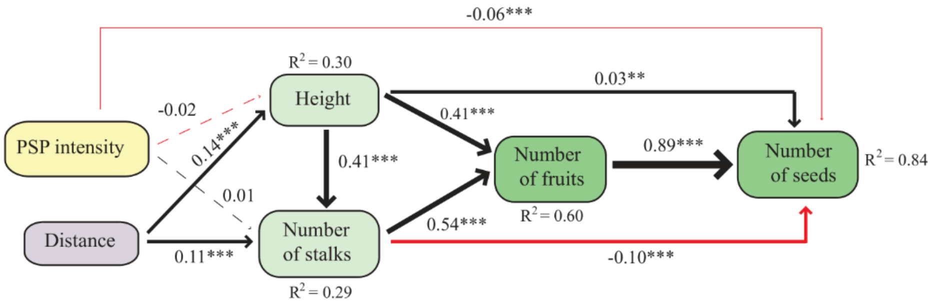


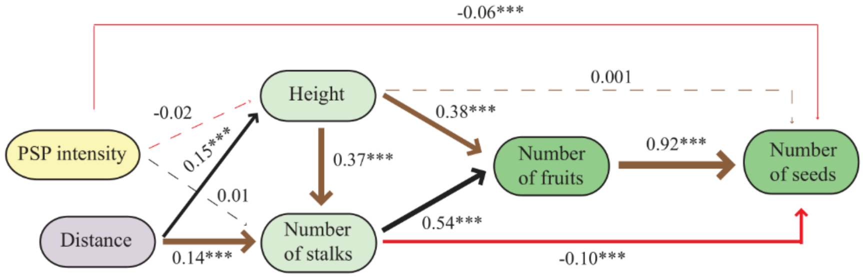
Figure 2.2. Structural equation models (SEMs) showing: A) the direct and indirect effects of pre-dispersal seed predator (PSP) incidence on plant performance (plant height, number of stalks) and fitness (number of fruits and of seeds per plant for all populations), and B) the direct and indirect effects of PSP incidence on plant performance and fitness in populations with and without ungulates. Distance refers to the space interval between conspecific *M. moricandioides* plants. Solid lines indicate significant paths and dashed lines non-significant ones. The red and black arrows denote negative and positive relationship, respectively. The numbers represent the coefficient estimates, and line thickness is proportional to the standardized effect size. R^2 value for each response variable is indicated. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Solid lines in brown color in models B models indicate significant differences in those paths between models.

A) PSP-*Moricandia moricandioides* interaction model



B) Ungulates effect on PSP-*Moricandia moricandioides* interaction

No ungulates: PSP net effect on the number of seeds = -0.07



Ungulates: PSP net effect on the number of seeds = -0.07

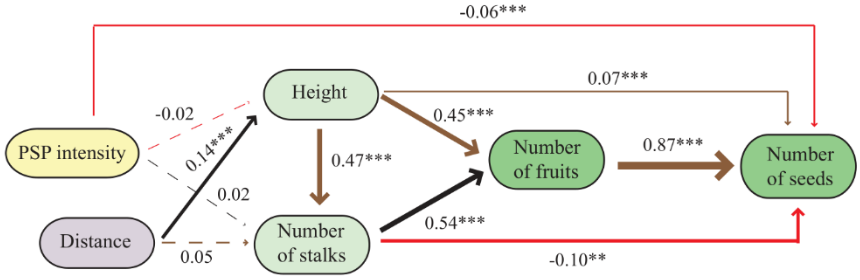


Figure 2.3. Structural equation models (SEMs) showing: A) the direct and indirect effects of pre-dispersal seed predator (PSP) intensity on plant performance (plant height, number of stalks) and fitness (number of fruits and of seeds per plant for all populations), and B) the direct and indirect effects of PSP intensity on plant performance and fitness in populations with and without ungulates. Distance refers to the space interval between conspecific *M. moricandioides* plants. Solid lines indicate significant paths and dashed lines non-significant ones. The red and black arrows denote negative and positive relationship, respectively. The numbers represent the coefficient estimates, and line thickness is proportional to the standardized effect size. R^2 value for each response variable is indicated. * *P* <0.05, ** *P* <0.01, *** *P* <0.001. Solid lines in brown color in models B models indicate significant differences in those paths between models.

Ungulate effects on PSPs and their interaction with *M. moricandioides*

As expected, our study shows that ungulates negatively affected PSP incidence and intensity. This type of asymmetrical interaction due to huge differences in size (no effect of PSPs on ungulates but a strongly negative effect of ungulates on PSPs) has been previously reported in other plants species (Roininen et al. 1997, Takagi and Miyashita 2014, Gish et al. 2017), including other Brassicaceae (Zamora and Gómez 1993, Gómez and González-Megías 2002, 2007b).

According to our prediction, ungulates altered the interaction between PSPs and *M. moricandioides*. However, and unexpectedly, ungulates enhanced the overcompensation response of *M. moricandioides* for PSPs. In the last 15 years, there have been at least three reviews focused on the combined effect of herbivores on plants (Denno and Kaplan 2007, Morris et al. 2007, Stephens et al. 2013). Stephens et al. (2013) concluded that in average the attack of multiple enemies had independent effects on plant performance. Moreover, these authors also predicted a combined antagonistic effect when the reproductive tissues are the ones consumed. Our results contrast with those conclusions since ungulates modulated PSP effect on plants, enhancing the overcompensation response of *M. moricandioides* for PSPs. A reproductive overcompensatory response when PSPs attack in combination with other herbivore guilds has been previously observed in the crop *Brassica napus* (Gagic et al. 2016). A potential explanation for our results would be that what we are observing is a compensatory response of plants not only to PSPs but also to ungulates. A typical compensatory respond of plants for ungulate damage is to increase the production of reproductive stalks (Ramula et al. 2019). However, we observed a negative effect of ungulates on plant height with no compensatory respond in the number of stalks, leading to a negligible effect on seed yield (-0.02; Suppl. 2.5, Fig. 2.S5; Suppl. 2.6, Fig. 2.S6). Another potential explanation would be that ungulates reduce the intraspecific competition between plants (by increasing the distance between individuals), facilitating the compensatory response of the plant to PSPs. As observed in our model, plants growing further apart from each other grew taller and produced more stalks. However, ungulates had no effect on the distance between conspecific plants, although they lessened the relationship between the distance to the closest neighbor and the production of reproductive stalks. Finally, the net effect of multiples herbivores feeding on plants is expected to depend on the intensity of damage provoked by each herbivore (Pilson 1996). Although, we effectively found a reduction in PSP incidence and intensity in populations with ungulates, ungulates did not affect the response of the plant to PSP intensity. Basically, by reducing PSP intensity, ungulates can indirectly enhance the compensatory response of the plants. Although a potential and feasible explanation, the real mechanism behind the enhanced tolerance response of plants to PSPs when dealing with both herbivores remains to be elucidated.

Conclusions

In summary, this long-term large-scale study showed that overcompensation for PSPs is the rule rather than the exception in *M. moricandioides* populations. However, the ability of the plants to compensate for PSP damage exhibits a temporal and spatial component, and depends on the intensity of the interaction. Additionally, we found that ungulates altered the interaction between PSPs and *M. moricandioides* by bolstering the interaction strength. This study focused on individual plant fitness, but it remains to be explored the isolated and combined effect of each herbivore at population level. Mainly because these two herbivores differ in their effect on seed quality, germination rate and seedling establishment (Chapter 3).

2.X Supplementary material

Supplementary material 2.1. Map of the experimental populations.

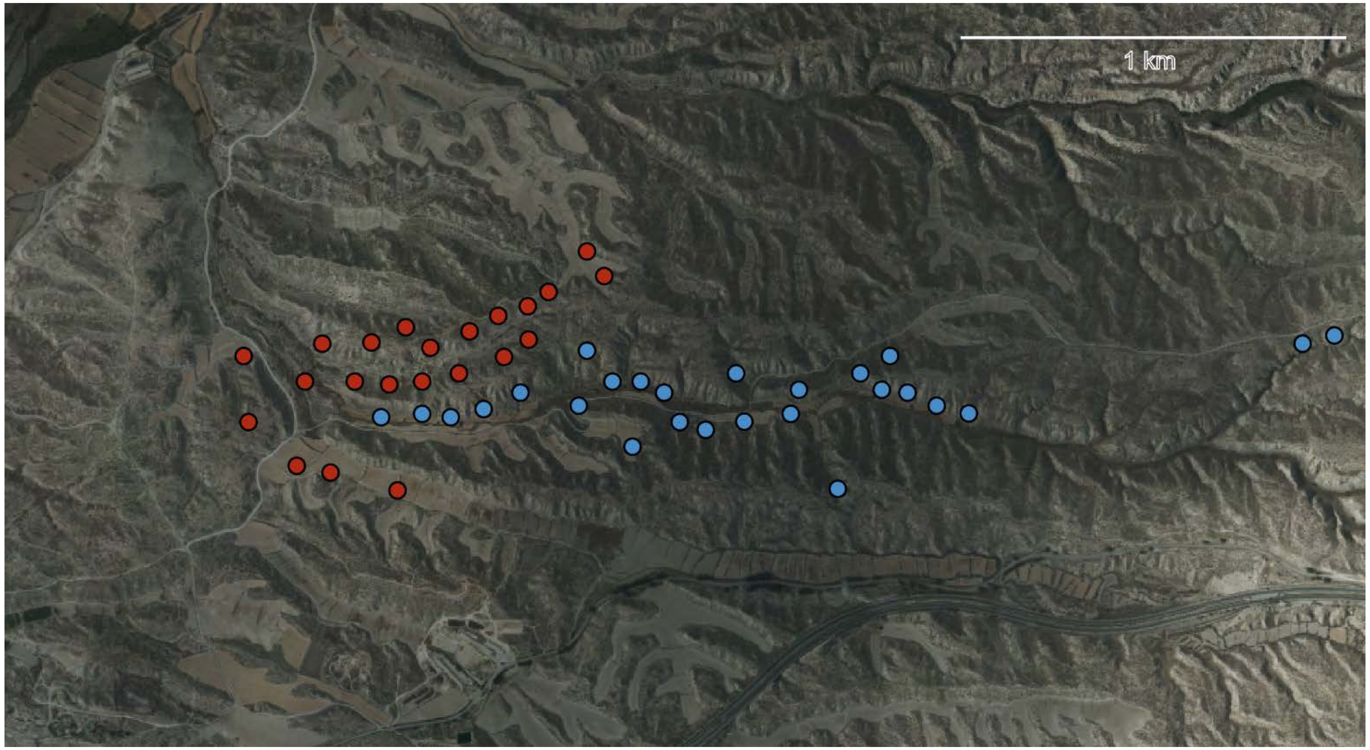


Figure 2.S1. Location map of the experimental populations of *Moricandia moricandioides* in the study area (Barranco del Espartal, geographical coordinates 37° 31´ 12´´ N 2° 42´ 12´´ W). Blue points denote populations excluded from ungulates, red points denote populations exposed to ungulates.

Supplementary material 2.2. Initial full SEM model.

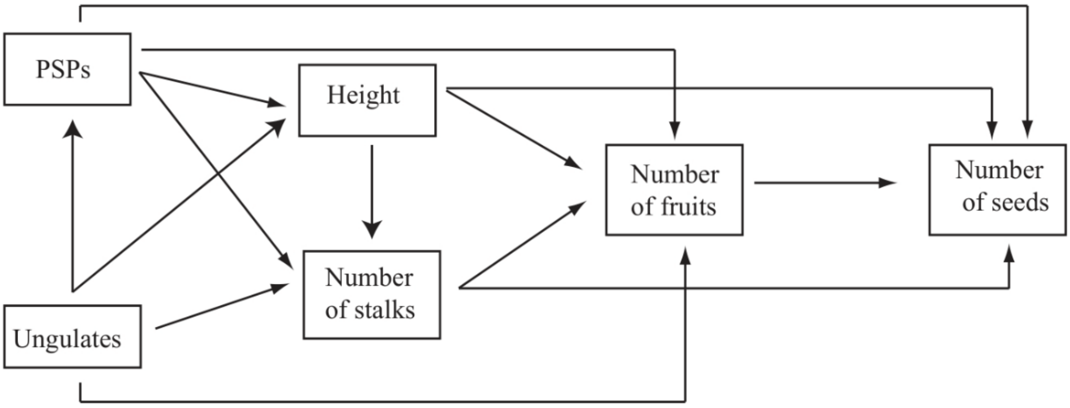


Figure 2.S2. Initial full structural equation model (SEM) to examine pre-dispersal seed predator (PSP) and ungulate effects on plant fitness.

Supplementary material 2.3. Ungulate effects on pre-dispersal seed predators.

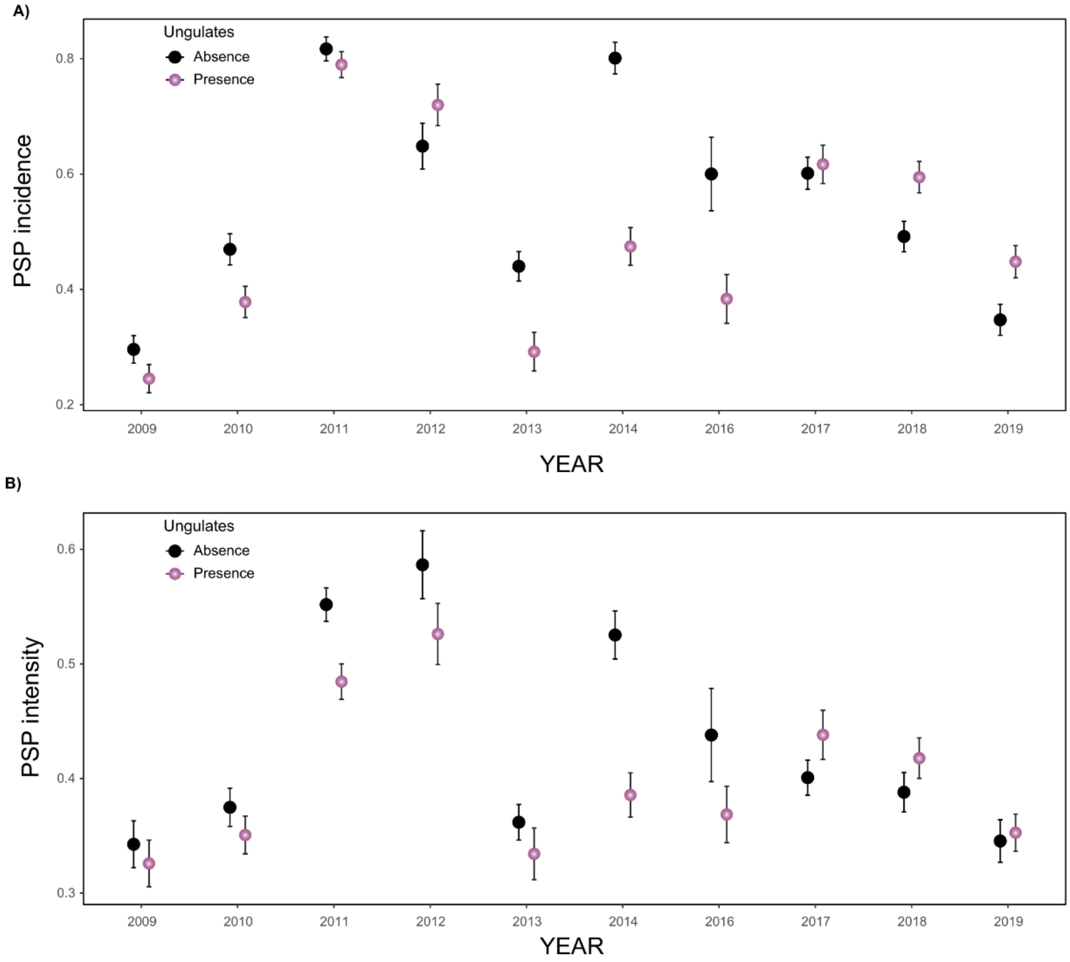


Figure 2.S3. Effect of ungulates on pre-dispersal seed predator (PSP) incidence (A) and intensity (B) each study year. Mean ± SE.

Supplementary material 2.4. Seeds per plant in studied popolations.

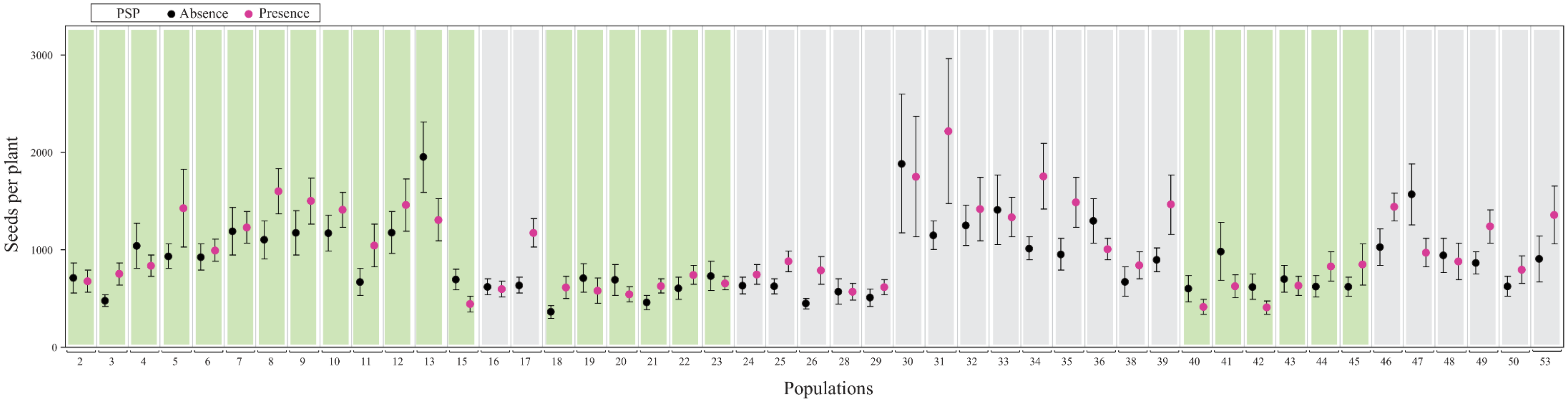


Figure 2.S4. Average seeds per plants with and without pre-dispersal seed predators (PSPs) at each *M. moricandioides* population. Green denotes populations excluded from ungulates, grey denotes populations exposed to ungulates. Mean ± SE.

Supplementary material 2.5. Ungulate effects on plant fitness.

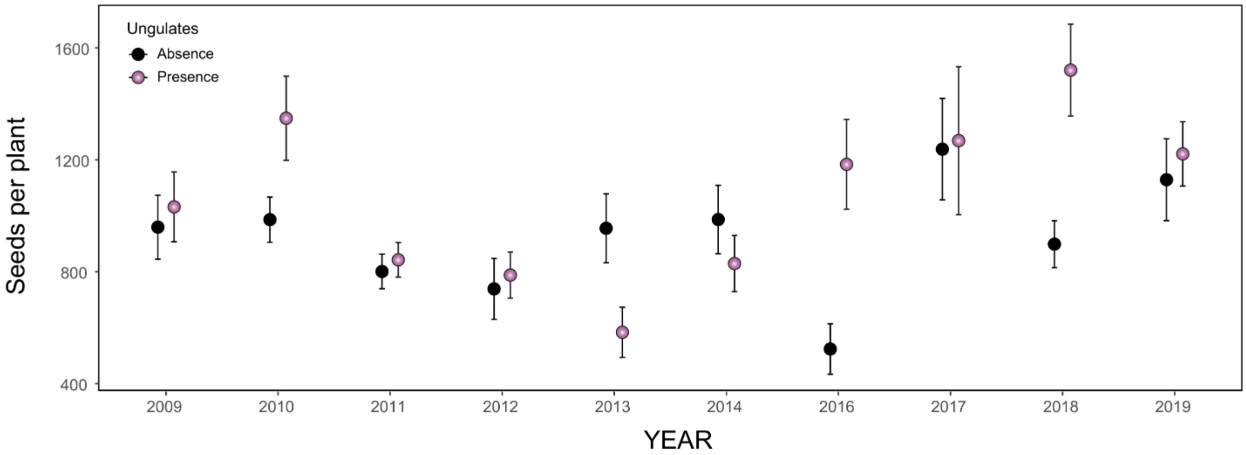
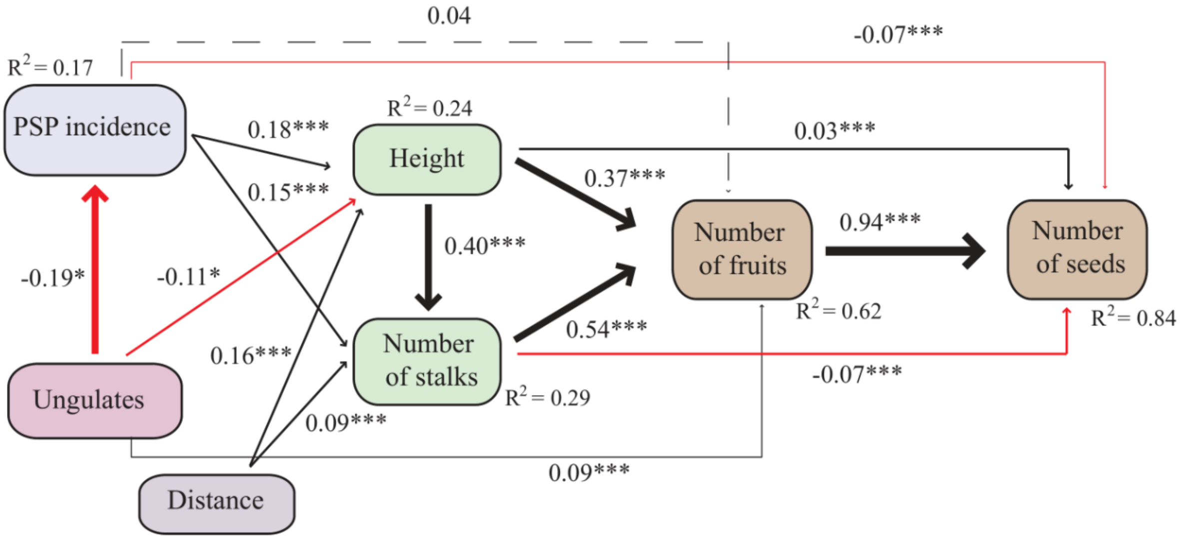


Figure 2.S5. Average seeds per plants produced by plants with and without ungulates each study year. Mean ± SE.

Supplementary material 2.6. SEM for pre-dispersal seed predator (PSP) and ungulate effects on plant fitness.

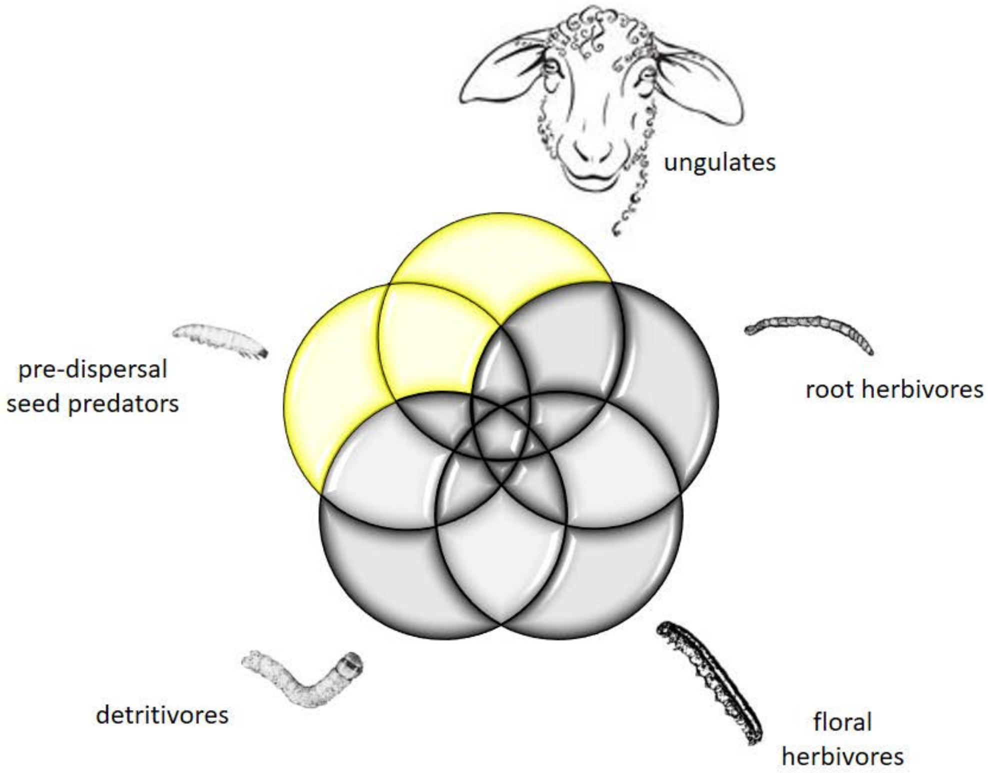


Ficher's $C = 11.53$, $P = 0.17$, AICc = 81.53, BIC = 311.98, k = 35

Figure 2.S6. Structural equation model (SEM) showing the direct and indirect effects of PSPs incidence and ungulates on plant performance (plant height, number of stalks) and fitness (number of fruits and seeds per plant for all populations). Distance refers to the space interval between conspecific *M. moricandioides* plants. Solid line indicates significant paths and dashed line non-significant ones. The red and black arrows denote negative and positive relationship, respectively. The numbers represent the coefficient estimates, and line thickness is proportional to the standardized effect size. R^2 value for each response variable is indicated. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Chapter 3

Transgenerational effects of ungulates and pre-dispersal seed predators on offspring success and resistance to herbivory



3.1 Abstract

Herbivorous mammals and insect pre-dispersal seed predators are two types of herbivores that, despite their functional and morphological differences, tend to severely impact many plant species, highly decreasing their seed production and even imperiling the performance of their offspring through transgenerational effects. However, how they influence offspring resistance to herbivory remains largely unknown. In this study we experimentally examined the effects of ungulates and pre-dispersal seed predators on seed quality as well as on the emergence, survival and resistance to herbivory of the seedlings of a semiarid herb. We found that ungulates reduced seedling recruitment but increased seedling resistance to leaf miners. These effects were probably a consequence of insufficient carbon provisioning in seeds that reduced seed viability and provoked carbon limitation in seedlings. Pre-dispersal seed predators did not influence seedling recruitment, but seedlings from mothers damaged by ungulates and by pre-dispersal seed predators suffered less herbivory by grasshoppers. Remarkably, intra-individual differences in damage by pre-dispersal seed predators affected the rate of damage underwent by seedlings. That is, seedlings derived from fruits attacked by seed predators were more resistant to herbivores than siblings derived from un-attacked fruits in plant populations exposed to ungulates. To our knowledge, this is the first study reporting variation in transgenerational-induced resistance of seedlings from the same maternal plant. This study is a valuable contribution to the understanding of transgenerational effects of multiple herbivores and their implications for a deeper comprehension of the natural systems in which they co-occur.

Keywords

Brassicaceae · Grazer · Herbivorous mammals · Insect herbivory · Seedling recruitment · Within-plant variation

3.2 Introduction

Plants are subjected to varying stresses during most of their life mainly because they are unable to escape by moving to another place. However, they have evolved multiple phenotypic traits enabling them to handle stressful conditions (Crawley 2009). It is well known that not only parental genotypes but also the parental environment determine the expression of traits in plants (Roach and Wulff 1987, Herman and Sultan 2011). When both parental and offspring environments are alike they may attenuate any detrimental effect on fitness caused by the stressful conditions (Roach and Wulff 1987, Herman and Sultan 2011). There is an intense debate trying to elucidate whether changes in the offspring phenotype are merely a response to the allocation of the resources made by the parents (maternal provisioning) or whether they are adaptive transgenerational effects (Herman and Sultan 2011, Uller et al. 2013, Latzel et al. 2014). Regardless of the mechanisms, the parental environment can potentially affect not only plant population dynamics but also evolutionary processes (Herman and Sultan 2011).

Transgenerational responses to the parental environment associated with abiotic stresses (e.g. nutrients, light, temperature) have been recognized for many years (Roach and Wulff 1987). More recently, the role of biotic stresses in altering offspring phenotype has also been highlighted (Agrawal 2001, Steets and Ashman 2010, Herman and Sultan 2011, Holeski et al. 2012, Colicchio 2017). In particular, several studies have shown that herbivores can alter offspring success through transgenerational effects (Agrawal 2001, 2002, Holeski 2007, Rasmann et al. 2012). Herbivore-mediated transgenerational effects can alter offspring phenotypes by affecting traits associated with offspring performance (e.g. height, biomass) or defense (e.g. trichome density, chemical defense) (Agrawal 2002, Holeski 2007, Holeski et al. 2012, Rasmann et al. 2012, Ballhorn et al. 2016, Colicchio 2017, Kellenberger et al. 2018). These biotic-mediated transgenerational effects can help offspring to better cope with herbivores, and consequently increase its fitness (Agrawal 2001, 2002, Holeski 2007, Rasmann et al. 2012). Therefore, the quantification of transgenerational effects of herbivory may provide a better understanding of the complex impact of herbivores on plant fitness (Ehrlén 2003).

During their lifespan plants are usually subjected to the attack of numerous herbivores. Herbivore effects on plants vary according to herbivore characteristics such as their specialization degree, size, or the part of the plant consumed (Karban and Baldwin 1997, Maron 1998, Maron and Crone 2006). Large herbivores such as mammals are important disturbance agents in most terrestrial ecosystems both by their direct and indirect impacts on plants (Huntly 1991). They alter nutrient cycling, modulate succession and vegetation dynamics and can generate spatial heterogeneity (Hobbs 1996, Horsley et al. 2003). Herbivorous mammals impact plants mostly by trampling and/or consuming them (Huntly 1991). These impacts entail a significant decrease in the number of seeds produced by damaged plants. Additionally, mammalian herbivores may diminish the quality of the seeds produced by the attacked plants, greatly lessening their germination, emergence and establishment (Tiffin 2000, Lecomte et al. 2016, 2017, Tadey and Souto 2016). Seed quality is related to the resource investment into the seeds by the maternal plant, and for many species it is critical for germination and seedling survival (Obeso 1993, Agrawal 2001, 2002, Steets and Ashman 2010, Zas et al. 2013).

Herbivore mammals also have important indirect effects on plants by consuming herbivorous insects associated with the plant (Gómez and González-Megías 2002, 2007b, Gish et al. 2017) or by disrupting the interaction between plants and their mutualists (Gómez 2003, Sakata and Yamasaki 2015). The interaction between herbivore mammals and phytophagous insects is generally asymmetric, due to their huge differences in size (Gómez and González-Megías 2002, 2007a). Herbivore mammals especially affect endophytic insects, including pre-dispersal seed predators (PSPs) (Gómez and González-Megías 2007b, Takagi and Miyashita 2014). PSPs are important components of ecological communities and usually have negative effects on plants by reducing seed production, seed germination and seedling establishment (Janzen 1971, Moles et al. 2003, Kolb et al. 2007). However, there are exceptions with some pre-dispersal seed predators having neutral effects (see Kolb et al. 2007) or even increasing host plant seed production (Chapter 1). Interestingly, plants often exhibit within-individual phenotypic variation in organs such as reproductive tissues, with seeds being one of the most variable structures (Herrera 2009, 2017). This can have important implications for the interactions between plants and PSPs. In fact, the action of PSPs may alter resource allocation processes within the plant and promote a local induction of defenses, and therefore enhance within-plant variability in seed attributes and entail significant transgenerational consequences.

Although there are some studies examining the effects of mammals and PSPs on seed quality and their consequences on seed germination and seedling survival, none than we are aware of go a bit further and examine the effect of both herbivores on offspring resistance to herbivory. In *Moricandia moricandioides*, a predominantly semelparous Brassicaceae herb inhabiting dryland environments in the Iberian Peninsula, PSPs induce a compensatory response by enhancing plant height and flower production which results in overcompensation in seed number (Chapter 1). In a 7-year study, we found that ungulates negatively affected *M. moricandioides* populations by reducing plant density and increasing the variability in population size among years. Similarly, ungulates decreased *M. moricandioides* fitness by reducing PSPs attack rate (Chapter 2). In the present study, we examined the effects of long-term ungulate pressure and of PSPs on seed quality and seedling emergence and survival. We hypothesized negative effects of both herbivores in line with a previous study on *M. moricandioides* that revealed that seeds derived from plants undergoing root and floral herbivory were of lower quality and performed worse during early establishment (González-Megías 2016). We then determined whether the exposure to both herbivore types by parental plants increased the resistance of the offspring to the damage by insect herbivores. We predicted distinct outcomes in response to both herbivore types due to their specialization degree (generalist versus specialist), size (the magnitude of damage and the part of the plant consumed), and net effect on the plant (negative for ungulates and positive by PSPs). Finally, because phenotypic difference can be found even between individual parts of single plant individual, we determined whether maternally-mediated effects occurred within plants by comparing the fate of seeds and siblings from fruit with and without PSPs, and whether these differences are influenced by the exposure of maternal plants to ungulates.

3.3 Materials and methods

Study area

The experimental study area is located at the Barranco del Espartal, a seasonal watercourse in the arid Guadix-Baza Basin (Granada Province, southeastern Spain). The climate at the study area is continental Mediterranean with strong temperature fluctuations (ranging from -14°C to up to 45°C) and high seasonality (hot summers, cold winters). Annual precipitation rarely exceeds 300 mm and potential evapotranspiration is 3–4 times higher than precipitation, severely conditioning vegetation diversity and cover (Sánchez-Piñero 2007). The soil is characterized by a sandy–loam texture, high pH, low water-retention capacity and high salinity. The vegetation is an arid open shrub-steppe dominated by *Artemisia herba-alba* Asso, *A. barrelieri* Bess. (Asteraceae), *Salsola oppositifolia* Desf. (Amaranthaceae), *Stipa tenacissima* L. (Poaceae), *Retama sphaerocarpa* L. (Fabaceae), *Ononis tridentata* L. (Fabaceae) and *Lygeum spartum* L. (Poaceae) (Sánchez-Piñero 2007).

The study area comprises private and governmental land and suffered different human pressure: some of the properties are used exclusively for hunting (mainly rabbits, hares and partridges) and grazing by domestic ungulates has not been permitted for at least the last 25 years. The rest of the area is open to be used by domestic ungulates (mainly sheep) and numbers has varied from 500 to 700 sheep (50 or more years ago) to around 100 sheep (since 2011 to today). In the areas to which they have access, ungulates cause a pronounced impact on annual and short-lived plant populations (Gómez 1996).

Model system

The short-lived Brassicaceae species *Moricandia moricandioides* (Boiss.) Heywood is highly abundant in the study area and was used as a model system. *Moricandia moricandioides* plants grow as a vegetative rosette during winter, and produce reproductive stalks during late spring (Gómez 1996). Most individuals reproduce only once, but few individuals are able to resprout the next season and reproduce more than one year (less than 10% of the population). As a Brassicales, *M. moricandioides* is provided with potent chemical defenses, with the glucosinolate-myrosinase system being probably the most important defense (Halkier and Gershenzon 2006). In particular, seeds are expected to be one of the most well defended tissues, due to their value in terms of fitness (Meldau et al. 2012).

The pre-dispersal seed predator *Crossobela trinotella* (Herrich-Schaffer, 1856; Lepidoptera, Gelechiidae) is among the most abundant herbivores of *M. moricandioides*. The attack rate of *C. trinotella* fluctuates between years and among populations (Chapter 2). This Brassicaceae specialist oviposits on flowers or immature fruits, and the caterpillar develops inside the fruits feeding on seeds (Li and Sattler 2012). There can be more than one caterpillar per fruit, each of them eating 8–12 seeds (fruits have usually 20–60 seeds).

Experimental sep-up and data collection

We selected 12 populations of *M. moricandioides* in the study area (Suppl. 3.1, Fig. 3.S1); six populations located in areas with ungulate presence (UNG+), and six populations in areas inaccessible for ungulates (UNG-). As populations, we refer to patches of *M. moricandioides* isolated from other M. moricandioides patches or individuals for at least 100 m.

During July-August 2013, we selected 15 random plants from each of the 12 experimental populations. We counted the total number of fruits per plant in the field and collected five fruits of each of the 15 plants per population (900 fruits in total). Once in the lab and during autumn, we recorded the presence (PSP+) or absence (PSP-) of the pre-dispersal seed predator at both plant and fruit level (see Suppl. 3.2, Fig. 3.S2 flow diagram). The percentage of fruits with seed predators found during the study year was 13% for the surveyed populations.

Crossobela trinotella was the main seed predator found in the fruits (84% of the PSPs), followed by an unidentified curculionid. Only in three fruits (0.33% of fruits) both PSP species were present together. Due to the predominance of *C. trinotella*, seed predator identity was not considered in the following analyses.

Seed quality

Seed quality was measured as seed mass, C and N contents and the concentrations of chemical defenses (glucosinolates) in the seed. Each of these attributes was estimated in a subset of plants (Suppl. 3.2, Fig. 3.S2). We weighed groups of 10 seeds from each fruit to estimate the mean weight of a seed for each fruit (with a 0.01 mg accuracy Sartorius Cubis MSE-125P precision balance). Mean seed weight from 209 fruits from 62 plants that belonged to populations with and without ungulates, and plants with and without PSPs was estimated. Fruit sample size across treatments varied between plants (see sample sizes in Suppl. 3.2, Fig. 3.S2) and at within-plants (fruits with and without PSPs from the same plant; Suppl. 3.2, Fig. 3.S2).

Carbon and nitrogen contents in seeds were measured at fruit level for another set of seeds in five UNG+ and five UNG- populations of *M. moricandioides*. A pool of ~15 seeds from each of 197 fruits from 64 plants were analyzed (range 1–5 fruits per plant), most of the plants being the same than those used for assessing seedling emergence (see below). Fruit selection was done to ensure a minimum of 10 fruits per treatment (see between-plant and within-plant sample sizes in Suppl. 3.2, Fig. 3.S2). Carbon and nitrogen contents in seeds were measured using a CHN Elemental Analyzer (Thermo Scientific Flash 2000) and the C/N ratio calculated.

Glucosinolate concentrations were quantified for another a set of seeds from five UNG+ and six UNG- populations of *M. moricandioides*. For glucosinolate concentrations at between-plant level, a pool of 20–25 seeds from all collected fruits of 99 plants was analyzed, many of the plants being the same than those used for assessing seedling emergence rate, and with a varying sample size across treatments (Suppl. 3.2, Fig. 3.S2). For glucosinolate concentrations in fruits within the same plant, a pool of 20–25 seeds from all fruits without PSPs was compared to a pool of 20–25 seeds from all fruits with PSPs from the same plant. Due to limitations in seed number, this analysis was restricted to 19 plants from 6 UNG- populations. Seeds were ground and extracted three times in 80% methanol, adding p-hydroxybenzyl glucosinolate (sinalbin) as an internal standard at the first extraction. Glucosinolate extraction and conversion to desulfoglucosinolates as well as measurement by high performance liquid chromatography coupled to a diode-array detector (1200 Series, Agilent Technologies) were done as previously described (González-Megías and Müller 2010). Desulfoglucosinolates were identified by comparison of UV-spectra and retention times to those identified in earlier studies (González-Megías and Müller 2010). Peaks were integrated at 229 nm and response factors of 1 for aliphatic glucosinolates and 0.26 for indolic glucosinolates were considered and related to the internal standard (response factor 0.5) and sample dry weight for calculation of concentrations.

Seedling emergence

To determine the effects of ungulates and PSPs on seedling emergence rate, ten *M. moricandioides* populations were selected (five UNG+ and five UNG-). In each population, up to eight plants were selected according to the presence of PSPs (four PSP+ and four PSP-). For each plant, we selected up to 25 intact seeds (range 8–25) from each of the five fruits collected from the field (Suppl. 3.2, Fig. 3.S2). A total of 6317 seeds were planted in black peat moss between 10 and 12-Dec-2013, in 34 seedbeds at a greenhouse with natural temperature and photoperiod conditions. Seedbeds were rotated in the same direction every other day to avoid possible location effects.

Seedling emergence was supervised every other day from the planting day until the end of January 2014 (last emergence was on 24-Jan-2014), when emergence of *M. moricandioides* stopped. Additionally, we calculated emergence time as the time lapse from planted to emerge.

Transgenerational effects of ungulates and pre-dispersal seed predators on seedlings: Field experiments

We carried out two experiments in the field to test transgenerational effects on seedling survival, performance and herbivory on *M. moricandioides*:

Experiment 1: Between-plant level

For this experiment, we selected seedlings belonging to six populations (three UNG+ and three UNG-). Ten seedlings from 6 mother plants (three PSP+ and three PSP-) per population were randomly positioned in 5 blocks in the study area (72 seedlings x block: 360 seedlings; Suppl. 3.2, Fig. 3.S2).

Experiment 2: Within-plant level

Following the same methodology as in experiment 1, we selected seedlings belonging to mother plants with PSPs from 8 populations (four UNG+ and four UNG-). From each plant (6 plants from UNG+ and 5 from UNG- populations), we selected 10 seedlings derived from fruits with PSPs and other 10 seedlings derived from fruits without PSPs. A total of 220 seedlings were randomly positioned in each of 5 designed blocks (44 seedlings x block; Suppl. 3.2, Fig. 3.S2).

Seedlings for both experiments were planted in the field between 12 and 14-Feb-2014. All selected seedlings had two young leaves when planted. During the experiment (from 17-Feb-2014 to 27-Apr-2014), the number of leaves attacked by herbivores, the identity of the herbivore, and plant survival were noted three times per week. The number of leaves per plant was counted once a week. Herbivory on seedlings corresponded almost exclusively to three herbivorous insect guilds, which were differentiated due to their distinguishable damage on leaves: generalist chewers (predominantly acridid grasshoppers, Acrididae, Orthoptera), specialist chewers (Phyllotreta spp., Chrysomelidae, Coleoptera) and leaf miners (Diptera). Herbivory was analyzed as the total number of leaves attacked by all herbivores, and by each individual guild. The number of attacked leaves was monitored rather than the amount of leaf consumed because leaves were too small, and because grasshopper and chrysomelid attacks on leaves many times implied the total or almost total consumption of leaves.

Statistical analyses

Effects of ungulates and pre-dispersal seed predators on seed quality and seedling emergence rate: Between-plant level

We analyzed the effects of both herbivore types (ungulates and PSPs) and their interaction on seed traits and seedling emergence. Seed quality traits (mass, carbon and nitrogen content, C/N ratio, and aliphatic and indolic glucosinolate concentrations) were analyzed using linear mixed models (LMMs) with REML-based estimations. Glucosinolate concentrations were log (x+1) transformed. Because both maternal lineage and population origin could influence the traits of interest, several models with different random structures were tested for all these variables to control for origin (Suppl. 3.3, Table 3.S1). Plant identity was always included as random factor, with the exception of glucosinolate concentrations because we had a single measure per plant.

Seedling emergence rate and time were analyzed using generalized linear mixed models (GLMMs), emergence rate with a binomial distribution with logit link function and emergence time with Poisson distribution with log link function. Several models with different random structures were also tested for these variables (Suppl. 3.3, Table 3.S1).

Effects of ungulates and pre-dispersal seed predators on seed quality and seedling emergence rate: Within-plant level

Within-plant level effects on seed traits and seedling emergence were estimated from the subsample of plants with PSPs from both UNG+ and UNG- populations, comparing fruits with and without PSPs from the same plants. Ungulate presence/absence was included as a factor in all analyses to determine whether ungulates affect the potential within-plant effect of PSPs. We were interested in the single effect of PSPs and the interactive effect of the two types of herbivores rather than in the single effect of ungulates (which cannot be tested within the same plant). However, to avoid confounding the readers, when the single effect of ungulates was significant in our analysis, it is indicated in the result section. Several models with different random structures were also tested for these variables with fruit nested within plant (Suppl. 3.3, Table 3.S1).

Transgenerational effects of ungulates and pre-dispersal seed predators on seedlings: Between-plant level

LMMs and GLMMs were also used to determine the effects of ungulates, PSPs and their interaction on variables from the between-plant level field experiment (Field Experiment 1). Maternal plant identity was always included as random factor to control for origin, and block was tested for all variables as a random factor and included when it contributed to model improvement (Suppl. 3.3, Table 3.S1). Seedling leaf production was analyzed with Gaussian distribution, and seedling survival rate with a binomial distribution. In the case of herbivore-related variables, zero-truncated Poisson and zero-inflated Poisson models with log link function were performed (Suppl. 3.3, Table 3.S1).

Transgenerational effects of ungulates and pre-dispersal seed predators on seedlings: Within-plant level

Within-plant level effects in offspring were estimated from the selected mother plants with PSPs from both UNG+ and UNG- populations for the within-plant level field experiment (Field Experiment 2), comparing siblings from fruits with and without PSPs. Maternal ungulate presence/absence was included as factor, maternal plant identity was included as random factor, fruit of origin was nested within plant and block was tested as a random factor for all variables (Suppl. 3.3, Tables 3.S1-2). Seedling leaf production, seedling survival rate and herbivore-related variables were analyzed with the same distributions described for between-plant level effects.

All models for each variable from the above sections were compared by three Information Criteria (IC), Akaike's Information Criterion (AIC), small sample size corrected Akaike's Information Criterion (AICc) and Bayesian Information Criterion (BIC), and weighed when there were >2 models (Suppl. 3.3, Tables 3.S1-2). ICs values were used for model selection on variables with 2 possible models, in which strictly best models (lowest IC values) were chosen. On variables for which there were >2 possible models, model weighing showed relatively high support for choosing the best model based on lowest IC values. Results were very similar for the three IC values and weights, and on the few cases in which there was divergence between them, BIC was used for model selection, as it tends to favor more parsimonious models (Grueber et al. 2011). All analyses were performed in R 3.1.1 (R Core Team 2014) using *nlme* (Pinheiro et al. 2014) and *lme4* (Bates et al. 2015) packages, and with the *glmmADMB* (Fournier et al. 2012) package in the case of zero-truncated and zero-inflated models.

3.4 Results

Effects of herbivores on seed quality and seedling emergence rate

There was no effect of herbivores on seed mass at neither between-plant level nor within-plant level (Table 3.1). Carbon content in seeds was negatively affected by ungulates at between-plant level and within-plant level (Fig 3.1a-b, Table 3.1). There was no effect of herbivores on nitrogen content and C/N ratio at between-plant level or at within-plant level (Table 3.1).

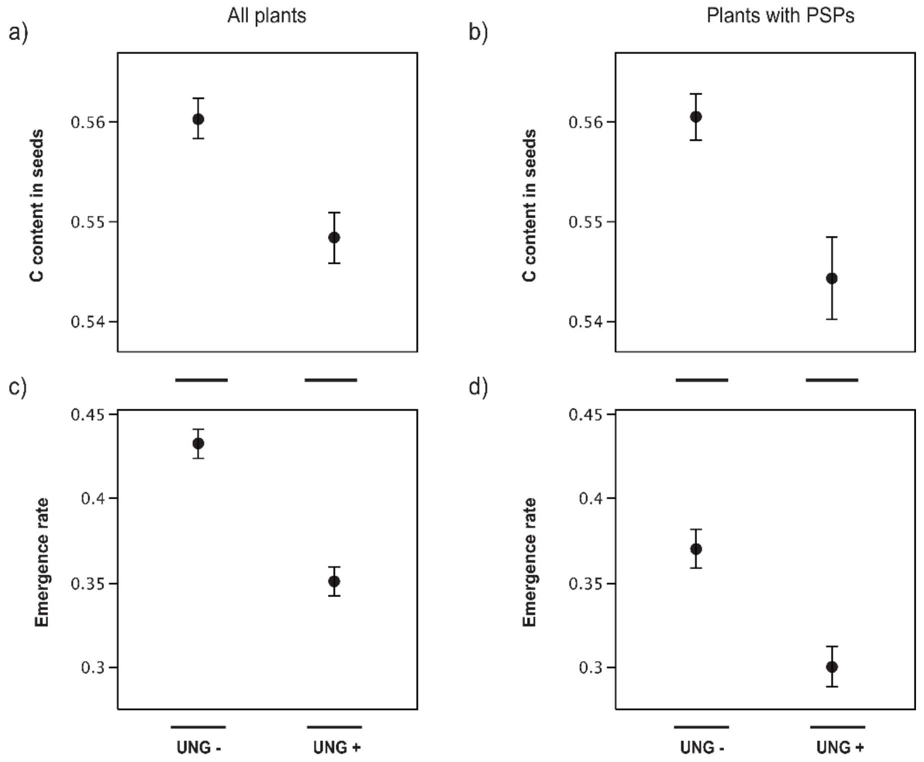


Figure 3.1. Effects of ungulates on seed carbon content and seedling emergence rate. Ungulate effect on seed carbon content of *Moricandia moricandioides* at between-plant (a) and at within-plant level (b). Ungulate effect on emergence rate at between-plant (c) and at within-plant level (d). Mean ± SE.

There was no effect of ungulates or PSPs on the concentration of total glucosinolates, aliphatic glucosinolates, or indolic glucosinolates in seeds at between-plant level or within-plant level (Table 3.1).

	Ungulate			PSP			Ungulate x PSP		
	F	*P*	df	*F*	*P*	df	*F*	*P*	df
Between-plant level									
Seed mass	0.00	0.97	1,58	0.49	0.48	1,58	1.92	0.17	1,58
Carbon in seeds	**4.90**	**0.031**	1,53	0.20	0.65	1,53	2.20	0.14	1,53
Nitrogen in seeds	0.00	0.97	1,8	0.11	0.74	1,45	1.00	0.32	1,45
C/N ratio in seeds	0.26	0.62	1,8	0.23	0.63	1,45	0.27	0.60	1,45
Aliphatic GLSs in seeds	0.00	0.97	1,95	0.45	0.50	1,95	0.39	0.53	1,95
Indolic GLSs in seeds	2.16	0.14	1,95	0.20	0.65	1,95	1.89	0.17	1,95
Within-plant level									
Seed mass	0.86	0.36	1,28	0.04	0.84	1,17	0.25	0.62	1,17
Carbon in seeds	**8.23**	**0.007**	1,27	0.30	0.59	1,14	0.00	0.97	1,14
Nitrogen in seeds	0.33	0.57	1,27	0.31	0.58	1,14	1.40	0.25	1,14
C/N ratio in seeds	0.09	0.76	1,27	0.15	0.70	1,14	1.43	0.25	1,14
Aliphatic GLSs in seeds	-	-		0.23	0.63	1,6	-	-	-
Indolic GLSs in seeds	-	-		0.16	0.69	1,6	-	-	-

Table 3.1. Ungulate and PSP effects on seed quality and glucosinolate (GLS) concentrations at between-plant and within-plant levels of *Moricandia moricandioides*. Significant effects ($P < 0.05$) are shown in bold.

Ungulates negatively affected emergence rate at between-plant level (Fig 3.1c) and within-plant level (Fig 3.1c, Table 3.2). Emergence time was not affected by ungulates or PSPs at any level (Table 3.2).

	Ungulate			PSP			Ungulate x PSP		
	χ^2	P	df	χ^2	P	df	F/χ^2	P	df
Between-plant level									
Emergence rate	**9.85**	**0.001**	1,57	2.90	0.08	1,57	0.97	0.32	1,57
Emergence time	0.62	0.43	1,56	0.14	0.70	1,56	0.02	0.88	1,56
Within-plant level									
Emergence rate	**4.59**	**0.032**	1,32	0.18	0.67	1,30	1.58	0.20	1,30
Emergence time	0.04	0.84	1,31	0.45	0.51	1,28	2.44	0.13	1,28

Table 3.2. Ungulate and PSP effects on seedling emergence and emergence time at between-plant and within-plant levels of *Moricandia moricandioides*. Significant effects ($P < 0.05$) are shown in bold.

Transgenerational effects of ungulates and pre-dispersal seed predators on seedlings

Experiment 1: Between-plant level

There was no transgenerational effect of ungulates or PSPs on the number of leaves produced per seedling or on seedling survival rate (Table 3.3).

There was no effect of previous generation herbivores on the number of leaves attacked in total or by chrysomelid beetles (Table 3.3). However, there was a negative transgenerational effect of ungulates on the number of leaves attacked by leaf miners (Fig 3.2a, Table 3.3). The number of leaves attacked by grasshoppers was lower in seedlings derived from maternal plants that suffered from both herbivores, as shown by the significant interaction term (Fig 3.2b, Table 3.3).

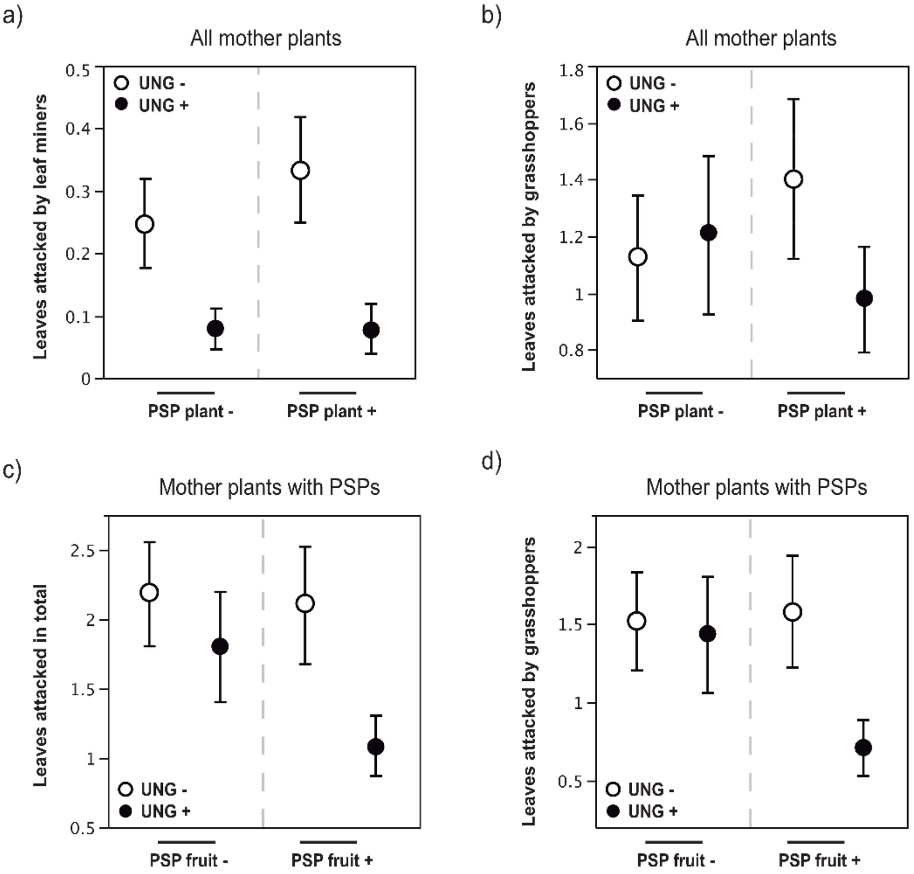


Figure 3.2. Transgenerational effects of ungulates and PSPs on next generation seedling herbivory. a) Transgenerational ungulate and PSP effects on attacked leaves of *Moricandia moricandioides* seedlings by leaf miners at between-plant level (Field Experiment 1). b) Transgenerational ungulate and PSP effects on total number of attacked leaves in seedlings at between-plant level (Field Experiment 1). c) Transgenerational ungulate and PSP effects on total number of attacked leaves in seedlings at within-plant level (Field Experiment 2). d) Transgenerational ungulate and PSP effects on total number of attacked leaves in seedlings by grasshoppers at within-plant level (Field Experiment 2). Mean ± SE.

Experiment 2: Between-plant level

There was no transgenerational effect of herbivores on the number of leaves produced per seedling (Table 3.3), but there was a negative transgenerational effect of ungulates on seedling survival rate in the field (UNG- = 0.64 ± 0.05, UNG+ = 0.45 ± 0.06; Table 3.3).

There was an interactive transgenerational effect of ungulates and PSPs on seedling herbivores (Table 3.3). The total number of leaves attacked by herbivores was lower in seedlings derived from maternal plants under both ungulates and PSPs exposure (Fig 3.2c). A similar but marginally significant transgenerational effect was found for the number of leaves attacked by grasshoppers (Fig 3.2d, Table 3.3). There was no effect of previous generation herbivores on the number of leaves attacked by chrysomelid beetles or leaf miners (Table 3.3).

	Ungulate			**PSP**			**Ungulate x PSP**		
	F/χ^2	P	df	F/χ^2	P	df	F/χ^2	P	df
Between-plant level (Exp. 1)									
Seedling performance									
Survival rate	0.00	0.97	1,32	0.00	0.97	1,32	0.42	0.51	1,32
Produced leaves	0.67	0.41	1,32	0.34	0.56	1,32	1.25	0.27	1,32
Seedling resistance									
Leaves attacked by chrysomelids	0.73	0.39	1,32	2.20	0.13	1,32	0.03	0.86	1,32
Leaves attacked by leaf miners	**4.61**	**0.031**	1,32	0.81	0.36	1,32	0.15	0.69	1,32
Leaves attacked by grasshoppers	0.00	0.97	1,32	1.41	0.23	1,32	**6.19**	**0.012**	1,32
Leaves attacked in total	0.65	0.42	1,32	0.05	0.82	1,32	0.27	0.60	1,32
Within-plant level (Exp. 2)									
Seedling performance									
Survival rate	**3.98**	**0.046**	1,9	0.07	0.79	1,9	0.10	0.75	1,9
Produced leaves	2.12	0.20	1,9	0.04	0.84	1,9	0.00	0.97	1,9
Seedling resistance									
Leaves attacked by chrysomelids	0.22	0.63	1,9	0.65	0.42	1,9	2.03	0.15	1,9
Leaves attacked by leaf miners	2.06	0.15	1,9	0.58	0.44	1,9	1.05	0.30	1,9
Leaves attacked by grasshoppers	0.03	0.86	1,9	0.03	0.86	1,9	3.51	0.06	1,9
Leaves attacked in total	1.65	0.19	1,9	2.66	0.10	1,9	**5.86**	**0.015**	1,9

Table 3.3. Ungulate and PSP effects on seedling performance and herbivory (seedling resistance) at between-plant and within-plant levels of *Moricandia moricandioides*. F is shown for produced leaves, χ^2 is shown for the rest. Significant effects ($P < 0.05$) are shown in bold.

3.5 Discussion

Effects of herbivores on seed quality and seedling emergence rate

The present study supports the idea that the maternal biotic environment has important consequences for the offspring. In particular, ungulates had strong and significant negative effects on *M. moricandioides* offspring. Ungulates effects on the mother plants significantly reduced the emergence rate of their seedlings in greenhouse conditions. This transgenerational effect of ungulates on emergence rate has been previously reported for other herbs and shrubs (Tiffin 2000, Baskin and Baskin 2014). We presume that this effect may even be underestimated, as any effects of herbivores on seedling emergence are expected to be much higher under field conditions (González-Megías 2016). Furthermore, damage by ungulates to mother plants also significantly lessened the survival of seedlings in field conditions. Interestingly, this effect was mostly evident in plants attacked also by PSPs, suggesting that PSPs may also impinge some, albeit weak, transgenerational effects. Therefore, our results suggest that ungulates may have much larger long-term effects on *M. moricandioides* population than inferred from their solely effects on seed production. The current study, thus, indicates that an accurate and precise estimate of ungulate effects on plant population dynamics requires not just quantifying their intra-generational impacts but also the transgenerational consequences of the damage. When these two processes act synergistically, the long-term negative effects on plant populations are greatly amplified and cannot be deduced from studies focusing exclusively on intra-generational effects.

Many theoretical and empirical studies have found that the effects of herbivores on plant performance are stronger in short-lived herbs than in perennials (Crawley 1989, Maron 1998). This is logical given that reductions in fecundity for short-lived plants directly translate into a reduction in lifetime fitness, whereas the effects of herbivores on perennial plant fitness are more difficult to determine because perennials can compensate for herbivore damage across years (Maron 1998). Recruitment is crucial for the fitness of short-lived plants, as the majority of plant mortality occurs at this stage (Moles and Leishman 2008). Our results on *M. moricandioides*, in which more than 90% of the individuals reproduce only once, support the evidence that herbivores can strongly influence the performance of short-lived plants.

Ungulates affected seed viability by decreasing seed mass in some arid and semiarid shrubs (Lecomte et al. 2016, Tadey and Souto 2016). In our system, ungulates affected seed quality by reducing the content of carbon in the seeds, but there was no effect in seed mass or nitrogen content. This reduction in carbon content in seeds might explain the lower seed viability, and even the higher mortality of seedlings. Net carbon balance is necessary for plant growth and survival, and modest changes in carbon allocation patterns may have large consequences for seedling emergence and seedling survival (Kitajima and Myers 2008). Indeed, recent studies in *Arabidopsis* reveal that carbon-dependent signaling pathways could be ubiquitous regulators of seed germination and as important as nitrogen content and seed mass in determining germination success (Palenchar et al. 2004, Osuna et al. 2015).

Carbon limitation in seeds could be a cost of resprouting after being grazed or trampled by ungulates, which immediately reduce the photosynthetic capacity and affect the rate of accumulation of water-soluble carbohydrates in the plant (Fulkerson and Donaghy 2001). Ungulate damage does not only lead to tissue loss and thus losses of carbon, but also affects the stored reserves, which mostly consist of carbon resources (Tiffin 2000). Thus, ungulates limit the amount of carbon that could be allocated to seeds, because the resources used for regrowth may translate to fewer resources allocated to reproduction (Suwa and Maherali 2008). Our previous work also showed that floral herbivores alter carbon and nitrogen content in seeds of *M. moricandioides* resulting in a reduction in seedling emergence and establishment (González-Megías 2016).

There was no prominent transgenerational effect of PSPs on *M. moricandioides*. In other systems, pre-dispersal seed predation reduces seedling emergence and recruitment in plant species differing in life cycle (Kolb et al. 2007). On the contrary, *M. moricandioides* plants attacked by PSPs overcompensated increasing seed production (Chapters 1 and 2). Therefore, the lack of any transgenerational effect of PSPs on seeds support the idea that there is a positive net effect of PSPs on *M. moricandioides* and opens an interesting debate about how this presumably antagonistic interaction has evolved to become a "mutualistic" one. This type of interaction is even more difficult to explain in resource-limited environments where both the plant and the insects undergo extreme and unpredictable abiotic conditions.

Effects of ungulates and seed predators on seedling herbivory and performance

Our study demonstrates significant transgenerational and interacting effects of ungulates and PSPs on plants by affecting the herbivory experienced by the offspring of the same host plant. Moreover, the transgenerational effects varied according to the herbivore feeding mode and their specialization degree, which suggests that multiple mechanisms are involved.

There are very few examples in the literature of maternal herbivory affecting the influences of other herbivore guilds on the offspring than the inducer herbivore (see Agrawal 2001). We found that when the parental generation was affected by ungulates, the offspring was less susceptible to leaf miner attack. Host specificity and adaptations to plant defenses tend to be very high in this herbivore guild (Novotny and Basset 2005, Giron et al. 2016), and leaf miners are also quite sensitive to leaf nutrient content and quality (Inbar et al. 2001, De Bruyn et al. 2002, Han et al. 2015). Hence, the ungulate transgenerational effect on leaf miners may be the result of the lower carbon content in seeds that could be inherited in seedlings. Analyses of seedling leaf nutrient content and defenses will be necessary to elucidate the specific mechanism behind our results.

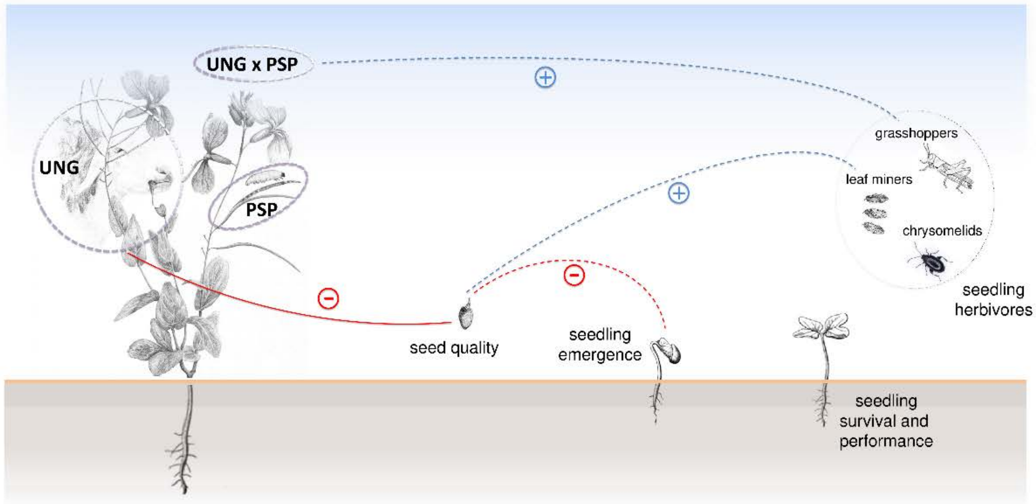
One of the most surprising results of our study is that PSPs and ungulates had an interacting transgenerational effect on *M. moricandioides* seedlings. When maternal plants suffered the pressure of both herbivores (ungulates and PSPs), seedlings increased resistance against generalist herbivores (grasshoppers). The fact that only generalist herbivores but not specialists ones (leaf miners and chrysomelids) were negatively affected by this interaction suggest that the combined impact of ungulate and PSP herbivory on maternal plants triggers a defense response in seedlings, because these are usually more effective against generalist than specialist herbivores (Núñez-Farfán et al. 2007, Hopkins et al. 2009). Several studies show that maternal herbivory can induce physical (trichomes) and chemical defenses (Holeski 2007, Ballhorn et al. 2016, Colicchio 2017).

Transgenerational defense induction can depend on the degree of predictability of future attack (Karasov et al. 2017). According to Agrawal (2002), transgenerational induced resistance to herbivores could be expected in those plants subjected to a non-predictable attack rate (i.e. PSPs). On the contrary, if the herbivore attack rate remains constant over time (i.e. ungulates) a constitutive resistance could have evolved. Transgenerational defense induction to reduce herbivory on offspring would therefore be expected for PSPs but not so clearly for ungulates that remain constant for decades and mainly affect plants by trampling. Indeed, ungulate constant impact could have also favored the development of tolerance that allows survival and reproduction (Tiffin 2000, du Toit and Olff 2014). Other authors suggest that seedlings of stressed plants are able to rapidly induce defenses in response to a stress similar to the one suffered by the parental plants (Beckers and Conrath 2007, Conrath 2011). Seedlings have limited structures required for resource acquisition, and thus, they might rely more on induced rather than constitutive resistance (Boege et al. 2007, Barton 2008). By which features herbivores were deterred in offspring is difficult to elucidate and needs further studies.

Biotic transgenerational effects on seeds and seedlings at within-plant level

Contrary to our prediction, we found no effect in seed traits and seedling performance of PSPs at within-plant level. Within-plant pre-dispersal seed predation effects have been observed in some trees (De Menezes et al. 2010, DeSoto et al. 2016). A higher emergence rate of un-attacked fruit seeds due to nitrogen allocation to these fruits has been reported in *Mimosa bimucronata* (De Menezes et al. 2010). Similarly, higher seed mass and emergence rate of un-attacked cone seeds have been observed in *Juniperus thurifera* (DeSoto et al. 2016). All examples in the literature of within-plant variation on seed quality due to PSP pressure are from long-lived plants. Plant capacity for localized response within the plant may thus depend on life cycle and predictability of seed predator attack rates.

Between-plant level effects



Within-plant level effects (plants with PSPs)

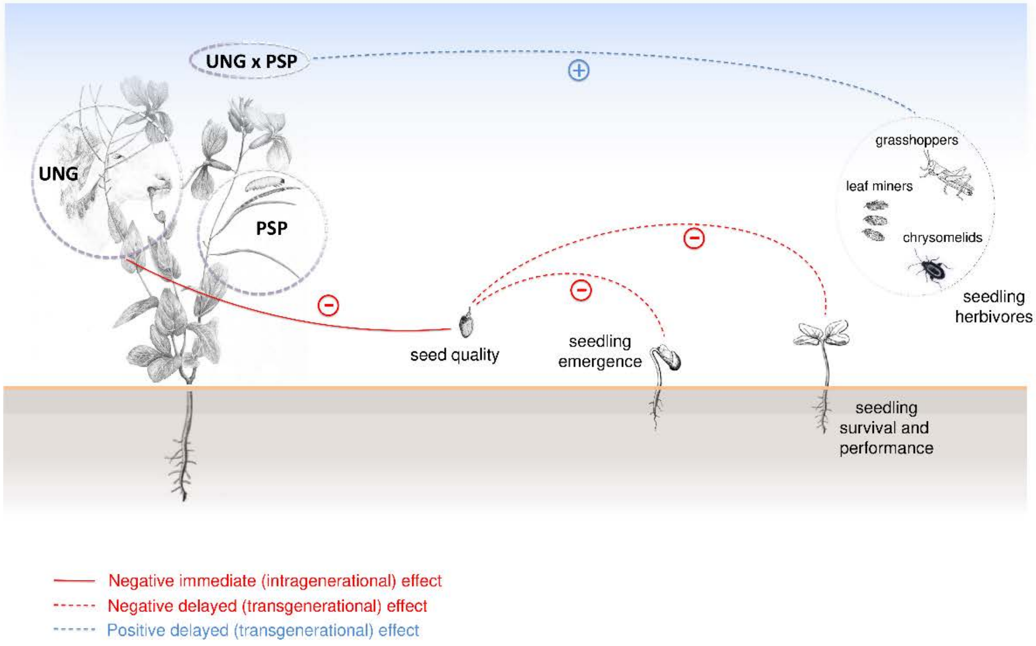


Figure 3.3. Illustrative figure of the observed transgenerational effects of ungulates and PSPs on *Moricandia moricandioides*. At between-plant level, we observed that ungulates reduced seed quality (carbon content) and seedling emergence rate, but these seedlings were more resistant to the attack by leaf miners. In addition, seedlings from mother plants exposed to ungulates and attacked by PSPs suffered less herbivory by grasshoppers. At within-plant level, the negative effect of ungulates was strengthened on plants with PSPs, as ungulates reduced seed quality and seedling emergence rate but also seedling survival rate. We observed variation in transgenerational-induced resistance among siblings, as seedlings from mother plants exposed to ungulates and from fruits attacked by PSPs were more resistant to herbivores than seedlings from the same plants from un-attacked fruits.

The most novel result arising from our study is that there was a within-plant transgenerational response to ungulates and PSPs. While some plant performance traits such as seedling emergence or survival were only affected at plant level, transgenerationally induced responses to herbivores occurred at both plant and within-plant levels, which may be related to within-plant variation in plant defense, i.e. glucosinolate induction in *M. moricandioides*. Defense induction in plants often occurs in the specific tissue damaged, and locally in the specific damaged part (Kessler and Baldwin 2002, Hopkins et al. 2009, van Dam et al. 2009). Regarding within-plant variation, a recent study shows that the patterns of DNA cytosine methylation in leaves are highly variable within individuals, and within-individual variance even surpasses the variance between individuals (Alonso et al. 2018). Through within-individual variation, plants probably better cope with the heterogeneous environment and optimize the exploitation of resources (Herrera 2017). Therefore, within-plant variation in transcriptional responses may be caused by within-plant transgenerational differences in gene expression regulation or defense-inducing hormones (Alonso et al. 2018).

Conclusions

In summary, our results reinforce the idea that the interplay of biotic factors can be especially relevant on plant recruitment. Additionally, this study reveals the crucial importance of biotic maternal environment on the outcome of biotic interactions in resource-limited environments. Two herbivore types, very different in size and feeding strategy, could have independent but also interactive effects on seedling recruitment and herbivore damage, for which seed nutrient provisioning and transgenerational defense induction might be the main mechanisms (see Fig 3.3). This study is in line with other studies in which the complexity of ensemble effects of species interactions were found to be transgenerationally transmitted (Irwin 2006, Gómez 2008, González-Megías 2016). Finally, our results highlight that biotic transgenerational effect occurred not only at plant level but also at within-plant level, with siblings differing on their transgenerational-induced resistance to insect herbivory. This result underlines the need to consider biotic transgenerational effects and the intra-individual variability when studying the interaction between herbivores and plants.

3.X Supplementary material

Supplementary material 3.1. Experimental *Moricandia moricandioides* populations

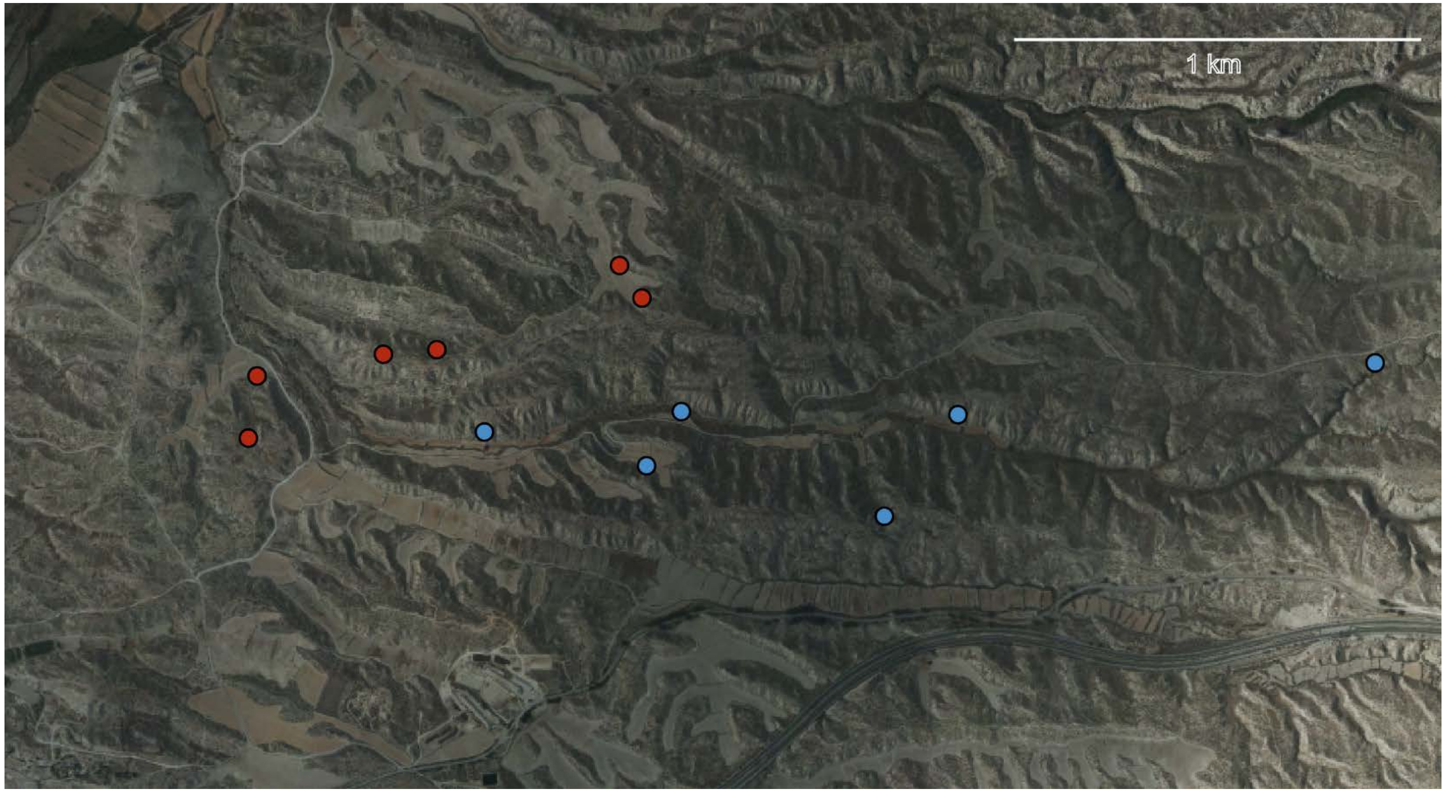
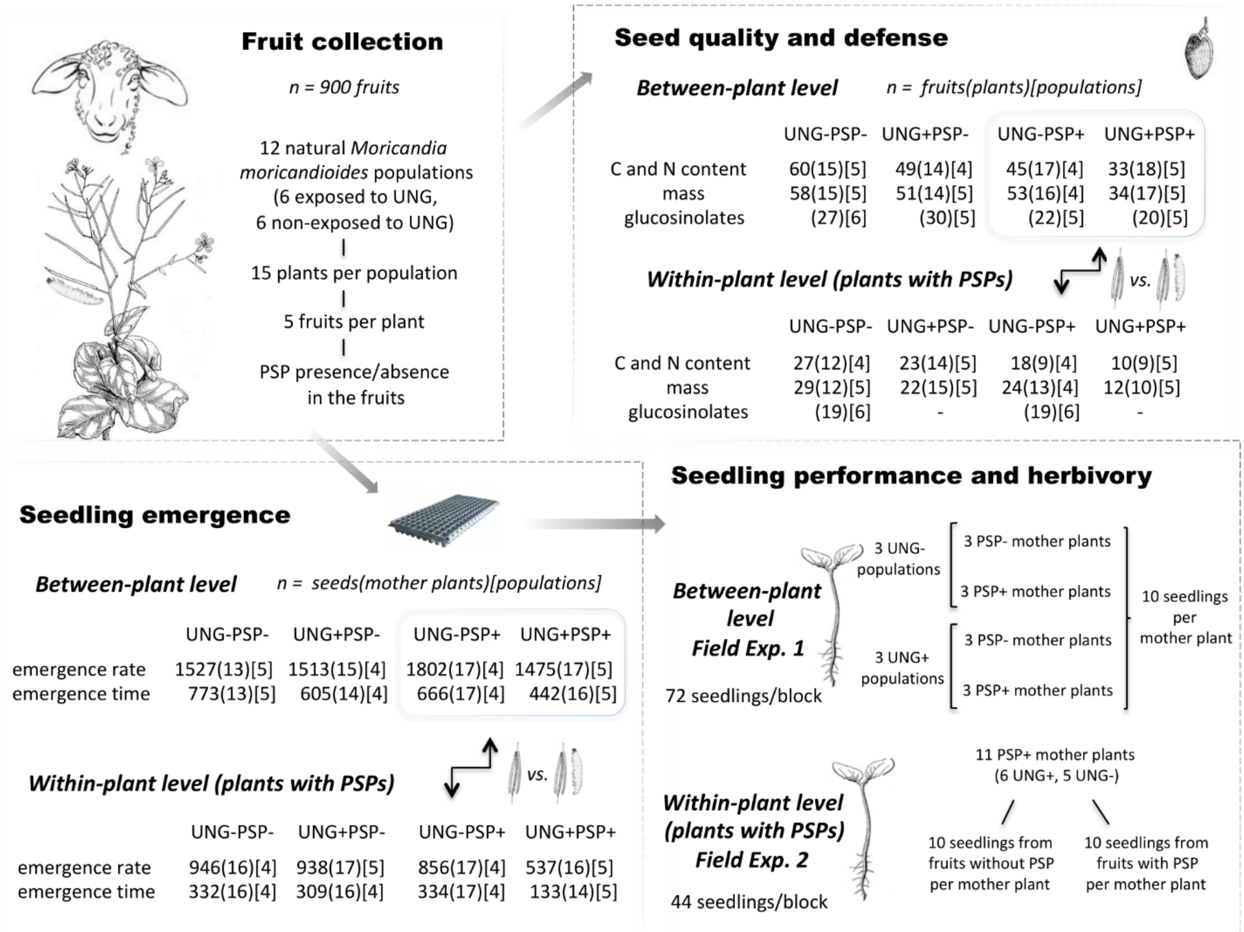


Figure 3.S1. Location map of the twelve experimental populations of *Moricandia moricandioides* in the study area (Barranco del Espartal, geographical coordinates 37° 31´ 12´´ N 2° 42´ 12´´ W). Blue points denote populations excluded from ungulates, red points denote populations exposed to ungulates.

Supplementary material 3.2. Flow diagram of the study



Seed quality and defense – Between-plant level (n = fruits(plants)[populations])

	UNG-PSP-	UNG+PSP-	UNG-PSP+	UNG+PSP+
C and N content	60(15)[5]	49(14)[4]	45(17)[4]	33(18)[5]
mass	58(15)[5]	51(14)[5]	53(16)[4]	34(17)[5]
glucosinolates	(27)[6]	(30)[5]	(22)[5]	(20)[5]

Seed quality and defense – Within-plant level (plants with PSPs)

	UNG-PSP-	UNG+PSP-	UNG-PSP+	UNG+PSP+
C and N content	27(12)[4]	23(14)[5]	18(9)[4]	10(9)[5]
mass	29(12)[5]	22(15)[5]	24(13)[4]	12(10)[5]
glucosinolates	(19)[6]	-	(19)[6]	-

Seedling emergence – Between-plant level (n = seeds(mother plants)[populations])

	UNG-PSP-	UNG+PSP-	UNG-PSP+	UNG+PSP+
emergence rate	1527(13)[5]	1513(15)[4]	1802(17)[4]	1475(17)[5]
emergence time	773(13)[5]	605(14)[4]	666(17)[4]	442(16)[5]

Seedling emergence – Within-plant level (plants with PSPs)

	UNG-PSP-	UNG+PSP-	UNG-PSP+	UNG+PSP+
emergence rate	946(16)[4]	938(17)[5]	856(17)[4]	537(16)[5]
emergence time	332(16)[4]	309(16)[4]	334(17)[4]	133(14)[5]

Figure 3.S2. Experimental design and sample sizes in each step of followed procedure: fruit collection, seed trait measurements, seedling emergence determination and field experiments with seedlings. Between-plant level effects refers to differences between (mother) plants, within-plant level effects refers to differences within each (mother) plant depending on whether they had PSPs in the fruits.

Supplementary material 3.3. Model selection at between-plant and within-plant levels

Both maternal lineage (e.g. within-plant variation is not distributed equally among individuals (Herrera 2017), genetic variation in fitness impacts of herbivory (Strauss and Agrawal 1999), genetic variation in offspring responses to herbivory (Agrawal 2002), genetic variation on epigenetic inducibility and/or phenotypic impact of epigenetic modifications (Holeski et al. 2013)), and population origin (e.g. differences in the strength of abiotic and biotic selection (Colautti et al. 2012), variation across populations in transgenerational phenotypic plasticity (Colicchio 2017)) could influence the traits of interest. Thus, we tested several models with different random structures for seed and seedling variables with the aim to control origin effects.

Plant-level models						
Random structure	**AIC**	**AIC w**	**AICc**	**AICc w**	**BIC**	**BIC w**
Seed mass						
Plant[Population] / Population[Ungulate]	-261.040	0.0259	-260.122	0.0211	-231.090	0.0003
Plant / Population[Ungulate]	-263.040	0.0705	-262.309	0.0630	-236.417	0.0042
Plant[Population]	-265.040	0.1915	-264.475	0.1861	-241.745	0.0609
Plant / Population	-265.040	0.1915	-264.475	0.1861	-241.745	0.0609
Plant	-267.040	0.5206	-266.618	0.5436	-247.073	0.8737
Carbon content in seeds						
Plant[Population] / Population[Ungulate]	840.428	0.0321	841.391	0.0261	869.977	0.0004
Plant / Population[Ungulate]	838.428	0.0873	839.194	0.0782	864.694	0.0059
Plant[Population]	837.060	0.1730	837.652	0.1690	860.042	0.0606
Plant / Population	836.428	0.2373	837.021	0.2310	859.411	0.0830
Plant	835.060	0.4703	835.502	0.4950	854.759	0.8501
Nitrogen content in seeds						
Plant[Population] / Population[Ungulate]	207.725	0.0838	208.687	0.0718	237.273	0.0033
Plant / Population[Ungulate]	205.725	0.2279	206.491	0.2152	231.990	0.0467

Plant[Population]	210.725	0.0185	211.340	0.0191	233.730	0.0196
Plant / Population	203.725	0.6195	204.317	0.6381	226.707	0.6556
Plant	208.746	0.0503	209.189	0.0558	228.446	0.2748
C/N ratio in seeds						
Plant[Population] / Population[Ungulate]	629.088	0.0782	630.051	0.0666	658.637	0.0025
Plant / Population[Ungulate]	627.088	0.2127	627.854	0.1997	653.354	0.0358
Plant[Population]	630.686	0.0352	631.279	0.0360	653.668	0.0306
Plant / Population	625.088	0.5782	625.681	0.5920	648.071	0.5021
Plant	628.686	0.0957	629.128	0.1056	648.385	0.4290
Aliphatics GLS in seeds						
Population[Ungulate]	-160.970	0.0000	-159.740	0	-142.805	0
Population	-162.970	0.0000	-162.057	0	-147.400	0
-	-183.064	0.9999	-182.419	1	-170.088	1
Indolic GLS in seeds						
Population[Ungulate]	-263.826	0	-262.595	0	-245.660	0
Population	-265.826	0	-264.913	0	-250.255	0
-	-295.755	1	-295.110	1	-282.780	1
Total GLS in seeds						
Population[Ungulate]	-161.871	0.0000	-160.640	0	-143.705	0
Population	-163.871	0.0000	-162.958	0	-148.301	0
-	-184.081	0.9999	-183.436	1	-171.106	1
Seedling emergence rate						
Plant[Population] / Population[Ungulate]	7020.923	0.0027	7020.946	0.0027	7074.931	0.0000
Plant / Population[Ungulate]	7018.923	0.0074	7018.941	0.0074	7066.180	0.0005
Plant[Population]	7033.025	0.0000	7033.039	0.0000	7073.531	0.0000
Plant / Population	7016.923	0.0201	7016.936	0.0202	7057.429	0.0412
Plant	7031.025	0.0000	7031.035	0.0000	7064.780	0.0010
Plant[Population] / Population[Ungulate] / Planting day	7016.035	0.0314	7016.064	0.0312	7076.794	0.0000
Plant / Population[Ungulate] / Planting day	7014.035	0.0853	7014.058	0.0850	7068.043	0.0002
Plant[Population] / Planting day	7012.690	0.1671	7012.708	0.1670	7059.947	0.0117

Plant / Population / Planting day	7012.035	0.2318	7012.053	0.2316	7059.292	0.0162
Plant / Planting day	7010.690	0.4542	7010.703	0.4549	7051.196	0.9291
Seedling emergence time						
Plant[Population] / Population[Ungulate]	15256.04	0.0292	15256.10	0.0289	15302.60	0.0001
Plant / Population[Ungulate]	15254.04	0.0794	15254.09	0.0792	15294.78	0.0033
Plant[Population]	15257.40	0.0148	15257.43	0.0149	15292.32	0.0114
Plant / Population	15252.04	0.2158	15252.08	0.2164	15286.96	0.1667
Plant	15255.40	0.0403	15255.43	0.0406	15284.50	0.5709
Plant[Population] / Population[Ungulate] / Planting day	15257.00	0.0181	15257.08	0.0178	15309.38	0.0000
Plant / Population[Ungulate] / Planting day	15255.00	0.0491	15255.06	0.0487	15301.56	0.0001
Plant[Population] / Planting day	15253.34	0.1129	15253.39	0.1126	15294.07	0.0048
Plant / Population / Planting day	15253.00	0.1335	15253.05	0.1332	15293.74	0.0056
Plant / Planting day	15251.34	0.3069	15251.37	0.3078	15286.26	0.2370
Survival rate (Exp. 1)						
Plant / Block	318.719		318.957		342.019	
Plant	316.820		316.990		336.237	
Produced leaves (Exp. 1)						
Plant / Block	1172.423		1172.742		1199.606	
Plant	1170.423		1170.661		1193.722	
Leaves attacked by chrysomelids (Exp. 1; zero-inflated Poisson)						
Plant / Block	438.482		438.801		465.665	
Plant	436.482		436.720		459.781	
Leaves attacked by leaf miners (Exp. 1; zero-inflated Poisson)						
Plant / Block	261.166		261.855		288.242	
Plant	259.168		259.701		282.859	
Leaves attacked by grasshoppers (Exp. 1; zero-inflated Negative binomial)						
Plant / Block	1007.254		1007.665		1038.321	
Plant	1007.446		1007.765		1034.629	
Leaves attacked in total (Exp. 1; zero-truncated Negative binomial)						
Plant / Block	-2804.06		-2803.741		-2776.877	

Plant	-2293.90	-2293.661	-2270.877

Table 3.S1. Model selection al plant level.

Within-plant level models						
Random structure	**AIC**	**AIC w**	**AICc**	**AICc w**	**BIC**	**BIC w**
Seed mass						
Plant[Population] / Population[Ungulate]	-101.061	0.0259	-97.955	0.0140	-72.739	0.0009
Plant / Population[Ungulate]	-103.061	0.0705	-100.503	0.0502	-77.314	0.0085
Plant[Population]	-105.061	0.1915	-102.992	0.1741	-81.889	0.0836
Plant / Population	-105.061	0.1915	-102.992	0.1741	-81.889	0.0836
Plant	-107.061	0.5206	-105.425	0.5876	-86.463	0.8234
Carbon content in seeds						
Plant[Population] / Population[Ungulate]	371.338	0.0649	374.812	0.0368	398.589	0.0041
Plant / Population[Ungulate]	369.338	0.1763	372.195	0.1360	394.111	0.0388
Plant[Population]	371.043	0.0752	373.351	0.0763	393.339	0.0571
Plant / Population	367.338	0.4793	369.646	0.4866	389.634	0.3641
Plant	369.043	0.2044	370.866	0.2644	388.862	0.5358
Nitrogen content in seeds						
Plant[Population] / Population[Ungulate]	133.222	0.0260	136.695	0.0130	160.472	0.0010
Plant / Population[Ungulate]	131.222	0.0707	134.079	0.0482	155.995	0.0093
Plant[Population]	129.229	0.1913	131.537	0.1717	151.525	0.0869
Plant / Population	129.222	0.1921	131.529	0.1724	151.518	0.0873
Plant	127.229	0.5200	129.052	0.5947	147.048	0.8155
C/N ratio in seeds						
Plant[Population] / Population[Ungulate]	314.001	0.0265	317.475	0.0133	341.252	0.0010
Plant / Population[Ungulate]	312.001	0.0721	314.858	0.0492	336.775	0.0095
Plant[Population]	310.066	0.1897	312.374	0.1705	332.362	0.0867
Plant / Population	310.001	0.1960	312.309	0.1762	332.297	0.0895
Plant	308.066	0.5157	309.889	0.5908	327.885	0.8132
Aliphatics GLS in seeds						
Plant[Population]	-52.750		-50.875		-44.562	

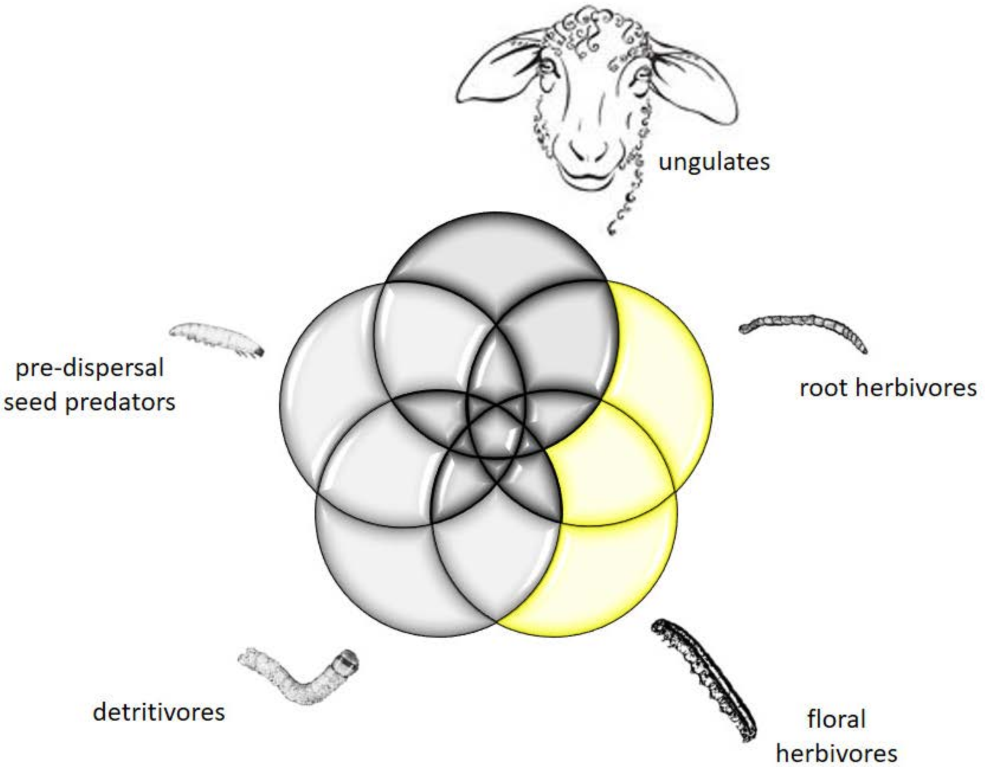
Plant	-54.750		-53.537		-48.199	
Indolic GLS in seeds						
Plant[Population]	-152.941		-151.066		-144.753	
Plant	-154.941		-153.729		-148.391	
Total GLS in seeds						
Plant[Population]	-52.939		-51.064		-44.751	
Plant	-54.939		-53.727		-48.389	
Seedling emergence rate						
Plant[Population] / Population[Ungulate]	3510.000	0.0006	3510.067	0.0006	3570.947	0.0000
Plant / Population[Ungulate]	3508.000	0.0017	3508.055	0.0017	3562.852	0.0002
Plant[Population]	3513.912	0.0001	3513.956	0.0001	3562.669	0.0002
Plant / Population	3506.000	0.0048	3506.044	0.0048	3554.758	0.0087
Plant	3511.912	0.0002	3511.946	0.0002	3554.574	0.0096
Plant[Population] / Population[Ungulate] / Planting day	3502.624	0.0257	3502.705	0.0253	3569.665	0.0000
Plant / Population[Ungulate] / Planting day	3500.624	0.0699	3500.691	0.0694	3561.571	0.0003
Plant[Population] / Planting day	3498.624	0.1901	3498.679	0.1897	3553.476	0.0166
Plant / Population / Planting day	3498.624	0.1901	3498.679	0.1897	3553.476	0.0166
Plant / Planting day	3496.624	0.5167	3496.668	0.5185	3545.381	0.9480
Seedling emergence time						
Plant[Population] / Population[Ungulate]	6801.210	0.0036	6801.374	0.0036	6846.303	0.0002
Plant / Population[Ungulate]	6798.755	0.0123	6798.919	0.0122	6843.848	0.0008
Plant[Population]	6797.534	0.0227	6797.665	0.0228	6837.616	0.0175
Plant / Population	6796.755	0.0334	6796.886	0.0337	6836.838	0.0259
Plant	6795.534	0.0616	6795.636	0.0630	6830.606	0.5833
Plant[Population] / Population[Ungulate] / Planting day	6798.020	0.0178	6798.221	0.0173	6848.124	0.0001
Plant / Population[Ungulate] / Planting day	6795.540	0.0614	6795.740	0.0597	6845.643	0.0003
Plant[Population] / Planting day	6793.540	0.1669	6793.704	0.1654	6838.633	0.0105

Plant / Population / Planting day	6793.540	0.1669	6793.704	0.1654	6838.633	0.0105
Plant / Planting day	6791.540	0.4536	6791.671	0.4570	6831.622	0.3509
Survival rate (Exp. 2)						
Plant / Block	304.150		304.839		331.226	
Plant	303.714		304.247		327.405	
Produced leaves (Exp. 2)						
Plant / Block	833.751		834.616		864.211	
Plant	835.084		835.773		862.160	
Leaves attacked by chrysomelids (Exp. 2; zero-inflated Poisson)						
Plant / Block	232.182		233.966		276.180	
Plant	242.256		242.945		269.332	
Leaves attacked by leaf miners (Exp. 2; zero-inflated Poisson)						
Plant / Block	262.104		262.969		292.564	
Plant	260.102		260.791		287.178	
Leaves attacked by grasshoppers (Exp. 2; zero-inflated Negative binomial)						
Plant / Block	658.120		658.985		688.580	
Plant	673.446		673.979		697.137	
Leaves attacked in total (Exp. 2; zero-truncated Negative binomial)						
Plant / Block	-1304.512		-1303.978		-1280.821	
Plant	-973.036		-972.637		-952.729	

Table 3.S2. Model selection al within-plant level.

Chapter 4

Additiveness and density-dependence in simultaneous root and floral herbivory



4.1 Abstract

Plants are often attacked by multiple herbivores, and depend on a precise regulation of responses to cope with a wide range of antagonists. Simultaneous herbivory may occur at different plant compartments, such as below- and aboveground, which may pose an acute threat to plant growth and reproductive output. In particular, plants often face co-occurring root and floral herbivory, but only few studies focus on such interactions. Using a field approach, we investigated the combined effects of root-feeding beetle larvae and floral-feeding pierid caterpillars on defense and reproduction of a semiarid herb. We manipulated the abundance of both herbivore groups in a full factorial density gradient, from absence to high density, focusing thus on density-dependent effects. We found that the fitness impact of both herbivore groups was independent, despite plant responses towards high floral herbivore density depended in a large extent on herbivore density belowground. Root herbivore density limited plant growth and flower production in a non-linear way, but its density-dependent effect on seed production was linear although non-significantly detrimental. Increasing floral herbivore density provoked compensatory investment in reproduction, and this tolerant response was combined with chemical defense induction when also root herbivore density was high. These responses did not enable to diminish the negative impact of high floral herbivore density on seed production, although entailed a non-linear relation between density and damage. Plants may thus prioritize specific trait combinations according to simultaneous herbivore density below- and aboveground to minimize their fitness impact.

Keywords

Brassicaceae · belowground-aboveground · Density-dependent · Defense · Resistance · Tolerance

4.2 Introduction

Herbivory imposes strong selection pressure on plants to replace lost tissues and to prevent subsequent losses in fitness (Marquis 1992, Mauricio and Rausher 1997). In response to herbivory, plants have developed various resistance and tolerance mechanisms, which imply reconfigurations of primary and secondary metabolism (Howe and Jander 2008, Schwachtje and Baldwin 2008). Many times resistance and tolerance are partially expressed together, since allocating resources to both defense strategies can be more than additive (Simms and Rausher 1987, Herms and Mattson 1992, Fornoni et al. 2004, Núñez-Farfán et al. 2007). Nonetheless, under natural conditions plants are simultaneously attacked by multiple herbivores, and plant's ability to resist or tolerate damage by an herbivore may be constrained by the cost of defense and the damage caused by the other herbivores (Strauss and Irwin 2004, Kaplan and Denno 2007, Morris et al. 2007, Strauss 2014, terHorst et al. 2018). However, in the majority of studies the combined effects of herbivore groups on plant fitness have been independent (Hambäck and Beckerman 2003, Stephens et al. 2013). When simultaneous herbivory occur at different plant parts, the effect tends to be additive (Stephens et al. 2013), although this conclusion is mostly based on folivores and suckers as aboveground herbivores (Zvereva and Kozlov 2012, Stephens et al. 2013). From the plant perspective, effects being independent or not mostly depended on the capacity to compensate damage, the plant parts consumed, and the temporal concurrence of herbivory (Stephens et al. 2013). From the herbivore perspective, it mostly depended on its density/intensity, and the strength of the direct or indirect interactions between herbivores (Stephens et al. 2013).

Herbivore density and the interactions among herbivore groups have played a determinant role in plant defense evolution (Stamp 2003). Since tolerance, resistance and attack intensity are linked, a simultaneous investigation of the three parameters is needed to disentangle the relationships among them. Herbivore effects can be density-dependent; that is, effects vary with their density. Examples abound in the case of large herbivorous mammals, in which the non-linear relationship between herbivore density/intensity and plant damage is well established (Bonenfant et al. 2009, Eschtruth and Battles 2009, Koda and Fujita 2011, Vicari et al. 2018, Lesser et al. 2019). Density-dependent effects are also thought to occur in insect herbivory, although in this case the existing studies are scarcer. Some studies have demonstrated the occurrence of density-dependence in insect herbivory not only in single but in dual herbivory scenarios (e.g. Masters 1995, Kroes et al. 2015, Ramirez and Eubanks 2016, Ponzio et al. 2017).

Root and floral herbivory are among the most damaging types of herbivory for plants (McCall and Irwin 2006, Zvereva and Kozlov 2012, Boaventura et al. 2021). Root herbivory can decrease water and nutrient uptake and thus reduce rates of photosynthesis. To deal with root herbivores, plants display diverse resistance and tolerance responses: increased chemical defense, regrowth of lost roots, and nutrient allocation from roots to aboveground tissues (Erb and Lu 2013, Robert et al. 2014, Johnson et al. 2016, Chapter 7). Nevertheless, aboveground biomass removal usually has larger effects on plant fitness than removal of an equivalent percentage of aboveground biomass, being plants rarely able to compensate for the damage caused by root herbivores (Zvereva and Kozlov 2012). Florivory in receiving increasing attention due to its generally negative impact on plant fitness (McCall and Irwin 2006, González-Browne et al. 2016, Boaventura et al. 2021). Plants may be under selection to decrease feeding by floral herbivores and/or mitigate the fitness costs associated with floral damage (Ehrlich and Raven 1964, Frame 2003, McCall and Irwin 2006). Plants may opt for increased chemical defenses against floral herbivores, although the success of this strategy when specialized herbivores are involved may depend on their counteradaptation degree (Siemens and Mitchell-Olds 1996, Núñez-Farfán et al. 2007, Muola et al. 2010). Compensatory responses to florivory by producing more flowers or by shunting resources to future flowers after damage may also occur (Olesen 1992, McCall and Irwin 2006, Wise et al. 2008). Tolerance towards florivory is even more likely than for folivory, because resource sinks (reproductive tissues) instead of resource sources are consumed (McCall and Irwin 2006). Through systemic induction of chemical defenses aboveground (Erb et al. 2009, Johnson et al. 2016), and nutrient allocation to reproductive tissues (Chapter 7), root herbivores can affect aboveground herbivore performance and damage on plants (Johnson et al. 2012, Soler et al. 2012). Less is known about the effects of floral herbivores on herbivores belowground, and about their joint effects on plants. Despite of probably being highly common in nature, (Johnson et al. 2015, Züst and Agrawal 2017), only few studies have investigated the effects of simultaneous root and floral herbivory (González-Megías and Müller 2010, González-Megías 2016). None that we know of has studied it in the framework of density-dependence.

In the wild Brassicaceae species *Moricandia moricandioides,* root herbivory by a single beetle larva and naturally occurring floral herbivory by pierid caterpillars independently affected plant defense and reproduction, but only florivory was detrimental for plant fitness (González-Megías and Müller 2010, González-Megías 2016). However, the effect of these two herbivore groups can be density-dependent (McCall and Irwin 2006, Zvereva and Kozlov 2012). Though, it remains to be disentangled whether herbivore damage, plant tolerance and resistance, and ultimately plant fitness, additively or non-additively vary according to the different density combinations of the two herbivore groups. With this aim, we carried out an experiment in the *M. moricandioides* system, in which the densities of both root herbivores and floral herbivores were manipulated in the field in a density gradient (absence, low or high densities). Several plant traits related to growth, defense and reproduction were measured. Given that caterpillar performance and growth rate determine the damage they cause on plants (Parry et al. 2003, Bukovinszky et al. 2009, Pashalidou et al. 2015a, 2015b, Veyrat et al. 2016), we also evaluated floral herbivore caterpillar development time in the different treatments. We predicted that (i) plant defense responses to both herbivore group densities would be independent, (ii) the impact on plant fitness of both herbivore group densities would be additive, and (iii) the impact on plant fitness of both herbivore groups would linearly rather than non-linearly increase with their density.

4.3 Material and methods

Study system

The experiment was conducted in 2013 at Barranco del Espartal, a semiarid open shrub-steppe located in the arid Guadix-Baza Basin (Granada, southeastern Spain). The climate is distinctively continental, with strong temperature fluctuations (ranging from -14ºC to up to 45ºC) and the high seasonality (hot summers, cold winters). Annual precipitation does not usually exceed 300 mm due to geographical isolation, originated by a chain of mountains.

We used the predominantly semelparous Brassicaceae species *Moricandia moricandioides* (Boiss.) Heywood as a model system, as it is abundant in semarid areas in the Iberian Peninsula such as the study site (Gómez 1996, González-Megías and Müller 2010, González-Megías and Menéndez 2012, Chapters 3 and 7). *Moricandia moricandioides* typically grows in patches, develops as a vegetative rosette during winter, and produces reproductive stalks during spring. The stalks remain photosynthetically active during the entire season (González-Megías and Müller 2010). After having reproduced, the vast majority of individuals die during summer (Chapter 7). As it occurs in Brassicaceae and related families, *M. moricandioides* produces the characteristic secondary defense metabolites named glucosinolates (Mithen et al. 2010).

Several insect herbivores are associated with *M. moricandioides*. The Brassicaceae specialists *Pontia daplidice* L. and *Euchloe crameri* Batler pierid caterpillars are among the most important aboveground herbivores (González-Megías and Müller 2010, Chapter 7). Ambient herbivory by *P. daplidice* and *E. crameri* is high in natural *M. moricandioides* plants in the study area; 1.4 ± 0.1 caterpillars of these species per plant were counted in samplings carried out in the study area from 2008 to 2018. The caterpillars of both pierid species feed on reproductive tissue (floral buds, flowers and immature fruits), have equivalent development times and cause similar type of damage on plants, which sometimes even implies total flower and fruit consumption of the plant (González-Megías 2016).

The most abundant root herbivore is *Cebrio gypsicola* Graells (Coleoptera, Cebrionidae), with an average density of 0.95 ± 0.2 larva/plant on natural *M. moricandioides* individual root samplings (Chapter 7). Both type of herbivores (root and floral herbivores) are capable of altering the production of the main defensive chemical compounds in Brassicaceae, that is glucosinolates (González-Megías and Müller 2010).

Experimental set-up

We manipulated the root herbivore and floral herbivore densities in a full factorial design with two factors (root and floral herbivore densities). The root herbivore (RH) density was manipulated at three levels: control plants with no root herbivores (RH_0, absence), treatments with one root herbivore individual (RH_1, low) and treatments with two root herbivore individuals (RH_2, high). The other factor was the floral herbivore (FH) density, again with three levels; control plants with no floral herbivores (FH_0, absence), treatments with one floral herbivore individual (FH_1, low) and treatments with two floral herbivore individuals (FH_2, high). The chosen density gradients for both root and floral herbivores are realistic based on natural *M. moricandioides* plant samplings in the study area.

We started the set up the experiment on 14-March-2013, when we moved the *M. moricandioides* seedlings to the study site. These plants came from seeds collected from the study area during the autumn of 2012. These seeds were germinated in pots with soil from the study area and grown in a common garden. In the field, we located 108 experimental plats in each of 6 blocks (2 replicates x 9 treatments/block), where plants were 30 cm apart from earch other. None of the plants had a reproductive stem at the moment of being moved to the field. During the first week in the field, and in the absence of natural rain, all plants were watered and net-covered to ensure their establishment.

To set up the RH treatments, plants were re-potted when moved to the field using mixed macroarthropod-free soil from the study site. The pots consisted of fiberglass-mesh cylinders (15 x 20 cm) of 1 mm mesh size to inhibit the entrance or escape of belowground macroinvertebrates. These pots were then buried with the upper surface level with the ground. The reliability of this methodology in recording root herbivory effects has been previously demonstrated in this system, as ~ 90% of the larvae can be recovered from the correspondent pots at the time of plant harvest (González-Megías and Müller 2010). Once the plants were established in the field, third-instar *C. gypsicola* larva/e, collected in the study area during winter 2012-spring 2013, were added to plants assigned to RH_1 and RH_2 treatments (25-March-2013).

To set up the FH treatments, we removed all *P. daplidice* and *E. crameri* eggs from FH_0 plants, but allowed natural oviposition by these species on reproductive stalks of FH_1 and FH_2 plants. In cases where no caterpillars had hatched on FH_1 or FH_2 plants when plants already had reproductive tissues, first instar caterpillars collected from the study area were added. Once FH_1 or FH_2 plants had the designated FH density level, additional pierid eggs laid by butterflies were thereafter removed from these the plants. When caterpillars died or disappeared before completing their larval cycle and moving to pupate, we replaced them by adding same-instar caterpillars collected from the study area to the plants.

Three plants died during the experiment, another 8 did not produce reproductive stalks, and on another 9 plants caterpillars did not remain on plants until completing their entire larval cycle, thus all these plants were excluded from the analyses. The final sample size was 88, and the sample size per treatment was RH_0FH_0 n = 11, RH_0FH_1 n = 11, RH_0FH_2 n = 9, RH_1FH_0 n = 12, RH_1FH_1 n = 10, RH_1FH_2 n = 8, RH_2FH_0 n = 9, RH_2FH_1 n = 10 and RH_2FH_2 n = 8.

Data collection

Plant reproductive traits (number of floral bud groups, flowers and fruits) were recorded on each experimental plant 3 times per week after the set-up of the experiment (from 27-March-2013, two days after the addition of RH larvae) until the end of the experiment (26-July_2013), resulting in a total of 40 surveys. At the end of the experiment, we counted the total number of flowers and fruits produced by each plant. Fruits were collected after complete maturation of seeds but before seed dispersal. All fruits were taken to the laboratory where the number of viable seeds (seeds hereafter) in each fruit was counted to quantify total seed production per plant, which we used as the estimate of fitness.

The entire aboveground tissue was collected and dried at 40°C for 48h to determine aboveground dry biomass and C/N ratio of leaf tissue. C/N ratio was also determined from seeds, in both cases with a CHN Elemental Analyser. Belowground tissue was not collected because we wanted to estimate resprouting rate at the next season. Resprouting rate was low (12.5%) and we observed no differences among treatments (data not shown).

To quantify glucosinolate (GLS) concentrations in leaves, the youngest leaf of one stem of each of the experimental plants was collected at the end of June, when plants had already interacted with both manipulated herbivores for weeks but prior to leaf senescence. Leaves were immediately frozen and freeze-dried. The dried material was ground and extracted three times in 80% methanol after the addition of *p*-hydroxybenzyl GLS (sinalbin) used as an internal standard. GLS extraction and conversion to desulfoglucosinolates were done following previously established methodology using high performance liquid chromatography coupled with a diode array detector (Chapter 7). Desulfoglucosinolates were identified by comparison of UV-spectra and retention times to those identified in earlier studies (González-Megías and Müller 2010, Chapter 7). Peaks were integrated at 229 nm and response factors of 1 for aliphatic and 0.26 for indolic GLSs were considered and related to the internal standard (response factor 0.5) and sample dry mass for calculation of concentrations.

We also calculated caterpillar development time (days) strictly for those caterpillars that completed their entire larval cycle without being replaced on the experimental plants (n = 53 caterpillars on n = 45 plants). When development time of both caterpillars on FH_2 plants could be measured, we used mean value per plant.

Statistical analyses

First, Generalized Estimation Equation models (GEE) were performed to test the effects of each continuous factor (RH and FH densities) and their interaction on plant reproductive development (number of floral bud groups, flowers and fruits) over time. It has been argued that plant reproduction is a hierarchical process with ephemeral structures (e.g. floral buds and flowers) that impede the assessment of florivory impact on plants (Breadmore and Kirk

1998). Considering that plant tolerance responses towards florivory may involve changes in the timing and the amount of reproductive tissue produced, we consider that GEE models which handle dependent observations in the same individual with a fitted correlation structure can help to delve into how the different treatments affected the reproductive process. GEEs, i.e. the marginal modelling approach, is a powerful and pragmatic tool for analyzing a variety of correlated data and can handle non-normal distribution and heteroscedasticity (Halekoh et al. 2006, Pekár and Brabec 2018). GEEs deal with dependencies by allowing correlations in residuals (i.e. repeated measurements made on the same individual in time) through a pre-specified correlation structure, which can be fitted in accordance with the correlation matrix structure (Halekoh et al. 2006, Pekár and Brabec 2018). Hence, GEEs produce high quality parameter estimates with asymptotically correct standard errors, providing correct subsequent inferences for marginal models (Pekár and Brabec 2018).

Due to sequential occurrence in reproductive traits and the ephemerality of some of them, we estimated the different reproductive traits over different time periods: 1) from the first floral bud production until final fruit number for floral bud groups (since initiation of reproduction to when there was no more investment in new reproductive tissue), 2) from the first floral bud production until no floral bud groups were left for flowers (floral buds are needed for flowering) and 3) from the first fruit production until final fruit number for fruits (since when fruiting began to when there were no more flowers that could pass to fruits). The FH density was fitted to the date interval they were present on plants. We tested different distributions and correlation structures for each of the variables. We used the Quasi Information Criterion (QIC) model fit for model selection, as it works well selecting the correlation structure in nonlikelihood-based methods, such as GEE (Pan 2001). Floral bud groups and fruits were modelled with a Poisson distribution, as they tended to decrease in number from the first observations, and an autoregressive model of the 1.St order correlation structure due to strong correlation between following surveys. In an autoregressive model the correlation declines with the distance between observations. Flowers were modelled with a Gaussian distribution and an exchangeable correlation structure, as there was no strong correlation between surveys, probably because of their short lifetime and because flowering could partially depend on the stochasticity of climatic conditions. An exchangeable model has a single correlation parameter identical for all pairs of measurements on the same individual, irrespective of how far in time the measurements are from each other. These analyses were performed using R (R Core Team 2017), with the package *geepack* (Halekoh et al. 2006).

Second, general or generalized linear mixed models (GLMMs) were performed to test the effects of each continuous factor (RH and FH densities) and their interaction on plant morphology (aboveground biomass), quality (nutrient content in leaves and seeds and GLS concentrations in leaves) and reproduction (number of flowers, fruits and seeds). GLMMs were also performed to test the effects of RH and FH densities and their interaction on FH caterpillar development time. Variables were modelled with Gaussian, Gamma or Poisson distributions, and were transformed when necessary (see Suppl. 4.1, Table 4.S1 for best models for each variable in detail). Models with Gamma distribution were analyzed with inverse link function and those with Poisson distribution were analyzed with log link function. Block was included as random factor for all the variables. When overdispersion was observed (Suppl. 4.1, Table 4.S1), GLMMs with observation-level random effects were run, which allowed for variation at plant level (Harrison 2014). Model selection was done according to Bayesian Information Criterion (BIC) model fit, as it tends to favor more parsimonious models (Grueber et al. 2011). These analyses were performed with the package *lme4* (Bates et al. 2015). When factor or interaction-level effects were significant, post hoc tests were performed with the same model structure but with RH and FH densities as categorical (absence, low or high density). As post hoc, we used the *P*-value adjusting multivariate test (see Suppl. 4.1, Table 4.S2 for detailed factor and interaction level post hoc results) with the *lsmeans* package (Lenth 2016). As a complement to linear models, we also performed generalized additive mixed models (GAMMs) to test for additivity in the effects of RH and FH densities on the above-mentioned variables. We used for each variable the same distribution and random structure specified in the GLMMs, being results obtained by GAMMs very similar to those of GLMMs (Suppl. 4.2, Table 4.S3, Fig. 4.S1). These analyses were performed with the package *mgcv* (Wood 2004).

In addition, we performed a permutational multivariate analysis of variance (PERMANOVA) to test whether RH and FH densities affected leaf GLS profiles, which was complemented by non-metric multidimensional scaling (NMDS) to test treatment dissimilarities in GLS profile composition. We used Horn dissimilarity and 10,000 permutations in both analyses for assessing significance, with 100 random starts in the NMDS. The analyses were performed with the *vegan* package (Oksanen et al. 2017).

Lastly, sets of component models were combined within a piecewise structural equation modelling (SEM) framework to parse the direct and indirect effects of RH and FH densities (as continuous) on plant fitness (number of seeds) through FH caterpillar development time (SEM 1) and through plant reproductive components (SEM 2). The SEMs were fitted using the R package *piecewiseSEM* (Lefcheck 2016). These models allow formulating hypotheses on pathways of interaction between parameters in the model, where all parameters could act as both predictor and response variables. As recommended, the SEMs had at least 10 times as many observations as variables (Shipley 2016). Variables were standardized (mean = 0, SD = 1) and we fitted the component models of the piecewise SEM as linear mixed models. For all component models, the random structure was the one used in GLMMs. We started with initial SEMs (whom hypothesized pathways were based on prior GLMM results; Suppl. 4.5, Fig. 4.S4; Suppl. 4.6, Fig. 4.S5) and improved them through the stepwise procedure by evaluating Akaike's information criterion corrected for small sample sizes (AICc) (Lefcheck 2016, Shipley 2016), until we procured the final SEMs with the lowest AICc scores (Suppl. 4.5, Table 4.S5; Suppl. 4.6, Table 4.S7). In *piecewiseSEM,* the optimization procedure is based on the removal of irrelevant paths and the inclusion (based on Shipley's test of d-separation) of any of the non-hypothesized biologically relevant paths that can improve the model (Lefcheck 2016, Shipley 2016).

4.4 Results

Plant reproductive trait development over time (GEEs)

With increasing density of RH, floral bud group and fruit production decreased on *M. moricandioides*, but not flower production (Table 4.1). With increasing density of FH, flower and fruit production was enhanced, but not floral bud group production (Table 4.1). No interactive effects of RH and FH densities on reproductive tissue production were observed (Table 4.1, see Fig. 4.1 for a graphic approximation).

Linear effects (GLMMs)

Plant morphology and reproduction

With increasing density of RH, aboveground biomass decreased (Table 4.2), although post hoc tests revealed only a marginally significant difference between absence of RH and high RH (Fig. 4.2A). FH density significantly affected aboveground biomass (Table 4.2). Plants with high FH had more aboveground biomass than plants with low FH (Fig. 4.2B).

The negative effect of RH on the number of flowers increased with RH density (only significant at high RH, Table 4.2, Fig. 4.2A) and fruits (only a marginally significant difference between absence of RH and high RH, Table 4.2, Fig. 4.2A), but there was no effect on the number of seeds (Table 4.2, Fig. 4.2A). FH density negatively affected the number of flowers and fruits but the effect did not vary between low and high densities (Table 4.2, Fig. 4.2B). The negative FH effect on seed number depended on FH density, and was only significantly lower at high FH (Table 4.2, Fig. 4.2B).

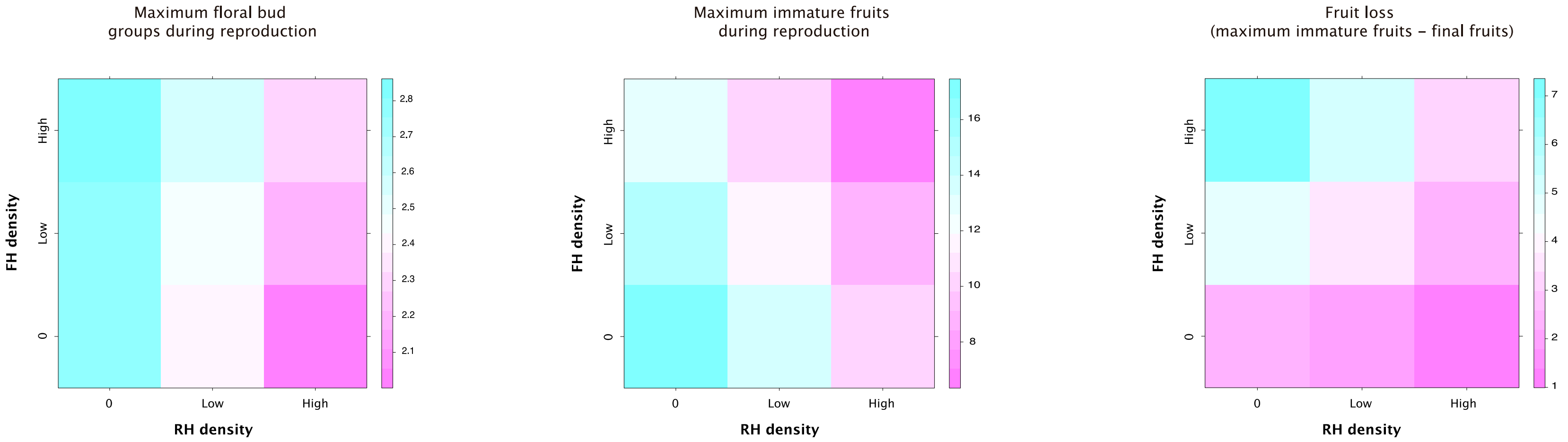


Figure 4.1. Contour plot of root herbivore (RH) and floral herbivore (FH) density effects on mean maximum number of floral bud groups and fruits produced, and on the mean number of immature fruits lost during reproduction.

	RH				**FH**				**RH x FH**			
	χ^2	z	P	df	χ^2	Z	P	df	χ^2	z	P	df
Floral bud production	**4.14**	**-2.05**	**0.04**	1,1704	0.03	0.16	0.86	1,1704	0.12	-0.34	0.73	1,1704
Flower production	0.50	-0.70	0.47	1,1389	**11.00**	**3.31**	**< 0.0001**	1,1389	0.33	0.57	0.56	1,1389
Fruit production	**8.15**	**-2.85**	**0.004**	1,1202	**76.89**	**8.76**	**< 0.0001**	1,1202	2.03	1.42	0.15	1,1202

Table 4.1. Results of generalized estimation equation models (GEEs) for the effect of root herbivores (RH) and floral herbivores (FH) on plant reproductive traits development over time. Significant values ($P < 0.05$) are highlighted in bold.

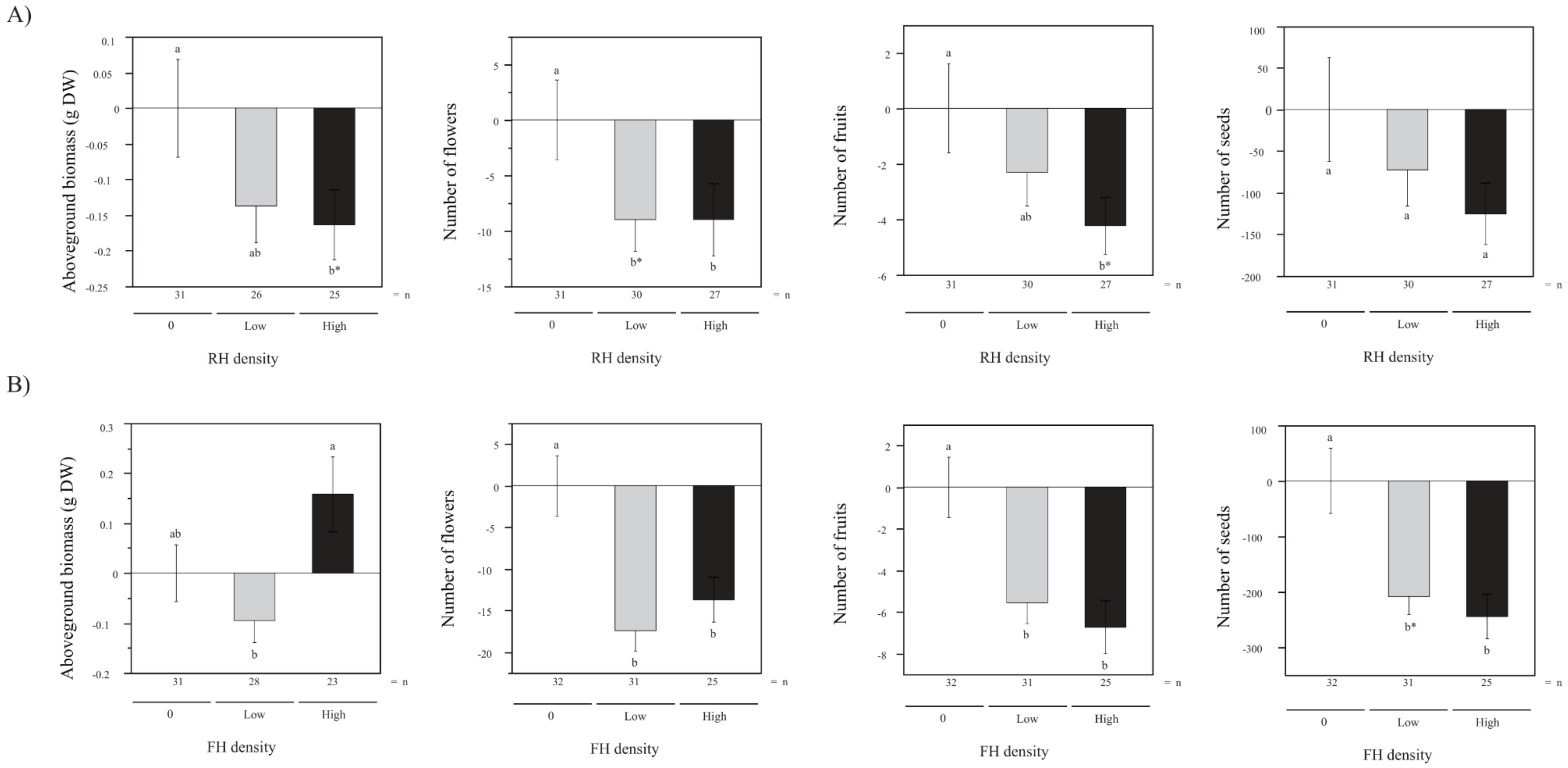


Figure 4.2. A) Root herbivore (RH) density effects on plant aboveground biomass and reproduction. Shown values (mean ± SE) are relative to mean RH$_0$ = 0. White bars are shown for RH$_0$ plants, grey bars are shown for RH$_1$ plants, black bars are shown for RH$_2$ plants. B) Floral herbivore (FH) density effects on plant aboveground biomass and reproduction. Shown values (mean ± SE) are relative to mean FH$_0$ = 0. White bars are shown for FH$_0$ plants, grey bars are shown for FH$_1$ plants, black bars are shown for FH$_2$ plants. For both A) and B), letters correspond to post hoc results (Suppl. 4.1, Table 4.S2a). Asterisk on *b* letter reveals that post hoc results are only marginally significant ($P < 0.08$) between *a* and *b*.

Plant quality

We observed no effect of RH and FH densities and their interaction for C/N ratio in leaves and seeds (Table 4.2). Regarding plant defense, ten glucosinolates were identified in leaves (mean of total concentration over all samples ± SE: 12.95 ± 1.15 µmol g^{-1} of dry weight); 4 indolic (0.59 ± 0.20 µmol g^{-1} of dry weight) and 6 aliphatic GLSs (12.36 ± 1.07 µmol g^{-1} of dry weight). Indol-3-yl-methyl GLS was the main indolic compound (0.52 ± 0.20 µmol g^{-1} of dry weight), while 3-butenyl GLS was the main aliphatic compound (11.45 ± 1.01 µmol g^{-1} of dry weight). RH and FH densities interactively affected total leaf GLS concentrations (Table 4.2). Total GLS concentrations in leaves were overally higher when both herbivore groups were present at high density: on high RH plants, plants with high FH had higher GLSs concentrations than plants without FH, and on high FH plants, plants with high RH had higher GLSs concentrations than plants without RH (Fig. 4.3; Suppl. 4.1, Table 4.S2b). The same was the case for aliphatics GLSs (Table 4.2), although the only significant post hoc difference was on high FH plants, between high RH plants and plants without RH (Suppl. 4.1, Table 4.S2b). Total indolic GLS concentrations were not affected by RH or FH densities (Table 4.2).

Permanova test revealed no significant effects of RH and FH densities in leaf GLS profile (RH density: $F = 1.66$, $P = 0.17$, $df = 1{,}82$; FH density: $F = 0.93$, $P = 0.40$, $df = 1{,}82$; interaction term of RH and FH densities: $F = 2.32$, $P = 0.08$, $df = 1{,}82$; see NMDS in Suppl. 4.3, Table 4.S4, Fig. 4.S2).

FH caterpillar development time

FH caterpillar development time was shortened with increasing density of RH ($\chi^2 = 7.54$, $P = 0.006$, $df = 1{,}38$). Caterpillars developed faster on high RH plants (14.86 ± 1.07 days) than on plants with no RH (17.27 ± 0.71 days), and than on low RH plants (16.03 ± 0.79 days; Suppl. 4.1, Table 4.S2a). FH density ($\chi^2 = 0.09$, $P = 0.76$, $df = 1{,}38$) and the interaction term of RH and FH densities ($\chi^2 = 1.40$, $P = 0.23$, $df = 1{,}38$) had no effect on caterpillar development time.

	RH			FH			RH x FH		
	F/χ^2	P	df	F/χ^2	P	df	F/χ^2	P	df
Aboveground biomass and reproduction									
Aboveground biomass	**6.54**	**0.03**	1,73	**3.92**	**0.04**	1,73	0.45	0.63	1,73
Number of flowers	**5.72**	**0.01**	1,79	**9.31**	**0.002**	1,79	0.21	0.81	1,79
Number of fruits	**5.40**	**0.02**	1,79	**18.44**	**< 0.0001**	1,79	0.10	0.90	1,79
Number of seeds	2.56	0.10	1,79	**12.03**	**0.0005**	1,79	0.19	0.90	1,79
Leaf and seed nutrient content									
C/N ratio in leaves	1.10	0.29	1,76	0.43	0.51	1,76	0.00	0.92	1,76
C/N ratio in seeds	0.04	0.82	1,59	2.04	0.15	1,59	0.44	0.50	1,59
Leaf glucosinolates (GLSs)									
Total GLSs	**5.08**	**0.02**	1,77	1.36	0.24	1,77	**4.03**	**0.04**	1,77
Aliphatic GLSs	**4.62**	**0.03**	1,77	1.16	0.27	1,77	**4.79**	**0.02**	1,77
Indolic GLSs	2.65	0.10	1,77	0.33	0.56	1,77	0.28	0.59	1,77

Table 4.2. Results of general and generalized linear models for the effect of root herbivores (RH) and floral herbivores (FH) on plant aboveground biomass, reproduction, leaf and seed nutrient content and leaf glucosinolates (GLSs). Significant values ($P < 0.05$) are highlighted in bold. F is shown for glucosinolates, χ^2 is shown for the rest.

Structural equation model of RH and FH density effects on plant fitness

In the SEM 1, RH and FH effects on plant fitness were ultimately independent (Fig. 4.4A; Suppl. 4.5, Table 4.S6). The number of flowers and total GLS concentrations influenced, positively and negatively, the number of days for FH caterpillar development, which in turn positively but not significantly affected plant fitness. RH density slightly reduced plant fitness, mainly by reducing the number of flowers (Fig. 4.4A; Suppl. 4.5, Table 4.S6). FH density severely reduced plant fitness also principally through reducing the number of flowers (Fig. 4.4A; Suppl. 4.5, Table 4.S6). The fitness consequences of the indirect effect of FH density and the combined RH-FH impact on FH caterpillar development time by altering GLSs were practically negligible (Fig. 4.4A; Suppl. 4.5, Table 4.S6).

In the SEM 2, RH and FH effects on plant fitness were also predominantly independent (Fig. 4.4B; Suppl. 4.6, Table 4.S8). RH density consequences through reducing aboveground biomass and through its interactive effect with FH density on GLS concentrations were minimal for plant fitness (Fig. 4.4B; Suppl. 4.6, Table 4.S8). FH density had a severe negative impact on plant fitness, mainly by reducing the number of flowers and the fruit set (Fig. 4.4B; Suppl. 4.6, Table 4.S8). The negative impact of FH density was partially loosened by the indirect consequences of boosting aboveground biomass (Fig. 4.4B; Suppl. 4.6, Table 4.S8).

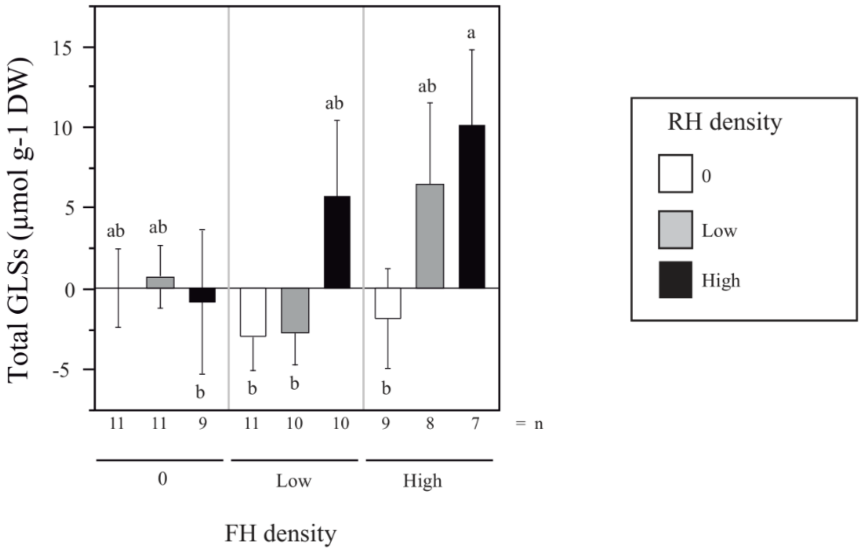


Figure 4.3. Root herbivore (RH) and floral herbivore (FH) density effects on total glucosinolate (GLS) concentrations. Shown values (mean ± SE) are relative to the focal treatment (mean RH_0FH_0 = 0). Letters correspond to Fischer Least Square Differences. Interaction level post hoc results are shown in Suppl. 4.1, Table 4.S2b.

4.5 Discussion

Density-dependent FH damage

Despite promoting a mixed resistance-tolerance response, reproductive tissue consumption by FH was detrimental for plant reproductive success, in agreement with other studies (McCall and Irwin 2006, González-Browne et al. 2016, West and Louda 2018). The negative effect of FH on seed number was evident on plants that had two caterpillars (high density), but such reduction was only marginally significant on plants with a single caterpillar (low density). The damage impinged on plant fitness did not significantly differ between low and high FH densities, what points to a non-linear relationship between herbivore density and damage in the case of florivory (a similar result was obtained in another experiment in this system with an equivalent manipulation of FH, see Chapter 5). Hence, the plant would have displaied an adaptive defensive phenotype that reduced the fitness costs of florivory (Mauricio et al. 1997, Núñez-Farfán et al. 2007, Züst and Agrawal 2017).

Plants, particularly those adapted to tolerate herbivory, seem to reproduce at the maximum rate when challenged by a severe threat to fitness (Arendt 1997, Garcia and Eubanks 2018). GEE results show that the plant exhibited a tolerance response when facing high FH density. Reproductive tissue production was boosted when FH caterpillars were actively feeding on plants (to a large extent eaten by FH caterpillars afterwards). The different reproductive tissue in which this effect is observed (flower and fruit production in current experiment versus floral bud group and flower production in Chapter 5) may be due to variation in environmental cues that caused differential temporal synchrony between the plant and the FH caterpillars (van Asch and Visser 2007, Abarca and Lill 2015), the hierarchical process of reproduction and the timing of effects within the plant life cycle (Agrawal 2000). That the plant promoted an intense tolerance response is reinforced by FH impact on aboveground biomass, which points to differential plant responses in dependence of FH density. When endured floral herbivory by a single caterpillar, the aboveground biomass was decreased, whereas when endured floral herbivory by two caterpillars, plants increased aboveground biomass. Probably higher aboveground biomass is a by-product of compensatory growth, and not a prerequisite for it (but see e.g. for the second case Kozłowski 1992, Adler et al. 2014, Chapter 7). As for the relation between FH density and plant resistance, it was mediated by RH density.

Density-dependent RH damage

As well as the florivory triggered the production of reproductive tissue, the GEE analysis showed the opposite for root herbivory. GEE effects on reproductive tissue coincided with those of GLMMs, in which the negative impact of RH on aboveground biomass, flower and fruit number increased with their density. Nevertheless, that reduction in biomass, flowers and fruits was non-linear as the impact tended to soften between low and high RH densities. Effect on seed yield was rather linear, but even high RH density did not substantially reduce it. The magnitude of RH damage is usually higher on growth than on reproduction in most plant species, which suggests up to a certain point a widespread compensatory capacity (Zvereva and Kozlov 2012). Compensatory capacity towards RH can be high in Brassicaceae, as reported for *M. moricandioides* (González-Megías 2016, Chapter 7) and wild mustard *Sinapis arvensis* (Poveda et al. 2003, 2005), but it is thought to be unusual in semiarid environments, where soil abiotic stress are predicted to exacerbate the negative impact of root removal by herbivores (Erb and Lu 2013).

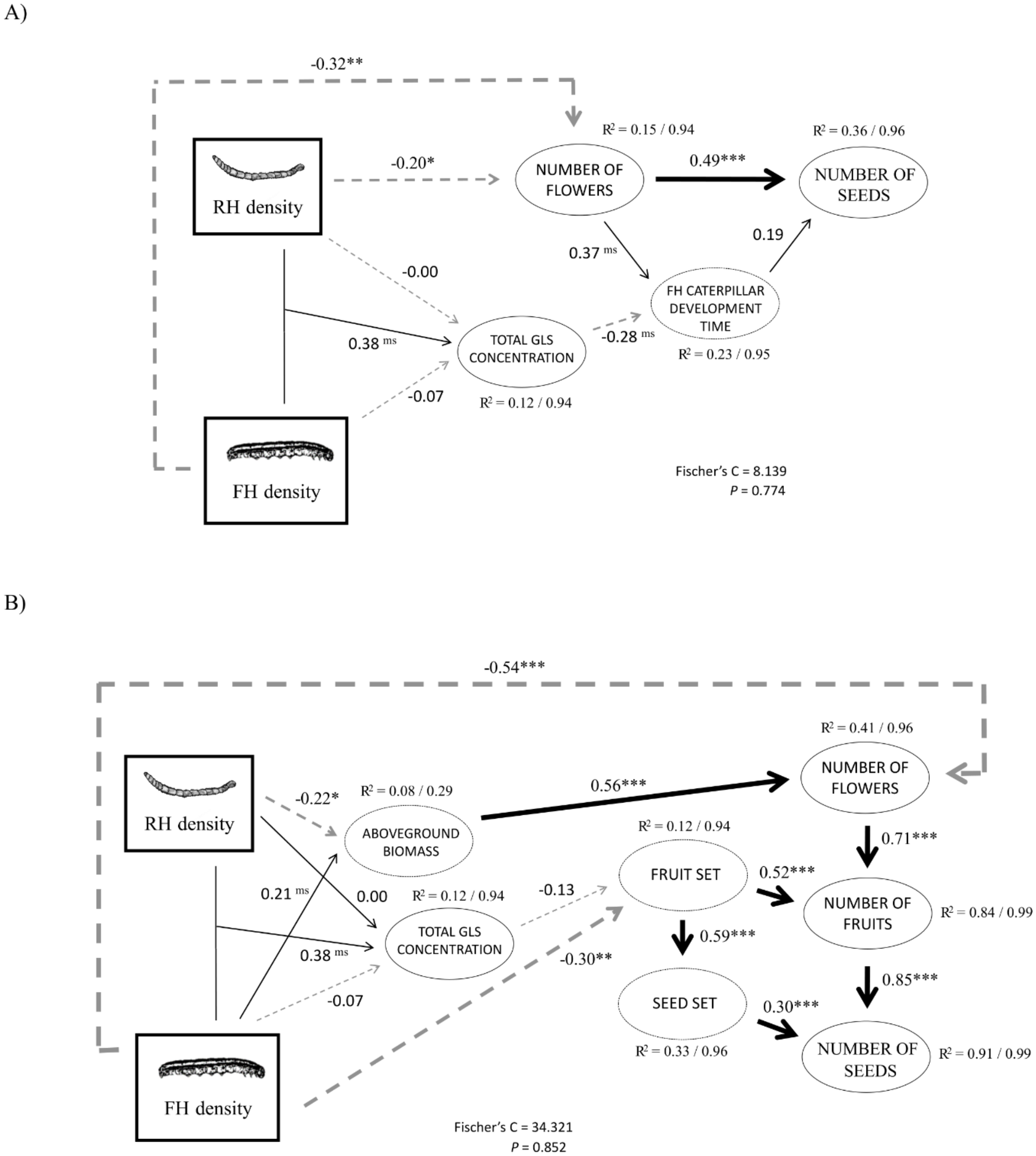


Figure 4.4. A) Final piecewise SEM 1 parsing the direct and indirect effects of RH and FH densities on plant fitness through FH caterpillar development time. B) Final piecewise SEM 2 parsing the direct and indirect effects of RH and FH densities on sequential plant reproductive components and fitness. Fruit set was calculated as the proportion of flowers that passed to fruits, and seed set as the proportion of ovules that passed to seeds. For both A) and B), standardized path coefficients are shown next to each path, and their significance level is shown as ^{ms} *P* <0.08,* *P* <0.05, ** *P* <0.01, *** *P* <0.001. Solid lines denote positive and dashed lines negative relationships, and their thickness is scaled to the magnitude of the path strength. Variance explained by the component models (R^2) is reported as marginal / conditional.

High RH density also reduced the development time of the FH caterpillars, whose potential consequences for the plants were analyzed by means of a SEM. The SEM pointed to a positive effect of flower number and a negative effect of GLS concentrations on FH caterpillar development time. RH density would have influenced these two paths (GLS induction jointly with high FH), both of which would have entailed a decrease in FH development time. Our GLSs measurement was made in leaves, but we predict that those plants with higher GLS concentrations in their leaves would have also higher concentrations in reproductive tissue. In fact, it has been widely observed that within the same plant the concentrations of secondary compounds are usually correlated across tissue types (Irwin and Adler 2006, Smallegange et al. 2007, Kessler and Halitschke 2009, Adler et al. 2012), even though higher in reproductive tissues than in leaves as predicted by the optimal defense theory (Strauss et al. 2004a, McCall and Fordyce 2010, Abdalsamee and Müller 2015). According to the SEM, a slower development of the caterpillars could increase the fitness of plants when coping with florivory. Therefore, rather than benefiting from the shorter time that the caterpillars would be consuming reproductive tissue, the effect of high RH density on the development time of the FH caterpillars could be detrimental to the plant. Possibly, the slower the caterpillar development, the lower their consumption rate and the greater plant capacity to produce new reproductive tissue and to mature its fruits, what would prevent its ingestion by caterpillars (slow-them-down strategy, see Kant et al. 2015). However, this indirect effect of RH did not aggravate the damage they caused to the plant. On the contrary, the density-dependent RH damage on the plant softened from the effect on biomass to seed production, which may be partly due to the proportionally greater number of flowers that were able to pass to fruits in these plants (see Fig. 1).

Consequences for FH caterpillar performance

From the caterpillar view, it is feasible that flower number and GLS concentrations can to some extent determine their development. Other pierid species such as *Pieris brassicae* preferably feed on GLS-rich flower tissues, in which they sustain higher growth rates (Smallegange et al. 2007), and probably increase their survival and fitness (Lucas-Barbosa et al. 2014). Indeed, faster development correlates with higher pupal mass in this species (Pashalidou et al. 2015a). For the same species, other authors suggest that shortened development time could also be due to food deprivation, and deprivation implies negative fitness consequences such as a reduction in pupal mass (Fei et al. 2016). Our results would be supportive of both hypotheses (Suppl. 4.4, Figure 4.S3a), although this should be empirically verified. Both potential factors influencing FH caterpillar development time seemed to interact (Suppl. 4.4, Figure 4.S3b), but the SEM dismissed that path. In any case, it is yet to be unravelled if root herbivory could have had an indirect facilitative effect (*in sensu* Ohgushi 2008) on FH caterpillars by increasing their growth rate, or if the consequences for caterpillar performance are negative due to resource (flower number) deprivation.

Additiveness and non-additiveness in simultaneous root and floral herbivory

Our results fulfil the general prediction that the fitness impact of simultaneous herbivory at different plant parts leans to be independent (Stephens et al. 2013), despite the joint effect size of high density of both herbivore groups tended to antagonism rather than to strict additivity (Suppl. 4.7, Fig. 4.S6). Nor were there any consequences on leaf and seed nutrient content, albeit herbivory on resource sources (roots) and resource sinks (reproductive tissues) can alter source-sink relations (White et al. 2016). On the contrary, the plant defensive response to FH and RH densities was non-additive. When the plant endured high FH compensatory growth prevailed, and only when accompanied by high root herbivory enhanced chemical defense was also evident. These trait combinations resulted in a linear although non-significatively detrimental fitness impact of RH density, and in a non-linear relationship between herbivore density and fitness damage in the case of florivory.

Plants employ a series of regulatory switches to prevent costly coexpression of high levels of growth and defense, which can be maladaptative, and must *opt* between the range of trait combinations to achieve the maximum fitness (Huot et al. 2014, Lozano-Durán and Zipfel 2015, Züst and Agrawal 2017). While the functionality of the tolerance response seemed undoubtedly beneficial for the plant in the present trophic framework, the non-additive resistance response (affecting GLS concentrations and profile) generates more uncertainties, both because of its effect on FH caterpillar development and due to the high photosynthetic requirement costs from the production of secondary metabolites such as GLSs (Bekaert et al. 2012, Yang et al. 2012, Campos et al. 2016).

Conclusions

Plants regulate their responses towards herbivore pressure below- and aboveground highly stress-specifically (Boege and Marquis 2005, van Dam 2009, Huber and Bauerle 2016). Our results with *M. moricandioides* provide evidence that plants prioritize differing trait combinations depending on herbivore density both below- and aboveground to achieve maximum (or minimize impact on) fitness. The optimal combination of resistance and tolerance would thus vary according to the type of herbivory the plant faces, herbivore density and the type of herbivory x density interaction. Therefore, we encourage the carrying out of more comparable studies, in which the density-dependent effect of several herbivores simultaneously is studied, and its relationship with the tolerance, resistance and fitness of the plant is evaluated. Our study also claims to consider a temporally-explicit approach when analyzing the effects of flovivory on plant defense and reproduction. This approach may be advantageous especially when florivory occur by caterpillars that stay for several weeks on plants, as plants may adjust their defensive response according to the herbivore lifetime relative to that of their own (Higginson et al. 2015).

4.X Supplementary material

Supplementary material 4.1. Model selection and post hoc tests.

Variable	Distribution	Random structure	BIC
FH caterpillar development time	Gamma	Block / Plant	-519.829
Aboveground biomass	Gamma	Block	10.030
Number of flowers	Poisson	Block / Plant	747.763
Number of fruits	Poisson	Block / Plant	575.836
Number of seeds	Poisson	Block / Plant	1141.135
C/N ratio in leaves	Gamma	Block / Plant	-756.116
C/N ratio in seeds	Gamma	Block / Plant	-851.526
Total GLSs*	Gaussian	Block / Plant	256.451
Aliphatic GLSs*	Gaussian	Block / Plant	256.304
Indolic GLSs*	Gaussian	Block / Plant	264.172

Table 4.S1. Model structure of selected GLMMs for analyzed variables. * GLS concentrations are range/(n-1) transformed.

	RH factor level effects		
	RH_0 – RH_1	**RH_0 – RH_2**	**RH_1 – RH_2**
FH caterpillar development time	-1.41	**-3.19****	**-2.50***
Aboveground biomass	-0.93	-2.21^{ms}	-1.18
Number of flowers	2.24^{ms}	**2.48***	0.27
Number of fruits	1.49	2.34^{ms}	0.82

	FH factor level effects		
	FH_0 – FH_1	**FH_0 – FH_2**	**FH_1 – FH_2**
Aboveground biomass	-1.01	1.71	**2.53***
Number of flowers	**4.39*****	**3.09****	-1.00
Number of fruits	**3.16****	**4.26*****	1.41
Number of seeds	2.21^{ms}	**3.62*****	1.59

Table 4.S2a. *P*-value adjusting multivariate post hoc tests for GLMM factor level effects. *z*-values are shown. ^{ms} *P* <0.08,* *P* <0.05, ** *P* <0.01, *** *P* <0.001.

	RH-FH interaction level effects								
	RH_0			**RH_1**			**RH_2**		
	$FH_0 - FH_1$	**$FH_0 - FH_2$**	**$FH_1 - FH_2$**	**$FH_0 - FH_1$**	**$FH_0 - FH_2$**	**$FH_1 - FH_2$**	**$FH_0 - FH_1$**	**$FH_0 - FH_2$**	**$FH_1 - FH_2$**
Total GLSs	0.67	0.84	0.20	0.81	-0.96	-1.65	-1.06	**-2.42***	-1.01
Aliphatic GLSs	0.60	0.64	0.06	0.88	-0.96	-1.71	-1.05	-2.27^{ms}	-0.98
	FH_0			**FH_1**			**FH_2**		
	$RH_0 - RH_1$	**$RH_0 - RH_2$**	**$RH_1 - RH_2$**	**$RH_0 - RH_1$**	**$RH_0 - RH_2$**	**$RH_1 - RH_2$**	**$RH_0 - RH_1$**	**$RH_0 - RH_2$**	**$RH_1 - RH_2$**
Total GLSs	-0.26	0.35	0.59	-0.11	-1.43	-1.29	-1.91	**-2.53***	-0.60
Aliphatic GLSs	-0.50	0.20	0.66	-0.20	-1.51	-1.28	-1.92	**-2.45***	-0.50

Table 4.S2b. *P*-value adjusting multivariate post hoc tests for GLMM interaction-level effects. *t*-values are shown. ^{ms} *P* <0.08,* *P* <0.05.

Supplementary material 4.2. Generalized additive linear models (GAMMs).

	RH			FH			RH x FH		
	F/χ^2	P	df	F/χ^2	P	df	F/χ^2	P	df
Aboveground biomass and reproduction									
Aboveground biomass	**5.82**	**0.01**	1,73	*3.25*	*0.07*	1,73	0.01	0.91	1,73
Number of flowers	**5.15**	**0.02**	1,79	**8.89**	**0.002**	1,79	0.15	0.69	1,79
Number of fruits	**5.05**	**0.02**	1,79	**18.22**	**<0.0001**	1,79	0.03	0.85	1,79
Number of seeds	2.17	0.14	1,79	**12.05**	**0.0005**	1,79	0.26	0.60	1,79
Leaf and seed nutrient content									
C/N ratio in leaves	0.96	0.32	1,76	2.93	0.08	1,76	1.12	0.28	1,76
C/N ratio in seeds	0.00	0.99	1,59	1.30	0.25	1,59	0.76	0.38	1,59
Leaf glucosinolates (GLSs)									
Total GLSs	**4.70**	**0.03**	1,77	1.17	0.27	1,77	**4.36**	**0.03**	1,77
Aliphatic GLSs	**5.12**	**0.02**	1,77	1.34	0.24	1,77	*3.64*	*0.05*	1,77
Indolic GLSs	2.53	0.11	1,77	0.32	0.57	1,77	0.26	0.61	1,77

Table 4.S3. GAMM results for the effect of root herbivores (RH) and floral herbivores (FH) on analyzed plant trait variables. No smoothing parameter was added to GAMMs as it is not possible for models with only three density levels. Significant values ($P < 0.05$) are highlighted in bold.

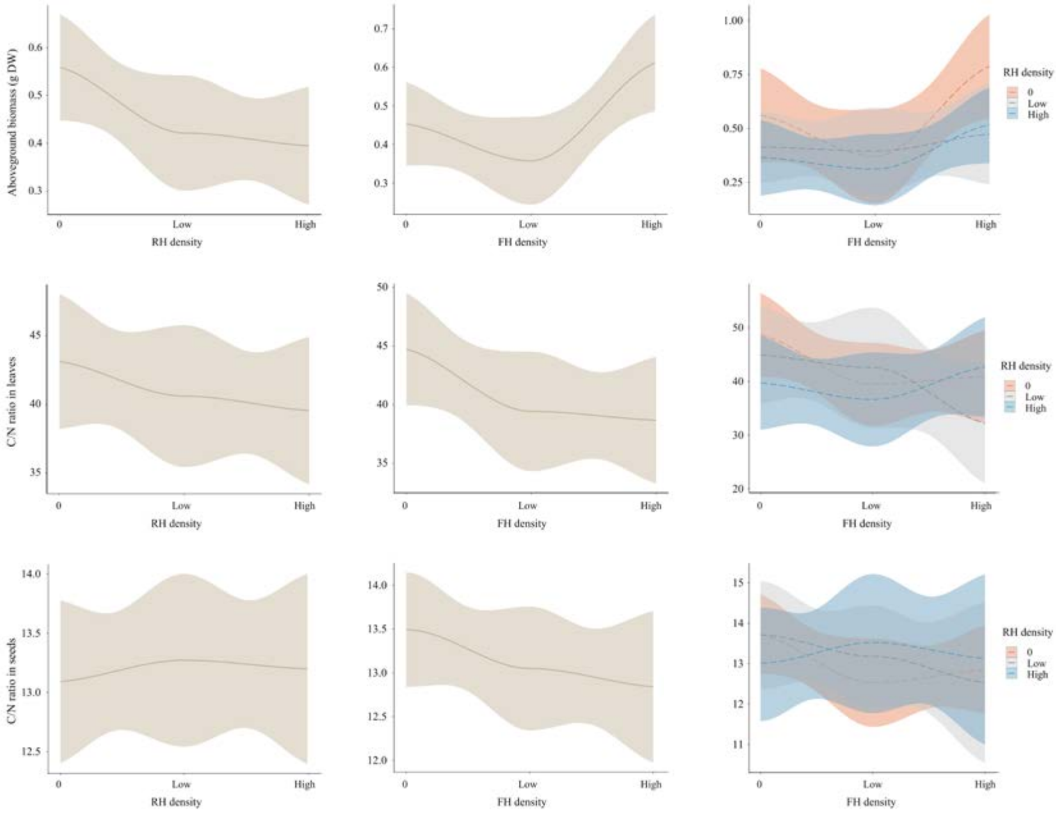
The results obtained by GAMM were very similar to those obtained by GLMMs. RH density had a significant effect on aboveground biomass and flower and fruit number (Table 4.S3). The number of fruits linearly decreased with RH density (Fig. 4.S1), while aboveground biomass and number of fruits non-linearly decreased with RH density, since the descending slope slowed down between the low and high densities (Fig. 4.S1).

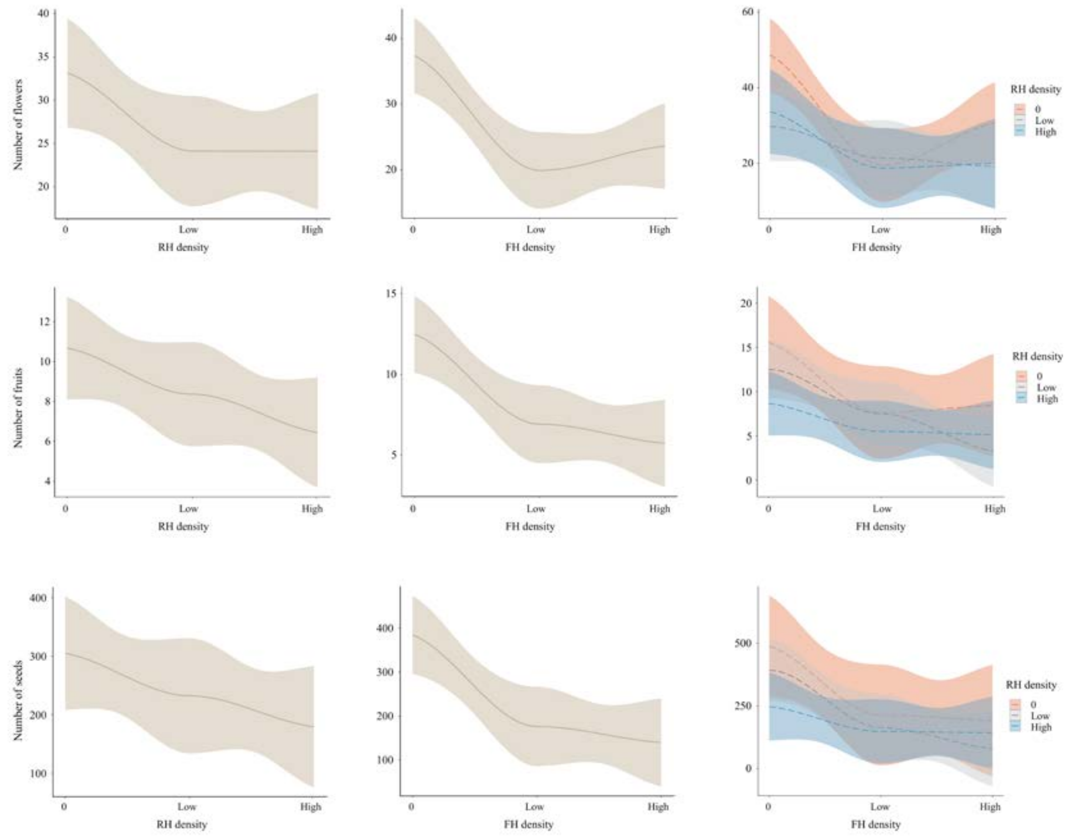
FH density had a significant effect on the number of flowers, fruits and seeds, while the effect was only marginally significant for aboveground biomass (Table 4.S3). For the three reproductive variables FH density effect was non-linear. The descending slope with increasing FH density slowed down between the low and high densities for number of fruits and seeds, while fully reversed and became ascending between the low and high densities for number of flowers (Fig. 4.S1).

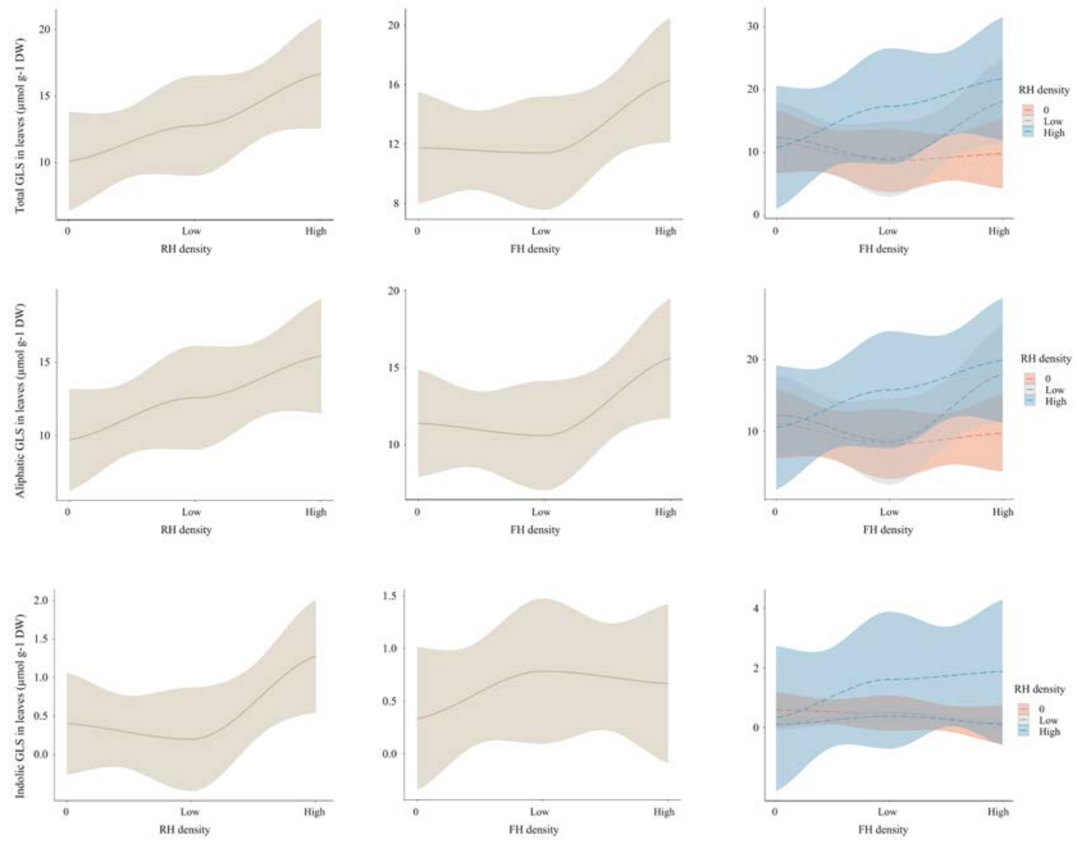
As in the GLMMs, RH and FH densities had an interactive effect on total leaf GLS concentrations (Table 4.S3, Fig. 4.S1).

Regarding FH caterpillar development time, RH density had a marginally significant effect (χ^2= 3.51, *P* = 0.06, *df* = 1,40). FH density (χ^2= 0.69, *P* = 0.40, *df* = 1,40) and the interaction term of RH and FH densities (χ^2= 0.65, *P* = 0.41, *df* = 1,40) had no effects on caterpillar development time.

Figure 4.S1. Below, locally estimated scatterplot smoothing (LOESS) plots for all analyzed plant variables regarding root herbivore (RH) density, floral herbivore (FH) density, and both herbivore group densities.




Number of flowers
40
35
30
25
20
0
Low
High
RH density
40
30
20
FH density
60
40
20
RH density
0
Low
High
Number of fruits
12
10
8
6
4
15
10
5
20
15
10
5
0
Number of seeds
400
300
200
100
500
250
0


Total GLS in leaves (µmol g-1 DW)
20
15
10
0
Low
High
RH density
20
16
12
8
FH density
30
20
10
0
RH density
0
Low
High
Aliphatic GLS in leaves (µmol g-1 DW)
15
10
20
Indolic GLS in leaves (µmol g-1 DW)
2.0
1.5
1.0
0.5
0.0
4
2

Supplementary material 4.3. Non-metric multidimensional scaling (NMDS) showing dissimilarity in the concentrations of the ten leaf glucosinolates.

	NMDS 1	**NMDS 2**	**R^2**	***P***
RH$_0$FH$_0$	-0.07711	-0.99702	0.0406	0.17
RH$_1$FH$_0$	0.08520	-0.99636	0.0102	0.65
RH$_2$FH$_0$	0.88671	-0.46233	0.0040	0.84
RH$_0$FH$_1$	0.97219	-0.23419	0.0297	0.28
RH$_1$FH$_1$	-0.32414	-0.94601	0.0065	0.76
RH$_2$FH$_1$	0.69755	-0.71654	0.0083	0.71
RH$_0$FH$_2$	0.00254	1.00000	0.1042	0.01
RH$_1$FH$_2$	-0.78123	0.62425	0.0249	0.35
RH$_2$FH$_2$	-0.71752	0.69654	0.0425	0.16

Table 4.S4. Contribution of each treatment to the leaf GLS profile NMDS plot axes and explained variation of dissimilarity.

The different RH and FH combinations explained 27% of dissimilarity in leaf GLS profiles between plants of different treatments (Table 4.S4). Plants with high FH independently of RH density showed the highest level of GLS profile dissimilarity with respect to other treatments (Fig. 4.S2). Regarding treatment alignment among the NMDS axes, 3 groups could be differentiated: GLS pattern of plants with high FH and absence of RH, with high FH and low or high RH, and absence or low FH density (Fig. 4.S2).

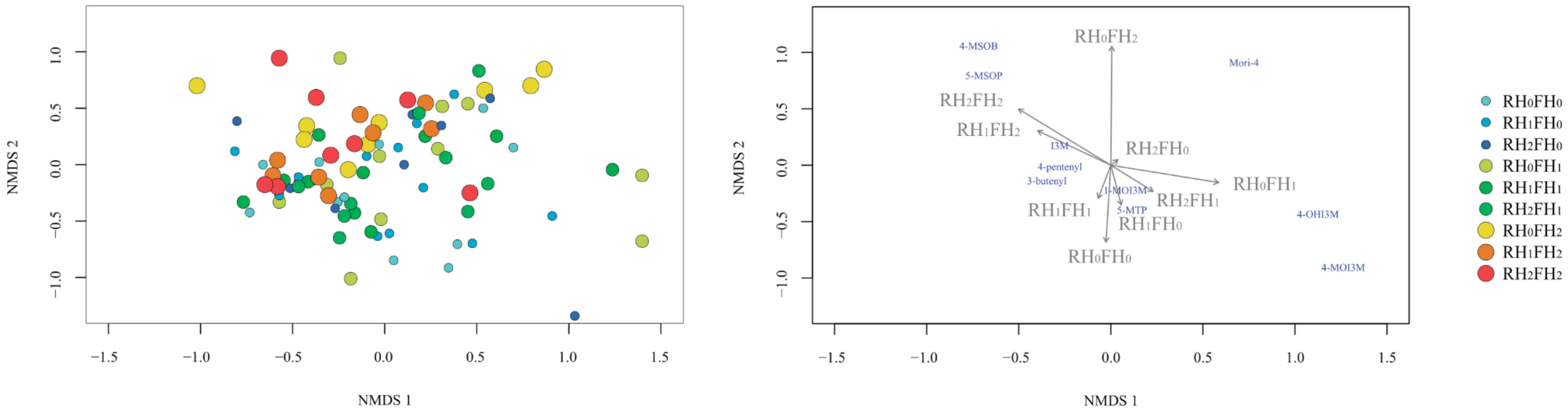


Figure 4.S2. Two dimensional NMDS plot showing dissimilarity in the concentrations of the ten glucosinolates (GLSs) between the different root herbivore (RH) and floral herbivore (FH) densities combinations. On the left, sample positions onto the NMDS plot. On the right, treatments as dissimilarity vectors and mean position of each GLS in the NMDS plot. 5-MTP = 5-methylthiopentyl GLS, Mori-4 = unidentified aliphatic GLS, 5-MSOP = 5-methylsulfinylpentyl GLS, 4-MSOB = 4-methylsulfinylbutyl GLS, 4-MOI3M = 4-methoxy-indol-3-yl-methyl GLS, 1-MOI3M = 1-methoxy-indol-3-yl-methyl GLS, 4-OHI3M = 4-hydroxy-indol-3-yl-methyl GLS, I3M = indol-3-yl-methyl GLS.

Supplementary material 4.4. FH caterpillar development time.

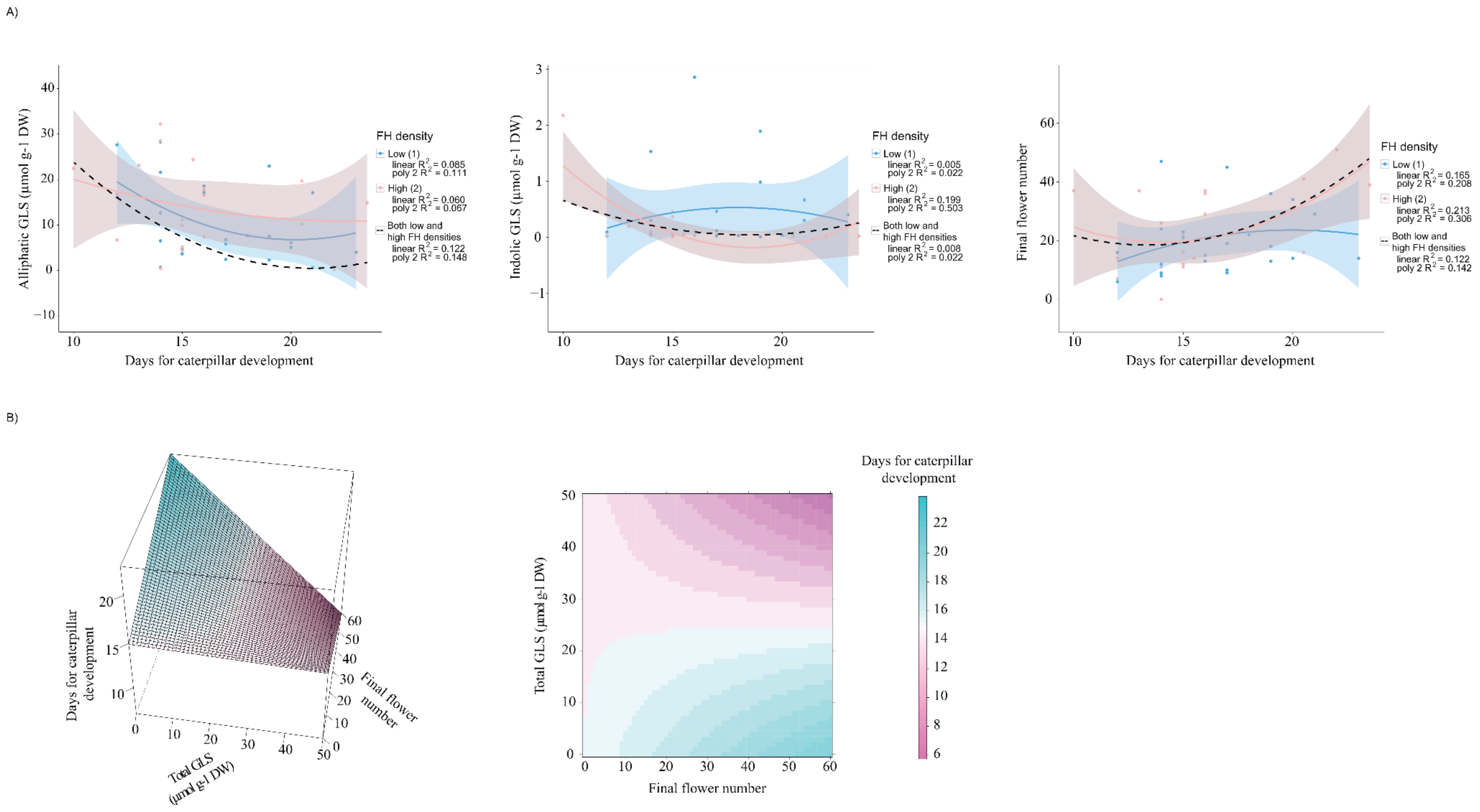


Figure 4.S3. (A) Linear and non-linear regression between the number of days for caterpillar development and aliphatic GLS concentration (plants with < 35 µmol aliphatic GLS g^{-1} of dry weight), indolic GLS concentration (plants with < 8 µmol indolic GLS g^{-1} of dry weight) and number of flowers. When development time for both caterpillars in FH_2 plants could be measured, mean value per plant is shown. (B) 3D and 2D plane of the interaction between total GLS concentration (plants shown in A) and number of flowers on the number of days for caterpillar development.

Supplementary material 4.5. Structural equation model (SEM) for RH and FH density effects through FH caterpillar development time on plant fitness.

Model	Removed paths	Added paths	AICc	ΔAICc	df	Fischer's *C*	*P*
Full initial model	-		172.785	25.728	10	6.274	0.792
model 2	FH density → Number of seeds	-	161.105	14.048	12	7.946	0.789
model 3	**Total GLS concentration x Number of flowers (interaction) → Seed set**	**-**	**147.057**	-	**12**	**8.139**	**0.774**

Table 4.S5. Stepwise SEM selection procedure for RH and FH density effects through FH caterpillar development time on plant fitness.

Cause	Effects on	Direct	Indirect	Total
RH density	Number of flowers	-0.20	-	-0.20
	Total GLSs	-0.00	-	-0.00
	FH caterpillar development time	-	-0.07	-0.07
	Number of seeds	**-**	**-0.11**	**-0.11**
FH density	Number of flowers	-0.32	-	-0.32
	Total GLSs	-0.07	-	-0.07
	FH caterpillar development time	-	-0.09	-0.09
	Number of seeds	**-**	**-**	**-0.16**
RH x FH densities	Number of flowers	-	-	-
	Total GLSs	0.38	-	0.38
	FH caterpillar development time	-	-0.10	-0.10
	Number of seeds	**-**	**-0.02**	**-0.02**

Table 4.S6. Standardized total, indirect and direct size effects of RH density, FH density and their interaction in the final SEM.

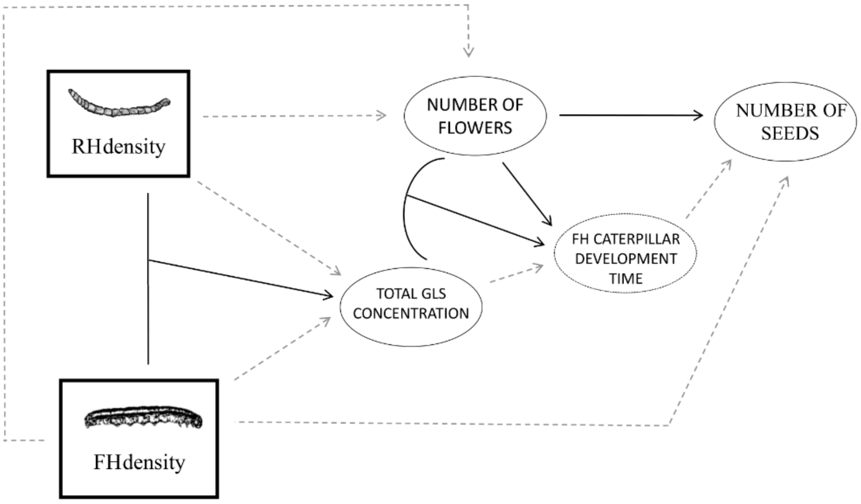


Figure 4.S4. Initially hypothesized SEM for FH and RH density effects through FH caterpillar development time on plant fitness. Solid lines denote positive and dashed lines negative relationships.

Supplementary material 4.6. Structural equation model (SEM) for RH and FH density on sequential plant reproductive components and fitness.

Model	Removed paths	Added paths	AICc	ΔAICc	df	Fischer's *C*	*P*
Full initial model			455.673	199.767	34	57.721	0.007
model 2	Aboveground biomass → Fruit set Number of flowers → Fruit set Aboveground biomass → Seed set Total GLS concentration → Seed set	-	309.762	53.856	42	52.778	0.123
model 3	RH density → Number of flowers	-	302.531	46.625	44	55.514	0.114
model 4	Total GLS concentration → Fruit set	-	302.591	46.685	46	61.471	0.063
model 5		Total GLS concentration → Fruit set Fruit set → Seed set	264.370	8.464	42	32.735	0.847
model 6	FH density → Seed set	-	**255.906**	-	**44**	**34.321**	**0.852**
model 7	-	Total GLS concentration → Number of flowers	257.300	1.394	42	29.613	0.925
model 8	Total GLS concentration → Number of flowers	Number of flowers → Number of seeds	280.685	24.779	42	39.939	0.562
model 9	Number of flowers → Number of seeds	Fruit set → Number of seeds	281.145	25.239	42	40.142	0.553

Table 4.S7. Stepwise SEM selection procedure for RH and FH density effects on sequential plant reproductive components and fitness. Fruit set was calculated as the proportion of flowers that passed to fruits, and seed set as the proportion of ovules that passed to seeds.

Cause	**Effects on**	**Direct**	**Indirect**	**Total**
RH density	Aboveground biomass	-0.22	-	-0.22
	Total GLSs	0.00	-	0.00
	Number of flowers	-	-0.12	-0.12
	Fruit set	-	-0.00	-0.00
	Number of fruits	-	-0.08	-0.08
	Seed set	-	-0.00	-0.00
	Number of seeds	**-**	**-0.06**	**-0.06**
FH density	Aboveground biomass	0.21	-	0.21
	Total GLSs	-0.07	-	-0.07
	Number of flowers	-0.54	0.11	-0.43
	Fruit set	-0.30	0.01	-0.29
	Number of fruits	-	-0.45	-0.45
	Seed set	-	-0.17	-0.17
	Number of seeds	**-**	**-0.43**	**-0.43**
RH x FH densities	Aboveground biomass	-	-	-
	Total GLSs	0.38	-	0.38
	Number of flowers	-	-	-
	Fruit set	-	-0.05	-0.05
	Number of fruits	-	-0.02	-0.02
	Seed set	-	-0.03	-0.03
	Number of seeds	**-**	**-0.05**	**-0.05**

Table 4.S8. Standardized total, indirect and direct size effects of RH density, FH density and their interaction in the final SEM.

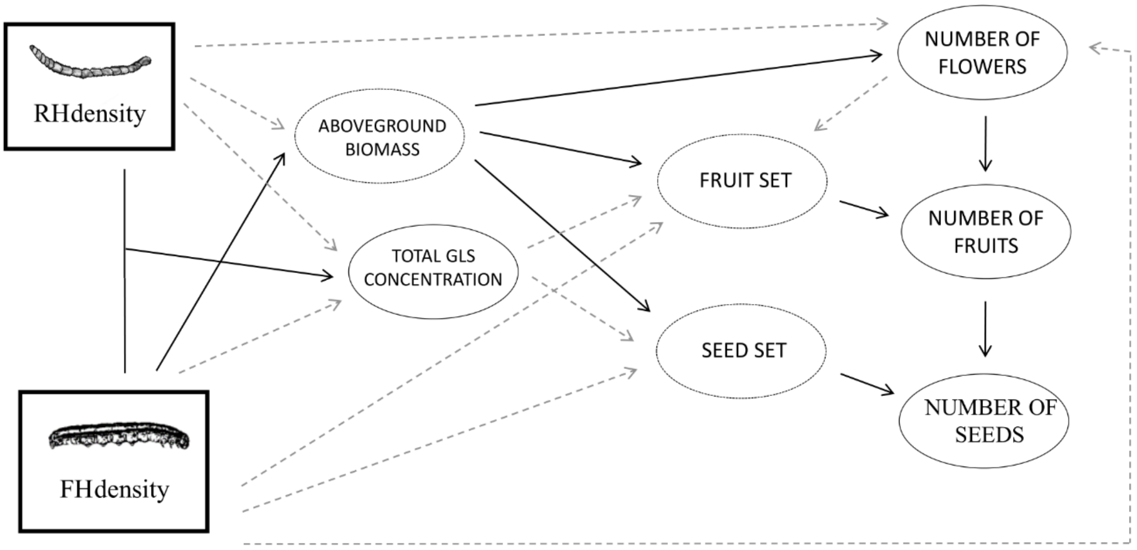


Figure 4.S5. Initially hypothesized SEM for the direct and indirect RH and FH density effects on sequential plant reproductive components and fitness. Fruit set was calculated as the proportion of flowers that passed to fruits, and seed set as the proportion of ovules that passed to seeds.

Supplementary material 4.7. RH and FH density effects on plant.

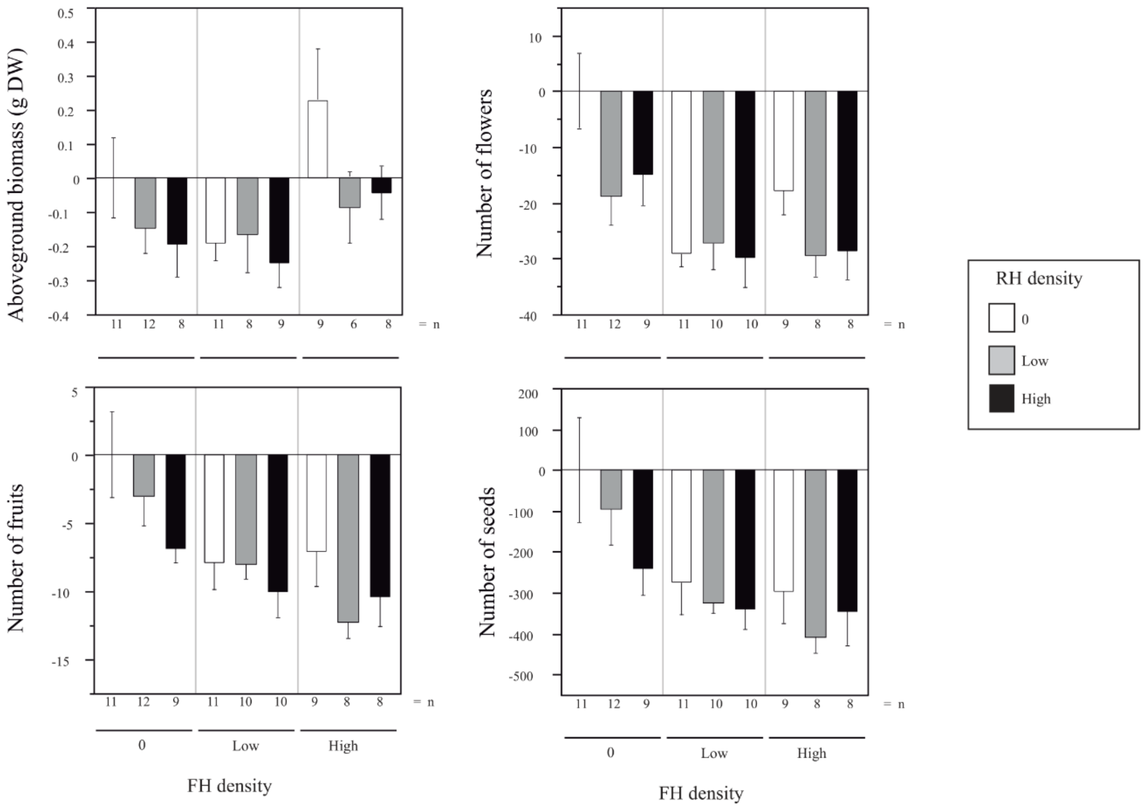
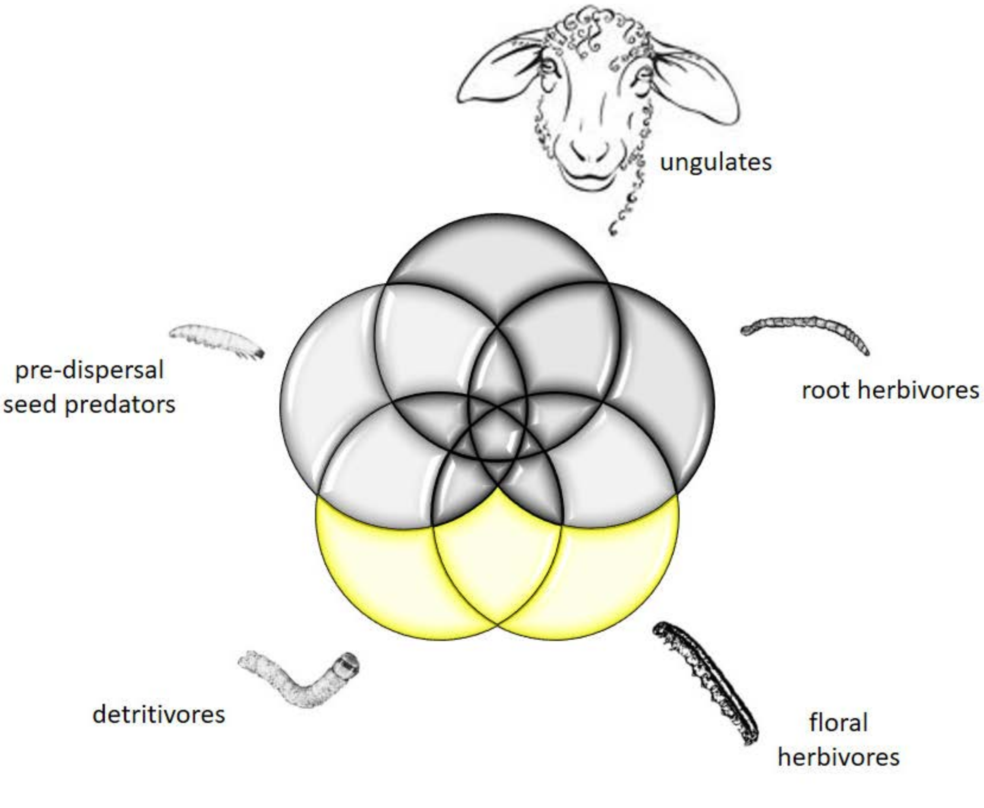


Figure 4.S6. Combined effects of root herbivore (RH) and floral herbivore (FH) densities on plant aboveground biomass and reproduction. Shown values (mean ± SE) are relative to the focal treatment (mean $RH_0FH_0 = 0$).

Chapter 5

Plants facing floral herbivory while interacting with detritivores: a density-dependent perspective



5.1 Abstract

Among all types of herbivory, florivory is undoubtedly one of the greatest threats to the sexual reproductive success of plants, both quantitatively by reducing seed yield and qualitatively by reducing seed nutritive value. Plant may then benefit from resistance, tolerance or mixed responses to decrease feeding by floral herbivores and/or mitigate the fitness costs associated with floral damage. Organisms such as detritivores can increase nutrient availability in plant rhizosphere, which can be used by plants to foster resistance and/or tolerance towards floral herbivores. The ecological relevance of this tritrophic interaction is thus particularly interesting in ecosystems in which plants are subject to limited resources. Here, we investigated in the field the combined effects of detritivore beetle larvae and specialist floral-feeding pierid caterpillars on a semiarid Brassicaceae herb. Going beyond the fixed paired tritrophic experimental designs, we manipulated the abundance of both detritivores and floral herbivores in a full factorial density gradient, from absence to high density. We found that despite promoting tolerance (surplus production of reproductive tissue) and slightly increasing resistance (chemical defense), high density of floral herbivores had a negative impact on seed production. However, that mixed response by the plant led to a non-linear relationship between herbivore density and damage, proving the validity of that defensive traits combination towards such harmful herbivory type. Increasing detritivore density did not affect seed production and neither enabled plants to better defend against or compensate for florivore damage. Instead, high detritivore density influenced other allocation processes within the plant, altering floral herbivore effects on seed quality.

Keywords

Brassicaceae · belowground-aboveground · Density-dependence · Defense · Resistance · Tolerance

5.2 Introduction

Herbivory is a major biotic interaction, and plants have evolved multiple mechanisms to defend against and compensate for herbivory damage (Agrawal 2011, Fornoni 2011). While significant research advances have been made in relation to leaf herbivory, the consequences of florivory have yet received less attention although in many cases it can surpass folivory in magnitude and impact (McCall and Irwin 2006). Florivory usually affects the quantity and quality of flower production (Ehrlich and Raven 1964, Louda and Potvin 1995, Mothershead and Marquis 2000), and negatively impacts plant fitness (González-Browne et al. 2016). Because florivory can limit plant reproduction, plants are under selection to decrease feeding by floral herbivores and/or mitigate the fitness costs associated with floral damage (McCall and Irwin 2006, Boaventura et al. 2021), influencing the evolution of defensive traits and heightening plant-herbivore coevolutionary antagonism (Ehrlich and Raven 1964, Frame 2003, McCall and Irwin 2006, Higginson et al. 2015). Plants may then defend against florivores by producing secondary toxic compounds, although these might not necessarily increase plant resistance in interactions involving specialist herbivores (Siemens and Mitchell-Olds 1996, Núñez-Farfán et al. 2007), as anti-herbivore defenses must not only reduce damage but to increase fitness in the presence of the herbivore (Karban and Myers 1989). Compensation to florivory may also occur, even more fully than for folivory as resource sinks instead of resource sources are consumed (McCall and Irwin 2006). Plants can try to escape from florivory by altering the time of reproduction (Kawagoe and Kudoh 2010), or by accelerating it (Wise et al. 2008, Lucas-Barbosa et al. 2013), but also by shunting resources to future flowers after damage (McCall and Irwin 2006, West and Louda 2018), or even tolerate it by producing surplus flowers (Islam and Crawley 1983, Piña et al. 2010). Alternatively, resistance and tolerance traits could also be partially expressed together in a growth–defense trade-off continuum, as both strategies are not mutually exclusive and their combined effect on plant fitness can be more than additive (Fornoni 2011, Züst and Agrawal 2017).

Plant capacity to defend against or compensate for florivory damage may be conditioned by resource availability, as plants have limited resources and time to invest in growth, reproduction and defense and should balance these to maximize fitness within the environmental constrains (Stearns 1989, Herms and Mattson 1992, De Deyn 2017). Increased resource availability could increase plant tolerance towards herbivores (De Deyn 2017) or loosen defense trade-offs, making unnecessary the prevention of costly coexpression of high levels of growth and resistance (Hahn and Maron 2016, Züst and Agrawal 2017). In this way, increased resource availability for plants could be provided by detritivores, as they enhance microbial turnover, nutrient recycling and the breakdown of organic matter (Bardgett 2005, De Deyn and Van der Putten 2005, Sagi et al. 2019). Plants generally benefit from detritivory, although the consequences of detritivore activity for them are far from being straightforward (Laossi et al. 2009). For example, high interspecific variability on the amount of nitrogen uptake due to detritivory has been observed (Kreuzer et al. 2004, Andriuzzi et al. 2016), and even between within related species such as in Brassicaceae, species largely differ in the use of detritivore-provided resources (Newington et al. 2004, Poveda et al. 2005, González-Megías and Müller 2010, González-Megías and Menéndez 2012, González-Megías 2016). However, positive effects of detritivores on plant performance are predicted on certain situations, such as at low levels of nutrient availability (Haase et al. 2008, Maron et al. 2014) and on stochastic environments where plants (specially short-lived ones) have short periods of ideal conditions for reproducing (González-Megías et al. 2011). Detritivores can also influence plant defense and by altering the concentration and profile of defensive secondary metabolites in aboveground plant parts (Wurst et al. 2004, Lohmann et al. 2009), what could potentially condition plants interactions with their associated (Bardgett and Wardle 2003, Poveda et al. 2005, González-Megías and Müller 2010). However, we undoubtedly need more studies addressing how detritivores affect plant performance and their plant-mediated effects on higher tropic levels (Wurst 2013). That need to gain knowledge is shared with florivory, whom surprisingly low attention received is aggravated by the lack of factorial experiments analyzing florivory impact in combination with other organisms (McCall and Irwin 2006, but see González-Megías and Müller 2010, González-Megías 2016).

In the wild Brassicaceae species *Moricandia moricandioides*, naturally occurring floral herbivory by pierid caterpillars has a detrimental effect on plant fitness (González-Megías 2016), although simultaneous resistance and tolerance by the plant led to a non-linear relationship between floral herbivore density and damage (Chapter 4). In this system, detritivory by a single beetle larva itself did not increase plant reproductive output, nor significantly lessened the fitness impact of floral herbivores (González-Megías 2016, Chapter 4). However, the presence of a detritivore larva did increase seed quality, and altered the glucosinolate profile in leaves when combined with floral herbivores (González-Megías and Müller 2010, González-Megías 2016). Since the relation between resource availability and defense appears to be mediated through herbivore pressure (Stamp 2003, Hahn and Maron 2016), it is to be unravelled whether a varying density of both detritivores and floral herbivores would result in additive or non-additive effects on plant tissue quality and the tolerance and resistance response, and how the covariance between plant traits determine florivore caterpillar performance, the damage caused and their fitness impact (see Hanley et al. 2007, Koussoroplis et al. 2019). Given that the magnitude and sign of biotic interactions often vary as a function of the interaction strength of a third interactor (van der Putten et al. 2001, Strauss and Irwin 2004, Morris et al. 2007, Chamberlain et al. 2014, terHorst et al. 2018), controlling for density-dependence, particularly in belowground-aboveground studies, supposes a step towards a better understanding of natural complexity (van der Putten et al. 2001, Soler et al. 2012). As examples, density-dependence has been already observed in nutrient mineralization by earthworms (Aira et al. 2008), nutrient uptake by plants (Timperley et al. 1970, Marschner 2011) or plant capacity itself to compensate for florivory (McCall and Irwin 2006, West and Louda 2018).

With the intention of giving light to the raised questions, we carried out an experiment in the *M. moricandioides* study system, in which the densities (absence, low or high) of both detritivores belowground and floral herbivores aboveground were manipulated in the field. We quantified several plant traits related to growth, defense, and reproduction testing for linearity and non-linearity in density-dependent effects. We also quantified the growth rate of the florivore caterpillars in the different treatments, and its derived potential consequences for the plant. We predicted that (i) the negative impact of floral herbivores on plant fitness will non-linearly increase with floral herbivore density as a function of increased resistance and tolerance, and that (ii) plant capacity to resist and compensate for floral herbivore damage will linearly increase with detritivore density, although this would be insufficient to downscale the damage inflicted by floral herbivores.

5.3 Material and methods

Study system

The experiment was conducted in 2015 at Barranco del Espartal, a gypsic semiarid open shrub-steppe located in the arid Guadix-Baza Basin (Granada, southeastern Spain). The climate is distinctively continental, with strong temperature fluctuations (ranging from -14ºC to up to 45ºC) and the high seasonality (hot summers, cold winters). Annual precipitation does not usually exceed 300 mm due to geographical isolation, originated by a chain of mountains.

The predominantly semelparous Brassicaceae species *Moricandia moricandioides* (Boiss.) Heywood is abundant in this habitat and was used as a model system (Gómez 1996, González-Megías and Müller 2010, González-Megías and Menéndez 2012, Chapters 3 and 7). *Moricandia moricandioides* plants are distributed in patches, and little litter accumulates underneath them. This species grows as a vegetative rosette during winter and produces reproductive stalks during spring, which remain photosynthetically active during the entire season (González-Megías and Müller 2010). After having reproduced, the vast majority of individuals die during summer (Chapter 7). As it occurs in Brassicaceae and related families, *M. moricandioides* produces the characteristic secondary defense metabolites named glucosinolates (Mithen et al. 2010).

Several insect herbivores are associated with *M. moricandioides*. The Brassicaceae specialists *Pontia daplidice* L. and *Euchloe crameri* Batler pierid caterpillars are among the most important aboveground herbivores (González-Megías and Müller 2010, Chapter 7). Ambient herbivory by *P. daplidice* and *E. crameri* is high in natural *M. moricandioides* plants in the study area; 1.4 ± 0.1 caterpillars of these species per plant were counted in samplings carried out in the study area from 2008 to 2018. The caterpillars of both pierid species feed on reproductive tissue (floral buds, flowers and immature fruits), have equivalent development times and cause similar type of damage on plants, which sometimes even implies total flower and fruit consumption of the plant (González-Megías 2016).

Macroinvertebrates play a major role in root and litter decomposition in the study area (Doblas-Miranda et al. 2009a, González-Megías et al. 2011), as it generally occurs in arid and semiarid ecosystems (Whitford 2000). Below-ground organisms such as *Morica hybrida* Charpentier, *Tentyria incerta* Solier and *Alphasida clementei* Pérez (all of them Coleoptera, Tenebrionidae) are among the most abundant generalist detritivores to be found in the study area, with an overall density of 7 individuals per soil m^2 (Doblas-Miranda et al. 2007).

Experimental set-up

We manipulated the detritivore and floral herbivore densities in a full factorial design with two factors (detritivore and floral herbivore densities). The detritivore (D) density was manipulated at three levels: control plants with no detritivores (D_0, absence), treatments with one detritivore individual (D_1, low) and treatments with two detritivore individuals (D_2, high). The other factor was the floral herbivore (FH) density, again with three levels; control plants with no floral herbivores (FH_0, absence), treatments with one floral herbivore individual (FH_1, low) and treatments with two floral herbivore individuals (FH_2, high). The chosen density gradients for both detritivores and floral herbivores are realistic based on natural *M. moricandioides* plant samplings in the study area.

We started the set up the experiment on 10 and 11-March-2015, when we moved 135 *M. moricandioides* seedlings to the study site. These plants came from seeds collected from the study area during the autumn of 2014. These seeds were germinated in pots with soil from the study area and grown in a common garden. In the field, we located 27 plants in each of 5 blocks, where plants were 30 cm apart from each other (3 replicates x 9 treatments/block). None of the plants had a reproductive stem at the moment of being moved to the field. During the first week in the field, and in the absence of natural rain, all plants were watered and net-covered to ensure their establishment.

To set up the D treatments, plants were re-potted when moved to the field using mixed macroarthropd-free soil from the study site. The pots consisted of fiberglass-mesh cylinders (15 x 20 cm) of 1 mm mesh size to inhibit the entrance or escape of belowground macroinvertebrates. These pots were then buried with the upper surface level with the ground. The reliability of this methodology in recording detritivory effects has been previously demonstrated in this system, as ~ 90% of the larvae can be recovered from the correspondent pots at the time of plant harvest (González-Megías and Müller 2010). As D, third-instar *Tentyria incerta* larvae were used, as they were the most abundant detritivores in the study area during autumn-winter 2014-2015, from which they were collected. The larvae were added to D_1 and D_2 plants once plants were established (27-March-2013).

To set up the FH treatments, naturally occurring egg oviposition by *P. daplidice* and *E. crameri* on reproductive stalks of FH_1 and FH_2 plants was allowed, but removed all eggs from FH_0 plants. In cases where no caterpillars had hatched on FH_1 or FH_2 plants when plants already had reproductive tissues, first instar caterpillars collected from the study area were added. Once FH_1 or FH_2 plants had the designated FH density level, additional pierid eggs laid by butterflies were thereafter removed from these plants. When caterpillars died or disappeared before completing their larval cycle and moving to pupate, we replaced them by adding same-instar caterpillars collected from the study area to the plants.

Twenty plants did not produce reproductive stalks, and on another 12 plants caterpillars did not remain on plants until completing their entire larval cycle, thus all these plants were excluded from the analyses. The final sample size was 105, and the sample size per treatment was D_0FH_0 n = 12, D_0FH_1 n = 12, D_0FH_2 n = 7, D_1FH_0 n = 12, D_1FH_1 n = 14, D_1FH_2 n = 9, D_2FH_0 n = 13, D_2FH_1 n = 13 and D_2FH_2 n = 11.

Data collection

Plant reproductive traits (number of floral bud groups, flowers and fruits) were recorded on each experimental plant 3 times per week after the set-up of the experiment (from 23-March-2015) until the end of the experiment (01-July 2015), resulting in a total of 44 surveys. At the end of the experiment, we counted the total number of flowers and fruits produced by each plant. Fruits were collected after complete maturation of seeds but before seed dispersal. All fruits were taken to the laboratory where the number of viable seeds (seeds hereafter) in each fruit was counted to quantify total seed production per plant, which we used as the estimate of fitness.

The entire aboveground tissue was collected to determine aboveground dry biomass and C/N ratio of leaf tissue. C/N ratio was also determined from seeds, in both cases with a CHN Elemental Analyser. Belowground tissue was not collected because we wanted to estimate resprouting rate at the next season, for what no differences among treatments were observed (data not shown).

To quantify glucosinolate (GLS) concentrations in leaves, the youngest leaf of one stem of each of the experimental plants was collected at mid-June, when plants had already interacted with florivores and detritivores for weeks but prior to leaf senescence. Leaves were immediately frozen and freeze-dried. The dried material was ground and extracted three times in 80% methanol after the addition of p-hydroxybenzyl GLS (sinalbin) used as an internal standard. GLS extraction and conversion to desulfoglucosinolates were done following previously established methodology using high performance liquid chromatography coupled with a diode array detector (Chapter 7). Desulfoglucosinolates were identified by comparison of UV-spectra and retention times to those identified in earlier studies (González-Megías and Müller 2010, Chapter 7). Peaks were integrated at 229 nm and response factors of 1 for aliphatic and 0.26 for indolic GLSs were considered and related to the internal standard (response factor 0.5) and sample dry mass for calculation of concentrations.

We also calculated caterpillar development time (days) strictly for those caterpillars that completed their entire larval cycle without being replaced on the experimental plants (n = 53 caterpillars on n = 45 plants). When development time of both caterpillars on FH_2 plants could be measured, we used mean value per plant.

Statistical analyses

First, Generalized Estimation Equation models (GEE) were performed to test the effects of each continuous factor (D and FH densities) and their interaction on plant reproductive development (number of floral bud groups, flowers and fruits) over time. It has been argued that plant reproduction is a hierarchical process with ephemeral structures (e.g. floral buds and flowers) that impede the assessment of florivory impact on plants (Breadmore and Kirk 1998). Considering that plant tolerance responses towards florivory may involve changes in the timing and the amount of reproductive tissue produced, we consider that GEE models which handle dependent observations in the same individual with a fitted correlation structure can help to delve into how the different treatments affected the reproductive process. GEEs, i.e. the marginal modelling approach, is a powerful and pragmatic tool for analyzing a variety of correlated data and can handle non-normal distribution and heteroscedasticity (Halekoh et al. 2006, Pekár and Brabec 2018). GEEs deal with dependencies by allowing correlations in residuals (i.e. repeated measurements made on the same individual in time) through a pre-specified correlation structure, which can be fitted in accordance with the correlation matrix structure (Halekoh et al. 2006, Pekár and Brabec 2018). Hence, GEEs produce high quality parameter estimates with asymptotically correct standard errors, providing correct subsequent inferences for marginal models (Pekár and Brabec 2018).

Due to sequential occurrence in reproductive traits and the ephemerality of some of them, we estimated the different reproductive traits over different time periods: 1) from the first floral bud production until final fruit number for floral bud groups (since initiation of reproduction to when there was no more investment in new reproductive tissue), 2) from the first floral bud production until no floral bud groups were left for flowers (floral buds are needed for flowering) and 3) from the first fruit production until final fruit number for fruits (since when fruiting began to when there were no more flowers that could pass to fruits). The FH density was fitted to the date interval they were present on plants. We tested different distributions and correlation structures for each of the variables. We used the Quasi Information Criterion (QIC) model fit for model selection, as it works well selecting the correlation structure in nonlikelihood-based methods, such as GEE (Pan 2001). Floral bud groups and fruits were modelled with a Poisson distribution, as they tended to decrease in number from the first observations, and an autoregressive model of the 1.St order correlation structure due to strong correlation between following surveys. In an autoregressive model the correlation declines with the distance between observations. Flowers were modelled with a Gaussian distribution and an exchangeable correlation structure, as there was no strong correlation between surveys, probably because of their short lifetime and because flowering could partially depend on the stochasticity of climatic conditions. An exchangeable model has a single correlation parameter identical for all pairs of measurements on the same individual, irrespective of how far in time the measurements are from each other. These analyses were performed using R (R Core Team 2017), with the package *geepack* (Halekoh et al. 2006).

Second, general or generalized linear mixed models (GLMMs) were performed to test the effects of each continuous factor (D and FH densities) and their interaction on plant morphology (aboveground biomass), quality (nutrient content in leaves and seeds and GLS concentrations in leaves) and reproduction (number of flowers, fruits and seeds). GLMMs were also performed to test the effects of D and FH densities and their interaction on FH caterpillar development time. Variables were modelled with Gaussian, Gamma, Poisson or Negative Binomial distributions, and were transformed when necessary (see Suppl. 5.1, Table 5.S1 for best models for each variable in detail). Models with Gamma distribution were analyzed with inverse link function and those with Poisson distribution were analyzed with log link function. When overdispersion was observed (Suppl. 5.1, Table 5.S1), GLMMs with observation-level random effects were run, which allowed for variation at plant level (Harrison 2014). Block was included as random factor for all the variables. Model selection was done according to Bayesian Information Criterion (BIC) model fit, as it tends to favor more parsimonious models (Grueber et al. 2011). These analyses were performed with the package *lme4* (Bates et al. 2015). When factor or interaction-level effects were significant, post hoc tests were performed with the same model structure but with D and FH densities as categorical (absence, low or high density). As post hoc, we used the *P*-value adjusting multivariate test (see Suppl. 5.1, Table 5.S2 for detailed factor and interaction level post hoc results) with the *lsmeans* package (Lenth 2016). As a complement to linear models, we also performed generalized additive mixed models (GAMMs) to test for additivity in the effects of D and FH densities on the above-mentioned variables. We used for each variable the same distribution and random structure specified in the GLMMs, being results obtained by GAMMs very similar to those of GLMMs (Suppl. 5.2, Table 5.S3, Fig. 5.S1). These analyses were performed with the package *mgcv* (Wood 2004).

In addition, we performed a permutational multivariate analysis of variance (PERMANOVA) to test whether D and FH densities affected leaf GLS profiles, which were complemented by used non-metric multidimensional scaling (NMDS) to test treatment dissimilarities in GLS profile composition. We used Horn dissimilarity and 10,000 permutations in both analyses for assessing significance, with 100 random starts in the NMDS. The analyses were performed with the *vegan* package (Oksanen et al. 2017).

Lastly, and based on previous results, sets of component models were combined within a piecewise structural equation modelling (SEM) framework to parse the direct and indirect effects of FH density (as continuous) on plant fitness (number of seeds) through FH caterpillar development time (SEM 1) and through plant reproductive components (SEM 2). The SEMs were fitted using the R package *piecewiseSEM* (Lefcheck 2016). These models allow formulating hypotheses on pathways of interaction between parameters in the model, where all parameters could act as both predictor and response variables. We limited to the maximum the number of variables, in order to have at least 10 times as many observations as variables (Shipley 2016). Variables were standardized (mean = 0, SD = 1) and we fitted the component models of the piecewise SEMs as linear mixed models. For all component models, the random structure was the same as for GLMMs. We started with initial SEMs (whom hypothesized pathways were based on prior GLMM results; Suppl. 5.5, Fig. 5.S4; Suppl. 5.6, Fig. 5.S5) and improved it through the stepwise procedure by evaluating Akaike's Information Criterion corrected for small sample sizes (AICc) (Lefcheck 2016, Shipley 2016), until we procured the final SEMs with the lowest AICc score (Suppl. 5.5, Table 5.S5; Suppl. 5.6, Table 5.S7). In *piecewiseSEM,* the optimization procedure is based on the removal of irrelevant paths and the inclusion (based on Shipley's d-separation test) of any of the non-hypothesized biologically relevant paths that can improve the model (Lefcheck 2016, Shipley 2016).

5.4 Results

Plant reproductive trait development over time (GEEs)

D density had no effect on floral bud group and flower production on *M. moricandioides*, while with increasing density of FH floral bud group and flower production was enhanced (Table 5.1). Fruit production seemed to be favored by D density, but when combined with FH density a negative non-additive effect was observed (Table 5.1, see Fig. 5.1 for a graphic approximation).

Linear effects (GLMMs)

FH caterpillar development time

FH caterpillar development time was shortened with increasing density of FH (F = 12.82, P = 0.0003, df = 1,55), as they developed faster on high FH than on low FH plants (16.31 ± 0.73 days on FH_1 plants vs. 12.86 ± 0.44 days on FH_2 plants; Suppl. 5.1, Table 5.S2a). D density (F = 1.06, P = 0.30, df = 1,55) and the interaction term of D and FH densities (F = 0.33, P = 0.56, df = 1,55) had no effect on caterpillar development time.

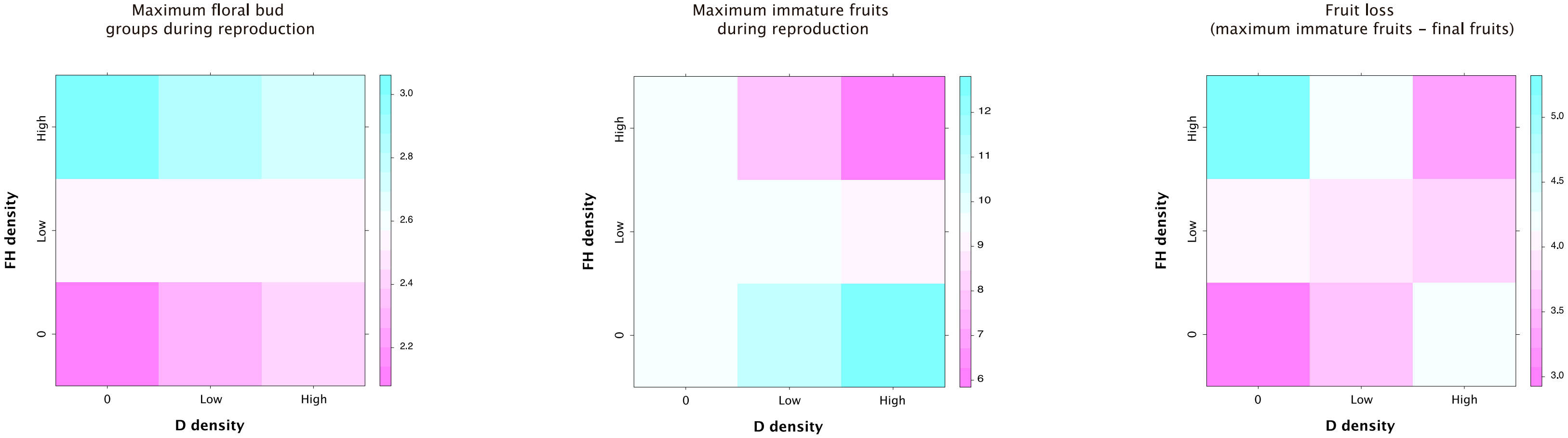


Figure 5.1. Contour plot of detritivore (D) and floral herbivore (FH) density effects on mean maximum number of floral bud groups and fruits produced, and on the mean number of immature fruits lost during reproduction.

	D				**FH**				**D x FH**			
	χ^2	*z*	*P*	df	χ^2	*z*	*P*	df	χ^2	*z*	*P*	df
Floral bud production	0.04	-0.19	0.24	1,1493	**6.85**	**2.61**	**0.008**	1,1493	0.01	0.10	0.91	1,1493
Flower production	2.22	1.49	0.13	1,1086	**12.28**	**3.50**	**0.0004**	1,1086	3.37	-1.83	0.06	1,1086
Fruit production	**3.93**	**1.98**	**0.04**	1,1034	1.14	1.06	0.28	1,1034	**4.06**	**-2.02**	**0.04**	1,1034

Table 5.1. Results of generalized estimation equation models (GEEs) for the effect of detritivores (D) and floral herbivores (FH) on plant reproductive traits development over time. Significant values ($P < 0.05$) are highlighted in bold.

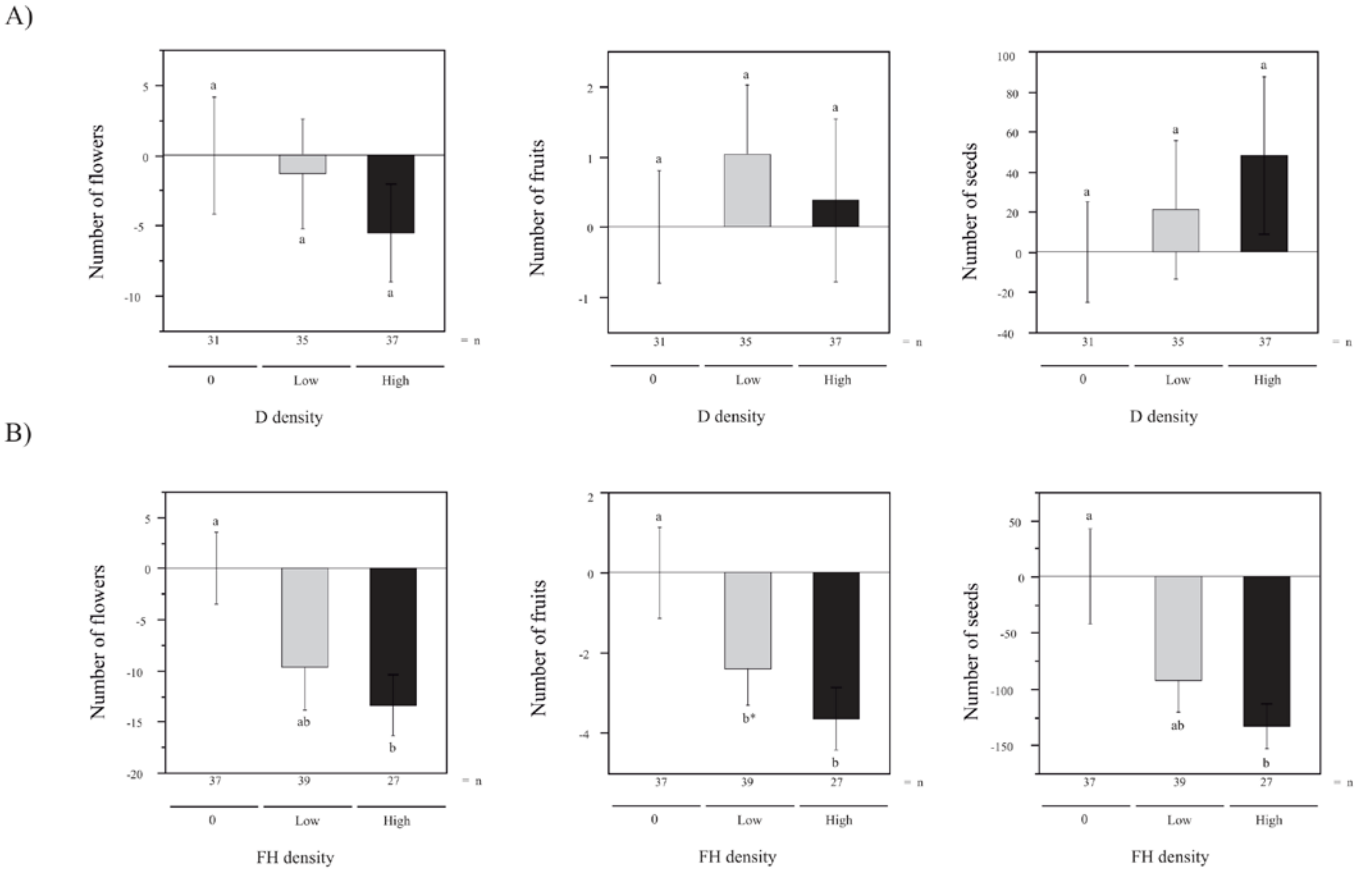


Figure 5.2. A) Detritivore (D) density effects on plant reproduction. Shown values (mean ± SE) are relative to mean $D_0 = 0$. White bars are shown for D_0 plants, grey bars are shown for D_1 plants, black bars are shown for D_2 plants. B) Floral herbivore (FH) density effects on plant reproduction. Shown values (mean ± SE) are relative to mean $FH_0 = 0$. White bars are shown for FH_0 plants, grey bars are shown for FH_1 plants, black bars are shown for FH_2 plants. For both A) and B), letters correspond to post hoc results (see Suppl. 5.1, Table 5.S2a). Asterisk on *b* letter reveal that post hoc results are only marginally significant ($P < 0.08$) between *a* and *b* (see Suppl. 5.1, Table 5.S2a).

Plant morphology and reproduction

D and FH densities did not have any significant effect aboveground biomass (Table 5.2). D density did not have either any effect on any measured reproductive trait (Table 5.2, Fig. 5.2A). The negative effect of FH on the number of flowers, fruits and seeds increased with FH density (Table 5.2). Plants with high FH had significantly fewer flowers, fruits and seeds than plants without FH, while low FH plants had no effect on the number of flowers and had only a marginally significant negative effect on the number of fruits and seeds when compared to plants without FH (Fig. 5.2B).

Plant quality

Both D and FH densities independently affected C/N ratio in leaves (Table 5.2). Plants with high D had higher C/N ratio in leaves (D_0 = 50.67 ± 2.46, D_1 = 50.23 ± 1.70, D_2 = 55.94 ± 2.25; Suppl. 5.1, Table 5.S2a). In the case of FH density, the effect was only significant between low FH and absence of FH (FH_0 = 56.04 ± 2.03, FH_1 = 50.39 ± 2.18, FH_2 = 49.88 ± 2.02; Suppl. 5.1, Table 5.S2a). C/N ratio in seeds was interactively affected by D and FH densities (Table 5.2): C/N ratio in seeds was lower on high FH plants, but the effect was cancelled at high D density (Fig. 3; Suppl. 5.1, Table 5.S2b).

In the case of GLS concentrations in leaves (mean of total concentration over all samples ± SE: 8.44 ± 0.98 µmol g^{-1} of dry weight) 10 compounds were identified, 4 indolic (1.33 ± 0.17 µmol g^{-1} of dry weight) and 6 aliphatic (7.11 ± 0.94 µmol g^{-1} of dry weight). Indol-3-yl-methyl GLS was the main indolic compound (0.60 ± 0.13 µmol g^{-1} of dry weight), while 3-butenyl was the main aliphatic compound (5.75 ± 0.86 µmol g^{-1} of dry weight). D density had no effect on GLS concentrations (Table 5.2). High FH density seemed to increase aliphatic, indolic and total GLS concentrations in relation to low or absence of FH (more than 50% in all cases; Table 5.2), but the factor-level test statistics were only marginally significant (Suppl. 5.1, Table 5.S2a).

Permanova test revealed no significant effects of D and FH densities in leaf GLS profile (D density: $F = 0.44$, $P = 0.78$, $df = 1{,}96$; FH density: $F = 1.32$, $P = 0.22$, $df = 1{,}96$; interaction term of D and FH densities: $F = 0.30$, $P = 0.91$, $df = 1{,}96$; see NMDS in Suppl. 5.3, Table 5.S4, Fig. 5.S2).

	D			FH			D x FH		
	F/χ^2	P	df	F/χ^2	P	df	F/χ^2	P	df
Aboveground biomass and reproduction									
Aboveground biomass	1.29	0.25	1,94	2.64	0.10	1,94	1.82	0.17	1,94
Number of flowers	2.45	0.11	1,94	**5.66**	**0.01**	1,94	0.31	0.57	1,94
Number of fruits	0.21	0.64	1,94	**7.24**	**0.007**	1,94	0.55	0.45	1,94
Number of seeds	1.56	0.21	1,95	**6.77**	**0.009**	1,95	0.41	0.52	1,95
Leaf and seed nutrient content									
C/N ratio in leaves	**13.05**	**0.0003**	1,83	**7.40**	**0.006**	1,83	0.02	0.88	1,83
C/N ratio in seeds	2.46	0.11	1,61	**6.96**	**0.08**	1,61	**5.10**	**0.02**	1,61
Leaf glucosinolates (GLSs)									
Total GLSs	0.11	0.73	1,92	**4.09**	**0.04**	1,92	0.07	0.78	1,92
Aliphatic GLSs	0.03	0.85	1,92	*3.60*	*0.05*	1,92	0.01	0.89	1,92
Indolic GLSs	0.16	0.68	1,92	*3.79*	*0.05*	1,92	0.72	0.39	1,92

Table 5.2. Results of general and generalized linear models for the effect of detritivores (D) and floral herbivores (FH) on plant aboveground biomass, reproduction, leaf and seed nutrient content and leaf glucosinolates (GLSs). F is shown for glucosinolates, χ^2 is shown for the rest. Significant values (P < 0.05) are highlighted in bold.

Structural equation model of RH and FH density effects on plant fitness

In the SEM 1, FH density negatively affected plant fitness (Fig. 5.4A). Fitness was affected principally due to the reduction in flower number with increasing FH density, and the negative consequences of reducing flower number were amplified by enlarging FH caterpillar development time, which limitedly but positively contributed to plant fitness (Fig. 4A; Suppl. 5.5, Table 5.S6). FH caterpillar development time was mainly affected by FH density itself rather than indirectly by reducing flower number (Fig. 5.4A).

In the SEM 2, both direct (through reducing number of seeds) and indirect (through reducing number of flowers and increasing GLS concentrations) paths showed to be important for the negative fitness consequences of FH density (Fig. 5.4B; Suppl. 5.6, Table 5.S8). FH density effect on fitness was partially ameliorated by the indirect consequences of moderately boosting aboveground biomass (Fig. 5.4B; Suppl. 5.6, Table 5.S8).

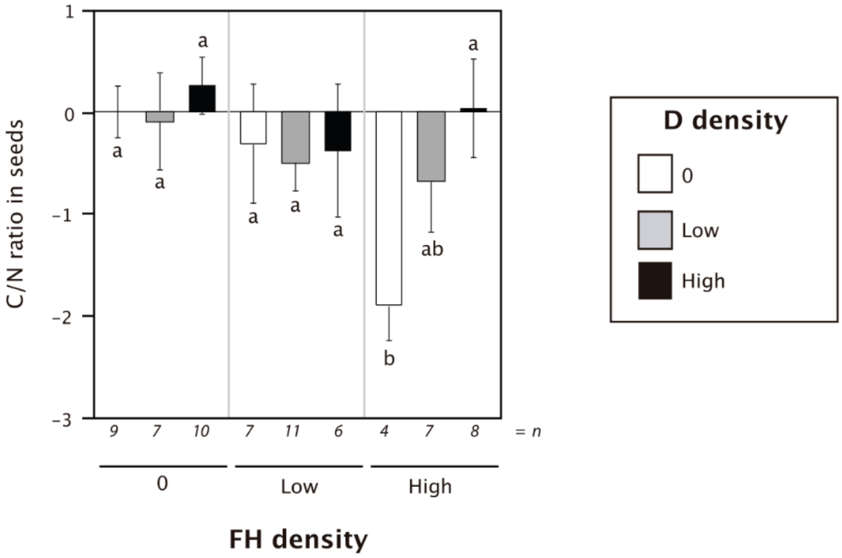


Figure 5.3. Detritivore (D) and floral herbivore (FH) density effects on C/N ratio in seeds. Shown values (mean ± SE) are relative to the focal treatment (mean $D_0FH_0 = 0$). Letters correspond to Fischer Least Square Differences. Interaction level post hoc results are detailed in Suppl. 5.1, Table 5.S2b.

5.5 Discussion

Density-dependent FH damage

Both resistance and tolerance can be successful responses minimizing florivory impact, as they have been observed in various plants differing in phylogenetic background and life cycle, and in response to both artificial and natural florivory (e.g. McCall and Irwin 2006, McCall and Karban 2006 for resistance; e.g. Wise et al. 2008, Lucas-Barbosa et al. 2013, Soper Gorden and Adler 2016 for tolerance). According to the present study, high FH density triggered both tolerance and slightly resistance in *M. moricandioides*. Tolerance to intense florivory was evident by temporally tracking plant reproduction through GEE analysis, since the highest production rate of floral bud groups and flowers occurred at that precise moment of FH presence on plants (to a large extent eaten by FH caterpillars afterwards). Plants thus promote this response in prevision of intense forthcoming damage on reproductive tissues (Wise et al. 2008, Lucas-Barbosa et al. 2013), as it occurred since the caterpillars hatched but prior to when severe damage was caused . In a previous experiment with a similar FH manipulation, *M. moricandioides* produced more flowers and fruits when coping with florivory, and in turn increased aboveground biomass more acutely that in the present experiment (Chapter 4). The fact that this tolerance response was observed in different sequential stages of reproduction in the two experiments is probably due to differential phenological mismatch across years between plants and caterpillars, as plants and insects do not respond equally to variation in environmental cues such as temperature and photoperiod (de Vries et al. 2011, Kharouba et al. 2015). According to this, in the present experiment caterpillars could have hatched and began to feed on initial ontogenic stages of plant reproduction, inducing compensatory production of floral buds. With regard to resistance, we observed a marginally-significant trend towards increasing both aliphatic and indolic GLS concentrations on plants with high FH.

Despite promoting tolerance and resistance, high FH density had a negative impact on plant fitness. High levels of coexpression for compensative growth and defense may be physiologically possible, although it could result a maladaptative strategy that does not necessarily imply fitness benefits (Züst and Agrawal 2017). In the case of *M. moricandioides*, however, the mixed resistance-tolerance response may be the best of possible trait combinations in that circumstance, since it reduced the fitness costs of florivory (Chapter 4). In this way, FH density had a non-linear impact on plant reproductive output, as there were no significant differences between high and low FH densities on their fitness impact (damage curve softened with increasing FH density). Hence, the combined defense strategy towards high FH density could be adaptive as it costs are non-linear (Mauricio et al. 1997, Fornoni et al. 2004, Fornoni 2011, Züst and Agrawal 2017), as previously seen in the system (Chapter4).

By means of a SEM we tested the direct and indirect paths by which FH density could have affected plant reproductive success, given that the development time of FH caterpillars was reduced when their density was high. The SEM suggested that FH density itself and the number of flowers accelerated and shortened, respectively, FH caterpillar development time. The relevance of flower number determining reproductive success would thus not only depend on the rate of those that passed to fruits, but also through conditioning food quantity for caterpillars (Awmack and Leather 2002). Despite its effect being moderate, a reduction on FH caterpillar development time would heighten the negative impact of high FH on plants. On the contrary, we observed that the harmful effects of FH non-linearly decreased with their density, highlighting that compensatory growth in reproductive tissue overly counteracted those effects.

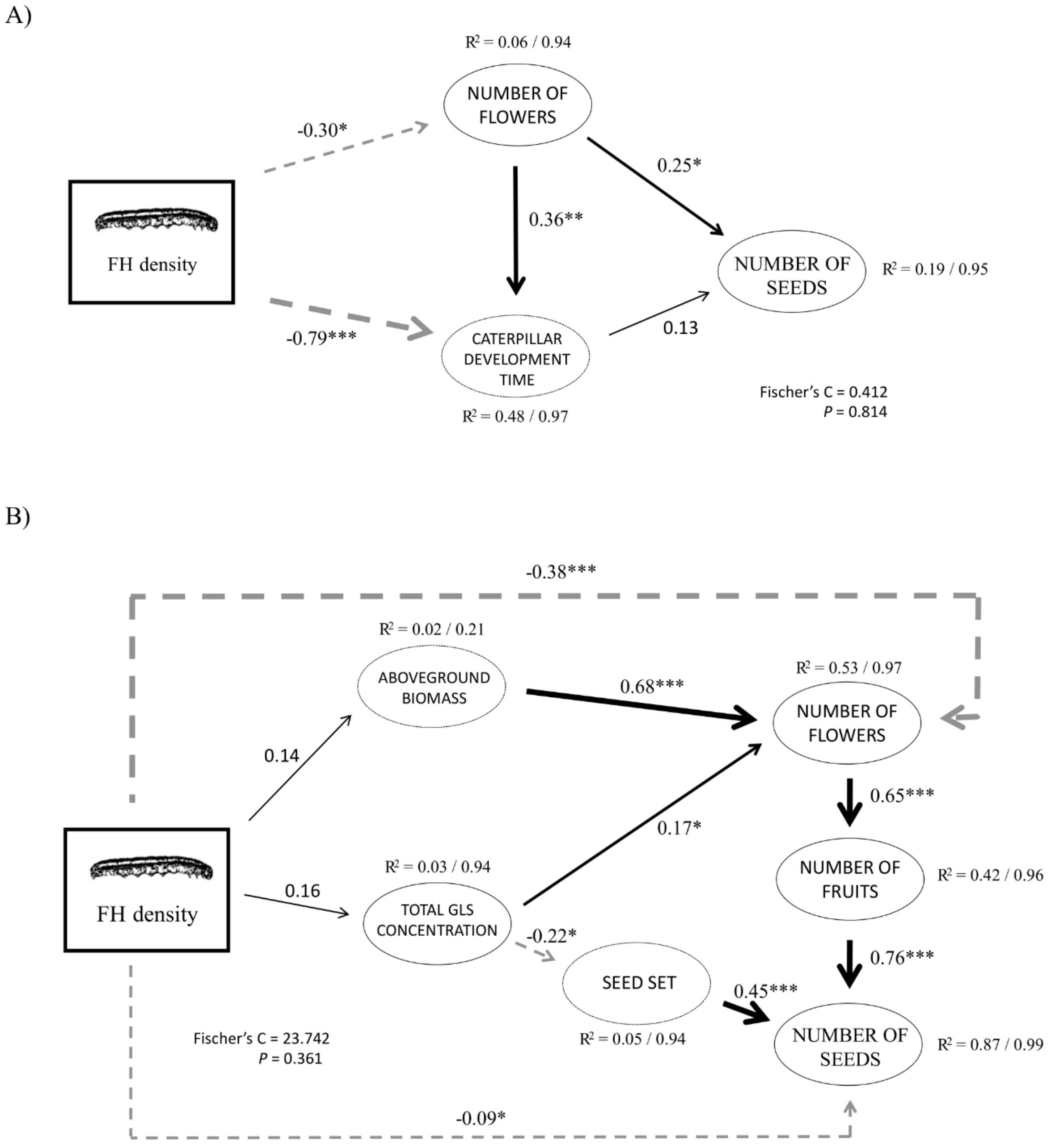


Figure 5.4. A) Final piecewise SEM parsing the direct and indirect effects of FH density on on plant fitness through FH caterpillar development time. B) Final piecewise SEM 2 parsing the direct and indirect effects of H and FH density on sequential plant reproductive components and fitness. Seed set refers to the proportion of ovules that passed to seeds. For both A) and B), standardized path coefficients are shown next to each path, and their significance level is shown as * *P* <0.05, *** *P* <0.001. Solid lines denote positive and dashed lines negative relationships, and their thickness is scaled to the magnitude of the path strength. Variance explained by the component models (R^2) is reported as marginal / conditional.

Consequences for FH caterpillar performance

From the caterpillar perspective, it has been shown for *Pieris brassicae* that food deprivation can shorten development time (Fei et al. 2016), a very plausible hypothesis in our system as it been observed in the present study (Suppl. 5.4, Fig. 5.S5a), and in a similar previous study with an equal FH manipulation (Chapter 4). That previous study also suggested that the induction of GLSs could also accelerate FH development time. This hypothesis was not that evident in the present experiment (Suppl. 5.4, Fig. 5.S5b), and the SEM actually dismissed that path, likely because glucosinolate concentrations could have exceeded in this case the counteradaptation threshold of these specialized herbivores, slowing and/or limiting their metabolization capacity (Siemens and Mitchell-Olds 1996, Ali and Agrawal 2012). Rather than through changes in GLS concentrations, in this case caterpillar density itself would have accelerated their own development through a reduction of food resources (flower number) and an augmentation in intraespecific competition (Kaplan and Denno 2007), which allows presuming a negative density-dependent effect on their own performance (Reader and Hochuli 2003, Fei et al. 2016).

D density-dependent effects and its consequences for FH damage

The present study complements previous ones (González-Megías 2016), showing that not even high density of D permitted *M. moricandioides* to increase its reproductive output nor significantly diminish FH impact on plant reproduction (Suppl. 5.7, Fig. 5.S6). It is intriguing that a non-micorrhyzal plant inhabiting a semiarid environment characterized by poor gypsum soils, in which calcium saturates the soil ion exchange complex and impedes plant nutrient uptake (Meyer et al. 1992, Palacio et al. 2014), is not apparently benefited by D to increase fitness nor to better defend against or compensate for herbivore damage. There are several reasons why this may occur. Regarding a hypothetical increase in tolerance, we did observe undercompensatory production of reproductive tissue towards FH, but plant capacity to regrowth after severe damage might be limited, especially in advanced ontogenic stages (Lowenberg 1994, Oguro and Sakai 2009, White et al. 2016). Moreover, if the plant would nearly reach their physiological maximum when facing severe herbivory (Arendt 1997, Garcia and Eubanks 2018), there would be little margin for increased production of new reproductive tissue. Such limitations after damage might be even strengthened in stochastic ecosystems such as semiarids where the severe and fluctuating environmental conditions make growing and reproductive seasons short and unpredictable (Hänel and Tielbörger 2015). We did yet observe that D density favored fruit production, but in combination with FH the interaction was negative and non-additive (GEE result), although with no consequences for final fruit number. With this set of arguments, and based on the actual data, it could be concluded that any benefit from increased resource availability would not entail a higher level of compensation (Hilbert et al. 1981). Regarding the functionality of a hypothetical stronger defense induction due to the extra resources provides by D, it may increase the cost/benefit balance when plants respond in such a mixed way to specialist chewing herbivores (Pilson 2000, Xiao et al. 2018). Nevertheless, it is worth mentioning that, as shown in earlier studies (González-Megías and Müller 2010), when plants faced floral herbivory in presence of low D we observed pronounced changes in GLSs profile. When the density of both D and FH was high, detritivore activity modulated FH impact on seed quality instead.

Some authors suggest that the breakdown of organic matter and nutrient mineralization due to detritivory is a slow process with delayed effects in soils, which do not necessarily benefit plants in short-term (Wurst and Ohgushi 2015). This could be critical for a predominantly semelparous herb with a short ideal period for reproducing due to the harsh environmental conditions, and limit its potential to largely influence growth and reproduction, restricting potential detritivore effects to nutrient and metabolite allocation. The most remarkable effects of D on *M. moricandioides* are indeed those occurring in late ontogenic stages, such as seed provisioning. Detritivore activity usually reduces C/N ratio in *M. moricandioides* seeds (González-Megías and Menéndez 2012, González-Megías 2016), which can ultimately increase seedling recruitment (González-Megías 2016). Nonetheless, D effects on seed quality are highly context-dependent since are modulated by precipitation regimes (González-Megías and Menéndez 2012), what may affect moisture and litter availability and quality (Doblas-Miranda et al. 2009a, 2009b, A'Bear et al. 2014), and by herbivory below- and aboveground (González-Megías, 2016). In the present study we observed that high D density allowed plants to counterbalance high FH-derived reduction on C/N ratio in seeds, which in turn could be due to the nitrogen released as a consequence of decreased photosynthetic activity (Schwachtje and Baldwin 2008). In the light of the recent findings in the model Brassicaceae *Arabidopsis* remarking the importance of C/N ratio-dependent signaling pathways for seed germination (Osuna et al. 2015), it would be interesting if future studies could discern its consequences on offspring viability, and therefore, on plant lifetime fitness. That would favor disentangling if the plant's resource optimization strategy in this case were to increase the quality rather than the quantity of the potential progeny.

5.X Supplementary material

Supplementary material 5.1. Model selection and post hoc tests.

Variable	Distribution	Random structure	BIC
FH caterpillar development time	Gaussian	Block	346.524
Aboveground biomass	Gamma	Block	97.729
Number of flowers	Poisson	Block / Plant	929.104
Number of fruits	Poisson	Block / Plant	605.641
Number of seeds	Negative binomial	Block	1001.520
C/N ratio in leaves	Gamma	Block / Plant	-798.431
C/N ratio in seeds	Gamma	Block	234.278
Total GLSs*	Gaussian	Block / Plant	303.343
Aliphatic GLSs*	Gaussian	Block / Plant	301.820
Indolic GLSs*	Gaussian	Block / Plant	303.363

Table 5.S1. Model structure of selected GLMMs for analyzed variables. * GLS concentrations are range/(n-1) transformed.

	D factor level effects		
	D_0 – D_1	**D_0 – D_2**	**D_1 – D_2**
C/N ratio in leaves	1.18	**2.56***	1.46
	FH factor level effects		
	FH_0 – FH_1	**FH_0 – FH_2**	**FH_1 – FH_2**
FH caterpillar development time	-	-	**3.44*** **
Number of flowers	2.01	**3.34** **	-1.08
Number of fruits	2.2z^{ms}	**2.46***	0.41
Number of seeds	1.50	**2.60***	1.27
C/N ratio in leaves	**-3.26** **	-1.32	1.66
Total GLSs	0.08	-2.11	-2.07

Table 5.S2a. *P*-value adjusting multivariate post hoc tests for GLMM factor level effects. *t*-values are shown for variables analyzed with Gaussian distribution (total GLSs), and *z*-values are shown for the rest. ^{ms} *P* <0.08,* *P* <0.05, ** *P* <0.01, *** *P* <0.001.

	D-FH interaction level effects								
	D$_0$			**D$_1$**			**D$_2$**		
	FH$_0$ – FH$_1$	**FH$_0$ – FH$_2$**	**FH$_1$ – FH$_2$**	**FH$_0$ – FH$_1$**	**FH$_0$ – FH$_2$**	**FH$_1$ – FH$_2$**	**FH$_0$ – FH$_1$**	**FH$_0$ – FH$_2$**	**FH$_1$ – FH$_2$**
C/N ratio in seeds	-0.91	**-3.46****	**-2.72***	-1.08	-1.33	-0.39	-1.60	-0.47	0.63
	FH$_0$			**FH$_1$**			**FH$_2$**		
	D$_0$ – D$_1$	**D$_0$ – D$_2$**	**D$_1$ – D$_2$**	**D$_0$ – D$_1$**	**D$_0$ – D$_2$**	**D$_1$ – D$_2$**	**D$_0$ – D$_1$**	**D$_0$ – D$_2$**	**D$_1$ – D$_2$**
C/N ratio in seeds	-0.29	0.05	0.35	-0.44	-0.11	0.30	2.09	**3.20****	1.31

Table 5.S2b. *P*-value adjusting multivariate post hoc tests for GLMM interaction-level effects. *t*-values are shown. ^{ms} *P* <0.08, * *P* <0.05, ** *P* <0.01.

Supplementary material 5.2. Generalized additive linear models (GAMMs).

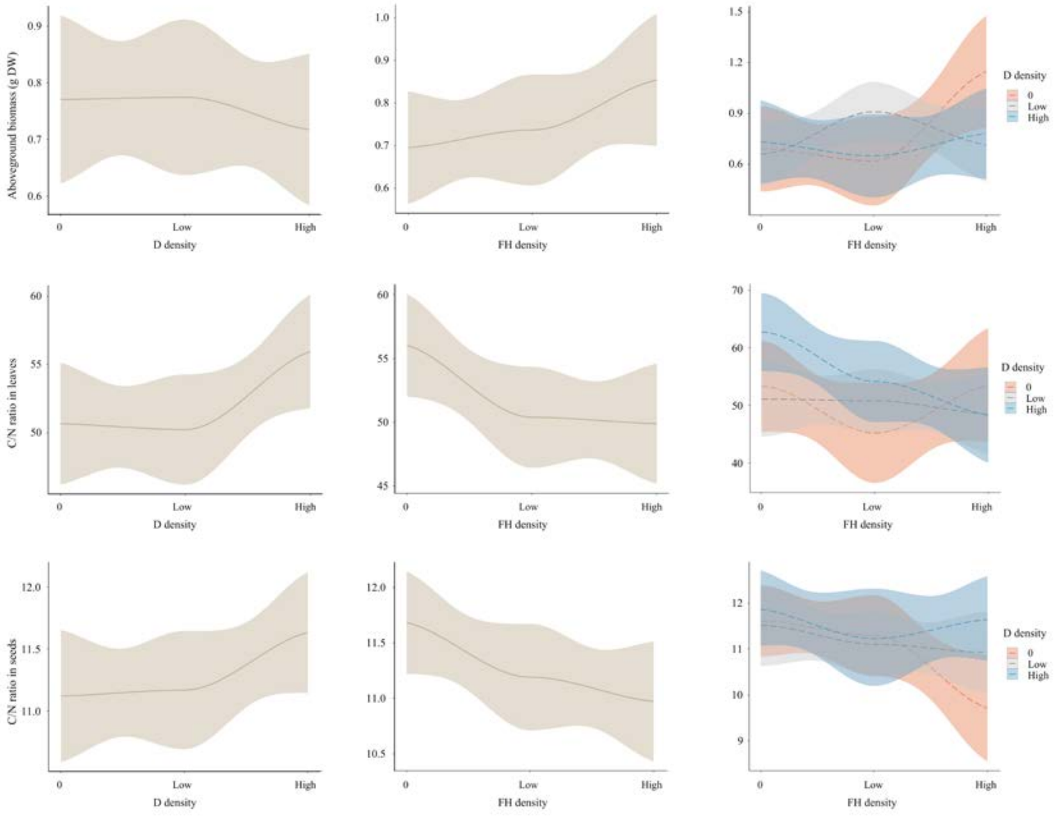
	D			**FH**			**D x FH**		
	F/χ^2	*P*	df	F/χ^2	*P*	Df	F/χ^2	*P*	df
Aboveground biomass and reproduction									
Aboveground biomass	1.34	0.24	1,94	2.72	0.09	1,94	1.88	0.17	1,94
Number of flowers	1.89	0.16	1,94	**5.33**	**0.02**	1,94	0.33	0.56	1,94
Number of fruits	0.08	0.76	1,94	**6.45**	**0.01**	1,94	0.49	0.48	1,94
Number of seeds	2.04	0.15	1,95	**9.38**	**0.002**	1,95	0.47	0.49	1,95
Leaf and seed nutrient content									
C/N ratio in leaves	2.82	0.09	1,94	**4.62**	**0.03**	1,94	2.91	0.08	1,94
C/N ratio in seeds	2.48	0.11	1,94	**5.95**	**0.01**	1,94	**4.03**	**0.04**	1,94
Leaf glucosinolates (GLSs)									
Total GLSs	0.09	0.75	1,92	*3.80*	*0.05*	1,92	0.05	0.81	1,92
Aliphatic GLSs	0.03	0.85	1,92	*3.45*	*0.06*	1,92	0.01	0.89	1,92
Indolic GLSs	0.16	0.68	1,92	*3.64*	*0.05*	1,92	0.69	0.40	1,92

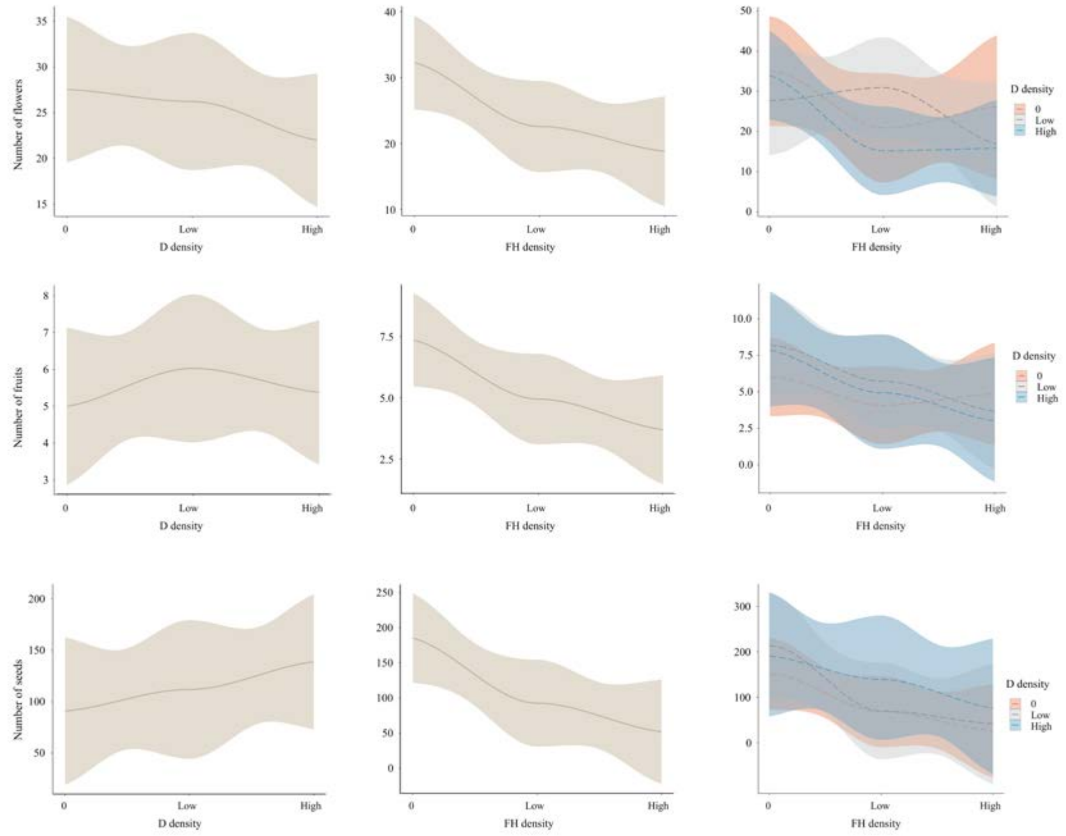
Table 5.S3. GAMM results for the effect of detritivores (D) and floral herbivores (FH) on analyzed plant trait variables. No smoothing parameter was added to GAMMs as it is not possible for models with only three density levels. Significant values ($P < 0.05$) are highlighted in bold.

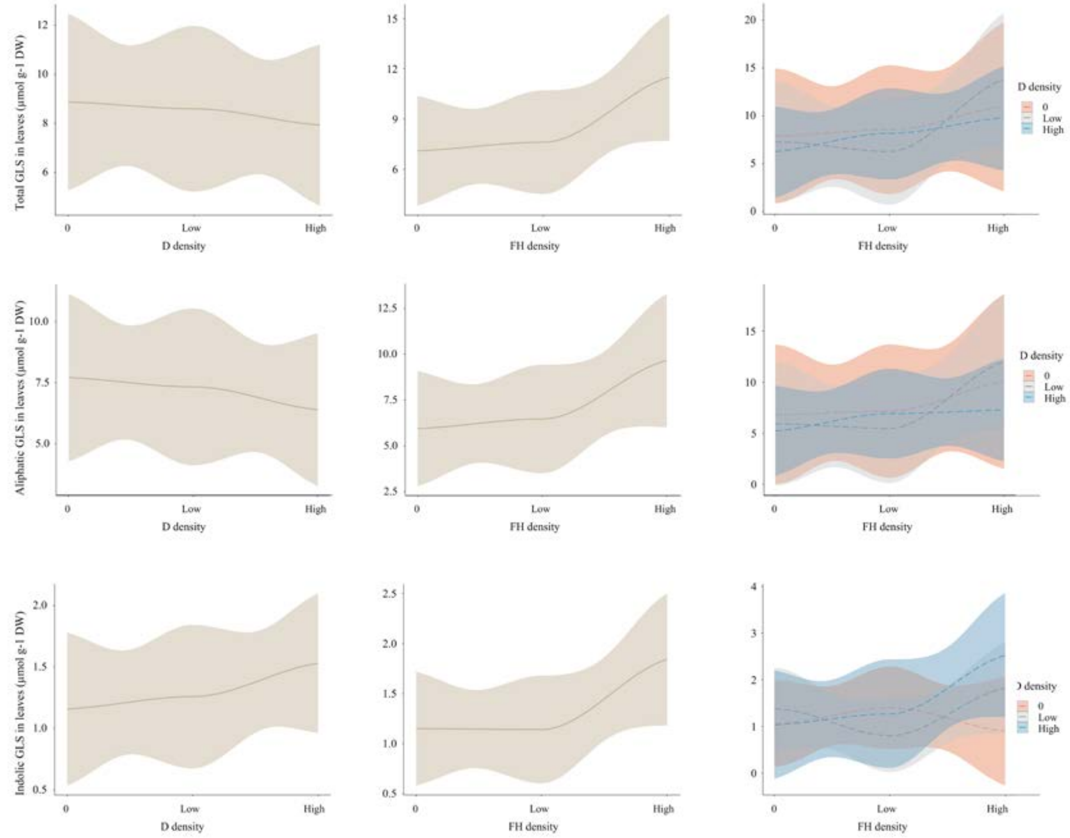
The results obtained by GAMM were very similar to those obtained by GLMMs. D density had no significant effect on any measured variable (Table 5.S3).

FH density had a significant effect on the number of flowers, fruits and seeds, and on C/N ratio in leaves, while FH density effect on GLS concentrations in leaves was only marginally significant (Table 5.S3). For the three reproductive variables the effect was not totally linear because descending slope with increasing FH density moderately slowed down between the low and high densities (Fig. 5.S1). For C/N ratio in leaves, it descended between absence of FH and low FH, but it was similar between low and high densities (Fig. 5.S1). As in the GLMMs, D and FH densities had an interactive effect on C/N ratio in seeds (Table 5.S3, Fig. 5.S1).

Figure 5.S1. Below, locally estimated scatterplot smoothing (LOESS) plots for all analyzed plant variables regarding root herbivore (D) density, floral herbivore (FH) density, and their combined densities.




Number of flowers
Number of fruits
Number of seeds
D density
FH density
0
Low
High
D density
0
Low
High
35
30
25
20
15
40
30
20
10
50
40
30
20
10
0
8
7
6
5
4
3
7.5
5.0
2.5
10.0
7.5
5.0
2.5
0.0
200
150
100
50
250
200
150
100
50
0
300
200
100
0


Total GLS in leaves (µmol g-1 DW)
Aliphatic GLS in leaves (µmol g-1 DW)
Indolic GLS in leaves (µmol g-1 DW)
D density
FH density
0
Low
High
D density
0
Low
High
12
10
8
6
15
12
9
6
20
15
10
5
0
10.0
7.5
5.0
12.5
10.0
7.5
5.0
2.5
15
10
5
0
2.0
1.5
1.0
0.5
2.5
2.0
1.5
1.0
0.5
4
3
2
1
0

Supplementary material 5.3. Non-metric multidimensional scaling (NMDS) showing dissimilarity in the concentrations of the ten leaf glucosinolates.

	NMDS 1	**NMDS 2**	**R^2**	***P***
D_0FH_0	0.38010	-0.92495	0.0047	0.79
D_1FH_0	-0.81829	-0.57481	0.0042	0.81
D_2FH_0	-0.31646	0.94861	0.0054	0.77
D_0FH_1	-0.71502	0.69910	0.0169	0.44
D_1FH_1	0.34500	0.93860	0.0334	0.19
D_2FH_1	-0.77803	0.62822	0.0067	0.71
D_0FH_2	0.99093	-0.13441	0.0091	0.64
D_1FH_2	0.15670	-0.98765	0.0679	0.03
D_2FH_2	0.41152	-0.91140	0.0050	0.78

Table 5.S4. Contribution of each treatment to the leaf GLS profile NMDS plot axes and explained variation of dissimilarity.

The different D and FH combinations provoked a dissimilar GLS profile, although treatment alignment among the NMDS axes did not show a clear pattern (Table 5.S4, Fig. 5.S2). Plants with low D and presence of FH had a relatively strong impact on GLS profile with respect to other treatments, and to a lesser extent plants with low or high FH and absence of D (Fig. 5.S2).

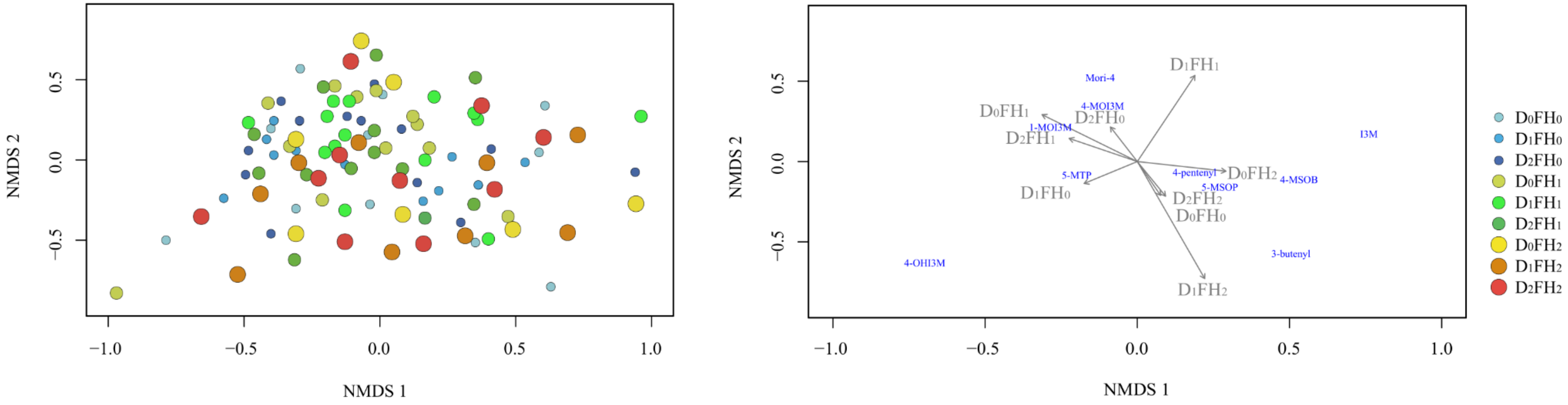


Figure 5.S2. Two dimensional NMDS plot showing dissimilarity in the concentrations of the ten glucosinolates (GLSs) between the different detritvore (D) and floral herbivore (FH) densities combinations. On the left, sample positions onto the NMDS plot. On the right, treatments as dissimilarity vectors and mean position of each GLS in the NMDS plot. 5-MTP = 5-methylthiopentyl GLS, Mori-4 = unidentified aliphatic GLS, 5-MSOP = 5-methylsulfinylpentyl GLS, 4-MSOB = 4-methylsulfinylbutyl GLS, 4-MOI3M = 4-methoxy-indol-3-yl-methyl GLS, 1-MOI3M = 1-methoxy-indol-3-yl-methyl GLS, 4-OHI3M = 4-hydroxy-indol-3-yl-methyl GLS, I3M = indol-3-yl-methyl GLS.

Supplementary material 5.4. FH caterpillar development time.

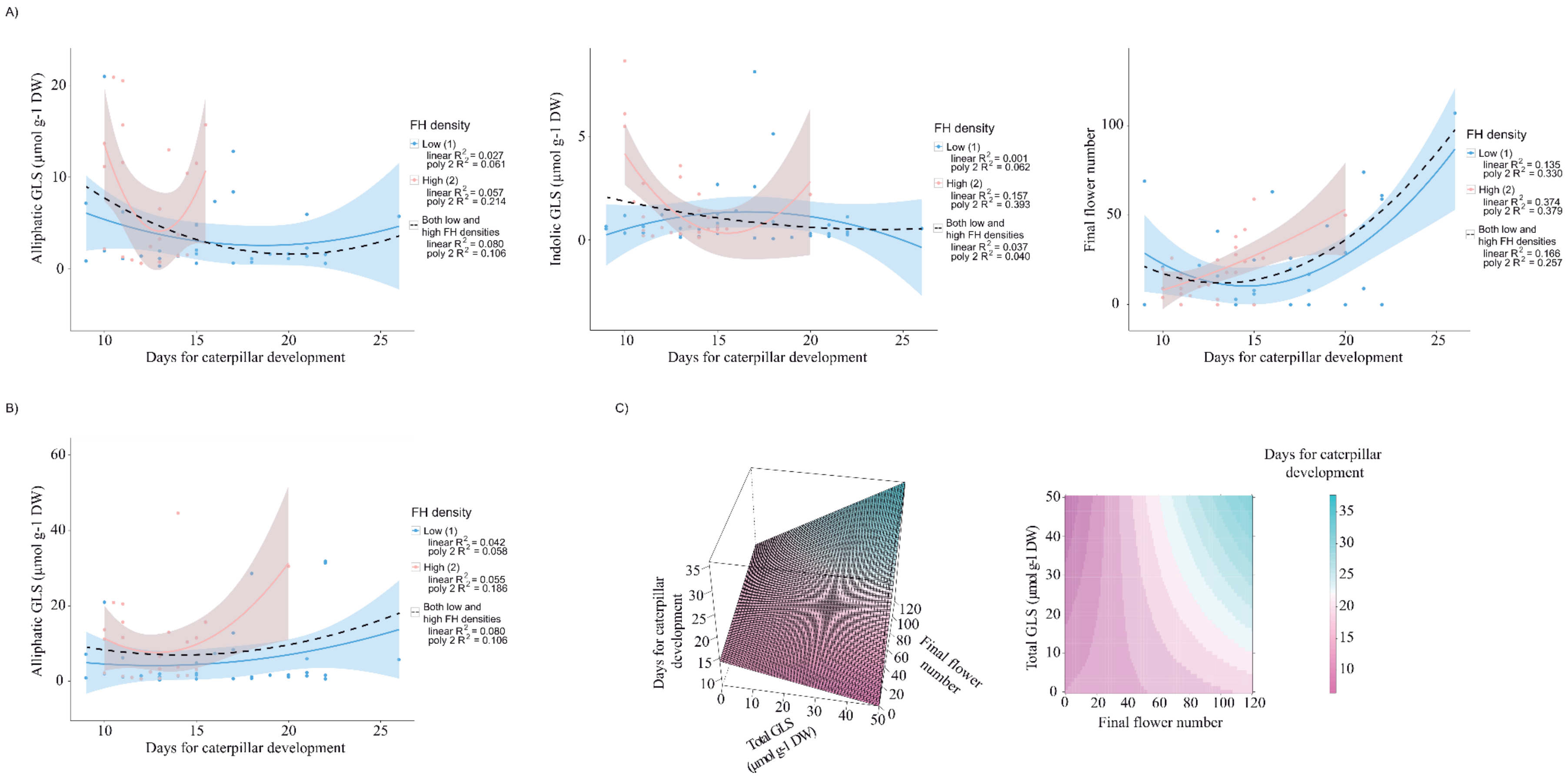


Figure 5.S3. (A) Linear and non-linear regression between the number of days for caterpillar development and aliphatic GLS concentration (plants with < 25 μmol aliphatic GLS g^{-1} of dry weight), indolic GLS concentration and number of flowers. When development time for both caterpillars in FH_2 plants could be measured, mean value per plant is shown. (B) Linear and non-linear regression between the number of days for caterpillar development and aliphatic GLS concentration (all plants). (C) 3D and 2D plane of the interaction between total GLS concentration (plants with < 25 μmol aliphatic GLS g^{-1} of dry weight) and number of flowers on the number of days for caterpillar development.

Supplementary material 5.5. Structural equation model (SEM) for FH density effects through FH caterpillar development time on plant fitness.

Model	Removed paths	Added paths	AICc	ΔAICc	df	Fischer's *C*	*P*
Full initial model			119.198	70.381	6	21.532	0.001
model 2	FH density → Number of seeds	-	113.240	64.423	8	21.944	0.005
model 3	-	FH density → FH caterpillar development time	97.065	48.248	6	8.621	0.196
model 4	Total GLS concentration → FH caterpillar development time	-	86.655	37.838	8	9.982	0.266
model 5	FH caterpillar development time → Number of seeds	-	93.023	44.206	10	17.474	0.065
model 6	-	FH density → Number of seeds	90.349	41.532	8	12.283	0.139
model 7	FH density → Total GLS concentration FH density → Number of seeds	FH caterpillar development time → Number of seeds	**48.817**	-	**2**	**0.412**	**0.814**
model 8	FH caterpillar development time → Number of seeds	-	55.001	6.184	4	7.673	0.104
model 9	-	FH density → Number of seeds	51.752	2.935	2	2.481	0.289

Table 5.S5. Stepwise SEM selection procedure for FH density effect through FH caterpillar development time on plant fitness.

Cause	Effects on	Direct	Indirect	Total
FH density	Number of flowers	-0.30	-	-0.30
	FH caterpillar development time	-0.79	-0.10	-0.89
	Number of seeds	-	-0.18	-0.18

Table 5.S6. Standardized total, indirect and direct size effects of FH density in the final SEM.

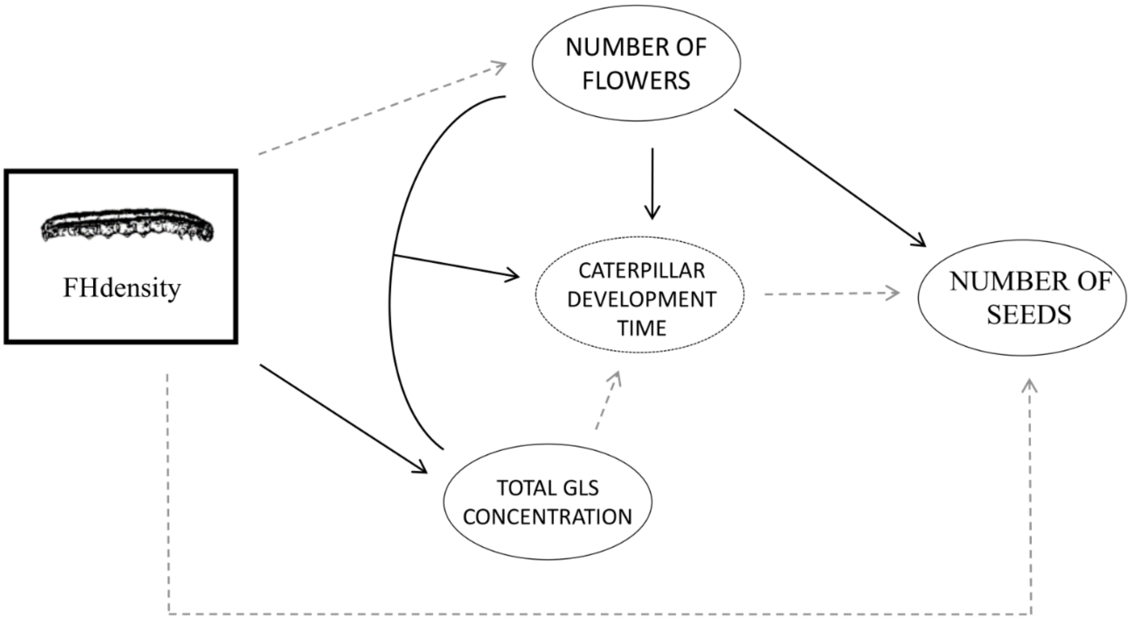


Figure 5.S4. Initially hypothesized SEM for FH density effect through FH caterpillar development time on plant fitness. Solid lines denote positive and dashed lines negative relationships.

Supplementary material 5.6. Structural equation model (SEM) for D and FH density on sequential plant reproductive components and fitness.

Model	Removed paths	Added paths	AICc	ΔAICc	df	Fischer's *C*	*P*
Full initial model			356.232	193.842	36	54.493	0.025
model 2	Aboveground biomass → Fruit set Number of flowers → Fruit set FH density → Fruit set FH density → Seed set	-	306.346	143.956	44	58.877	0.066
model 3	Total GLS concentration → Fruit set D density → Fruit set Fruit set → No. of flowers	-	201.139	38.749	34	43.956	0.118
model 4	Aboveground biomass → Seed set D density → Seed set	-	172.966	10.576	26	38.138	0.059
model 5	-	FH density → Seed set	178.853	16.463	24	37.193	0.042
model 6	FH density → Seed set	FH density → Number of seeds	169.123	6.733	24	31.688	0.135
model 7	-	Total GLS concentration → Number of flowers	**162.390**	-	**22**	**23.742**	**0.361**

Table 5.S7. Stepwise SEM selection procedure for D and FH density effects on sequential plant reproductive components and fitness. Fruit set was calculated as the proportion of flowers that passed to fruits, and seed set as the proportion of ovules that passed to seeds.

Cause	Effects on	Direct	Indirect	Total
FH density	Aboveground biomass	0.14	-	0.14
	Total GLSs	0.16	-	0.16
	Number of flowers	-0.38	0.11	-0.27
	Number of fruits	-	-0.17	-0.17
	Seed set	-	-0.03	-0.03
	Number of seeds	**-0.09**	**-0.14**	**-0.23**

Table 5.S8. Standardized total, indirect and direct size effects of FH density in the final SEM.

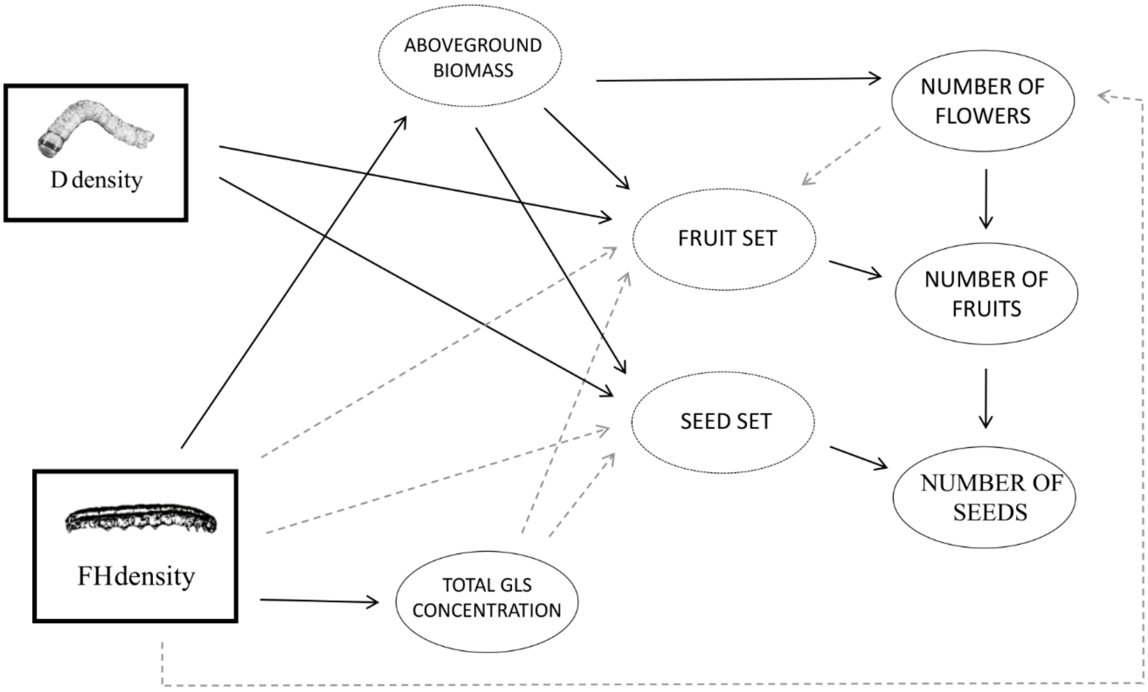


Figure 5.S5. Initially hypothesized SEM for the direct and indirect RH and FH density effects on sequential plant reproductive components and fitness. Fruit set was calculated as the proportion of flowers that passed to fruits, and seed set as the proportion of ovules that passed to seeds.

Supplementary material 5.7. D and FH density effects on plant.

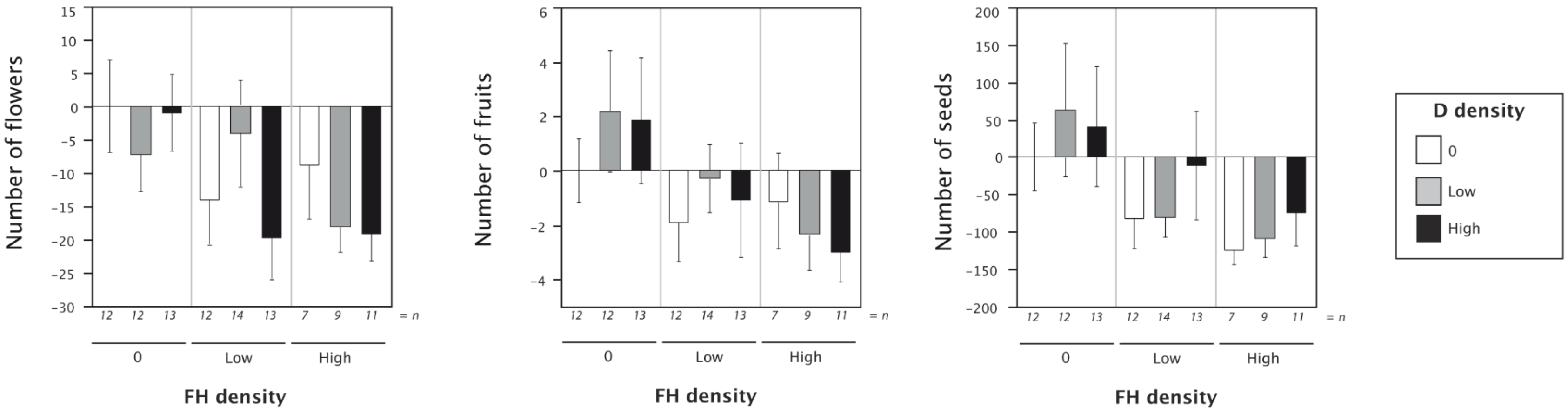
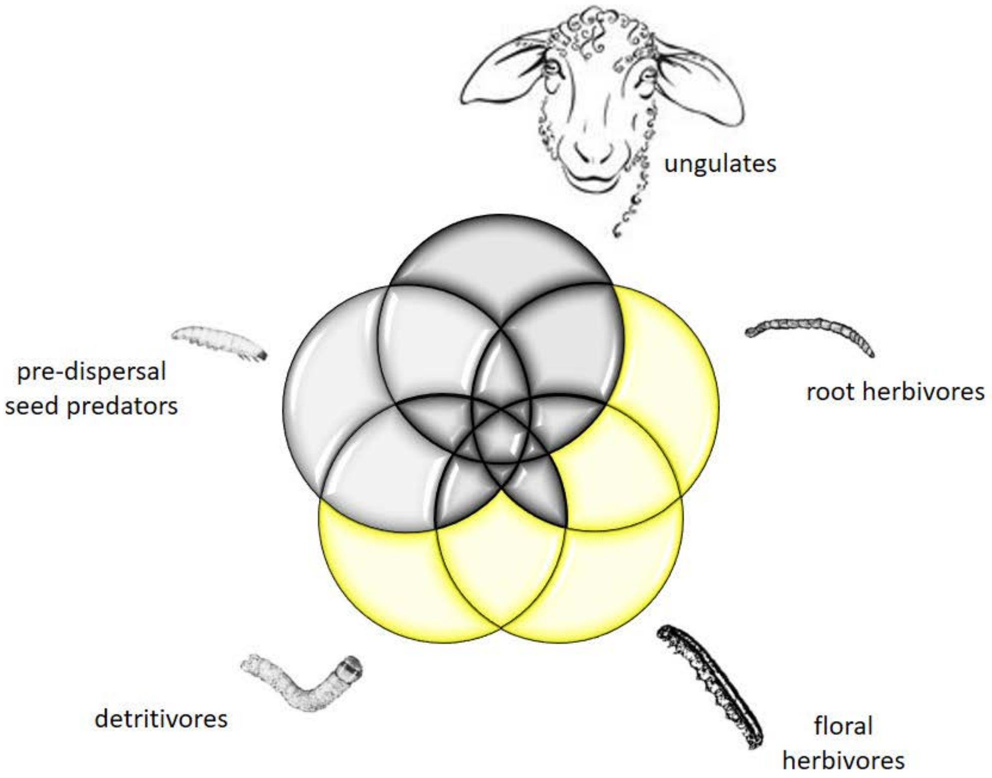


Figure 5.S6. Combined effects of detritivore (D) and floral herbivore (FH) densities on plant reproduction. Shown values (mean ± SE) are relative to the focal treatment (mean $D_0FH_0 = 0$).

Chapter 6

Density- and context-dependent transgenerational effects of detritivores, root herbivores and floral herbivores



6.1 Abstract

The increasing evidence that parental abiotic and biotic environment can determine offspring phenotypes is redefining many research areas in ecology. Particularly in plants, we are gathering knowledge that almost any direct or indirect interaction can have transgenerational consequences on offspring, despite they are often complex due to context dependence. It is to be elucidated if transgenerational effects depend also on the strength of interactions in parental generation. Here, we examined from two cohorts both the context-dependent and the density-dependent transgenerational effects of detritivores, root herbivores and floral herbivores on the emergence, performance and fitness of a semiarid herb. We found that all three interactions had transgenerational effects on offspring, although they depended both on the context and on the density with which they interacted with the mother plant. Maternal seed provisioning was moderately important for seedling emergence, while offspring reproductive output was more conditioned by the inherited growth-defense phenotypes and their interplay with the associated community, such as floral herbivore caterpillars themselves and their parasitoids. Some of the observed effects were thus up to the mark, such as lower seedling emergence due to maternal florivory and higher emergence due to high maternal detritivory, which even counteracted the negative effect of florivory. On the contrary, several findings were surprising: chemical defenses in offspring leaves decreased with increasing maternal root herbivory, and the transgenerational effects of florivory on offspring and even on florivores feeding on them highly depended on maternal belowground interactions. Our results attest the need for more field studies within natural systems in which the transgenerational consequences of a wide variety of direct and indirect interactions are considered.

Keywords

Brassicaceae · belowground-aboveground · Insect herbivory · Maternal effect · Semiarid environment · Transgenerational inheritance

6.2 Introduction

Herbivory-mediated selection is central to the evolution of plant traits that impact growth, defense and reproduction (Strauss et al. 2002, Carmona et al. 2011, Agrawal et al. 2012, Uesugi et al. 2017). In the last years, herbivore capacity to determine the progeny phenotypes through transgenerational effects has also been acknowledged (Agrawal 2001, 2002). Transgenerational effects can affect the outcome of plant-herbivore interactions when the environment experienced by the mother conditions offspring traits and performance (Roach and Wulff 1987). These transgenerational effects can occur across a single generation, as a result of either parental effects or induced genetic changes (Rossiter 1996). Therefore, accounting for possible transgenerational effects provides a better estimate of interaction strength and their impact on the fitness of the interacting species (Ehrlén 2003). Transgenerational effects in plants are often the result of maternal seed provisioning and not of apparent adaptive plasticity (carry-over effects; Uller et al. 2013, Engqvist and Reinhold 2016), and can highly depend on within-generational herbivory effects on plant reproduction. Seed mass is the trait that has been more broadly used as a proxy of seed provisioning (Moles and Leishman 2008), and its transgenerational effects conditioning offspring performance are broadly demonstrated (Obeso 1993, Agrawal 2001, 2002, Steets and Ashman 2010, Zas et al. 2013). Although less used as a seed provisioning proxy, maternal herbivory effects on seed quality in terms of nutrient content have been also shown to have transgenerational consequences (González-Megías 2016, Chapter 3). Herbivore-induced transgenerational effects go beyond seed provisioning and include several mechanisms such as allocation of defensive chemicals, defense-inducing hormones and/or epigenetically inherited regulation in gene expression, and can be adaptive when they provide reliable cues about the environment that the offspring will experience (Herman and Sultan 2011, Holeski et al. 2012, Auge et al. 2017, Balao et al. 2018, Yin et al. 2019). Consequently, when herbivory experienced by mothers and offspring is similar, inherited induction of physical or chemical defenses may result beneficial (Agrawal 2001, 2002, Ballhorn et al. 2016, Colicchio 2017, Sobral et al. 2021).

Besides, transgenerational effects in plants and their ecological implications are still a great challenge (see Richards et al. 2017 for a recent review focused on plant epigenetics). Transgenerational memory to abiotic and biotic stressors is not universal in plants, as it may depend upon the nature of the specific stress or the specific host plant, and the selective advantages of transgenerational effects among species depending on habitat predictability and life history characteristics (Herman et al. 2014). Field experiments provide thus unique ecological contexts to gain information about transgenerational inheritance, although the lack of high-resolution genomic tools in non-model species difficulties to establish links among genotype, transgenerational effects, environment and phenotype (Richards et al. 2017). Field experiments also provide the opportunity to test context-dependent (indirect) transgenerational effects, which may contribute to the understanding of ecological patterns in species-rich, natural communities where indirect effects are prevalent (Miller and Travis 1996, terHorst and Lau 2012, terHorst et al. 2018). Context-dependent transgenerational effects have been already observed in a wide variety of mutualistic (Irwin 2006), competitive (Lau 2008, terHorst and Lau 2012, Van Allen and Rudolf 2015) and trophic interactions (Gómez 2008, González-Megías 2016, Chapter 3), and may even result maladaptive if they contribute to diffuse coevolution and offspring habitat unpredictability (Herman et al. 2014). On the other hand, the consequences of transgenerational effects can even become more complex if the effects of abiotic and biotic stressors are intensity/density-dependent. There is already evidence of intensity-dependent transgenerational effects of drought (Rendina Gonzalez et al. 2016), disease pressure (López Sánchez et al. 2021) and temperature in plants (Rahavi and Kovalchuk 2013), and intraspecific competition in animals (Allen et al. 2008), but as far as we know they have not been broadly tested in the context of plant-herbivore interactions.

We have previously studied context-dependent transgenerational effects on early vital stages such as seedling emergence and survival in the predominantly semelparous Brassicaceae herb *Moricandia moricandioides*. In one of the studies, seeds from plants undergoing root and floral herbivory were of lower quality with consequent negative effects on seedling emergence time and survival, but the effects on seed quality and their transgenerational effects were overally counteracted in presence of detritivores (González-Megías 2016). In another study, we found that seeds from plants undergoing grazing were of lower quality with consequent negative effects on seedling emergence, but seedling resistance to herbivores was higher on plants undergoing both grazing and seed predation (Chapter 3). These studies suggest that maternal resource allocation to both defense and tolerance can have costs in seed provisioning and seedling performance (Herman and Sultan 2011), but also that seedlings from plants undergoing high herbivore pressure might inherit a higher defense inducibility upon herbivory. In the present study, we investigated whether the transgenerational effects of root herbivores, floral herbivores and detritivores are also density-dependent by observing their effects on seedling emergence and performance in the field. We used seeds from two experimental cohorts: in the first one (RH x FH cohort), we manipulated both root herbivore and floral herbivore groups in a full-factorial density gradient of absence (no individuals), low density (a single individual) and high density (two individuals). In the second one (D x FH cohort) we manipulated detritivores and floral herbivores in a similar way. We analyzed not only transgenerational effects on plant offspring fitness, but also their interaction with floral herbivores and their parasitoids. We measured several offspring traits related to growth, reproduction and chemical defenses to elucidate whether these were inherited traits or resultant of interactions with their community.

6.3 Materials and methods

Study system

Moricandia moricandioides

We used the short-lived Brassicaceae species *M. moricandioides* (Boiss.) Heywood as a model system. *Moricandia moricandioides* is diploid and with C_3 photosynthetic metabolism, and it is highly abundant in semi-arid environments of the eastern Spain (Perfectti et al. 2017). This species has inferior growth rates although thicker leaves with more chlorophyll and slightly greater rates of photosynthesis per unit leaf area than several cultivated *Brassica* species (McVetty et al. 1989). It grows as a vegetative rosette during winter, and produces reproductive stalks during spring. The stalks remain photosynthetically active during the entire season (González-Megías and Müller 2010). The plants produce glucosinolates, which are the characteristic defense compounds occurring in the order Brassicales (Fahey et al. 2001).

Moricandia moricandioides – floral herbivore pierids interaction

The Brassicaceae specialists *Pontia daplidice* L. and *Euchloe crameri* Batler pierid caterpillars are among the most important aboveground herbivores of *M. moricandioides* (González-Megías and Müller 2010, Chapter 7). Oviposition by these pierids is usually higher on more vigorous plants, although some other factors such avoiding intraguild competition, and oviposition choices to minimize predation and parasitation risk make caterpillars of these species to be distributed among most *M. moricandioides* and other Brassicaceae plants (Aguirrebengoa, personal observation). Caterpillars of these two species have similar growth rates and cause increasing damage on plant reproductive structures within consequent caterpillar instars. Unlike for *Pieris rapae* and *Pieris brassicae*, *E. crameri* and *P. daplidice*'s counteradaptations to glucosinolates have not been yet studied, but they surely have as pierid species radiation occurred through adaptation to glucosinolate metabolization (Winde and Wittstock 2011, Edger et al. 2015). Pashalidou et al. (2015a) showed that oviposition by *P. brassicae* on *M. moricandioides* did not induce a hypersensitive response-like necrosis, nor influence larval development and pupal mass. That result fits with our previous work in the system (Chapter 5), in which glucosinolates are induced upon caterpillar feeding but not upon oviposition (plants in which florivory was not allowed had much more eggs laid on them, but glucosinolate concentrations were lower). *Moricandia moricandioides*' response towards these florivore caterpillars combines both tolerance (increase in aboveground biomass and compensatory investment in reproductive tissue) and resistance traits (induction of glucosinolates), despite which high density of florivores negatively impact fitness (Chapters 4 and 5).

Floral herbivore pierids – pierid parasitoids interaction

Caterpillars of both *P. daplidice* and *E. crameri* are usually parasitized in the study area (see below) by the koinobiont *Cotesia kazak* Telenga (Braconidae, Hymenoptera) (González-Megías and Müller 2010). We have no records of egg parasitism after many years of egg collection in the field, but parasitism is frequent in collected first instar caterpillars of these species (González-Megías, personal observation). Hence, we know that parasitization occurs at or from first caterpillar instar. However, parasitization is only evident from late-third caterpillar instar, when the parasitoid larva definitely kills the caterpillar and makes a cocoon immediately next. Therefore, parasitism cannot be determined when caterpillars die or disappear on former instars.

Seedling emergence

Seeds from both cohorts (RH x FH densities experiment and D x FH densities experiment) were used to determine whether the combined densities of above- and belowground organisms influence seedling emergence. In the case of RH x FH, we planted around 30 seeds (mean ± SE: 29.90 ± 0.04 seeds per mother plant, 1,854 seeds in total) from each of 62 mother plants (at least 6 mothers from each of the 9 treatments). From D x FH cohort, we also planted around 30 seeds (28.32 ± 0.71 seeds per mother plant, 1,614 seeds in total) from each of 56 mother plants (again at least 6 mothers from each of the 9 treatments). Seeds were planted in black peat moss in 11 x 20 seedbeds at a greenhouse with natural temperature and photoperiod conditions and protected from herbivory by a 250 µm size antitrip mesh. Seedbeds were rotated in the same direction every two days, to avoid possible location effects.

Seeds from the RH x FH experiment were planted between 29-September and 3-October 2016, while seeds from the D x FH experiment were planted on following days (4 an 5-October). We supervised seedling emergence every other day from the planting day until the end of November of that year (last observed emergence occurred on 7-November). From that date on, seedlings were transplanted to pots with a mix of black peat moss and soil from their natural habitat. Pots were placed at the same greenhouse and kept at equal conditions as seedbeds.

Seedling performance in the field

Study area

The field experiment was conducted with emerged seedlings in 2017 at the semiarid basin Barranco del Espartal (Guadix-Baza Basin, Granada Province, southeastern Spain), at the same exact location where the experiments with their mothers were carried out, and where

M. moricandioides is highly abundant (Gómez 1996, González-Megías and Müller 2010, González-Megías and Menéndez 2012). The climate at the study area is continental Mediterranean with strong temperature fluctuations (ranging from -14°C to up to 45°C) and high seasonality (hot summers, cold winters). Annual precipitation rarely exceeds 300 mm.

Experimental set up

In the beginning of March 2017, we selected for the field experiments the 8 most suitable seedlings from each of the maternal treatments from both cohorts. This experiment consisted in a transgenerational common garden experiment (*in sensu* Colicchio 2017), in which seedlings were placed in their natural habitat with no manipulation at all. With these field experiments we pretended to disentangle the transgenerational effects of maternal biotic environment on offspring morphology, quality (nutrient content and chemical defenses) and reproduction. The experiment also pretended to discern how these potential transgenerational effects could shape the interaction between the plant and florivore pierids and determine the performance of both. Prior biomass acquisition exerts a great selection in the probability of reproduction of *M. moricandioides* in its natural conditions, and many plants even die without having reproduced (Chapter 7). Therefore, we selected vigorous seedlings with high probability to reproduce and, as a result, to interact with floral herbivores. Even so, the selected seedlings from each maternal treatment were descendants from at least two mother plants in all cases (range 2-6) to avoid excessive maternal lineage-derived (genotypic) effects.

Seedlings (offspring hereafter) from both cohorts were moved to the field on 7-March. None of those seedlings had reproductive stems at that time. Each cohort was planted separately in 4 blocks with 18 plants each and separated 30 cm apart from each other (72 plants per cohort, 2 replicates per maternal treatment/block). During the first week in the field, and in the absence of natural rain, all plants were watered and net-covered to ensure their establishment. 8 offspring from the RH x FH cohort and 9 offspring from the D x FH cohort did not produce reproductive stalks during the experiment, thus all these samples were excluded from the posterior analyses due to their impossibility to interact with floral herbivores. Final sample size for the offspring from the RH x FH experiment was: RH_0FH_0 n = 8, RH_0FH_1 n = 8, RH_0FH_2 n = 8, RH_1FH_0 n = 5, RH_1FH_1 n = 6, RH_1FH_2 n = 8, RH_2FH_0 n = 7, RH_2FH_1 n = 7 and RH_2FH_2 n = 7. Final sample size for the offspring from the D x FH experiment was: D_0FH_0 n = 6, D_0FH_1 n = 8, D_0FH_2 n = 7, D_1FH_0 n = 6, D_1FH_1 n = 8, D_1FH_2 n = 6, D_2FH_0 n = 8, D_2FH_1 n = 7 and D_2FH_2 n = 7.

Data collection

We surveyed plants from 15-March to 23-June, when all fruits produced by the plants had matured and no florivore caterpillar remained feeding on plants. During these 15 weeks we surveyed plants twice a week (30 surveys) and counted the number of pierid eggs and the total number and instar stage of *E. crameri* and *P. daplidice* caterpillars on plants, which were posteriorly summed (from first to fourth instar stage, fifth instar caterpillars are rarely found because they leave the plant to pupate). Egg development does not usually last more than a week for these pierid species, and we considered the position and the maturation stage of each egg at each survey on each plant to avoid counting the same eggs twice when we calculated the total number of laid eggs per plant (eggs are white when oviposited, and they change to yellow and finally to orange just before the caterpillar hatches). In a similar way, caterpillars of these species have very low within-plant mobility and we considered each individual to be the same when was found on the same plant at consecutive caterpillar instars within following surveys. During the experiment, we only found a single pierid caterpillar (*Pieris rapae*) that was not *E. crameri* or *P. daplidice*. We also counted at each survey the number and position of *C. kazak* parasitoid cocoons to avoid summing them repeatedly. We observed no other pierid parasitoid during the entire experiment. At the end of June, we counted the total number of flowers and fruits produced by each plant. Fruits were collected after complete maturation of seeds but before seed dispersal. For fitness estimation, we counted the total number of viable seeds per plant.

The entire aboveground tissue was collected to determine aboveground dry biomass and C and N content in leaf tissue. To quantify glucosinolate (GLS) concentrations in leaves, the youngest leaf of one stem of each of the experimental plants was collected before leaf senescence. Leaves were stored and immediately frozen and freeze-dried, and the dried material was ground and extracted three times in 80% methanol after the addition of p-hydroxybenzyl GLS (sinalbin) as an internal standard. GLS extraction and conversion to desulfoGLSs was done following previously established methodology (González-Megías and Müller 2010). DesulfoGLSs were identified by comparison of UV-spectra and retention times to those identified in earlier studies (González-Megías and Müller 2010). Peaks were integrated at 229 nm and response factors of 1 for aliphatic and 0.26 for indolic GLSs were considered and related to the internal standard (response factor 0.5) for calculation of concentrations.

Statistical analyses

Univariate GLMMs

General and generalized linear mixed models (GLMMs) were performed to test the potential density-dependent transgenerational effects of each continuous maternal biotic factor (RH and FH densities, or D and FH densities) and their interaction on offspring traits by analyzing each cohort separately (see Suppl. 6.1, Table 6.S1 for each analysis in detail). We analyzed their possible effect on seedling emergence and offspring reproduction (number of flowers, fruits and seeds), offspring interaction with florivore pierids (number of pierid eggs and number of caterpillars per instar) and florivore pierid parasitism rate. GLMMs were also performed to elucidate the transgenerational effects of the maternal biotic environment on other offspring morphological (aboveground biomass) and qualitative traits (N and C content and GLS concentrations in leaves), which could potentially determine offspring reproductive success and their interaction with florivore pierids.

Because maternal lineage could influence the traits of interest (e.g. genetic variation in fitness impacts of herbivory, Strauss and Agrawal 1999; genetic variation in offspring responses to herbivory, Agrawal 2002; genetic control of epigenetic variation, Becker et al. 2011, Dubin et al. 2015), mother identity was always included as random for all (emergence and field) variables. Seedling emergence was analyzed with binomial distribution and planting day as random, as it may have conditioned the microclimatic conditions that severely affect the germination and emergence process. Field experiment variables were analyzed with the best fitting distribution (Gaussian, Gamma, Poisson or Negative Binomial), and variables were transformed when necessary (Suppl. 6.1, Table 6.S1). Models analyzed with Gaussian distribution did not improve when we tried to model heteroscedasticity with generalized least squares, so we kept them as GLMMs. Those variables fitting Gamma distribution were analyzed with inverse link function and variables fitting Poisson or Negative Binomial distribution with log link function. When overdispersion was observed, GLMMs with observation-level random effects were run (Harrison 2014), which allowed for variation at plant level (Suppl. 6.1, Table 6.S1). Due to the reduced sample size, block was tested for all variables as an additional random factor and only included when it contributed to model improvement (Suppl. 6.1, Table 6.S1). Model selection on all measured variables was done according to Bayesian Information Criterion (BIC) model fit, as it tends to favor more parsimonious models (Grueber et al. 2011). GLMMs were performed using R (R Core Team 2017), with the package *lme4* (Bates et al. 2015). When factor-level or interaction-level effects were significant, post hoc tests were performed with the same model structure but with the RH and FH or D and FH densities as categorical predictors (absence, low or high density). As post hoc, we used a *P*-value adjusting multivariate test (see Suppl. 6.2, Table 6.S2 for detailed factor and interaction level post hoc results) with the *lsmeans* package (Lenth 2016).

Structural equation model

We fitted a piecewise structural equation model (SEM) for both cohorts to link transgenerational effects, offspring reproduction and florivore caterpillar performance, using the R package *piecewiseSEM* (Lefcheck 2016). SEM allows formulating hypotheses on pathways of interaction between parameters in the model, where all parameters could act as both predictor and response variables. We limited to the maximum the number of variables, as it is suggested that for having reliable goodness of fit, a SEM model should contain at least 5-10 times as many observations as variables (Lefcheck 2016). We used standardized variables (mean = 0, SD = 1) and fitted the component models of the piecewise SEM as linear mixed models. For all component models mother identity was included as random, and block and/or plant (offspring) identity were also included as random when they contributed to model improvement (identical random structure as in the above mentioned GLMMS; Suppl. 6.1, Table 6.S1). We started with an initial SEM and improved it through the stepwise procedure by evaluating Akaike's information criterion corrected for small sample sizes (AICc) for model selection (Lefcheck 2016, Shipley 2016), until we procured the final SEM with the lowest AICc score (Suppl. 6.3, Table 6.S3). In piecewise SEM, the optimization procedure is based on the removal of irrelevant paths and the inclusion (based on Shipley's test of *d*-separation) of any of the non-hypothesized biologically relevant paths that improve the model (Lefcheck 2016, Shipley 2016).

Our initial SEMs for both cohorts contemplated the observed trangenerational effects on aboveground biomass and aliphatic GLS concentrations, and their direct and indirect consequences for plant reproduction (with fruit number as a proxy of seed number to reduce the number of paths), number of oviposited pierid eggs on plants, pierid caterpillar development on plants and caterpillar parasitism rate.

6.4 Results

Seedling emergence: RH x FH cohort

Maternal FH density influenced seedling emergence (df = 2,51, χ^2 = 7.14, P = 0.007), as it decreased in both low and high FH with respect to seedling from mothers without FH (Fig. 6.1; Suppl. 6.2, Table 6.S2a). Seedling emergence was unaffected by maternal RH density (df = 2,51, χ^2 = 0.98, P = 0.32) and by the interaction term between maternal RH and FH densities (df = 4,51, χ^2 = 0.32, P = 0.57).

Seedling emergence: D x FH cohort

Maternal D density influenced seedling emergence (df = 2,48, χ^2 = 18.89, $P < 0.001$), as it was higher at high maternal D than at low or absence of D (Fig. 6.1; Suppl. 6.2, Table 6.S2b). Seedling emergence was unaffected by maternal FH density (df = 2,48, χ^2 = 0.43, P = 0.51) and by the interaction term between maternal D and FH densities (df = 4,48, χ^2 = 1.31, P = 0.25).

Offspring performance in the field: RH x FH cohort

Morphology and reproduction

Maternal RH and FH densities did not affect offspring aboveground biomass (Table 6.1), nor offspring reproduction (number of flowers, fruits and seeds, Table 6.1, Fig. 6.2).

	RH			**FH**			**RH x FH**		
	F/χ^2	P	df	F/χ^2	P	df	F/χ^2	P	df
Morphological and reproductive traits									
Aboveground biomass	0.03	0.86	1,45	0.21	0.64	1,45	2.01	0.15	1,45
Number of flowers	0.91	0.34	1,45	0.23	0.63	1,45	0.07	0.79	1,45
Number of flowers	0.46	0.49	1,45	0.01	0.92	1,45	0.18	0.67	1,45
Number of seeds	0.08	0.77	1,23	0.14	0.68	1,23	2.39	0.12	1,23
Glucosinolate (GLS) concentration in leaves									
Total GLSs	**8.63**	**0.005**	1,43	0.11	0.74	1,43	0.00	0.99	1,43
Aliphatic GLSs	**9.78**	**0.0017**	1,43	0.26	0.61	1,43	0.00	0.99	1,43
Indolic GLSs	0.26	0.61	1,43	1.40	0.24	1,43	0.63	0.43	1,43
Pierid oviposition									
Number of pierid eggs	0.51	0.47	1,45	0.24	0.62	1,45	**11.40**	**0.0015**	1,45
Pierid caterpillar number per instar									
1st – 2nd instar caterpillars	0.03	0.86	1,45	1.10	0.29	1,45	1.82	0.17	1,45
3rd instar caterpillars	0.13	0.71	1,45	1.47	0.22	1,45	2.48	0.11	1,45
late 3rd instar caterpillars	1.11	0.29	1,45	*3.67*	*0.069*	1,45	0.47	0.49	1,45
4th instar caterpillars	0.05	0.82	1,45	**7.70**	**0.005**	1,45	0.82	0.36	1,45
Pierid caterpillar parasitism rate									
Parasitism rate	0.00	0.99	1,22	*3.30*	*0.082*	1,22	0.67	0.42	1,22

Table 6.1. GLMM results for maternal root herbivore (RH) and floral herbivore (FH) density effects on *Moricandia moricandioides* offspring morphology, reproduction, leaf glucosinolate concentrations, and on pierid egg oviposition, pierid caterpillar performance and parasitism on offspring. *F* is shown for glucosinolates and pierid caterpillar parasitism rate, χ^2 is shown for the rest. Significant results ($P < 0.05$) are highlighted in bold.

Glucosinolate (GLS) concentrations and nutrient content in leaves

Maternal RH density limitedly influenced total GLS concentrations on offspring (Table 6.1), as it was marginally lower on offspring with high maternal RH than on absence of RH ($RH_0 = 30.15 \pm 7.65$, $RH_1 = 19.92 \pm 5.99$, $RH_2 = 11.08 \pm 3.68$ µmol g^{-1} of dry weight; Suppl. 6.2, Table

6.S2a). This effect was mainly due to aliphatic GLSs (Table 6.1), with significant differences between high maternal RH and absence of maternal RH (Fig. 6.2; Suppl. 6.2, Table 6.S2a). Maternal RH or FH densities did not affect indolic GLS concentrations on offspring (Table 6.1). Leaf quality in terms of C and N was unaffected by maternal RH and FH densities (data not shown).

	D			**FH**			**D x FH**		
	F/χ^2	P	df	F/χ^2	P	df	F/χ^2	P	df
Morphological and reproductive traits									
Aboveground biomass	0.80	0.37	1,49	0.12	0.72	1,49	**6.97**	**0.008**	1,49
Number of flowers	0.28	0.59	1,49	0.24	0.62	1,49	**9.26**	**0.002**	1,49
Number of flowers	0.00	0.99	1,49	0.00	0.99	1,49	**9.71**	**0.001**	1,49
Number of seeds	1.98	0.15	1,31	0.09	0.76	1,31	**7.87**	**0.005**	1,31
Glucosinolate (GLS) concentration in leaves									
Total GLSs	0.34	0.56	1,47	1.13	0.29	1,47	1.62	0.20	1,47
Aliphatic GLSs	0.86	0.35	1,47	*4.00*	*0.051*	1,47	**4.22**	**0.045**	1,47
Indolic GLSs	0.25	0.61	1,47	0.93	0.86	1,47	1.23	0.27	1,47
Pierid oviposition									
Number of pierid eggs	0.13	0.71	1,49	**8.47**	**0.003**	1,49	**7.07**	**0.007**	1,45
Pierid caterpillar number per instar									
1st – 2nd instar caterpillars	0.07	0.79	1,45	1.56	0.21	1,45	**9.13**	**0.002**	1,45
3rd instar caterpillars	0.07	0.79	1,45	0.19	0.66	1,45	*2.82*	*0.093*	1,45
late 3rd instar caterpillars	0.39	0.53	1,45	1.11	0.29	1,45	1.88	0.17	1,45
4th instar caterpillars	1.60	0.20	1,45	0.97	0.32	1,45	0.29	0.60	1,45
Pierid caterpillar parasitism rate									
Parasitism rate	0.33	0.57	1,22	1.03	0.32	1,22	0.65	0.42	1,22

Table 6.2. GLMM results for maternal detritivore (D) and floral herbivore (FH) density effects on *Moricandia moricandioides* offspring morphology, reproduction, leaf glucosinolate concentrations, and on pierid egg oviposition, pierid caterpillar performance and parasitism on offspring. F is shown for glucosinolates and pierid caterpillar parasitism rate, χ^2 is shown for the rest. Significant results ($P < 0.05$) are highlighted in bold.

Florivore pierid oviposition, performance and parasitism

The number of pierid eggs laid on offspring was interactively affected by maternal RH and FH densities (Table 6.1): (1) in absence of FH, the number of eggs increased with RH density, (2) at high RH, the number of eggs decreased with FH density and (3) at high FH, the number of eggs decreased at high RH (Fig. 6.2; Suppl. 6.2, Table 6.S2a).

We observed no differences in the number of 1st to 3rd instar caterpillars on offspring (Table 6.1). There was a marginally significant effect of maternal FH density on the number of late 3rd instar caterpillars (Table 6.1), although with no significant post hoc differences among FH levels (Suppl. 6.2, Table 6.S2a). The effect of maternal FH density on the number of 4th instar caterpillars on offspring yes that was significant (Table 6.1), as it was lower in low and high FH than in absence of FH (Fig. 6.2; Suppl. 6.2, Table 6.S2a). Pierid parasitism rate on offspring was marginally higher at low maternal FH than in absence of FH (Table 6.1, Fig. 6.2; Suppl. 6.2, Table 6.S2a).

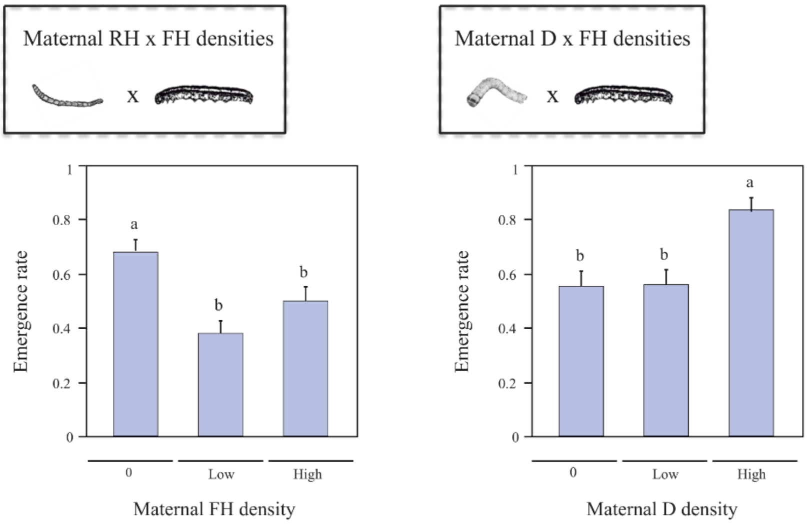


Figure 6.1. On the left, maternal floral herbivore (FH) density effect on seedling emergence (RH x FH cohort). On the right, maternal detritivore (D) density effect on seedling emergence (D x FH cohort). Mean ± SE are shown. Letters correspond to post hoc results. Asterisks on letters reveal that post hoc results are only marginally significant (see Suppl. 6.2, Table 6.S2a).

Offspring from the D x FH cohort

Morphology and reproduction

Maternal D and FH densities interactively affected aboveground biomass on offspring (Table 6.2): (1) in absence of D, it increased with FH density, (2) at high D, it was higher in absence of FH than at high FH and (3) at high FH, it increased with D density (Fig. 6.3; Suppl. 6.2, Table 6.S2b). Maternal D and FH densities also interactively affected the number of flowers, fruits and seeds on offspring (Table 6.2): in the three cases (1) at high D, the number of flowers, fruits and seeds was higher in absence of FH; in the case of flowers (2) in absence of FH, the number of flowers increased at high D; and in the case of fruits and seeds (3) at high FH, the number of fruits and seeds was lower at high D (Fig. 6.3; Suppl. 6.2, Table 6.S2b).

Glucosinolate (GLS) concentrations and nutrient content in leaves

Maternal D and FH densities interactively affected aliphatic GLS concentrations on offspring (Table 6.2): (1) in absence of D, it was higher at high FH and (2) at high FH, it was higher in absence of D than at low D (Fig. 6.3; Suppl. 6.2, Table 6.S2b). We observed no effects for indolic GLSs or for total GLS concentrations (Table 6.2). Leaf quality in terms of C and N was unaffected by maternal D and FH densities (data not shown).

Florivore pierid oviposition, performance and parasitism

The number of pierid eggs laid on offspring and the number of 1st – 2nd instar caterpillars on them was interactively affected by maternal D and FH densities (Table 6.2): in both cases, at high D their number decreased with FH density (Table 6.2; Suppl. 6.2, Table S2b). We found no differences in the number of caterpillars from 3rd instar on (Table 6.2). There was neither any effect in pierid caterpillar parasitism rate on offspring (Table 6.2).

SEM: Transgenerational effects, offspring fitness and florivore performance

Final SEM for RH x FH cohort

Aboveground biomass had a positive effect on flower production and indirectly on reproductive output (Fig. 6.4, see also Fig. 6.6 for the standardized values of all used variables). Flower number would have increased plant reproductive output, the number of late 3rd instar caterpillars and caterpillar parasitism rate. Caterpillar parasitism rate on offspring was also negatively affected by maternal FH density. Aliphatic GLSs (influenced by maternal RH density) would not have had much influence on plant reproduction nor caterpillar development.

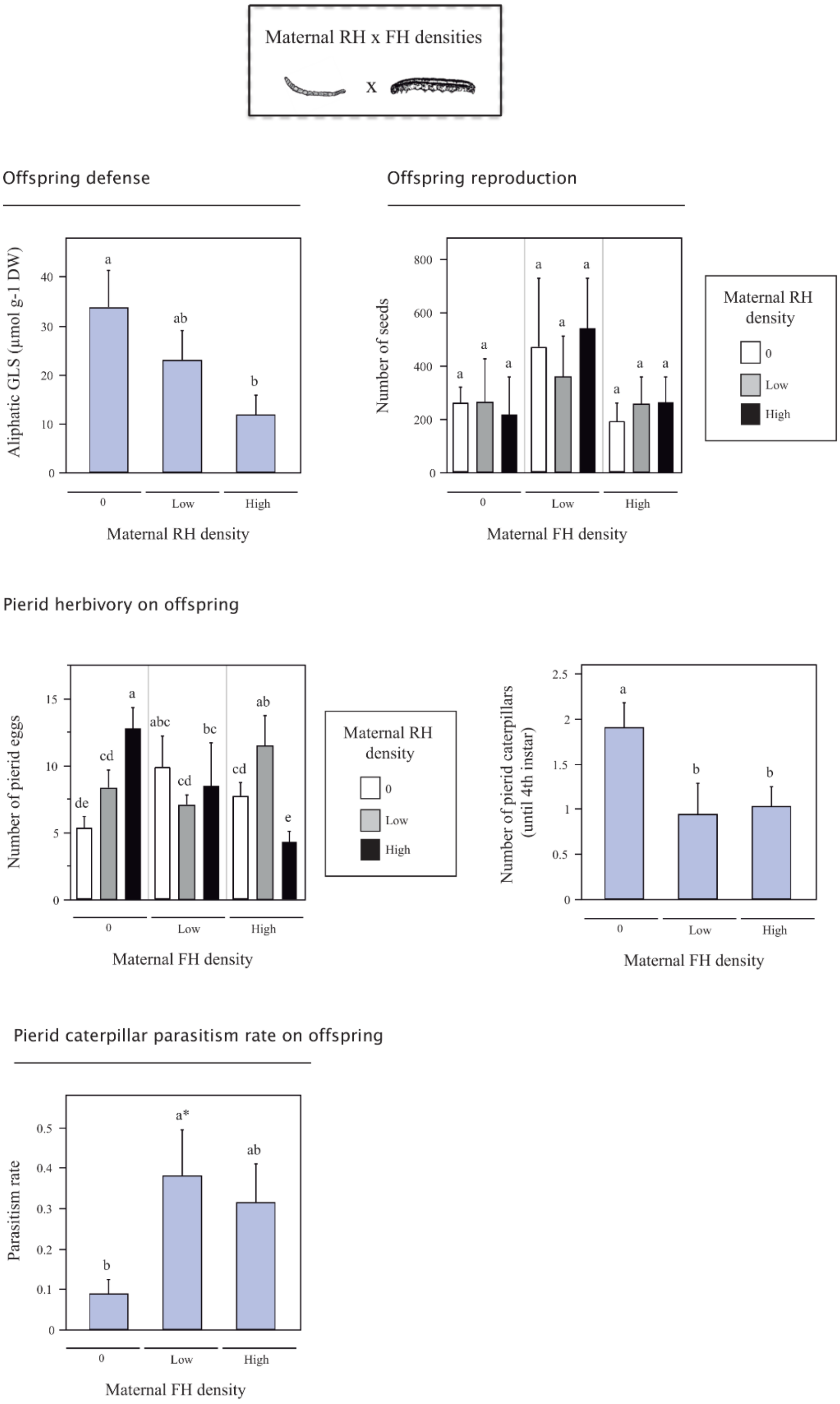
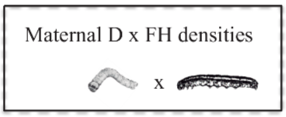
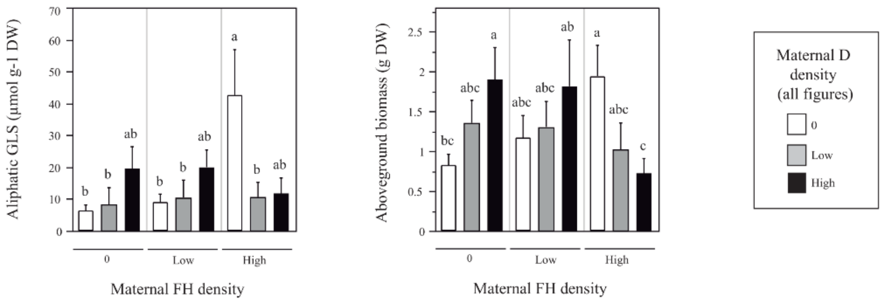


Figure 6.2. Maternal root herbivore (RH) and floral herbivore (FH) density effects on aliphatic GLS concentration, number of seeds, number of pierid eggs, pierid caterpillar parasitism rate and number of caterpillars that reached 4th instar. Letters at factor-level results correspond to post hoc tests (*a** reveals that significant difference from *b* is only marginal, see Suppl. 6.2, table 6.S2a). Letters at interaction-level results correspond to Fischer Least Square Differences. Interaction-level post hoc results are shown in Suppl. 6.2, Table 6.S2. Shown values (mean ± SE) are weighted by mother identity in all cases for a more correct visual approximation to GLMM results.

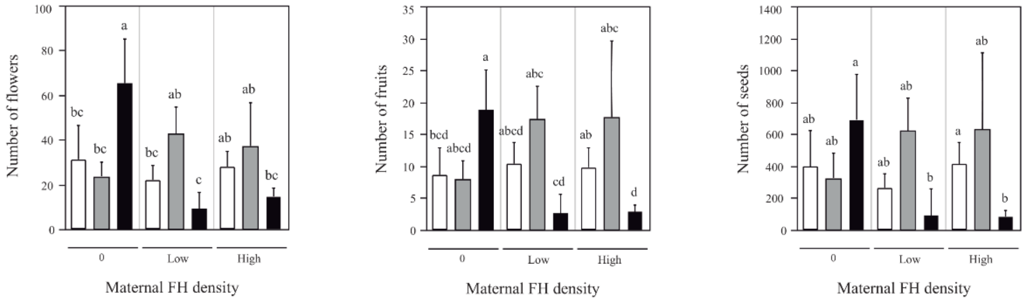


Offspring defense

Offspring morphology



Offspring reproduction



Pierid herbivory on offspring

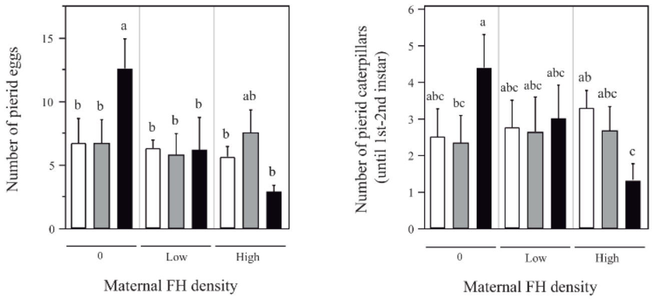


Figure 6.3. Maternal detritivore (D) and floral herbivore (FH) density effects on aliphatic GLS concentration, aboveground biomass, number of flowers, fruits and seeds, number of pierid eggs and number of 1st-2nd instar caterpillars. Letters at factor-level results correspond to post hoc tests (*a** reveals that significant difference from *b* is only marginal, see Suppl. 6.2, table 6.S2b). Letters at interaction-level results correspond to Fischer Least Square Differences. Interaction-level post hoc results are shown in Suppl. 6.2, Table 6.S2. Shown values (mean ± SE) are weighted by mother identity in all cases for a more correct visual approximation to GLMM results.

Final SEM for D x FH cohort

Maternal D and FH densities interactively influenced aboveground biomass, and biomass favored higher concentrations of aliphatic GLSs (Fig. 6.5, see also Fig. 6.6 for the standardized values of all used variables). Aboveground biomass had a positive effect on flower production, with consequent increment in reproductive output. Flower number could have favored and maternal FH density unfavored pierid egg oviposition. Aliphatic GLSs would have positively influenced the number of late 3rd instar caterpillars. Finally, fourth instar caterpillar number on offspring would have been detrimental for fruit number.

6.5 Discussion

Context-dependence in density-dependent transgenerational effects

Seedling emergence and recruitment exerts a strong selective pressure in short-lived plants' fitness (Silvertown et al. 1993, Moles and Leishman 2008), and it is invariably affected by the environmental conditions undergone by the mother (Gutterman 2000, Donohue et al. 2010). In the present study we found density-dependent but also context-dependent effects of maternal biotic environment on seedling emergence, as the effect of florivore density depended on the trophic guild and density of belowground organisms interacting with the plant. Like this, low and high maternal florivore densities had a detrimental effect on seedling emergence (RH x FH cohort), but high density of detritivores in maternal plant rhizosphere increased seedling emergence and counteracted the negative effect of florivory (D x FH cohort). Maternal seed provisioning (carry-over effects) seems to have at least partially influenced seedling emergence (Suppl. 6.4, Table 6.S4), which is an important restricting factor in resource-limited environments (Maron et al. 2014). Beyond the importance of seed mass and nitrogen content in seedling emergence (Gutterman 2000), an optimal content and ratio of carbon and nitrogen in seeds has strong influence in emergence, as it is been recently shown for the model Brassicaceae species *Arabidopsis thaliana* (Palenchar et al. 2004, Osuna et al. 2015), and it is recurrently evident in our system (González-Megías 2016, Chapter 3).

In any event, emergence success does not necessarily predict a posterior greater performance of individuals (Donohue et al. 2010). Allocation costs can be more magnified in seedlings, which initially depend on the reserves stored in their seeds for growth, than in posterior vital stages (Hanley et al. 2004). In the present study, maternal seed quality seemed to be less influential on offspring later performance and reproduction, which was apparently more determined by the interplay between inherited phenotypes and the offspring biotic environment. We found that both density-dependent and context-dependent transgenerational effects complexly determined offspring fitness, but also florivore caterpillar performance feeding on offspring. In one of the cohorts (RH x FH), we found no differences on offspring fitness among maternal treatments, but a lower survival of florivore caterpillars on offspring whom mothers faced this same type of herbivory. On the contrary, in the other cohort (D x FH) we found that offspring from mothers with high detritivore density and florivory had the lowest performance and fitness.

Complex ensemble of transgenerational effects and adaptiveness

Chemical defenses such as GLSs are costly for plants in terms of resources (Bekaert et al. 2012), and theory predicts resource-based trade-offs between growth and defense (Coley et al. 1985, Herms and Mattson 1992). However, a negative covariance between growth and defense is not that often empirically observed (Mauricio and Rausher 1997, Núñez-Farfán et al. 2007, Hahn and Maron 2016). Between other reasons, this can be because bigger plants provide more resources that can be diverted to defense (Stamp 2003). In the present study we did not observe a negative covariance between chemical defense (GLS concentrations in leaves) and growth (aboveground biomass) on offspring, although these traits did not show a collinear expression. More than by florivore pressure on F_1 generation, the specific biotic environment undergone by the mother complexly modulated aliphatic GLS concentrations and growth on offspring. However, it is remarkable that mean total GLS concentrations on offspring were almost doubled in relation to mothers for both cohorts (from mean ± SE 12.00 ± 1.78 to 20.23 ± 3.60 µmol g^{-1} of dry weight in the RH x FH cohort, and from 8.46 ± 2.04 to 16.27 ± 3.60 µmol g^{-1} of dry weight in the D x FH cohort). High GLS concentrations can potentially accelerate larval development and consequently mitigate florivore damage when florivory occur by counteradapted larvae that can use these metabolites in their own benefit (Smallegange et al. 2007, Bandeili and Müller 2010). Besides, there can be a counteradaptation threshold, from which higher concentrations of GLSs would not favor larval development (Chapter 5). Considering that investment in GLSs can to an extend trade-off with the reproductive effort (Herms and Mattson 1992, Huot et al. 2014, Karasov et al. 2017, Züst and Agrawal 2017), the fitness impact of GLS induction would be contingent upon florivore density on offspring, and a disproportionate induction in relation to florivore density could swing the optimal cost-benefit balance. When plants can defend against but also compensate for herbivory damage, allocating resources to defense can even result a cost rather than a benefit (Pilson 2000). In the present study, an increase in offspring aboveground biomass seemed to be undoubtedly adaptive, as its benefits allowing more investment in reproductive tissue and higher compensation to florivore damage full well counterbalanced the more pierid eggs laid and the higher caterpillar survival on these plants. The most adaptive inherited phenotype would thus be the one that combines high biomass and high but not disproportionate concentration of GLSs (Suppl. 6.4, Table 6.S4; Suppl. 6.5, Fig. 6.S1), although optimal trait covariance can also be strongly influenced by microenvironmental biotic and abiotic conditions (Suppl. 6.6, Fig. 6.S2).

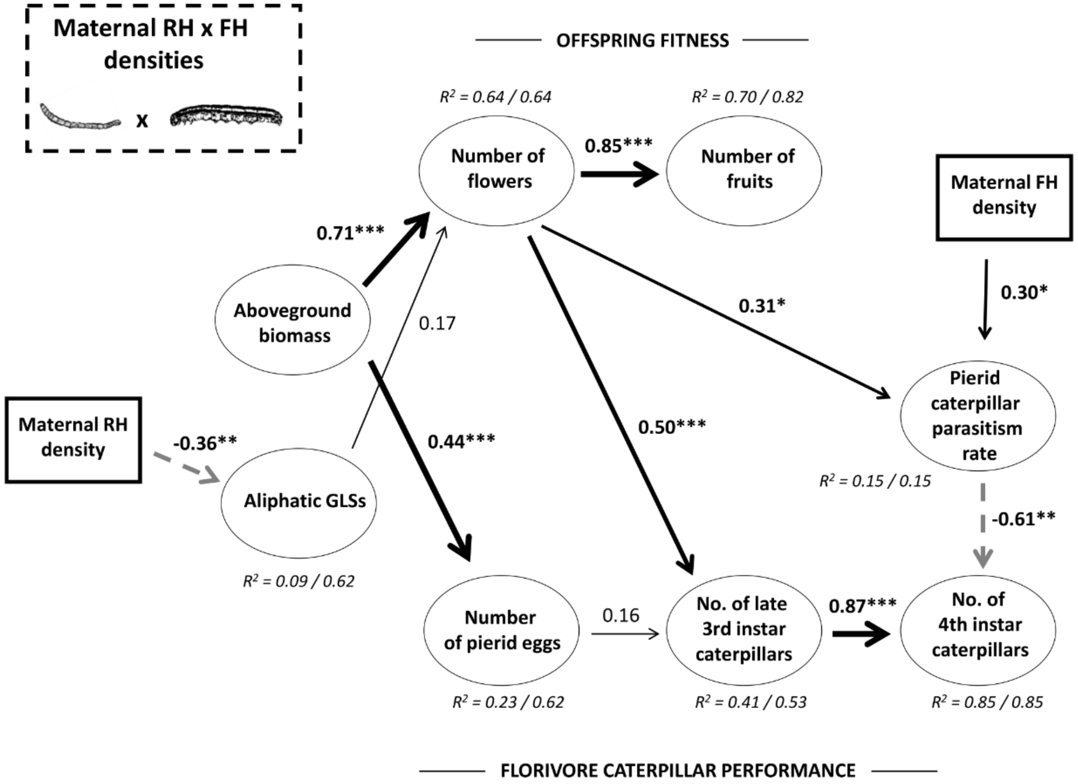


Figure 6.4. Final piecewise SEMs linking transgenerational effects, offspring reproduction and florivore caterpillar performance on offspring in the RH x FH cohort. Standardized path coefficients are shown next to each path, and their significance level is shown as ^{ms} *P* <0.08,* *P* <0.05, ** *P* <0.01, *** *P* <0.001. Solid lines denote positive and dashed lines negative relationships, and their thickness is scaled to the magnitude of the path strength. Marginal/conditional R^2 values for each component model are given. AICc = 655.948, Fischer's C = 39.052, *P* = 0.683.

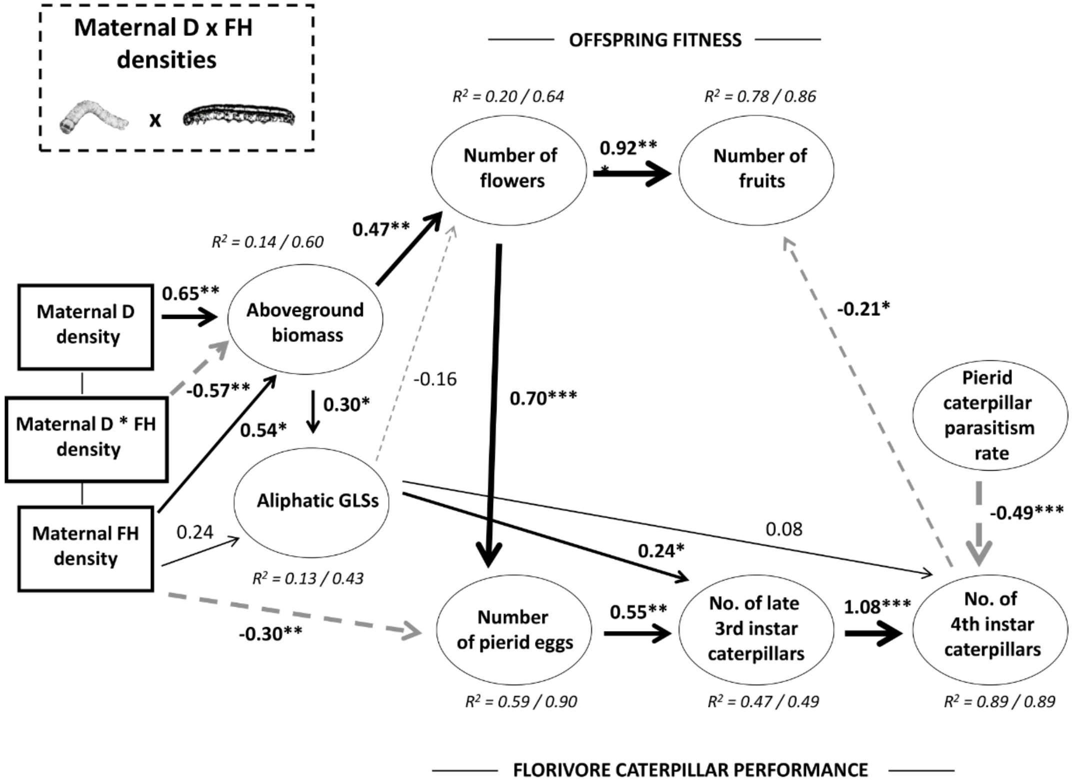


Figure 6.5. Final piecewise SEMs linking transgenerational effects, offspring reproduction and florivore caterpillar performance on offspring in the D x FH cohort. Standardized path coefficients are shown next to each path, and their significance level is shown as * *P* <0.05, ** *P* <0.01, *** *P* <0.001. Solid lines denote positive and dashed lines negative relationships, and their thickness is scaled to the magnitude of the path strength. Marginal/conditional R^2 values for each component model are given. AICc = 526.373, Fischer's C = 60.694, *P* = 0.242.

The transgenerational effects of root herbivores, detritivores and floral herbivores on plant offspring performance have been poorly studied so far (see González-Megías 2016), what impedes any type of comparison. For the RH x FH cohort, we observed that those offspring from plants facing high root herbivory had the lowest GLS concentrations, although concentrations were similar to that of their mothers. On the contrary, GLS concentrations vastly increased on offspring from low and especially absence of root herbivory plants. That is the opposite to the transgenerational effects of the most extensively studied herbivore guild, foliar herbivores, which usually induce physical or chemical defenses in offspring (Agrawal 2001, 2002, Ballhorn et al. 2016, Colicchio 2017). This reduction in GLSs was not due to differential florivory in offspring but a transgenerational effect, although changes in GLS concentrations did not apparently entail notable fitness consequences. In the same cohort (RH x FH), offspring from plants undergoing floral herbivory maintained fitness (in relation to offspring from mothers with no floral herbivory) probably due to a lower survival of florivore caterpillars on them. This lower survival on offspring whom mothers had the same type of herbivory was not apparently the classical defense induction transgenerational effect (Agrawal 2001), but could have been partially mediated by indirect effects such as an increase in parasitism.

The transgenerational effects of florivory differed in the other cohort (D x FH), thus could be dependent of the identity and density of belowground organisms interacting with the plant. Surprisingly, we found for this cohort that offspring from mothers with high detritivore or high florivore density had the greatest fitness due to the adaptive value of their growth and defense trait combinations. However, offspring from mothers with high detritivore density and low or high florivore density were the ones with the lowest fitness, and this was not due to differential florivore pressure on offspring but to a lower capacity to produce reproductive tissue. A possible explanation for this interactive effect in offspring fitness could be related with the fact that the negative effect of maternal florivory on seedling emergence was counterbalanced by the presence of high maternal detritivory, but that compensation could provoke an ontogenic trade-off on those emerged seedlings that arise in further vital stages. Life history events are not isolated episodes but are indeed connected ontogenetically, physiologically and morphologically (Yang and Rudolf 2010, Sobral et al. 2021).

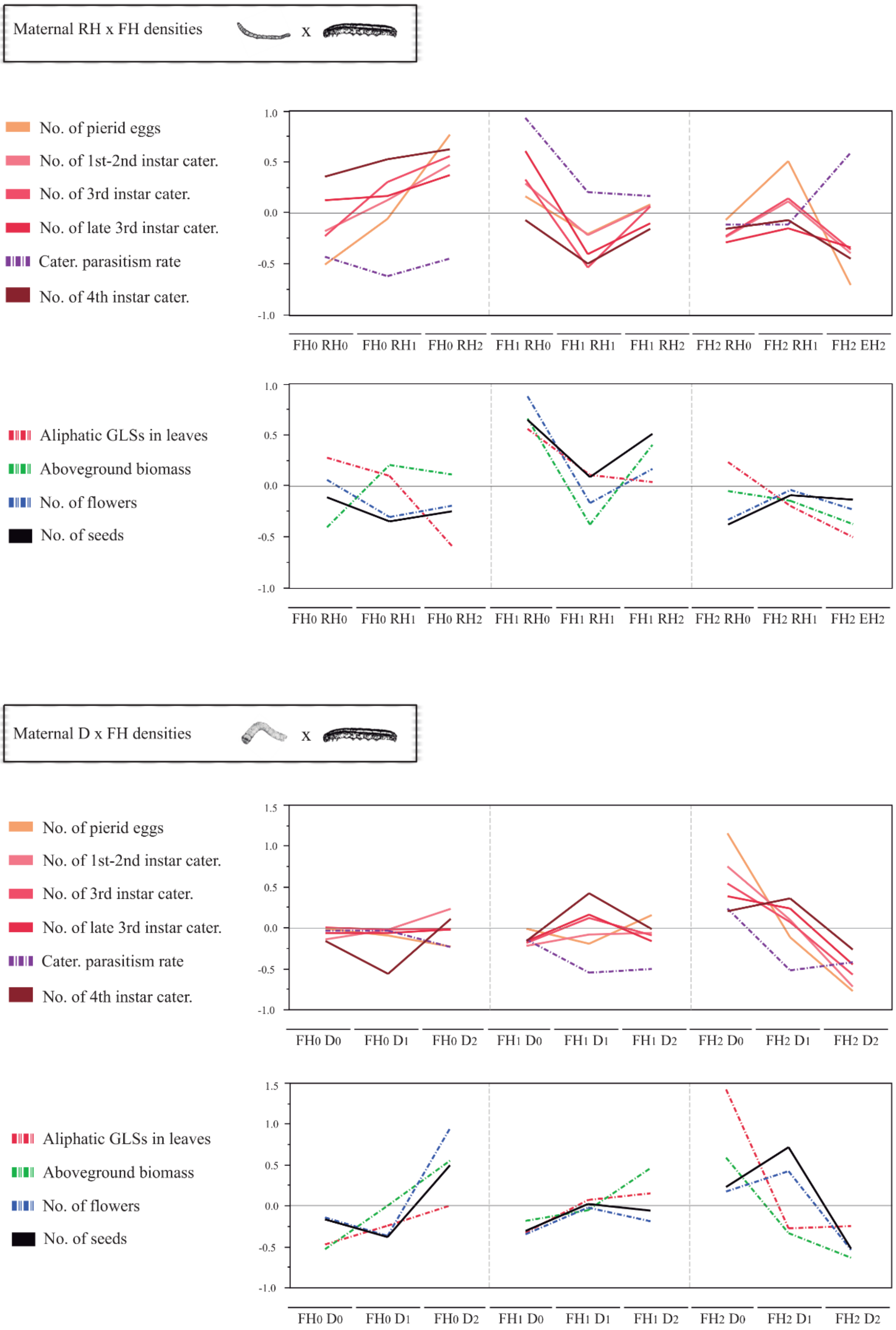


Figure 6.6. Line plot of mean standardized values (mean = 0, SD = 1) per treatment of offspring traits and fitness, pierid egg oviposition, pierid caterpillar parasitism rate and caterpillar performance on offspring. Above, offspring from the RH x FH cohort. Below, offspring from the D x FH cohort.

Transgenerational effects cascading up to the third trophic level

Natural enemies such as parasitoids play a major role in plant-herbivore interaction and in the evolution of plant defenses (Mauricio and Rausher 1997, Züst et al. 2012), being considered as plants' indirect defense in many systems (Turlings et al. 1990, Pashalidou et al. 2015b). However, to be part of plant strategies to deal with specific herbivores there has to be a coevolution between the plant and the parasitoids, for which plants have to provide successful cues about potential hosts (Kessler and Heil 2011). For this, it is usually essential a specific blend of volatiles from plants damaged by a certain herbivore and sensory perception of these volatiles by its parasitoids (Clavijo McCormick et al. 2012). In the present study, we observed a trend for higher pierid caterpillar parasitism rate on offspring whom mothers faced herbivory by the same species, although only in the RH x FH cohort. The mechanisms for that apparent indirect transgenerational effect remain unclear though. In this cohort, parasitism rate was also higher on plants with more flowers, in which plants could increase visual and chemical conspicuousness towards parasitoids for foraging (Vet 2001, Awmack and Leather 2002). Being an involuntary consequence of transgenerational effects or not, an increase in pierid caterpillar parasitism in offspring could have reduced the negative fitness consequences of maternal florivory in the RH x FH cohort, increasing the intrinsic complexity and the reach of transgenerational effects.

Conclusions

Here we demonstrate that the interaction with both antagonists and detritivores can have transgenerational consequences in a short-lived plant. These effects can be context- and density dependent, and are far from being intuitive based on their within-generational effects. Importantly, these transgenerational effects can differentially affect offspring vital transitional stages such as emergence and posterior reproduction. As previously shown in this system, maternal seed provisioning moderately determined seedling emergence, but its transgenerational relevance lessened in further ontogenic stages, in which inherited phenotypes and their interplay with the associated community complexly shapes offspring fitness (Chapter 3). Surprisingly, we also found that transgenerational effects induced by florivore caterpillars could condition their own parasitism rates when feeding on offspring. In line with other authors, and despite our reduced sample sizes, this study leads to conclude that the predictability of transgenerational effects decrease with increasing experimental complexity and ecological realism (terHorst and Lau 2012, Herman et al. 2014). Together with the awaited advances in the understanding of epigenetic inheritance and its persistence in model species, and how these mechanisms shape offspring phenotypes together with carry-over effects, genotype and the environment, we consider that there is a need for more field studies within natural systems in which the transgenerational consequences of a wide variety of unexplored direct and indirect interactions are considered. These field studies would allow delving into the ecological and evolutionary implications of transgenerational effects, and a deeper comprehension of those natural systems themselves.

6.X Supplementary material

Supplementary material 6.1. Model selection.

Variable	Distribution	Random structure	BIC
Emergence rate	Binomial	Planting day / Mother	2300.329
Aboveground biomass	Gamma	Mother / Block	120.523
Number of flowers	Negative binomial	Mother / Block	576.403
Number of fruits	Negative binomial	Mother / Block	432.786
Number of seeds	Poisson	Mother / Plant	837.729
Total GLSs*	Gaussian	Mother / Block	169.260
Aliphatic GLSs*	Gaussian	Mother / Block	168.613
Indolic GLSs*	Gaussian	Mother / Block	180.050
Carbon content in leaves	Gamma	Mother / Plant	-683.259
Nitrogen content in leaves	Gamma	Mother / Plant	-980.224
C/N ratio in leaves	Gamma	Mother / Plant	-560.585
Number of pierid eggs	Negative binomial	Mother / Block	382.607
1st – 2nd instar pierid caterpillars	Poisson	Mother / Block	268.737
3rd instar pierid caterpillars	Poisson	Mother / Block	264.735
late 3rd instar pierid caterpillars	Poisson	Mother / Block	234.294
4th instar pierid caterpillars	Poisson	Mother / Block	210.294
Parasitism rate	Gaussian	Mother	82.885

Table 6.S1a. Full specification of selected GLMMs for analyzed variables for the RH x FH cohort. * GLS concentrations are range/(n-1) transformed.

Variable	**Distribution**	**Random structure**	**BIC**
Emergence rate	Binomial	Planting day / Mother	1761.339
Aboveground biomass	Gamma	Mother / Block	156.674
Number of flowers	Negative binomial	Mother / Block	582.653
Number of fruits	Negative binomial	Mother / Block	451.956
Number of seeds	Poisson	Mother / Plant	855.798
Total GLSs*	Gaussian	Mother / Block	193.502
Aliphatic GLSs*	Gaussian	Mother	196.244
Indolic GLSs*	Gaussian	Mother	193.092
Carbon content in leaves	Gamma	Mother / Plant	-702.702
Nitrogen content in leaves	Gamma	Mother / Plant	-1078.055
C/N ratio in leaves	Gamma	Mother / Plant	-555.696
Number of pierid eggs	Negative binomial	Mother / Block	368.277
1st – 2nd instar pierid caterpillars	Poisson	Mother / Block	259.030
3rd instar pierid caterpillars	Poisson	Mother / Block	241.612
late 3rd instar pierid caterpillars	Poisson	Mother / Block	229.458
4th instar pierid caterpillars	Poisson	Mother	190.011
Parasitism rate	Gaussian	Mother	75.158

Table 6.S1b. Full specification of selected GLMMs for analyzed variables for the D x FH cohort.
* GLS concentrations are range/(n-1) transformed.

Supplementary material 6.2. Post hoc tests.

RH x FH cohort

	RH factor level effects		
	RH_0 – RH_1	**RH_0 – RH_2**	**RH_1 – RH_2**
Total GLSs in leaves	1.21	2.26^{ms}	0.92
Aliphatic GLSs in leaves	1.37	2.41*	0.91
	FH factor level effects		
	FH_0 – FH_1	**FH_0 – FH_1**	**FH_1 – FH_2**
Emergence rate	4.49***	2.99**	-1.55
Number of late 3rd instar pierid caterpillars	1.01	1.89	0.83
Number of 4th instar pierid caterpillars	3.01**	2.98**	-0.10
Pierid caterpillar parasitism rate	-2.51^{ms}	-1.92	0.75

D x FH cohort

	D factor level effects		
	D_0 – D_1	**D_0 – D_2**	**D_1 – D_2**
Emergence rate	-0.25	-4.58***	-4.08***

Table 6.S2a. *P*-value adjusting multivariate post hoc tests for GLMM factor level effects for both RH x FH and D x FH cohorts. *t*-values are shown for variables analyzed with Gaussian distribution (glucosinolates (GLS) and pierid caterpillar parasitism rate), and *z*-values are shown for the rest. ^{ms} *P* <0.08,* *P* <0.05, ** *P* <0.01, *** *P* <0.001.

	RH x FH cohort: RH-FH interaction level effects								
	RH_0			RH_1			RH_2		
	FH_0 – FH_1	FH_0 – FH_2	FH_1 – FH_2	FH_0 – FH_1	FH_0 – FH_2	FH_1 – FH_2	FH_0 – FH_1	FH_0 – FH_2	FH_1 – FH_2
Number of pierid eggs	-2.03	-1.42	0.62	0.22	-1.63	-1.95	1.98	4.24***	2.43*
	FH_0			FH_1			FH_2		
	RH_0 – RH_1	RH_0 – RH_2	RH_1 – RH_2	RH_0 – RH_1	RH_0 – RH_2	RH_1 – RH_2	RH_0 – RH_1	RH_0 – RH_2	RH_1 – RH_2
Number of pierid eggs	-0.98	-3.53**	-2.23^{ms}	1.13	0.41	-0.72	-1.52	2.30^{ms}	3.67***

	D x FH cohort: D-FH interaction level effects								
	D_0			D_1			D_2		
	FH_0 – FH_1	FH_0 – FH_2	FH_1 – FH_2	FH_0 – FH_1	FH_0 – FH_2	FH_1 – FH_2	FH_0 – FH_1	FH_0 – FH_2	FH_1 – FH_2
Aboveground biomass	1.25	2.16^{ms}	1.14	0.33	1.24	0.93	-1.70	-2.26^{ms}	-0.80
Number of flowers	0.27	-1.33	-1.69	-0.68	-0.92	-0.30	2.67*	2.71*	0.03
Number of fruits	-0.66	-1.66	-1.11	-0.70	-1.11	-0.52	2.16^{ms}	2.95**	0.83
Number of seeds	-0.60	-1.58	-1.09	-0.96	-0.42	0.49	2.15^{ms}	2.37*	0.21
Aliphatic GLSs in leaves	-0.44	-3.20**	-2.00	-0.47	-0.08	0.37	-0.89	0.11	0.93
Number of pierid eggs	0.23	0.61	0.41	0.61	-0.12	-0.75	2.71*	4.32**	1.80

Number of 1st – 2nd instar caterpillars	-0.32	-0.82	-0.55	-0.61	-0.36	0.23	1.41	3.05**	1.88

	FH_0			**FH_1**			**FH_2**		
	$D_0 - D_1$	**$D_0 - D_2$**	**$D_1 - D_2$**	**$D_0 - D_1$**	**$D_0 - D_2$**	**$D_1 - D_2$**	**$D_0 - D_1$**	**$D_0 - D_2$**	**$D_1 - D_2$**
Aboveground biomass	1.01	2.03	1.35	0.00	-0.82	-0.84	-0.31	-2.38*	-2.16^{ms}
Number of flowers	0.07	-2.15^{ms}	-2.22^{ms}	-0.95	0.17	1.07	0.43	1.85	1.30
Number of fruits	-0.53	-2.12	-1.56	-0.68	0.67	1.32	-0.08	2.56*	2.45*
Number of seeds	0.02	-1.32	-1.37	-0.35	1.41	1.74	1.16	2.61*	1.35
Aliphatic GLSs in leaves	0.01	-0.84	-0.85	0.00	-1.25	-1.28	2.40*	1.68	-0.58
Number of pierid eggs	0.04	-2.03	-2.10	0.45	0.32	-0.11	-0.69	1.72	2.34^{ms}
Number of 1st – 2nd instar caterpillars	0.40	-1.85	-2.29	0.15	-0.24	-0.39	0.89	2.13	1.32

Table 6.S2b. *P*-value adjusting multivariate post hoc tests for GLMM interaction-level effects for both RH x FH and D x FH cohorts. *t*-values are shown for variables analyzed with Gaussian distribution (glucosinolates (GLS) and pierid caterpillar parasitism rate), and *z*-values are shown for the rest. ^{ms} *P* <0.08,* *P* <0.05, ** *P* <0.01, *** *P* <0.001 .

Supplementary material 6.3. Structural equation model (SEM) for both RH x FH and D x FH cohorts.

RH x FH cohort							
Model	**Removed paths**	**Added paths**	**AICc**	**ΔAICc**	**df**	**Fischer's *C***	***P***
Full initial model			629.833	52.113	62	67.346	0.299
model 2	Number of fourth instar caterpillars → Number of fruits	-	654.968	77.248	60	56.551	0.529
model 3	-	Caterpillar parasitism rate → Number of fruits	676.425	98.705	60	63.090	0.368
model 4	Caterpillar parasitism rate → Number of fruits	Aboveground biomass → Number of fruits	675.144	97.424	60	62.819	0.377
model 5	Aboveground biomass → Number of fruits	Maternal FH density → Number of late third instar caterpillars	665.567	87.847	60	60.793	0.447
model 6	Maternal FH density → Number of late third instar caterpillars	Number of flowers → Number of late third instar caterpillars	628.147	50.427	62	66.957	0.311
model 7	Aboveground biomass → Number of late third instar caterpillars	-	577.720	-	64	68.430	0.329
model 8	Number of pierid eggs → Number of late third instar caterpillars	-	**629.833**	**52.113**	**62**	**67.346**	**0.299**

Table 6.S3a. Stepwise SEM selection procedure for RH x FH cohort SEM.

D x FH cohort							
Model	**Removed paths**	**Added paths**	**AICc**	**ΔAICc**	**df**	**Fischer's *C***	***P***
Full initial model			1138.26	611.887	54	86.600	0.003
model 2	Aliphatic GLSs → Number of pierid eggs Aliphatic GLSs → Caterpillar parasitism rate	-	937.585	411.212	58	88.443	0.006
model 3	-	Maternal FH density → Number of pierid eggs	976.338	449.965	56	78.061	0.027
model 4	Number of flowers → Caterpillar parasitism rate	-	630.385	104.012	54	77.347	0.020
model 5	Number of flowers → Number of late-third instar caterpillars	-	593.900	67.527	56	79.513	0.021
model 6	Aliphatic GLSs → Number of late-third instar caterpillars	Number of flowers → Number of late-third instar caterpillars	638.267	111.894	52	66.951	0.079
model 7	Number of flowers → Number of late-third instar caterpillars	-	597.670	71.297	54	68.766	0.085
model 8	-	Aboveground biomass → Aliphatic GLSs	610.394	84.021	52	60.097	0.206
model 9	Maternal D density → Aliphatic GLSs Maternal D x FH density → Aliphatic GLSs	-	**526.373**	-	**54**	**60.694**	**0.247**

Table 6.S3b. Stepwise SEM selection procedure for D x FH cohort SEM.

Supplementary material 6.4. Offspring performance and fitness correlation with mother plant traits.

RH x FH cohort					
	Mother plant traits				
Offpsring traits	C content in seeds	N content in seeds	C/N ratio in seeds	C and N content in seeds (trait combination)	**Mother-offspring correlation for the same trait**
Emergence rate	0.29*	0.26*	-0.23^{ms}	0.24^{ms}	-
Aboveground biomass	-0.09	-0.06	0.09	-0.07	-0.024
C and N content in leaves (trait combination)	0.13	0.06	-0.03	0.06	-0.062
Aliphatic GLSs in leaves	-0.06	0.16	-0.12	0.15	0.297
Indolic GLSs in leaves	0.02	-0.29	0.33	-0.28	0.158
Number of flowers	0.00	0.19	-0.18	0.19	0.021
Number of fruits	-0.18	0.06	-0.10	0.05	-0.337
Number of seeds	-0.14	0.02	-0.12	0.07	-0.384*

Table 6.S4a. Pearson's correlation coefficients among maternal seed quality and offspring emergence, offspring aboveground biomass, leaf quality and reproduction, and mother- mean offspring correlations for the same traits in the RH x FH cohort. ^{ms} *P* <0.08,* *P* <0.05.

D x FH cohort					
	Mother plant traits				
Offpsring traits	C content in seeds	N content in seeds	C/N ratio in seeds	C and N content in seeds (trait combination)	**Mother-offspring correlation for the same trait**
Emergence rate	-0.17	0.22	0.19	0.22	-
Aboveground biomass	-0.15	0.08	-0.04	0.10	0.562**
C and N content in leaves (trait combination)	0.10	-0.04	0.08	-0.06	0.012
Aliphatic GLSs in leaves	-0.02	0.22	-0.13	0.18	-0.126
Indolic GLSs in leaves	0.14	0.08	-0.03	0.03	0.042
Number of flowers	0.10	-0.18	0.19	-0.17	0.094
Number of fruits	0.19	-0.24	0.26	-0.24	0.326
Number of seeds	0.23	-0.28	0.30	-0.28	0.369*

Table 6.S4b. Pearson's correlation coefficients among maternal seed quality and offspring emergence, offspring aboveground biomass, leaf quality and reproduction, and mother- mean offspring correlations for the same traits in the D x FH cohort. ^{ms} *P* <0.08,* *P* <0.05, ,** *P* <0.01.

Supplementary material 6.5. Mother and offspring seed number.

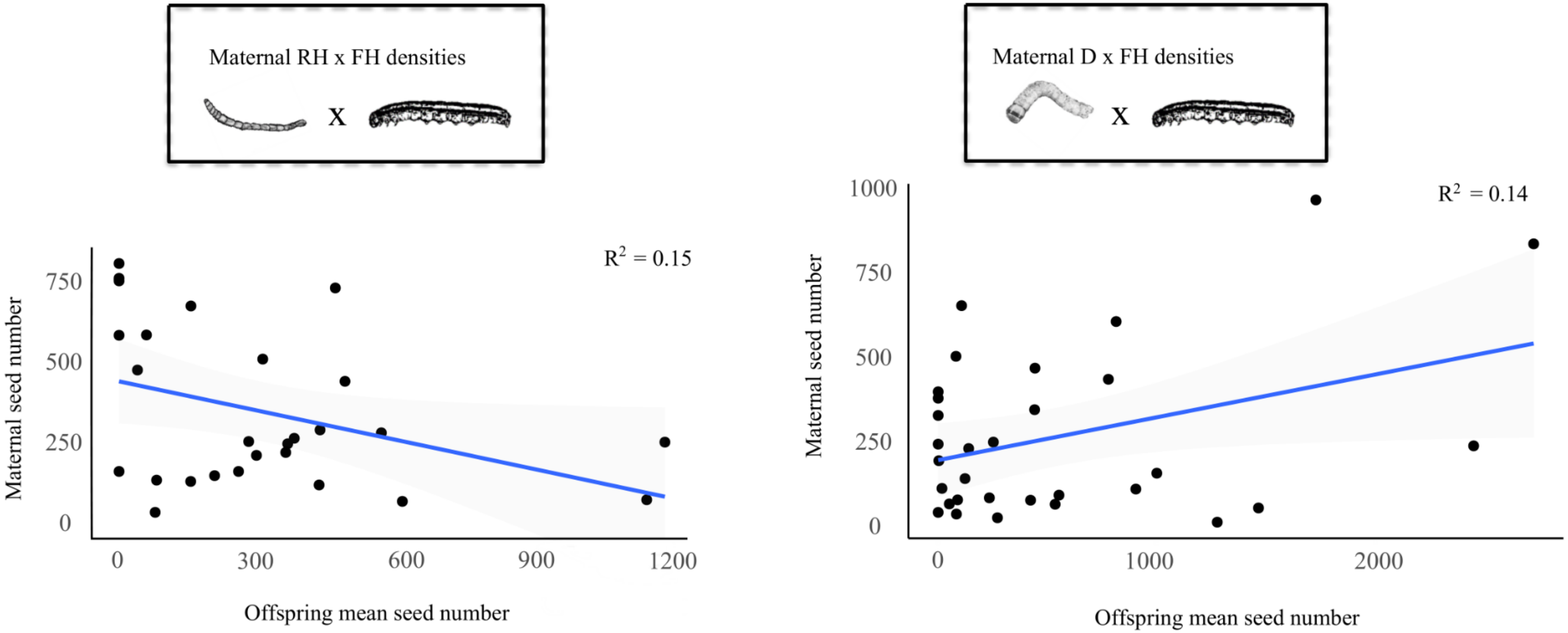


Figure 6.S1. Linear regression plot of mother plant seed number and mean offspring seed number.

Supplementary material 6.6. Relationship among offspring variables.

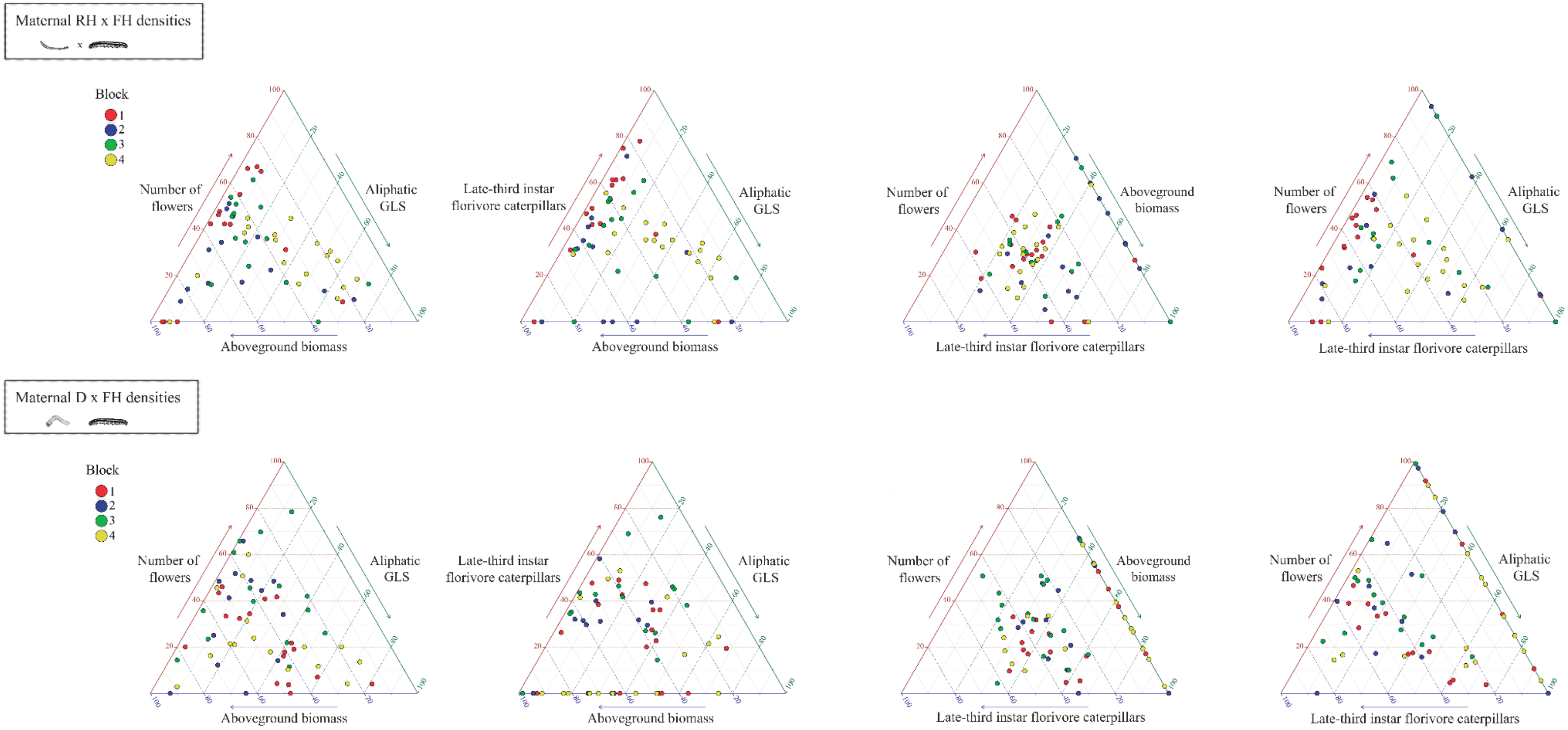


Figure 6.S2. Ternary diagrams of proportional three-way values of offspring aboveground biomass, aliphatic GLS concentrations, number of flowers and number of late-third instar florivore caterpillars. Colors correspond to each of the four blocks used for both cohorts.

Chapter 7

Altered rainfall patterns reduce plant fitness and disrupt interactions between below- and aboveground insect herbivores

7.1 Abstract

Evidence is accumulating of the disruptive effects of climate change on species interactions. However, little is known about how changes in climate patterns, such as temporal shifts in rainfall events, will affect multitrophic interactions. Here, we investigated the effects of changes in rainfall patterns on the interactions between root herbivores, a plant and its associated aboveground insects in a semiarid region by experimentally manipulating in the field rainfall intensity and frequency. We found that a shift in rainfall severely constrained biomass acquisition and flowering of the plant *Moricandia moricandioides*, resulting in fitness reduction. Importantly, enhanced rainfall affected the interactions between below- and some aboveground herbivores, disrupting the positive effects of root herbivores on chewing insects. The shifts in precipitation had also plant-mediated consequences for planthoppers, the dominant sap suckers in our study system. A combination of mechanisms involving biomass acquisition and plant defenses seemed to be responsible for the different responses of insects and their interactions with the plant. This study provides evidence that altered rainfall patterns due to climate change affect not only trophic groups differentially but also their interactions.

Keywords

Altered rainfall · Brassicaceae · Climate change · Herbivory · Root herbivore · Semiarid environment · Simulated precipitation

7.2 Introduction

Climate change is considered one of the most significant global change drivers (Tylianakis et al. 2008, Valladares et al. 2015). In the last decade, many studies have shown the impact of climate change on species diversity and distribution (Parmesan 2006, Siepielski et al. 2017). Climate change also alters species coexistence and biotic interactions (see Valladares et al. 2015 for a review), with consequences at community and ecosystem levels affecting even evolutionary trajectories (Parmesan 2006, Siepielski et al. 2017). However, understanding and predicting how biotic interactions will respond to climate change remains a great challenge (Tylianakis et al. 2008, Barnett and Facey 2016), since both disrupting (Durant et al. 2007, Memmott et al. 2007, Singer and Parmesan 2010) and strengthening effects of climate change on species interactions have been observed (O'Connor 2009). This variety of results arises partly since responses to climate change differ between trophic levels (Voigt et al. 2003, Tylianakis et al. 2008, González-Megías and Menéndez 2012).

Climate change models predict altered precipitation patterns and an increased number of extreme precipitation events in the future (IPCC, 2014). In the Mediterranean region, rainfall is expected to change seasonality, with peaks of rainfall shifting from autumn-winter to late spring-early summer and to show more frequent extreme events (IPCC, 2014). According to Solomon et al. (2007), climate models for drylands also forecast an increase in temperature, and significant alterations in rainfall patterns by the late 21.St century. Because arid and semiarid ecosystems represent 41% of the terrestrial surface and account for more than 25% of global soil organic carbon (Safriel and Adeel 2005), it is imperative to gain a clear understanding of the responses of these biomes to climate change (Maestre et al. 2013, Nielsen and Ball 2015). Although arid and semiarid plants are up to a certain point resilient to changes in rainfall frequency and intensity (Miranda et al. 2009), the effects vary among plants depending on life cycle, rooting system, phenological state and physiological and morphological readiness for water uptake and growth (Ogle and Reynolds 2004, Reynolds et al. 2004, Yahdjian and Sala 2010, Zhang et al. 2019).

Studies based on regional climate projections and expected seasonal changes can also provide valuable insights into climate change effects on multitrophic interactions (Jamieson et al. 2012). Precipitation changes due to climate change are predicted to have short-term impacts on insect communities, likely having cascading effects through the food web (Barnett and Facey 2016, Torode et al. 2016). Climate change has also the potential to disrupt belowground-aboveground interactions, such as altering root herbivore effects on plants and consequently on aboveground organisms (van der Putten et al. 2009). It is well known that under drought conditions root herbivores increase plant susceptibility to aboveground herbivory (Zvereva and Kozlov 2012, McKenzie et al. 2013, Guyer et al. 2018). Root herbivory can decrease water and nutrient uptake and therefore reduce rates of photosynthesis, deplete stored resources in the roots and cause photoassimilates to be diverted for root regrowth and repair (Blossey and Hunt-Joshi 2003, Johnson et al. 2016). Plant strategies to deal with root herbivores are diverse, and can indirectly affect other above- and/or belowground herbivores. Plants can regrow damaged tissue, invest in chemical defenses, allocate nutrients from roots to aboveground tissues, and phenologically escape by dispersing seeds earlier (Newingham et al. 2007, Robert et al. 2014). Studies regarding the effects of precipitation changes on interactions between root herbivores, plants and aboveground organisms have mostly focused on summer drought simulation scenarios (Johnson et al. 2011, Tariq et al. 2013). However, little is known yet about the effects of shifts in precipitation patterns on such interactions. Moreover, only a handful of studies investigated these complex interactions by manipulating precipitation in the field (Barnett and Facey 2016, Wade et al. 2017).

In the *Moricandia moricandioides* system, a Brassicaceae herb inhabiting semiarid environments, root herbivory has been shown to modulate induced plant defenses and the interaction between the plant and aboveground organisms (González-Megías and Müller 2010). In addition, shifts in rainfall patterns in this system have been shown to provoke changes in the strength and/or the sign of the interactions between below- and aboveground organisms. For example, disrupting the positive effect of detritivores on the abundance of chewing and sucking insects (González-Megías and Menéndez 2012). In the present study we focus on understanding the impact of changes in rainfall patterns on the interactions between root herbivores, its host plant, and its associated aboveground herbivorous insects. We experimentally manipulated rainfall intensity and frequency during late spring / early summer period, based on future projections for the study area, and measured the effects of changing rainfall patterns on trophic interactions and the underlying mechanisms. We predicted that i) altered rainfall pattern will negatively affect the reproductive success of the semiarid herb *M. moricandioides* due to reduced soil water holding. Changes in the timing and magnitude of water pulse events, such as extreme rainfall events, can provoke a greater fraction of water to be lost as runoff (Siteur et al. 2014); ii) plant responses to altered rainfall will be mediated by the interaction of root herbivores with the aboveground insect community and iii) the underlying mechanisms will be associated with plant strategies to deal with root herbivores, including increased chemical defense and nutrient reallocation.

7.3 Materials and methods

Study sistem

The experiment was conducted at Barranco del Espartal, a seasonal watercourse located in the semiarid Guadix-Baza Basin (southeastern Spain). The climate at the study area is continental Mediterranean with pronounced temperature fluctuations (ranging from -14ºC to up to 45ºC) and high seasonality. Annual precipitation rarely exceeds 300 mm.

The plant species *Moricandia moricandioides* (Boiss.) Heywood (Brassicaceae) is highly abundant in this habitat and was used as a study system. *Moricandia moricandioides* plants are distributed in monospecific stands. This species germinates in autumn, grows as a vegetative rosette during winter, and produce reproductive stalks in spring when it receives sufficient amount of water. The stalks remain photosynthetically active during the entire season (González-Megías and Müller 2010). The plants produce glucosinolates, which are the characteristic defense compounds occurring in the order Brassicales (Fahey et al. 2001). After having reproduced, the vast majority of individuals die during summer (approx. 93% of the individuals).

The aboveground insect herbivores associated with *M. moricandioides* include specialist and generalist species belonging to several trophic guilds (see González-Megías and Müller, 2010). One of the most abundant root herbivores is *Cebrio gypsicola* Graells (Coleoptera, Cebrionidae), representing ~ 25% of belowground macroinvertebrate biomass (Doblas-Miranda et al. 2007). We found 0.95 ± 0.2 larva/plant of *C. gypsicola* when sampling random individual during the study years.

Experimental set-up

To explore the individual and combined effects of a future scenario of rainfall changes and root herbivores on the interaction between *M. moricandioides* plants and aboveground insects, we conducted a field experiment in 2013. The experiment consisted of a split-plot design with two factors and two levels per factor. The two factors were rainfall, in which late spring/early summer rainfall was enhanced, and root herbivores, in which the absence/presence of this guild was manipulated. The enhanced rainfall treatment was applied to 10 randomized blocks with five blocks per level. Each block had 20 plants located at 30 cm apart from each other (200 plants in total). A similar experimental design has been previously tested in the study area manipulating rainfall and other belowground organisms (see González-Megías and Menéndez, 2012).

During the winter of 2012-2013, seeds of *M. moricandioides* collected from the study area were germinated in pots with soil from the study area and grown in a common garden. Plants without reproductive stalk were kept in these pots until beginning of May when they were moved to the field. The absence of rain during early spring constrains plant flowering during that period (authors' previous observation). Therefore, we started our experiment in May because we were interested in measuring the response of the plants to the delay of rainfall from early to late spring (a period with also higher temperatures). Once in the field, plants were re-potted using mixed soil from the study site from which macro-invertebrates were removed by hand. The pots consisted of fiber-glass-mesh cylinders (10 x 15 cm) of 1 mm mesh size to inhibit the entrance or escape of belowground macroinvertebrates. These pots were then buried with the upper surface level with the ground. During the first week in the field all plants were watered and net-covered to ensure their establishment.

Metric	Period A	Period B	Total	Delayed period 1	Delayed period 2	Total
Total precipitation	91.89 ± 12.24	31.12 ± 5.02	123.93			
Number of rainy days	17.90 ± 1.54	5.60 ± 0.82	23.5			
Daily precipitation	5.00 ± 0.40	5.60 ± 0.98	5.3			
Supplemented precipitation				64 (+20.40)	32 (+0.30)	117
Supplemented rain events				4 (+8)	2 (+1)	15
Supplemented precipitation/rain event				16 (+2.27)	16 (+0.30)	16

Table 7.1. Total precipitation (mm), rainy days, and precipitation per day (mm) observed in the study area during the previous 15 yr (2003–2012) during two periods (mean ± SE); Supplemented and natural (in parentheses) precipitation, rainy days, and precipitation per day during the experimental year 2013. Notes: Delayed periods corresponded to the rainfall treatment (R+) in which blocks assigned to this treatment received natural precipitation plus the supplemented precipitation (simulating delayed rainfall of periods A and B predicted by IPCC for the study region). Period A, March to mid-May; Period B, mid-May to June. Delayed period 1, mid-May to June; Delayed period 2, July.

The two levels of the rainfall treatment (R) were a control without manipulation (R-) and an enhanced rainfall level (R+). The rain level applied was based on a projected precipitation model in which rainfall during late spring / early summer was increased compared with current conditions (a delay in precipitation scenario), but it was concentrated in few events that were more extreme in intensity (lower frequency of rain episodes but higher rainfall quantity per event, Table 7.1). This scenario was designed according to the future predictions for this type of Mediterranean dryland ecosystems (IPCC, 2014, Solomon et al., 2007). To simulate this future climatic scenario, we calculated the mean precipitation in the study area of the last 15 years during two periods, (A) the beginning of spring (March to mid-May) and (B) late spring to the beginning of summer (mid-May to June; Table 7.1). For our experiment, we define two alternative periods: 1) Delayed period 1, in which plants associated with R+ treatment were supplemented with water in mid-May to end June to the level of the mean precipitation recorded for the period (A) (Table 7.1), and 2) Delayed period 2, in which plants associated with R+ treatment were supplemented with water in July to the level of the mean precipitation recorded for the period (B) (Table 7.1). The amount of water needed to be added to plants under the R+ treatment was around 123 mm (Table 7.1). Because it was impossible to predict natural precipitation during 2013 (the year we carry out the experiment), we roughly calculated the amount of water to be added based on the "predicted" precipitation for that particular time of the year of the previous 15 years. The total final amount of water received by the experimental plants in the R+ treatment during the delayed periods was similar than the expected (Table 7.1). The frequency of the rain events was reduced by 36.2 percent of the recorded events during the last 15 years (Table 7.1). The occurring natural precipitation during the experiment was taken into account to adjust the amount of precipitation supplemented as explained above (Table 1). August was not included in the experiment because no plants survive either in natural or under experimental conditions to this month. To simulate cloud cover during the experimental rainfall events, we covered the blocks with a net that filtered radiation by 50%. This net was used during the day of the experimental rainfall event and the day after, simulating the mean reduction of solar radiation that has been observed under natural rainfall conditions in the study area. The net was located more than 1 m above the top of the plants to ensure no effect on insect visitation. Environmental data was provided by a weather station in the study area (Embalse del Negratín, Confederación Hidrográfica del Guadalquivir, Granada).

The root herbivore treatment consisted of a control with no root herbivore (RH-) and the addition of one larva of *C. gypsicola* (RH+). Each of the 20 plants within each block was allocated randomly to one of the two root herbivore levels. Two weeks after the plants were moved to the field, one larva of *C. gypsicola* (second or third-instar) was added to the soil of each plant assigned to the RH+ treatment. In previous experiments with the same root herbivore, a very high larvae recovery rate was achieved at the time of plant harvest (~ 90%, González-Megías and Müller, 2010), confirming the reliability of this methodology in recording root herbivory effects.

Data collection

Herbivorous insect abundance

To score the abundance of aboveground herbivores, the number of naturally occurring individuals of each insect species found on each experimental plant was recorded 3 times per week after the set-up of the experiment (from 2-May-2013), resulting in a total of 31 surveys across which numbers were summed per species. Insect species were assigned to trophic guilds (see González-Megías and Müller, 2010 for a similar procedure).

Plant trait measurements

We recorded the number of open flowers per plant three times per week to calculate flowering phenology (number of days with open flowers). At the end of the experiment (29-July-2013), we counted the number of reproductive stalks and the total number of flowers and fruits produced by each plant. All fruits were taken to the laboratory where the total number of seeds per fruit was counted for each plant. The entire shoots of each plant were collected individually from the field and oven-dried at 40 ºC for 72 h (until complete desiccation). Shoot tissue was weighed to calculate aboveground biomass. C and N concentrations, and their ratio in leaves were determined for plants using a CHN Elemental Analyser. The following variables were used in our analyses: 1) for plant performance: aboveground biomass, and the number of flowers, 2) for plant phenology: the number of days the plant display flowers (flowering duration), 3) for plant quality: glucosinolate concentration and C/N ratio, and 4) for plant fitness: plants that produced flowering stalks (as a binary variable), and the total number of seeds produced by the plant.

To quantify glucosinolate (GLS) concentrations of the aboveground plant tissue, the youngest leaf of one stem of each of the experimental plants was collected before leaf senescence (a total of 173 plants; R-RH- = 42 plants, R-RH+ = 43 plants, R+RH- = 43 plants, R+RH+ = 45 plants). Leaves were stored and immediately freeze-dried, and the dried material was ground and extracted three times in 80% methanol after the addition of *p*-hydroxybenzyl GLS (sinalbin) as an internal standard. GLS extraction was done following previously established methodology (Müller and Sieling 2006, González-Megías and Müller 2010). Desulfoglucosinolates were identified by comparison of UV-spectra and retention times to those identified in earlier studies (Müller and Sieling 2006, González-Megías and Müller 2010). Peaks were integrated at 229 nm, response factors of 1 for aliphatic and 0.26 for indolic GLSs considered, and areas related to the internal standard (response factor 0.5) and sample dry weight for calculation of concentrations.

Statistical analyses

Analyses were performed at two levels: 1) with all plants in the experiment: to determine the effect of each experimental factor (rainfall and root herbivores) on plant success and aboveground herbivore abundance, and 2) with flowering plants only (plants that produced reproductive stalks): to determine if the effect of experimental factors varied once the plant initiated reproduction.

Linear and generalized linear mixed models for treatment effects

Because this experiment was designed from the beginning as a full factorial experiment with two levels using blocks (a typical split-plot design), we analyzed our data using linear mixed models. The success or failure of plants to produce reproductive stalks was also analyzed as a binomial variable when all experimental plants were included in the analysis.

Due to the specific requirements of each response variable in terms of data distribution, homoscedasticity, overdispersion and zero-inflation, we used univariate general (LMMs) and generalized linear mixed models (GLMMs; Suppl. 7.1, Table 7.S1) to test the effects of each factor (rainfall and root herbivores) and their interaction on plant variables and insect abundance. These models provide a flexible and accurate approach for analyzing these kinds of data with random effects (Bolker et al. 2009). Treatment effects on herbivore abundance were analyzed at trophic guild level (chewers and sap suckers). For sap suckers, we also performed the analysis separately for planthoppers because they represented the vast majority of this guild. We did not perform analysis on other separate taxonomic groups due to their low abundance. We fitted the most appropriate distribution for each variable according to the error conformation (Gaussian, binomial, Poisson or Negative Binomial). When variables analyzed with normal distribution were not homoscedastic, we modeled heteroscedasticity using generalized least squares. Block was always included in the model as a random factor nested within rainfall except when overdispersion was found. In those cases, GLMMs with observation-level random effects were run, which allowed for variation at plant level (Harrison 2014). Zero-inflation was also modeled when necessary. Model selection was based on Bayesian Information Criterion (BIC). All analyses were performed in R 3.1.2 (R Core Team 2020) using *nlme* (Pinheiro et al. 2014) and *lme4* (Bates et al. 2015) packages, and with *glmmADMB* (Fournier et al. 2012) in the case of zero-inflated models.

Multivariate analysis on chemical defense profile

We used non-metric multidimensional scaling (NMDS) to test for dissimilarities in GLS profiles between the treatments. NMDS is an indirect gradient analysis approach that produces an ordination based on a distance or dissimilarity matrix. Treatments were assigned as vectors onto a two-dimension plot, and we utilized Horn dissimilarity, 10,000 permutations and 100 random starts for assessing significance. The analysis was performed with the R package *vegan* (Oksanen et al. 2017).

7.4 Results

Effects of the experimental treatments on plant traits

Effects on plant performance and fitness

For all plants, enhanced rainfall reduced aboveground vegetative biomass by 66% (Table 7.2, Fig. 7.1A) and reduced more than half the proportion of plants that produced reproductive stalks (Table 7.2, Fig. 7.1B). Enhanced rainfall also reduced the number of flowers (Table 7.2, Fig. 7.1C) and the number of seeds produced per plant (Table 7.2, Fig. 7.1D). There were no significant effects of root herbivores on any of the plant traits analyzed (Table 7.2).

For flowering plants only, enhanced rainfall marginally reduced the number of flowers (Table 7.3, Fig. 7.2A) but had no significant effect on any other plant trait. Presence of root herbivores significantly increased flowering duration (Table 7.3, Fig. 7.2B). No experimental effect was observed on the number of seeds when considering only flowering plants (Table 7.3).

	R			RH			R x RH		
	F/χ^2	P	df	F/χ^2	P	df	F/χ^2	P	df
Plant traits									
Aboveground biomass	**19.88**	**0.002**	1,8	2.63	0.10	1,180	0.06	0.80	1,180
Plants with reproductive stalks	**13.68**	**0.006**	1,8	0.24	0.62	1,187	0.18	0.67	1,187
Number of flowers	**6.28**	**0.012**	1,8	0.29	0.59	1,186	0.74	0.38	1,186
Number of seeds	**4.55**	**0.032**	1,8	0.24	0.62	1,187	0.62	0.43	1,187
Herbivore abundance									
Total sap suckers	2.00	0.15	1,8	0.84	1,187	1,187	0.19	0.66	1,187
Planthoppers	2.87	0.09	1,8	0.44	1,187	1,187	0.08	0.77	1,187
Total chewers	0.19	0.66	1,8	**0.037**	1,187	1,187	**4.52**	**0.033**	1,187

Table 7.1. LMM and GLMM results for the effect of rainfall (R), root herbivores (RH) and their interaction (R x RH) on plant traits and herbivore abundance for all plants. F is shown for aboveground biomass, $\chi 2$ value is shown for the rest of variables. Significant results ($P < 0.05$) are indicated in bold.

Effects on plant quality: C/N content and GLSs

No treatment effects were observed on C content, N content or C/N ratio in leaves for either all plants or flowering plants only.

Ten GLSs were found in *M. moricandioides* leaves, six aliphatic and four indolic. The total GLSs concentration was on average 7.73 ± 0.77 μmol g^{-1} of dry weight, with a prevalence of aliphatic (89.65%) over indolic GLSs (10.35%). No treatment effects on GLSs were observed when considering all plants. The NMDS also showed that treatments explained little of the observed variation in the GLS profiles (Suppl. 7.2, Table 7.S2).

For flowering plants only, enhanced rainfall induced the production of the main indolic compound, indol-3-yl-methyl GLS (Fig. 7.2C). No significant effect was observed for total indolic or total aliphatic GLS concentrations. NMDS results showed that the GLS profile composition of the enhanced rainfall with no root herbivory treatment was significantly dissimilar from the rest of treatments (Suppl. 7.2, Table 7.S2, Fig. 7.S1).

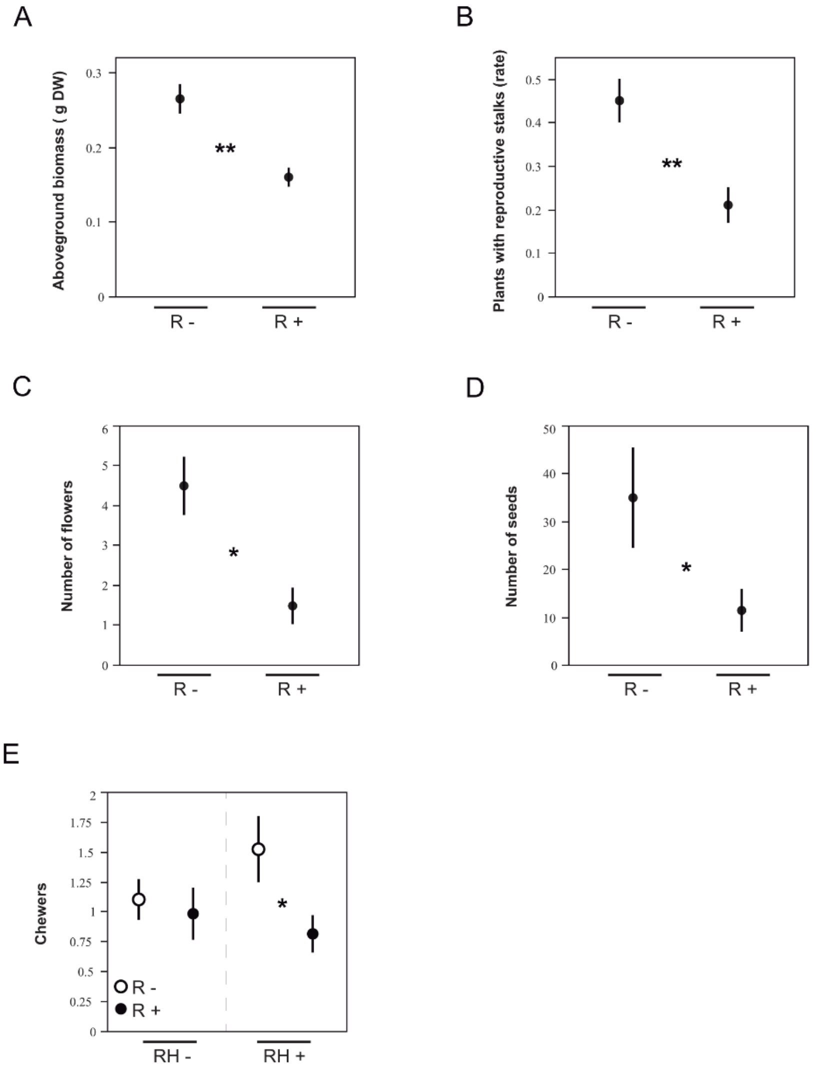


Figure 7.1. Rainfall (R) effect on aboveground biomass (A), the rate of plants that produced reproductive stalks (B), the number of flowers (C) and the number of seeds (D) when considering all plants. Rainfall (R) and root herbivore (RH) interacting effect on chewer abundance (C) when considering all plants. **$P < 0.01$, * $P < 0.05$. Mean ± SE are shown.

Effects of the experimental treatments on aboveground herbivorous insects

During the study period, several species of chewing and sap sucking insects visited and fed on the experimental plants. Most chewers (93%) were caterpillars of specialist species, the pierid butterflies *Pieris rapae* L., *Pontia daplidice* L. and *Euchloe crameri* Batler L., the diamondback moth (*Plutella xylostella* L., Plutellidae) and an unidentified gelechiid moth species. Generalist chewers belonged to three species of beetles, *Galeruca angusta* (Kuster), *Mylabris quadripunctata* L. and *Mylabris hieracii* (Graells) as well as some unidentified species of Orthoptera. Several sap suckers were recorded feeding predominantly on stems and reproductive stalks, they were represented mainly by phloem-feeding generalist planthopper species (80% of all sap suckers; *Agalmatium bilobum* Fieber Hemiptera, Issidae, and an unidentified cicadelid), aphids (*Myzus persicae* Sulzer, *Aphis fabae* Scopoli, *Brevicoryne brassicae* L. and *Lipaphis erysimi* Kaltenbach), and shield bugs (such as *Ventocoris* spp.).

	R			**RH**			**R x RH**		
	F/χ^2	P	df	F/χ^2	P	df	F/χ^2	P	df
Plant traits									
Flowering days	0.11	0.75	1,8	**4.26**	**0.045**	1,43	1.40	0.24	1,43
Number of flowers	3.19	0.07	1,8	0.13	0.71	1,52	0.10	0.75	1,52
Number of seeds	0.51	0.47	1,8	0.31	0.57	1,53	0.00	0.99	1,53
Herbivore abundance									
Total sap suckers	1.67	0.19	1,8	0.12	0.72	1,54	0.19	0.66	1,54
Planthoppers	**3.91**	**0.048**	1,8	0.26	0.61	1,54	0.00	0.99	1,54
Total chewers	0.15	0.69	1,8	**5.21**	**0.022**	1,54	1.40	0.23	1,54

Table 7.2. LMM and GLMM results for the effect of rainfall (R), root herbivores (RH) and their interaction (R x RH) on plant traits and herbivore abundance for flowering plants only. *F* is shown for flowering days, $\chi 2$ value is shown for the rest of variables. Significant results ($P < 0.05$) are indicated in bold.

For all plants, there was a significant interaction between treatments (root herbivores and rainfall) on the abundance of chewers (Table 7.2), with enhanced rainfall reducing the abundance of chewers but only in the presence of root herbivores (Fig. 7.1E). No effect of the experimental treatments was observed for all sap suckers or planthoppers only (Table 7.2).

For flowering plants only, enhanced rainfall reduced by nearly 50% the abundance of planthoppers (Fig. 7.2D, Table 7.3), but there was no effect on all sap suckers (Table 7.3). Chewers were positively affected by root herbivores (Fig. 7.2E, Table 7.3), with no significant effects of rainfall or the interaction between the two treatments.

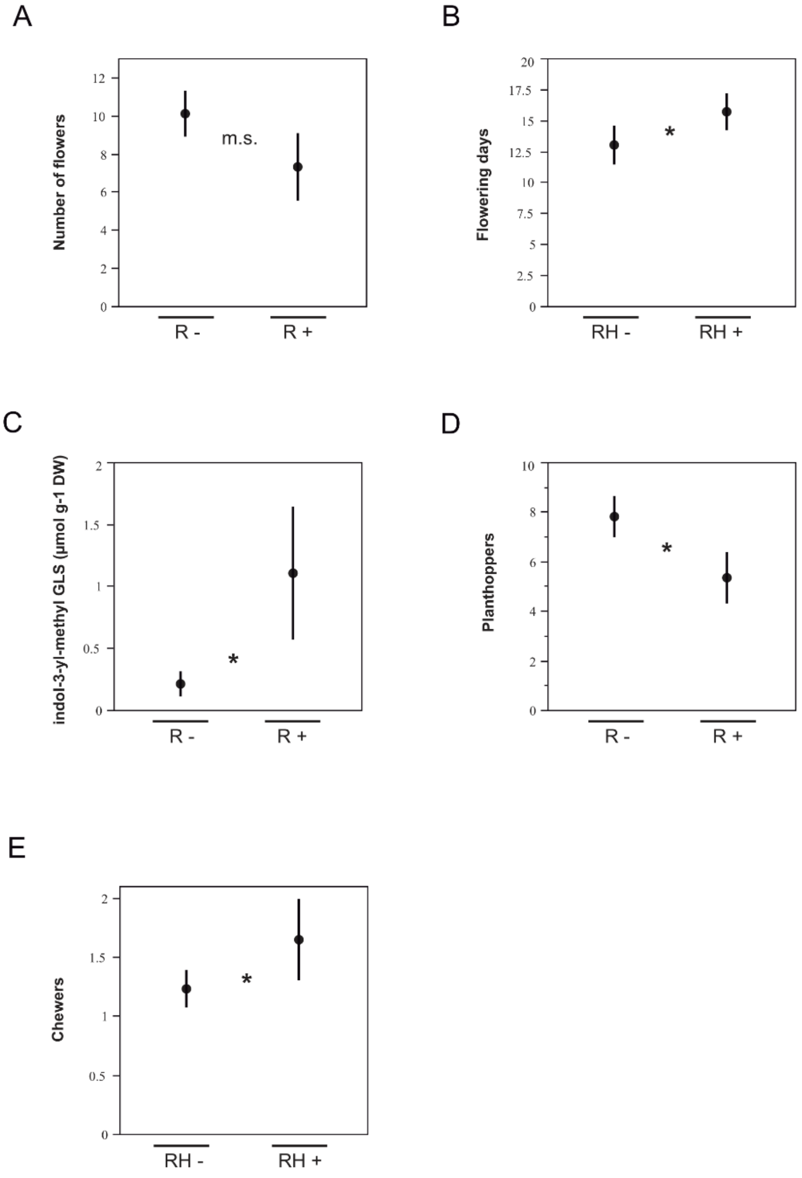


Figure 7.2. Rainfall (R) effect on the number of flowers (A), indol-3-yl-methyl GLS concentration (C), and planthopper abundance (D) when considering only the flowering plants. Root herbivore (RH) effect on the number of flowering days (B), and chewer abundance (E) when considering only the flowering plants. ^{ms} $P = 0.07$, *$P < 0.05$. Mean ± SE are shown.

7.5 Discussion

Effects of the experimental treatments on plant traits

One of the most important results found in this study is that enhanced rainfall severely affected *M. moricandioides* by reducing by half the number of plants that produced reproductive tissue. Multiple environmental and endogenous signals (e.g., photoperiod, temperature, resource availability) are known to induce and regulate plant flowering (Simpson and Dean 2002, Putterill et al. 2004). As a result, plants may adjust flowering to changing environmental conditions (Putterill et al. 2004). In Mediterranean dryland ecosystems, drought and enhanced rainfall have been shown to affect flowering of several short-lived species by decreasing plant biomass (Hänel and Tielbörger 2015, Nielsen and Ball 2015). In a similar way, enhanced rainfall affected *M. moricandioides* by reducing biomass, and the number of reproductive stalks in our experiment. *Moricandia moricandioides* is probably adapted to flower during hot and dry conditions in the study area, so unexpected precipitation could have triggered physiological changes resulting in reduced plant growth. In this way, not only the amount of precipitation but also its timing and intensity would have determined the performance of this semiarid herb (Lázaro et al. 2001). In our experiment, the combination of high temperatures during the raining period with extreme rainfall events probably diminished soil water holding reducing water availability for the plants. A reduction in size can result in lower survival, competitiveness and reproductive output in plants (Kozłowski 1992). In the case of *M. moricandioides* in the study area, plants with insufficient biomass unable to reproduce may alternatively reallocate resources to roots and favor next season re-sprouting. However, only ~ 6% of non-flowering *M. moricandioides* plants re-sprouted in the next season, indicating that resource reallocation to roots may have been limited. This idea is reinforced because there was no effect of enhanced rainfall on C/N content in leaves, what would have been expected if nutrient reallocation to roots had occurred.

Enhanced rainfall also reduced the number of flowers in the subset of plants that produced reproductive tissue. Previous experiments on the system with a similar rainfall simulation (González-Megías and Menéndez 2012) showed a negative effect of enhanced rainfall on the number of flowers but not on plant size or on the overall reproductive output. However, in the present study the enhanced rainfall simulation reduced plant fitness, measured as the number of seeds produced by the plants. Hence, the effects of rainfall shifts on plants such as *M. moricandioides* may be year-dependent and vary in intensity in environments with severe and fluctuating conditions such as arid and semiarid ecosystems, in which plant growing seasons are short and unpredictable (Hänel and Tielbörger 2015 and references therein). In this case, the change in rainfall also provoked the induction of some GLSs in the plants. These results could indicate that plants were investing in chemical defense although GLS concentration is also known to respond to different water regimes in other Brassicaceae (for a review see Metz et al. 2014).

The root herbivore treatment only affected *M. moricandioides* flowering pattern by influencing flowering duration. Belowground herbivory may have provoked nutrient flows from roots to the aboveground tissues, which often occur in short-lived plants when root defenses are ineffective (Moore and Johnson 2017). This reallocation may have led to a prolonged flowering duration at the expense of a less intense flowering. Despite the effect of root herbivores on flowering pattern there was no effect on plant reproductive output, what could also be due to a higher abundance of chewers on these plants. Neutral effects of root herbivores on plant reproductive success have been reported previously for our system (González-Megías 2016) and in other Brassicaceae species (Poveda et al. 2005), in contrast to most plant species that cannot fully compensate after root herbivore attack (Zvereva and Kozlov 2012).

Effects of rainfall and root herbivores on herbivorous insects

On flowering plants, chewers, mainly pierid caterpillars, were positively affected by root herbivores. This positive effect can be related to the longer flowering duration triggered by root herbivores that could increase the feeding time for chewers in reproductive tissue. Most pierid species prefer to feed on reproductive tissues, which increase their survival (Lucas-Barbosa et al. 2014). Nevertheless, when considering all experimental plants, enhanced rainfall disrupted the positive effect of root herbivores on chewers. The enhanced rainfall effect was likely the result of a density-mediated indirect interaction, i.e, a reduction of resources (smaller plants with less flowers). A change in plant architecture is well known to affect the abundance of insect herbivores associated to a particular plant (Haysom and Coulson 1998).

Planthoppers were negatively affected by enhanced rainfall, although this effect was only detectable in plants that have produced flowering stalks. Reduction in flower number in plants exposed to enhanced rainfall may have been one of the causes of reduced planthoppers abundance, as these herbivores seem to prefer sucking on reproductive tissues (Poveda et al. 2005, González-Megías and Müller 2010). However, the main mechanism is likely to be the induction of plant chemical defenses (GLSs) in plants under the enhanced rainfall treatment, which are transported by the phloem (Chen and Andreasson 2001). Plant defenses are usually more effective against generalist than specialist herbivores (Núñez-Farfán et al. 2007, Hopkins et al. 2009). In particular, indolic GLSs are often detrimental to non-specialist sap suckers (Pfalz et al. 2009). In semiarid environments, where there is low potential for plants to compensate for the loss of biomass to herbivory, any plant trait that reduces herbivore damage is fundamental (Herms and Mattson 1992).

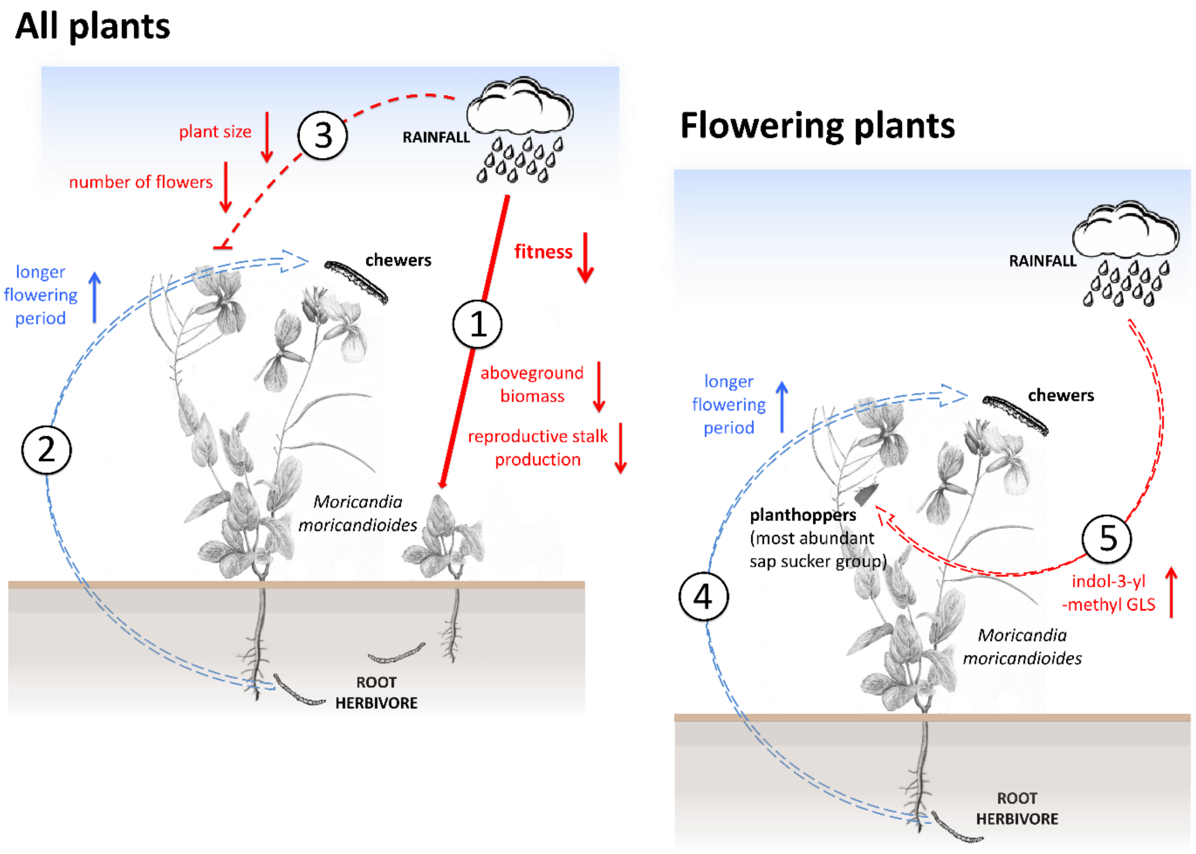


Figure 7.3. Illustration of the main effects of altered rainfall (red) and root herbivores (blue) on *M. moricandioides* and indirectly on aboveground herbivorous insects. For all plants, (1) enhanced rainfall negatively affected aboveground biomass as well as the number of reproducing plants and the number of seeds. (2) Plants facing root herbivory flowered for a longer period, elongating feeding time for chewers on reproductive tissue, and thus probably increasing their survival and abundance. (3) Root herbivore effect on chewers was disrupted by enhanced rainfall, likely because plants were smaller and produced less flowers. For flowering plants, (4) there was a positive effect of root herbivores on chewers with no influence of rainfall, supporting the assumption that enhanced rainfall disrupted the interaction through its effect on plant performance. (5) Enhanced rainfall reduced the abundance of the main sapsucker guild, planthoppers. This effect likely occurred because enhanced rainfall increased the concentration of indolic GLSs. These chemical defenses are usually detrimental to nonspecialist suckers.

Conclusions

Our results highlight that a likely change in precipitation patterns, with a rainfall shift from spring to summer due to climate change, could strongly affect *M. moricandioides* flowering process and reproductive output, for which prior biomass acquisition may be determinant. This study thus supports the hypothesis that short-lived plants are highly sensitive to changing climatic conditions (Voigt et al. 2003, Morris et al. 2008, Jamieson et al. 2012). The present study also provides robust evidence that change in rainfall patterns could affect insect groups in different ways, causing changes in the strength and/or the sign of insect interactions. Further experiments are necessary to determine how belowground herbivores will be affected by changes in rainfall patterns. A key result of our study is that several mechanisms are involved in those responses, likely acting at different life stages of the plant (vegetative and flowering stage, see Fig. 3). One mechanism is related to biomass acquisition by plants, which directly affected plant fitness and herbivore abundance. Another mechanism is related to the induction of chemical defenses, which affected generalist herbivores and was evident on plants that managed to reproduce. Therefore, a future climate change scenario in which plant reproductive phenology could be severely constrained by changes in precipitation may disrupt plant-insect synchrony and trigger trophic cascade effects.

7.X Supplementary material

7.1 **Supplementary material.** LMM and GLMM structure for analyzed variables

Variables	Distribution	Overdispersion	Heteroscedasticity
All plants			
Aboveground biomass	Gaussian	Yes	
Plant with reproductive stalks	Binomial	-	
Number of flowers	Zero-inflated Poisson	Implicit in the model	
Number of seeds	Zero-inflated Negative Binomial	Implicit in the model	
C content in leaves	Gaussian	-	
N content in leaves	Gaussian	-	
C/N ratio in leaves	Gaussian	-	R – CVF*
Total aliphatic GLSs in leaves$^+$	Gaussian	-	
3-butenyl GLS in leaves$^+$	Gaussian	-	
Total indolic GLSs in leaves$^+$	Gaussian	-	
Indol-3-yl-methyl GLS in leaves$^+$	Gaussian	-	
Total sap suckers	Zero-inflated Poisson	Implicit in the model	
Planthoppers	Zero-inflated Poisson	Implicit in the model	
Total chewers	Zero-inflated Poisson	Implicit in the model	

Flowering plants			
Flowering days	Gaussian	-	
Number of flowers	Poisson	Yes	
Number of seeds	Poisson	Yes	
C content in leaves	Gaussian	-	
N content in leaves	Gaussian	-	
C/N ratio in leaves	Gaussian	-	R – CVF*
Total aliphatic GLSs in leaves^{+}	Gaussian	-	
3-butenyl GLS in leaves^{+}	Gaussian	-	
Total indolic GLSs in leaves^{+}	Gaussian	-	
Indol-3-yl-methyl GLS in leaves^{+}	Gaussian	-	
Total sap suckers	Poisson	Yes	
Planthoppers	Poisson	Yes	
Total chewers	Zero-altered Poisson	Implicit in the model	

Table 7.S1. Model structure for analyzed variables. *R – CVF: Constant variance function to Rainfall treatment (*varIdent* function, *nlme* R package). $^{+}$ log (x + 1) transformation.

7.2 Supplementary material. Non-metric multidimensional scaling (NMDS) showing dissimilarity in the concentrations of the ten leaf glucosinolates.

	NMDS 1	**NMDS 2**	**R^2**	***P***
All plants				
R- RH-	-0.58015	-0.81451	0.0326	0.06
R- RH+	0.43612	0.89989	0.0085	0.48
R+ RH-	-0.15316	0.98820	0.0113	0.37
R+ RH+	0.85427	-0.51983	0.0105	0.41
Flowering plants				
R- RH-	-0.81273	-0.58264	0.0486	0.28
R- RH+	-0.80953	-0.58708	0.0096	0.78
R+ RH-	0.33518	0.94215	0.1107	**0.04**
R+ RH+	0.87740	-0.47977	0.0568	0.23

Table 7.S2. Contribution of each treatment to the leaf GLS profile NMDS plot axes and explained variation of dissimilarity.

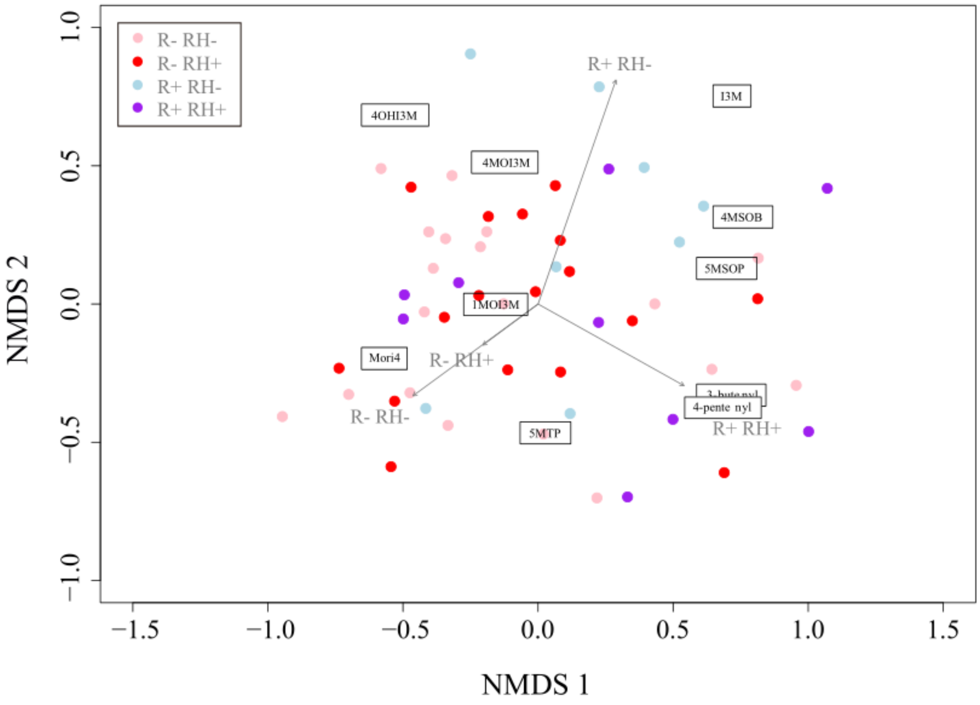


Figure 7.S1. Two dimensional NMDS plot showing dissimilarity in the concentrations of the ten leaf glucosinolate (GLS) compounds with treatments as dissimilarity vectors in flowering plants. Individual samples (points) and mean position of each GLS in the NMDS plot are shown. Stress = 0.15. Aliphatic GLSs: 5MTP = 5-methylthiopentyl GLS, Mori4 = unidentified aliphatic compound, 5MSOP = 5-methylsulfinylpentyl GLS, 4MSOB = 4-methylsulfinylbutyl GLS, 4-pentenyl GLS and 3-butenyl GLS. Indolic GLSs: 4MOI3M = 4-methoxy-indol-3-yl-methyl GLS, 1MOI3M = 1-methoxy-indol-3-yl-methyl GLS, 4OHI3M = 4-hydroxy-indol-3-yl-methyl GLS, I3M = indol-3-yl-methyl GLS.

General discussion

In this thesis, we experimentally investigated the context-dependency in the interaction between the plant *Moricandia moricandioides* and its most relevant herbivores. Here we proceed to synthesize the most relevant results obtained, and discuss them in a broader sense.

Direct effect of herbivores and detritivores on plant fitness

When considering both within-generational and transgenerational effects of herbivores and detritivores, it has been evident in this study that herbivory by ungulates (Chapters 2 and 3; Fig. GD1) and floral herbivores (Chapters 4, 5 and 6; Fig. GD1), and to a lesser extent by root herbivores (Chapters 4, 6 and 7; Fig. GD1), had a detrimental effect on plant fitness (Chapters 2, 3, 4, 5 and 6; Fig. GD1). This was not the case for other indirect interactions such as the one with detritivores (Chapters 1, 5 and 6; Fig. GD1), nor was it the case for all plant-herbivore interactions, as it has been shown for pre-dispersal seed predators (Chapters 1, 2 and 3; Fig. GD1). Ungulates, in addition to damaging plants through trampling, vastly affected plants through consumption: decreased seedling emergence and survival by reducing the carbon content of the seeds, and strongly limited seedling recruitment capacity (Chapters 2 and 3). Given the importance of recruitment on short-lived plants' fitness (Silvertown et al. 1993, Moles and Leishman 2008, Donohue et al. 2010), it is not surprising the negative ungulate impact on the plant's population dynamics. Increasing densities of floral herbivores had a negative impact on plant reproductive output, although the relation between floral herbivore density and reduction in seed number was non-linear (Chapters 4 and 5). Despite not having indisputable effects on seed quality (Chapters 4 and 5, but see González-Megías 2016), floral herbivores had also negative although non-linear transgenerational effects, since both low and high densities of floral herbivores decreased seedling emergence (Chapter 6). A linear trend to reduce plant reproductive output with increasing densities of root herbivores could be observed, although it was not significant (Chapter 4). The absence of a clear effect of this type of herbivore on plant fitness is somehow surprising, since other plants in more benign environments suffer greater damage, despite the fact that root herbivory and abiotic stress have been predicted to have synergistic effect (Blossey and Hunt-Joshi 2003, Zvereva and Kozlov 2012, Erb and Lu 2013). In the same way, no notable density-dependent transgenerational fitness consequences of root herbivores were observed (Chapter 6).

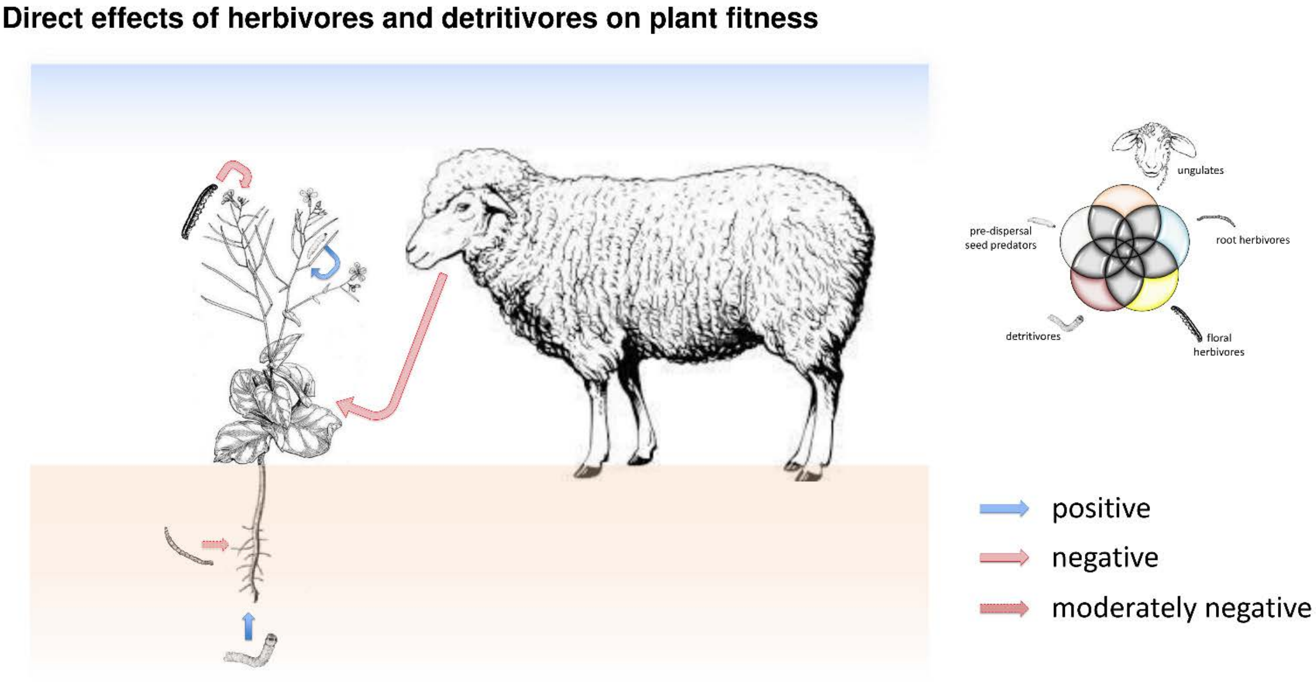


Figure GD1. Herbivore and detritivore effects on *M. moricandioides* fitness.

A totally different story was observed for pre-dispersal seed predators, whose effect on the fitness of the plant was positive. Pre-dispersal seed predation boosted plant seed production (Chapters 1 and 2). Increased seed production did not have negative transgenerational fitness consequences: seeds were apparently of poorer quality (Chapter 1, but see Chapter 3), but seedling emergence was not significantly reduced and seedling recruitment was not affected (Chapter 3). Fornoni (2011) indicated that many plants are capable of a generalized response to compensate for the loss of tissues. *Moricandia moricandioides* defends itself through tolerance (sometimes a mixed resistance-tolerance response) to pre-dispersal seed predation, ungulate herbivory and trampling, florivory and root herbivory. So, it is possible that tolerance mechanisms in this species coevolved in a diffuse way with multiple herbivores. Compensation for pre-dispersal seed predators and not the other herbivores feeding on reproductive tissues would have been possible due the type of herbivory and amount of tissue consumed. Plants determinately respond when valuable tissues in terms of fitness are jeopardized (see Karban and Baldwin 1997, Meldau et al. 2012). However, ungulates and florivore caterpillars consume a vast amount of stalks, floral buds, flowers and immature fruits, and the plant cannot fully compensate the loss of these tissues. The plant would not have enough time within the season and capacity to provision resources to efficiently replace these lost tissues (Boege and Marquis 2005, Ehrlén 2015). Pre-dispersal seed predators also predate valuable tissues such as seeds, and when seed predation rates are high can also negatively impact plant reproductive output (Chapter 2). However, when seed predation rates were moderate, overcompensation could be given (Chapters 1 and 2). Our results indicate that plant overcompensation for pre-dispersal seed predators is triggered from the moment they are detected, and not on later stages of reproduction such as fruit and seed maturation, when seed predation *de facto* occurs. Besides, the covariance between tolerance and resistance should be further studied, since leaf chemical defenses decreased when plants faced pre-dispersal seed predation (although that occurred in absence of root and floral herbivory; Chapter 1), but not when faced florivory (Chapters 4 and 5). As opposed to pre-dispersal seed predators, tolerance was not effective enough to defend against floral herbivores, hence chemical resistance would be also required to minimize fitness impact of these herbivores.

Finally, an indirect interaction such as detritivory did not influence plant seed production (Chapters 1 and 5), but favored seedling emergence in a non-linear way: seedling emergence increased when mother plants had high density of detritivores with respect to absence or low density of detritivores (Chapter 6). Examples of positive detritivore effects on plant performance and fitness abound, but few studies have contemplated their net effects on plants by quantifying transgenerational effects (De Deyn and Van der Putten 2005).

Context-dependent effects of herbivores and detritivores on plant

It has been demonstrated that both the magnitude and sign of biotic interactions vary in space and time, and context-dependency is not consistent among mutualistic, competitive and antagonistic interactions (Chamberlain et al. 2014). Interactions such as the ones between plants and herbivores also vary as a function of a third interacting organism, for example another herbivore. In our study system, context-dependence could be observed in all interactions studied (Fig. GD2). The fact that context-dependency could be transgenerationally transmitted (Chapters 3 and 6), and that it could even occur at within-plant level (Chapter 3) as is disentangled from our study, greatly hinders our capacity to understand natural complexity.

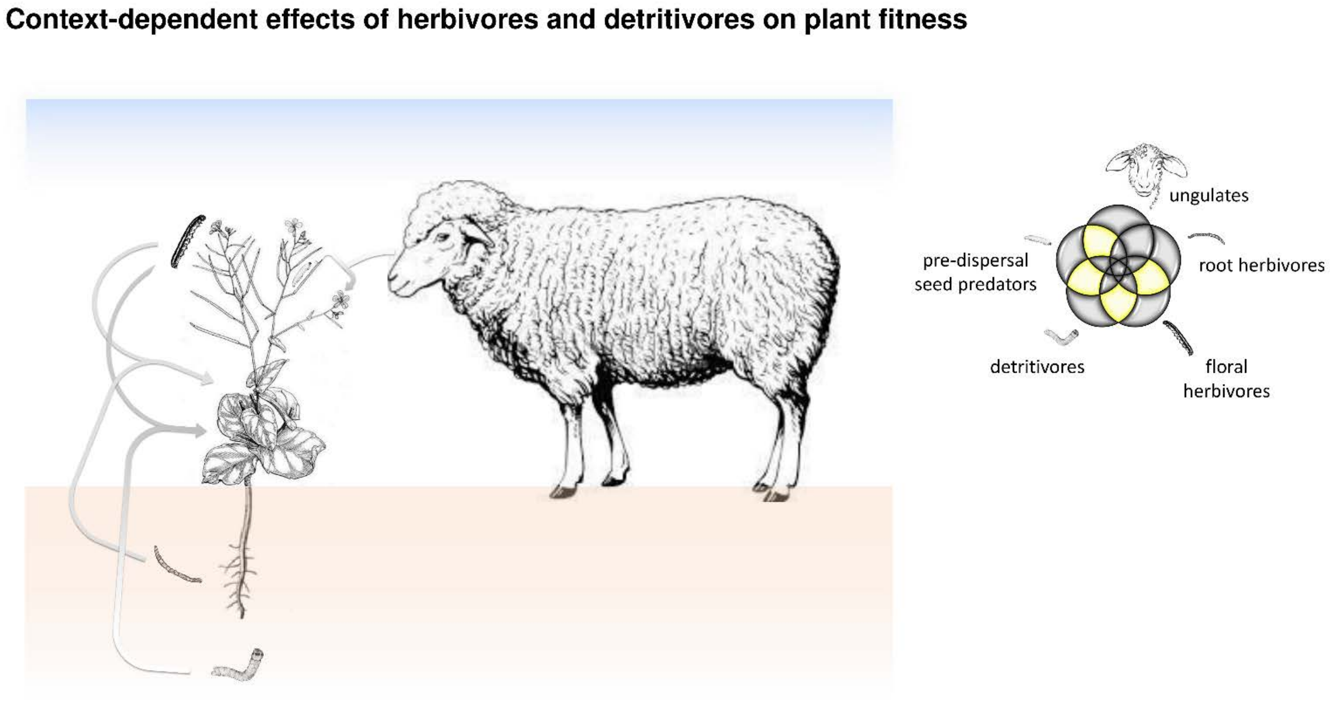


Figure GD2. Context-dependent effects between ungulates and pre-dispersal seed predators, detritivores and floral herbivores, and root and floral herbivores on *M. moricandioides* fitness.

Interestingly, plant tissue quality in terms of nutrient content and chemical defenses (referring to both leaves and seeds) have been observed to vary depending on the identity and density of the interacting organisms. Interactive effects even exceeded a single plant generation and affected progeny (and their herbivory rates) though intricate transgenerational effects such as changes in seed nutrient provisioning and/or epigenetic effects that complexly modulated plant defense and the plant nutritive value (Chapters 3 and 6). Plant morphology, reproduction and ultimately fitness were in most of the experiments independently affected by each of the plant-interacting organisms, as previously shown for this system (Chapters 1, 4 and 5, González-Megías 2016, but see Chapter 2). However, it should be emphasized that the absence of interactive effects on plant fitness does not mean that the exerted selection by each plant-interactor would not be influenced by other interacting organisms (Strauss et al. 2004b). The two most exhaustive reviews to date on additive- and non-additiveness in the effect of simultaneous herbivores on plant fitness found that in most systems the effects are usually independent (Morris et al. 2007, Stephens et al. 2013), although Stephens et al. (2013) realized that when various herbivores concurrently fed on plant reproductive structures, herbivore effects were lower than each herbivore effect in isolation (in other words, were antagonistically non-additive). Nevertheless, these theoretical and analytical approaches did not contemplate the more recently studied transgenerational effects of herbivory (Herman and Sultan 2011), and the possibility of interactive ttransgenerational effects of herbivores (Chapter 3).

Novel results have arisen from the study of simultaneous ungulate and pre-dispersal seed predator impacts on plants. On the one hand, despite their effect on plants and competitors, ungulates increased pre-dispersal seed predators' positive effects on plant seed production (Chapter 2). Besides, ungulate negative effects on seedling emergence and survival were strengthened by the presence of pre-dispersal seed predators (Chapter 3). That is, the sign of the effect was maintained but the magnitude increased, as the impact of both herbivores were reinforced by the presence of the other. On the other hand, maternal presence of both herbivores transgenerationally increased seedling resistance at plant but also at within-plant level. That occurred without noticeable changes in maternal seed traits such as mass, chemical defense and carbon and nutrient, scaling the inherent complexity of transgenerationally induced resistance (Chapter 3). Such effects could thus be related with gene expression regulation or defense-inducing hormones, and not with seed provisioning (Chapter 3). Like this, the ensemble effects of two very different types of herbivores would be transgenerationally transmitted (see also examples of transgenerational effects of mutiple herbivores Gómez 2008, González-Megías 2016), and that could even occur at within-individual level. It has recently become evident that herbivores can select within the same tissues of an individual plant, and thus differentially affect consumed and unconsumed tissues within the same plant (Herrera 2009, Jakobs et al. 2019). Even so, it has to be demonstrated that other herbivores besides pre-dispersal seed predators could have within-plant effects on fitness, or determine within-sibling differences in defense traits beyond *per se* variability in progeny traits (bet hedging strategies; Simons 2009, Moore et al. 2013, Herman et al. 2014, Sultan 2017). Pre-dispersal seed predators affect the intergenerational nexus that are embryos (seeds), and seed predation prior to seed maturation would strongly determine progeny phenotypes through both epigenetic modifications and altered seed provisioning (Herman and Sultan 2011, Herrera 2017). In addition, on detritivore presence, seeds of plants with pre-dispersal seed predators had higher glucosinolate concentrations than plants without pre-dispersal seed predators (Chapter 1). The consequences of chemical defense upregulation in seeds by pre-dispersal seed predators in combination with other members of the community and its transgenerational effects need to be further explored.

Some of the multiple and diverse trait changes provoked by floral herbivores were conditioned by the belowground organisms interacting with the plant. Leaf chemical defense was non-additively affected by root and floral herbivore densities, as at high densities of both herbivores glucosinolate concentrations were considerably increased (Chapter 4). On the contrary, these two herbivores did not seem to have notable interactive transgenerational effects, beyond conditioning the number of florivore pierid eggs laid on the offspring (Chapter 6). Instead, detritivore and floral herbivore densities non-additively affected seed quality: C/N ratio in seeds was lower on plants with high density of floral herbivores, but the effect was cancelled when detritivore density was also high (Chapter 5). Whether it was partially due to the changes observed in seed quality or not, floral herbivore and detritivore densities had interactive transgenerational effects on offspring morphology, reproduction and florivory rate without affecting offspring defenses, a fact that cannot be easily interpreted (Chapter 6). It is also remarkable that high maternal density of detritivores was even able to counteract the reduction in seedling emergence provoked by floral herbivores (Chapter 6).

Direct and indirect interactions among herbivores and detritivores

The net effect of any organism on the plant is contingent on its consequences for the rest of the plant-interacting organisms. The present study has enabled to observe the direct and plant-mediated indirect interactions between distinct associated organisms such as herbivores and detritivores, many of which have been widely ignored in the existing literature to date despite their potential biological relevance (e.g. belowground organisms on floral herbivores and pre-dispersal seed predators and vice versa). As pointed by Kaplan and Denno (2007) in their extensive meta-analysis, indirect and asymmetrical interactions between herbivores would be usual, as it occurred in our study system (Fig. GD3). Indeed, indirect interactions would predominate, although there were also relevant direct interactions (Fig. GD3). Direct interactions between herbivores were mostly asymmetrical due to differences in size, and implied accidental or intraguild predation. Logically, accidental or intraguild predation will occur with greater intensity on herbivores with less mobility such as caterpillars, even more if their development is endophyte (Bonal and Muñoz 2007, Gómez and González-Megías 2007b, Takagi and Miyashita 2014). Kaplan and Denno (2007) as well noted that facilitative interactions between herbivores may abound (see Ohgushi 2008, 2016). The stress-gradient hypothesis also postulates a high frequency of net positive interactions between competitors (herbivores in this case) in stressful environments (Bertness and Callaway 1994), although evidence to date mostly fit for plants (He et al. 2013). In our multiherbivore system, facilitative or positive interactions between herbivores would be rather scarce (Fig. GD3). The stronger (negative) impact on the herbivore community would be caused by those herbivores that consume vast amounts of vegetative and reproductive tissue. Like this, plant fitness-reducing herbivores such as ungulates and floral herbivores could be considered the keystone herbivore species in the system due to their consequences for plant fitness and phenotypes, their role as dominant agents of selection on plant defense traits and their capacity to override and canalize the likelihood of attack by other concurring herbivores (Hunter 1992, Van Zandt and Agrawal 2004, Gómez and González-Megías 2007a, Agrawal 2011, Stam et al. 2014, Strauss 2014, Poelman and Kessler 2016). We found that ungulates and floral herbivores by diminishing the amount of reproductive tissue, and to a lesser extent pre-dispersal seed predators by increasing it, conditioned the abundance of concurrent herbivores. Hence, direct (intraguild predation) and indirect density-mediated effects (decrease/increase in the shared resource) would have prevailed over trait-mediated indirect effects such as changes in chemical defenses (Gómez and González-Megías 2007a). Some studies have suggested that plant morphological, phenological and reproductive traits can be more influential determining herbivorous insect abundance than resistance traits such as chemical defense (Carmona et al. 2011, Fornoni 2011, Loranger et al. 2012, Ohgushi 2016). Nevertheless, the importance of these secondary metabolites determining the abundance of herbivores should not be underestimated, as their induction could not only reduce the abundance of the associated herbivores as seen in our system (González-Megías and Müller 2010, González-Megías and Menéndez 2012, Chapters 3 and 7), but their mere constitutive presence would also ubiquitously narrow the number of occurring herbivorous species (Feeny 1976, Swain 1977, Becerra 1997, Futuyma and Agrawal 2009, Utsumi et al. 2009, Moore et al. 2013, Agrawal and Weber 2015).

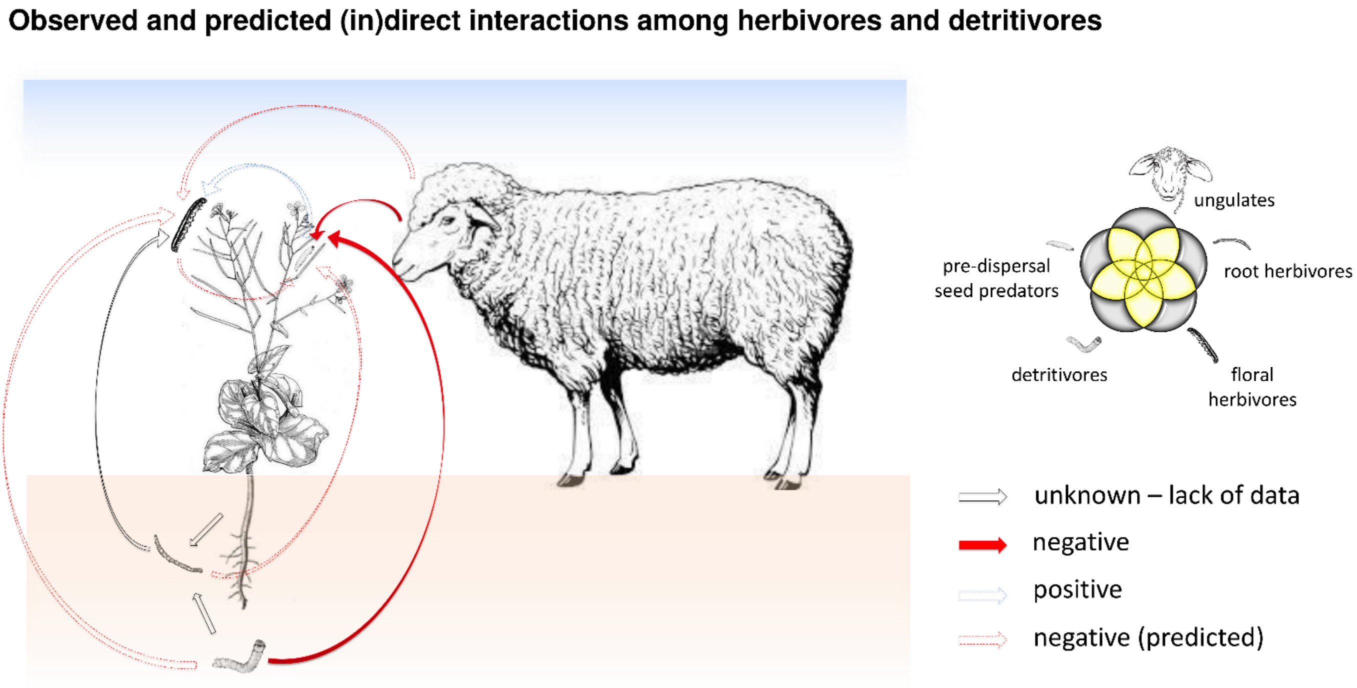


Figure GD3. Observed and expected direct and indirect interactions between herbivores and detritivores.

Obviously, the interaction between herbivore mammals such as ungulates and herbivore insects is thought to be asymmetric, due to their huge differences in size (Zamora and Gómez 1993, Gómez and González-Megías 2002, 2007a). Ungulates would affect herbivore insects, but not the other way. First, ungulates would have a direct negative effect on aboveground herbivores such as floral herbivores and pre-dispersal seed predators (Chapter 1) by the mentioned intraguild predation. And second, ungulate negative impact on traits such as plant density and recruitment, plant regrowth capacity after damage and/or plant quality could have a synergistic negative consequences on herbivores, particularly on aboveground ones, even transgenerationally affecting progeny herbivores (Chapters 2 and 3). Ungulate effects altering plant quality following regrowth for subsequent insect feeding and reducing herbivorous insect abundance and diversity is widely supported by the literature (González-Megías et al. 2004, Gómez and González-Megías 2007b, Takagi and Miyashita 2014, van Klink et al. 2015, Gish et al. 2017, Mesa et al. 2019, Filazzola et al. 2020).

The relation between floral herbivores and pre-dispersal seed predators would also be asymmetric: floral herbivores would benefit for pre-dispersal seed predators, but florivory would be harmful for pre-dispersal seed predators. Floral herbivores would benefit from the plant overcompensation for pre-dispersal seed predation, although we do not know how the changes in the quality of the tissues caused by pre-dispersal seed predators, such as in nutrient content and chemical defenses, would affect specialist herbivores like florivore pierids (Chapter 1). The abundance of sap suckers was increased on plants enduring pre-dispersal seed predation, probably due to both changes in compensative regrowth and chemical defenses (Chapter 1). On the contrary, ungulates and floral herbivores are expected to harm sucker insects by reducing plant reproductive tissue where they fed on. Back to florivores, they would be detrimental for pre-dispersal seed predators in two ways. One would be intraguild predation, as floral herbivores would directly feed on eggs laid on flowers and fruits, and on larvae feeding in immature fruits (Chapters 4 and 5). The other would be indirect, by reducing plant reproductive tissue where pre-dispersal seed predators lay eggs and develop, and probably by disrupting plant overcompensation for this type of herbivory and thus also limiting the potential benefits pre-dispersal seed predators obtain from that tolerance response (Chapters 4 and 5).

Belowground herbivores such as detritivores and root herbivores could indirectly affect aboveground herbivores like floral herbivores and pre-dispersal seed predators. Detritivore effect on pre-dispersal seed predators is thought to be negative, as provoked changes on plant reproductive traits and on chemical defense profile would diminish seed predation attack rates (González-Megías and Müller 2010, González-Megías and Menéndez 2012). It is to be explored if detritivore-mediated changes in seed defenses could affect pre-dispersal seed predators' survival and performance (Chapter 1). Detritivore effects on floral herbivores would be subtle but rather negative, since when combined with floral herbivores a negative non-additive effect on compensative fruit production was observed, limiting the number of immature fruits they could feed on (Chapter 5). For root herbivores, we speculate that in any case pre-dispersal seed predators would be negatively affected, as increasing densities of root herbivores moderately decrease the production of reproductive tissues by the plant (Chapter 4). Regarding root herbivore effects on floral herbivores, it remains unknown whether they could have an indirect facilitative effect on floral herbivores by increasing their growth rate, or if the consequences for florivore caterpillar performance would be negative due to resource deprivation (Chapter 4).

When moving to aboveground-belowground effects, we still lack data regarding how aboveground herbivory affects root traits. Aboveground herbivory can modify root traits by inducing chemical defense in roots and/or changing allocation patterns between above- and belowground plant parts, facilitating or limiting root herbivory (Kaplan et al. 2008, Kutyniok and Müller 2012, Kafle et al. 2014, Kleine and Müller 2014, Thomas et al. 2017, Machado et al. 2018, Heinze 2020). Kafle and Wurst (2019) showed that aboveground herbivory can even alter the odds of enduring root herbivory on progeny. A deeper knowledge of the root-feeding cebrionid larvae preferences and their capacity to tolerate or resist chemical defenses such as glucosinolates would be necessary. Detritivore effects on root herbivores would be also difficult to predict. As previously highlighted, detritivores modulate plant defenses in aerial plant parts such as leaves and seeds (González-Megías and Müller 2010, Chapters 1 and 5), so it is very likely that they also modulate root defenses, in addition to the diverse changes that they cause in the soil characteristics of the rhizosphere that could somehow affect root herbivores (see Erb and Lu 2013). Nor do we know how detritivores can be affected by herbivores above- and belowground. Apparently, glucosinolates can be harmful for tenebrionid larvae, as shown for *Tenebrio molitor* feeding on glucosinolate-rich *Brassica napus* rapeseed (Pracros et al. 1997). Thus, detritivores could be harmed by herbivore-induced soil glucosinolate hydrolysis products such as volatiles from living or decomposing plants (Bending and Lincoln 1999, Hanschen et al. 2015).

Effects on other trophic levels: herbivore and detritivore indirect interactions with pollinators and pierid caterpillar parasitoids

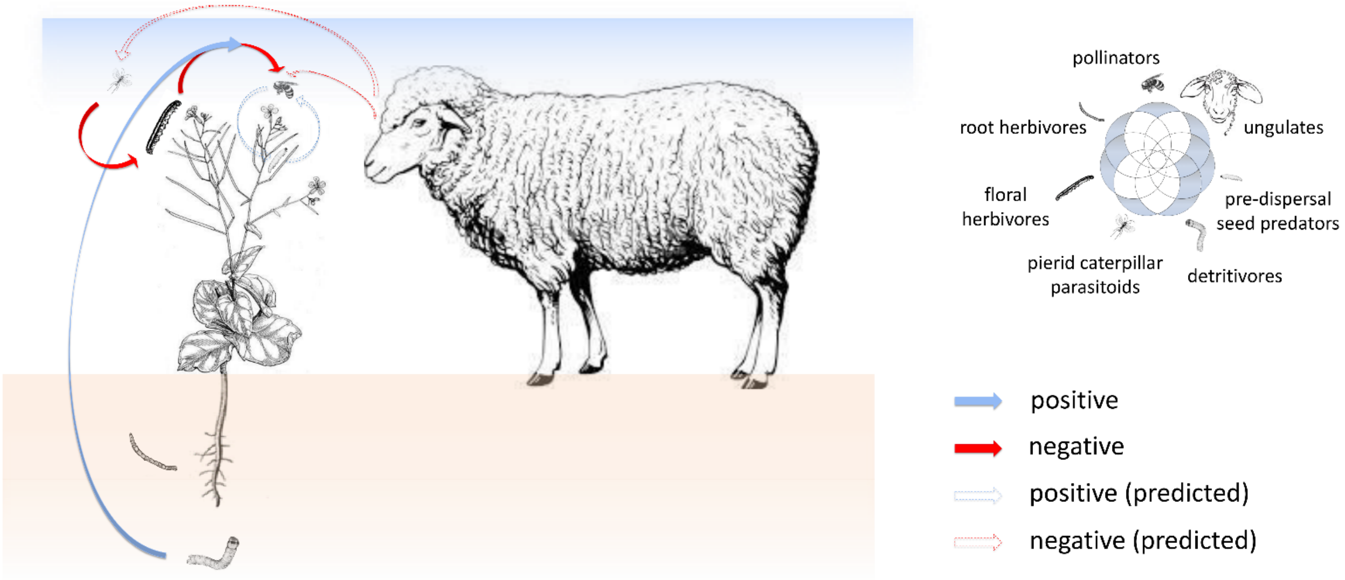

Observed and predicted (in)direct interactions with pollinators and pierid caterpillar parasitoids

Figure GD4. Observed and expected direct and indirect effects of herbivores and detritivores on pollinators and pierid caterpillar parasitoids.

Plant-herbivore relationships are usually shaped by more complex indirect interaction webs, and thus multitrophic perspectives usually provide a more accurate picture of the real situation in nature (van der Putten et al. 2001, Ohgushi et al. 2012, Ohgushi 2016, terHorst et al. 2018). From the experiments carried out in this study we could partially infer the effect of detritivores and the different herbivores on important biotic interactions of the plant such as pollination and parasitism. Regarding pollination, the sexual reproduction mode and the type of pollination must be taken into account, and in the case of zoophilic pollination, the quantity, variety and effectiveness of pollinators that determine whether the plant is pollen-limited or not (Gómez et al. 2007, 2016, 2020, Lucas-Barbosa 2016, Valverde et al. 2019). In our self-incompatible system, but with a bunch of highly-efficient pollinator species, there is a positive association between floral displays (flower abundance) and pollinator visitation (Gómez 1996, Gómez et al. 2016, Torices et al. 2018, 2021), common in plenty other plant-pollinator systems (Cohen and Shmida 1993, Conner and Rush 1996, Chittka and Raine 2006, Hegland and Boeke 2006, Huang et al. 2006, Soper Gorden and Adler 2013, Schlinkert et al. 2015). Therefore, we would expect that those herbivores that diminish flower amount (ungulates and floral herbivores) would reduce subsequent pollinator visitation due to resource dilution (Karban and Strauss 1993, Krupnick et al. 1999, Gómez 2003, 2008, Strauss and Irwin 2004, Moreira et al. 2019; Fig. GD4). These herbivores could also diminish flower attractiveness by reducing floral rewards and inducing deterrent floral defense traits, influencing pollinator performance and foraging (Adler et al. 2001, Strauss et al. 2004a, Schiestl et al. 2014, Jacobsen and Raguso 2018, Soper Gorden and Adler 2018, Tsuji and Ohgushi 2018, Haas and Lortie 2020, but see Carper et al. 2016, Rusman et al. 2019). Along this line, we observed that the number of pollinators per flower was lower in plants with high density of floral herbivores (Chapter 5, data not shown). However, the presence of detritivores, without having altered the number of flowers, nullified the negative effect of floral herbivores on pollinator visitation (General Discussion annexus, Fig. GDA1). This effect of the detritivores, therefore, could be mediated by changes in the flower quality and reward (Krupnick and Weis 1999, Adler et al. 2001). Pollinator visitation does not counteract the negative effects of florivory on female fitness (Bronstein et al. 2003, McCall 2008, Rodríguez-Rodríguez et al. 2015, Rusman et al. 2018, but see Rodríguez-Rodríguez et al. 2017, Soper Gorden and Adler 2018), even more if the plant is not pollen limited, as it is the case of *M. moricandioides* (Chapter 1, Sõber et al. unpublished). Nevertheless, a reduction in overall pollinator visitation may compromise the often-neglected male fitness on entomophilous outcrossing species such as our study plant (Krupnick and Weis 1999, but see Carper et al. 2016). Detritivores could thus ameliorate the negative impact florivores cause on plant male fitness through reduced pollinator visistation, supposing another means through detritivores could result beneficial to plants. Root herbivores had no effect on pollinator visitation in our system (Chapter 4, data not shown). Root herbivores have the potential to affect pollinator visitation in some systems (Poveda et al. 2003, Barber and Soper Gorden 2015, Barber et al. 2015), but not in others (Soler et al. 2012, Moreira et al. 2019). The relationship between pollinators and pre-dispersal seed predators seems to be very different from the rest of herbivores in our study system (Fig. GD4). Pollinators and pre-dispersal seed predators have been broadly considered to exert conflicting selective pressures on flowering phenology (Brody 1997, Elzinga et al. 2007, Parachnowitsch and Caruso 2008, Vanhoenacker et al. 2013). Nonetheless, in the *M. moricandioides* system, pre-dispersal seed predators (and the plant) would benefit from pollination (Herrera 2000, Cariveau et al. 2004), necessary for the formation of fruits in which to develop, and pollinators (and the plant) would benefit from increasing flower production triggered by overcompensation, giving rise to a positive tritrophic feedback loop between plant, herbivores and pollinators.

The attraction of herbivore parasitoids as an indirect defense plays a major role in many plant systems (Price et al. 1980, Turlings et al. 1990, van Loon et al. 2000, Heil 2008, Pashalidou et al. 2015b, Pearse et al. 2020), many times exerting stronger selective pressure on herbivores than plant defensive traits themselves (top-down forces surpassing bottom-up forces; Mauricio and Rausher 1997, Züst et al. 2012, Vidal and Murphy 2018). In our study system, with regard to the pierid caterpillar parasitoids, we can only expect a negative effect of ungulates on them. Ungulates would accidentally predate floral herbivores and consequently the koinobiont endoparasitoid, and diminish the quantity of potential hosts for parasitoids (Fig. GD4). However, the density of potential hosts (pierid caterpillars) did not influence parasitism rates in our system (Chapters 4 and 5, data not shown), despite it has been proposed that it would increase volatile emissions (Vet 2001, Dicke and Baldwin 2010, De Rijk et al. 2016), key cues on parasitoid host-search (Dicke et al. 1990, Turlings et al. 1990). Perhaps more than the number of potential hosts, it is caterpillar quality what mostly determines the rate of parasitism (Bukovinszky et al. 2008). Further studies should determine the scale of coevolution between the plant and the parasitoids, for which plants have to provide successful cues (e.g. volatiles) about potential hosts (Kessler and Heil 2011, Clavijo McCormick et al. 2012, Aartsma et al. 2019). Volatile release tends to be low in *M. moricandioides* (González-Megías, personal observation). Rowen and Kaplan (2016) found that inducible volatile emissions tend to higher in cultivated rather than in wild plants, which is somehow counterintuitive due to the cost-effectiveness of this strategy to get rid of harmful herbivores by wild plants. We cannot forget that, in turn, the pierid caterpillars are also under selection to escape predation and parasitism (see for example Singer and Stireman 2005), which can lead to antiparasitoid strategies such as aposematism (Soler et al. 2005, Harvey et al. 2007, Higginson et al. 2015). Finally, it is also noteworthy that in part of the descendants of the plants that faced floral herbivory we observed a threefold increase in the parasitism rate of these same herbivores (Chapter 6), apparently a transgenerational effect cascading up to the third trophic level that would deserve further research.

A semiarid herb and a multiherbivore system: plant defense fine-tuning

Herbivores such as ungulates, floral herbivores, pre-dispersal seed predators, and root herbivores negatively or positively affected plant fitness, and induced or suppressed resistance and/or tolerance mechanisms in the plant (Fig. GD5). These herbivores have been revealed as relevant biotic interactions acting on plant defense selection. Other specialist herbivores such as leaf miners, flea beetles (*Phyllotreta* spp.), the diamondback moth (*Plutella xylostella*) and specialist aphids (*Brevicoryne brassicae* and *Lipaphis erysimi*), could also play a certain role in the defensive response of the plant; we estimate that they would have little influence on tolerance, and with regard to chemical resistance they likely trigger changes in the profile rather than vast modulations of total glucosinolate concentrations (Fig. GD5). Conversely, we surmise from this study that the rest of the biotic interactions would not be so relevant in the defensive configuration of the plant. The relevance of generalist herbivorous insects in the system would be limited, largely because the low abundance and diversity of plants in the area maintains scant populations of these species (Fig. GD5). Other antagonists such as pathogens do not seem to substantially affect the plant (at least as visible features of disease). The constant low relative humidity prevents the colonization of, among others, pathogenic fungi (Fig. GD5). The pollinator community is relatively abundant throughout the flowering season, which may be due to the low spatial abundance of any type of flowers. As our study plant is not pollen limited, there would not be excessive competition for the attraction of pollinators and the flowers may not require so many resources, limiting the potential occurrence of trade-offs between the mating and the defensive system (Fig. GD5). Concrete studies should address the reach of indirect defense in *M. moricandioides*. Apparently, it might be limited by the intrinsic stochasticity of semiarid environments in terms of growing seasons, population dynamics and phenological synchrony between plant, herbivore and parasitoid (Fig. GD5). Finally, detritivores and the greater resource availability derived from their activity do not seem to noticeably influence plant defensive responses (Fig. GD5). Again, environmental stochasticity would play a major role in that. Plants in these environments would be adapted to low nutrient availability (extra nutrients provided by detritivores would not be an evolutionary stable signal), and their defensive performance would have evolved in such way. Nonetheless, the extra nutrients provided by detritivores would be used in allocation processes, and even in modifications in the chemical defense profile. Changes in chemical defense profile can have substantial community wide consequences (Moore et al. 2013, Stam et al. 2014, Richards et al. 2015, Speed et al. 2015, Kessler and Kalske 2018), as it apparently occurs in our system (González-Megías and Müller 2010, González-Megías and Menéndez 2012).

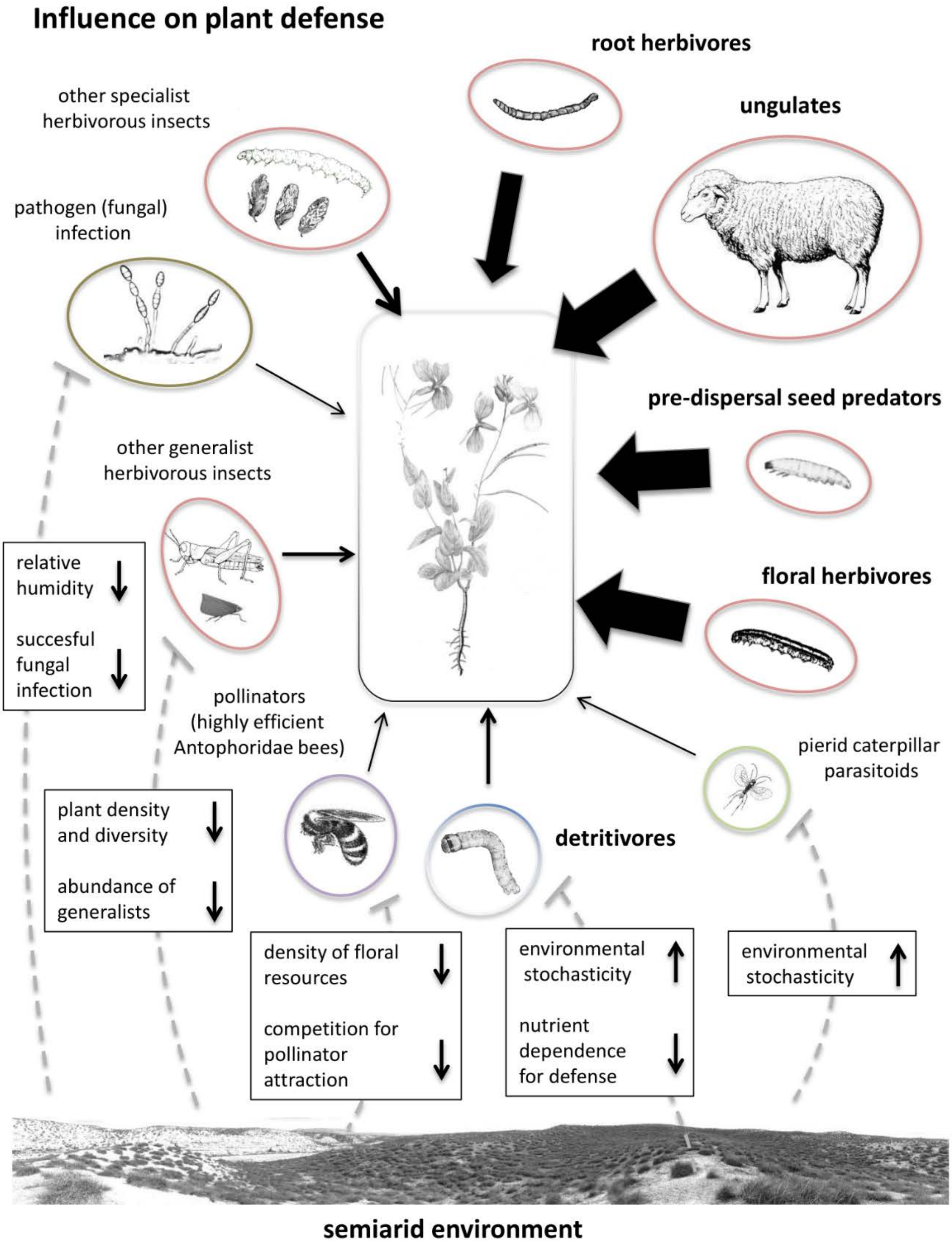


Figure GD5. Relative predicted influence of the different biotic interactions on *Moricandia moricandioides*' defense.

When contextualizing what occurs with our model plant with respect to the different plant defense hypotheses, we can first conclude that it does not fit well with most of the predictions made for low resource environments. Plant growth and defense would not trade-off over a resource availability gradient, (expanded growth-differentiation balance hypothesis), plants would not have low capacity to compensate for herbivore damage in these environments (resource availability hypothesis, plant stress hypothesis), and tolerance should not be necessarily greater when resources are increased (compensatory continuum hypothesis). On the contrary, our model plant's defense fits well with the growth rate model and the limiting resource model (Table I1), which state that plants grow below their maximum growth rate under stressful conditions, so may have higher capacity to tolerate and compensate upon herbivory (Hilbert et al. 1981, Arendt 1997, Wise and Abrahamson 2005, Olejniczak 2011). Indeed, many plant traits that are adaptive in stressful and stochastic environments would also favor greater tolerance to herbivory (Hahn and Maron 2016). Our results also fit with some other postulates of the limiting resource model: as in our case, fitness loss would occur if we consider the plant is carbon-limited, herbivory increases this limitation and impedes the acquisition of this resource (see in Chapter 3 carbon limitation exerted by ungulate herbivory). Optimal defense theory would also have great empirical support in our study (Table I1). Like this, most valuable tissues such as seeds were provided with potent constitutive defenses, and the cost-effectiveness of defense at high herbivore pressure was proven true. However, the prediction that damage early on ontogeny and/or early in the season would be more detrimental would require clarification. As stated by Boege and Marquis (2005), that function would be non-linear: herbivory would be detrimental on early ontogenic stages because the plant would not yet acquired the necessary resources for defense, and on late stages because the reproductive season would be ending and most of resource investment would have been already done. At intermediate stages, instead, plants would still have time and resources to defend against herbivores.

Moricandia moricandioides system: particularities and generalities

Plant defense theories offer many predictions about differential patterns of defense along gradients of environmental stress and resource availability, herbivore intensity and predictability, and lifespan (Grime 2001, Stamp 2003, Hanley et al. 2007, Hahn and Maron 2016). *Moricandia moricandioides* is a non-mycorrhizal plant that tolerates low nutrient levels, has great rates of photosynthesis, and is provided with potent chemical defenses and tolerance mechanisms. Several traits could be highlighted as the ones that mostly condition the plant's individual performance and fitness (adaptive or functional traits, although practically any trait would be relevant): chemical defense, dormant meristem activation, flowering onset and duration, seed set, and seed provisioning. All these singularities, and possibly others not described here, may limit the commonality of the findings observed in this work, in line with the conception that particularities are the norm rather than the exception. However, we consider that several findings regarding our study system can be generalizable to other plant-herbivore systems.

Ungulate's detrimental effects on *M. moricandioides* fitness and population dynamics may be common in semiarid environments, in which aridity and overgrazing could have synergistic effects (Gaitán et al. 2018, Pelliza et al. 2020, 2021, Chapters 2 and 3). But in turn, communities subjected to a long history of grazing/browsing are usually dominated by tolerant species and genotypes (Rosenthal and Kotanen 1994, Del-Val and Crawley 2005, Tahmasebi Kohyani et al. 2009, Martin et al. 2015). Ungulates are indeed considered a strong selection force on Brassicaceae family (Gómez and Zamora 2000a, 2000b, González-Megías et al. 2004, Gómez and González-Megías 2007a), and may have favored the development of tolerance.

Regarding plant tolerance traits, common mechanisms of tolerance such as compensatory growth and meristem activation would have allowed the plant to regrowth after damage, or to boost the production of reproductive tissue at the expectation of forthcoming damage. At moderate herbivore damage, as in the case of pre-dispersal seed predators, these tolerance responses could even result in overcompensation, in line with previous hypotheses (McNaughton 1983, van der Meijden et al. 1988, Chapters 1 and 2). A meta-analysis concluded that low resource environments would not necessarily restrict plant ability to overcompensate for herbivore damage (Hawkes and Sullivan 2001), as shown in our study. Overcompensation may not be such an exception after fruit consumption and/or in response to chewing insects as previously thought, in Brassicaceae and many other plant families (Garcia and Eubanks 2018). Our study has been the first demonstration of overcompensation for pre-dispersal seed predation, but it is at the same time one of the few studies in which this type of herbivory has been experimentally manipulated. Therefore, and given the large set of studies showing that several morphological, phenological and reproductive plant traits, especially extensive floral displays, contribute to both increased pre-dispersal seed predation and plant fitness (Campbell 1991, Nakamura et al. 1995, Lortie and Aarssen 2000, Pilson 2000, Leimu et al. 2002, Fenner et al. 2002, Gómez 2008, Ehrlén and Münzbergová 2009, Kolb and Ehrlén 2010, Bello-Bedoy et al. 2011, Sims et al. 2012, Brody and Irwin 2012, Ehrlén et al. 2012, Matesanz et al. 2015, Gagic et al. 2016, Abdala-Roberts et al. 2017, Chen et al. 2017), we speculate that overcompensation for pre-dispersal seed predation can be widespread.

Plant tolerance encompasses other traits such as increments in photosynthetic activity, and modifications in resource allocation processes. Plants may up-regulate resource acquisition via increased photosynthetic capacity and thus minimize or entirely avoid costs of defense and growth-defense trade-offs (Núñez-Farfán et al. 2007, Moore et al. 2013). This would be necessary against herbivores such as floral herbivores for which a mixed resistance-tolerance response is necessary, making the relationship between floral herbivore intensity and impact on plant fitness non-linear (Chapters 4 and 5). In addition to the high rates of photosynthesis in *M. moricandioides*, it would be necessary to unravel if the photosynthetic capacity in reproductive structures such as stalks (Raven and Griffiths 2015) and the seeds themselves (Bazzaz et al. 1979) is sufficient to supply some or almost all of the organic carbon used in their growth. Stalks are indeed photosynthetically active in *M. moricandioides* (González-Megías and Müller 2010).

On the other hand, in our understanding of the system, resource allocation processes would be essential in *M. moricandioides* interactions with belowground organisms. Belowground-aboveground nutrient allocation in response to root herbivory may occur in

M. moricandioides (Chapter 7). This mechanism would be crucial when root defenses are ineffective, particularly in annual and short-lived plants (Moore and Johnson 2017). In this way, the fitness impact caused by root herbivores would be lowered in our study plant (González-Megías 2016, Chapters 4, 6 and 7). Similar effects would have been observed in other Brassicaceae (Poveda et al. 2005), but not in most plant species that could not fully compensate after root herbivore attack (Zvereva and Kozlov 2012). Regarding detritivore activity, it seems that the extra resources provided by detritivores would be used to improve the qualitative status of leaves and seeds (Chapters 1 and 5), which could ultimately increase individual plant lifetime fitness (González-Megías 2016, Chapter 6), rather than substantially increasing plant resistance and tolerance. This may be because plants in resource-poor environments have evolved towards resource non-dependent defense mechanisms, and because in stochastic environments such as semiarids with short and unpredictable reproductive seasons this could be the best resource optimization strategy.

With regard to the consequences of altered rainfall patterns, we found that it could modulate plant-herbivore interactions. The simulated climate change scenario limited plant growth and reproduction, whereby disrupting the positive effects of root herbivores through elongation of the flowering period on aboveground chewer insects. We believe it is possible that enhanced rainfall at the reproductive season could harm other short-lived plants adapted to live and reproduce in dry environments. In fact, there is already some study indicating that in Mediterranean dryland ecosystems, enhanced rainfall can reduce the biomass of several short-lived plants (Hänel and Tielbörger 2015). Therefore, changes in plant-herbivore interactions and plant-mediated disruptions in herbivore-herbivore interactions are to be expected in other systems. More studies addressing this issue in addition to multifactorial studies that simultaneously manipulate multiple climatic factors will enable more robust predictions of how climate change will affect plant–herbivore interactions (Hamann et al. 2021).

Finally, much more studies investigating the transgenerational effects of multiple biotic interactions would be needed to obtain comparable results and to evaluate them in a broader context. In essence, the transgenerational effects detailed for this study system would not have any intrinsic particularity that may limit their potential ubiquity.

Conclusiones

1. *Moricandia moricandioides* (Brassicaceae) muestra una alta plasticidad en la defensa frente a sus herbívoros más importantes. La planta exhibe diferentes combinaciones de rasgos de resistencia y tolerancia que son específicas de la identidad del herbívoro, la intensidad del daño y la parte de la planta consumida. Estos resultados apoyan diferentes hipótesis propuestas sobre los mecanismos usados por las plantas en su defensa frente a los herbívoros como son *optimal defense theory*, *growth rate model* y *limiting resource model*, y van en contra de hipótesis planteadas asociadas a la disponibilidad de recursos.

2. *Moricandia moricandioides* sobrecompensa el daño producido por los depredadores de semillas pre-dispersivos mediante una estimulación en la producción de tejidos tanto reproductivos como vegetativos. La planta parece compensar el daño producido por los ungulados, pero estos disminuyen la viabilidad de la descendencia. La planta también compensa el daño provocado por radicícolas mediante una re-asignación de recursos de las raíces a los tejidos de la parte aérea. Por el contrario, la planta responde a través de una estrategia mixta de resistencia y tolerancia cuando se enfrenta a otros herbívoros asociados a tejidos reproductivos como los florícolas, pese a lo cual estos reducen de manera importante la producción de semillas y su viabilidad.

3. Las respuestas de la planta a los diferentes herbívoros dependen de la presencia de otros herbívoros concurrentes y no pueden inferirse de las respuestas individuales a cada tipo de herbívoro. A su vez, el hecho de que la combinación de dos tipos de herbívoros tenga un impacto aditivo o no aditivo sobre el éxito reproductivo de la planta depende de la identidad y de la densidad de los herbívoros que interaccionan. La alta densidad simultánea de radicícolas y florícolas induce la defensa química de la planta. A pesar de

ello, los radicícolas y florícolas tienen efectos aditivos sobre el éxito reproductivo de la planta. Por contra, la capacidad de la planta para sobrecompensar el daño de los depredadores de semillas pre-dispersivos a través de la producción de tallos reproductivos, y por consiguiente su efecto neto positivo sobre el éxito reproductivo de la planta, es mayor en las poblaciones de *M. moricandioides* expuestas a ungulados.

4. La planta habita en un entorno de baja disponibilidad de recursos y parece haber desarrollado mecanismos de defensa no dependientes de los recursos. Sin embargo, la actividad de los detritívoros afecta a la respuesta de *M. moricandioides* frente a los herbívoros. Los detritívoros de forma interactiva con los herbívoros modulan el perfil de defensa química de la planta. Además, los detritívoros alteran los patrones de asignación de recursos en la planta, y consecuentemente incrementan la viabilidad de las semillas, pudiendo así contrarrestar los efectos perjudiciales de los herbívoros sobre el éxito reproductivo de la planta.

5. Los herbívoros tienen efectos transgeneracionales sobre la progenie de *M. moricandioides*. Estos efectos transgeneracionales dependen del contexto y la densidad de los organismos que interaccionan con la planta madre, y pueden afectar de manera compleja el establecimiento, la defensa y el desarrollo de la progenie. El entorno biótico materno predispone de manera compleja la emergencia de las plántulas y los mecanismos de defensa frente a herbívoros en la siguiente generación de adultos, aumentando su resistencia a herbívoros vegetativos. Definitivamente, es necesaria mucha más investigación para incrementar el conocimiento sobre las consecuencias ecológicas y evolutivas de los efectos transgeneracionales bióticos.

6. Los efectos transgeneracionales no ocurren sólo a nivel de individuo (la madre) sino también a nivel de dentro de individuo (el fruto), de forma que los descendientes de una misma planta en igualdad de condiciones abióticas difieren en su resistencia a los herbívoros.

7. Un escenario simulado de cambio climático en el que el momento y la frecuencia de la precipitación son alterados afecta negativamente el éxito reproductivo de la planta y altera las interacciones herbívoro-herbívoro mediadas por la planta. Un escenario futuro de cambio climático nos hace prever no sólo cambios en la intensidad de las interacciones de *M. moricandioides* con su comunidad de herbívoros, sino también en la persistencia de esta especie en las zonas más extremas de su distribución.

Conclusions

1. *Moricandia moricandioides* (Brassicaceae) shows high plasticity in the defense against its most important herbivores. The plant exhibits different combinations of resistance and tolerance traits that are specific to the identity of the herbivore, the intensity of the damage, and the part of the plant consumed. These results support different hypotheses proposed about the mechanisms used by plants in their defense against herbivores, such as *optimal defense theory*, *growth rate model* and *limiting resource model*, and go against the hypotheses associated with resource availability dependence in plant defense.

2. *Moricandia moricandioides* overcompensates for the damage produced by pre-dispersal seed predators by stimulating the production of both reproductive and vegetative tissues. The plant seems to compensate for the damage produced by the ungulates, but these diminish the viability of the offspring. The plant also compensates for the damage caused by root herbivores by re-allocating resources from the roots to aboveground tissues. On the contrary, the plant responds through a mixed resistance-tolerance strategy when faces other herbivores associated with reproductive tissues such as floral herbivores, despite which these herbivores significantly reduce seed production and viability.

3. Plant responses to the different herbivores depend on the presence of other concurrent herbivores and cannot be inferred from the individual responses to each type of herbivore. In turn, whether the combination of two types of herbivores has an additive or non-additive impact on plant fitness depends on the identity and density of the interacting herbivores. The simultaneous high density of root and floral herbivores induces the chemical defense of the plant. Despite this, root and floral herbivores have additive effects on the plant. In contrast, the ability of the plant to overcompensate for pre-dispersal

seed predators through the production of reproductive stalks, and therefore its net positive effect on the reproductive success of the plant, is greater in M. *moricandioides* populations exposed to ungulates.

4. The plant inhabits a low resource environment and seems to have developed resource non-dependent defense mechanisms. Nevertheless, detritivore activity affects the plant response towards herbivores. Detritivores interactively with herbivores modulate plant chemical defense profile. In addition, detritivores alter resource allocation patterns in the plant, and consequently increase the viability of the seeds, thus being able to counteract the detrimental effects of herbivores on plant fitness.

5. Herbivores have transgenerational effects on the plant's progeny. These transgenerational effects depend on the context and density of the organisms interacting with the maternal plant, and can complexly affect the establishment, defense and performance of the progeny. The maternal biotic environment predisposes the emergence of seedlings and defense mechanisms against herbivores in the next generation of adults in a complex way, increasing their resistance to vegetative herbivores. Definitively, much more research is imperative to gain knowledge about the ecological and evolutionary consequences of biotic transgenerational effects.

6. Transgenerational effects do not only occur at the individual level (the mother plant) but also at within-individual level (the fruit), so that descendant siblings under equal abiotic conditions differ in their resistance to herbivores.

7. A simulated climate change scenario in which the timing and frequency of precipitation are altered negatively affects the plant fitness and disrupts plant-mediated herbivore-herbivore interactions. Future climate change associated scenarios make us foresee not only changes in the intensity of the

interactions of *M. moricandioides* with its herbivore community, but also in the persistence of this species in the most extreme zones of its distribution.

General discussion annexus

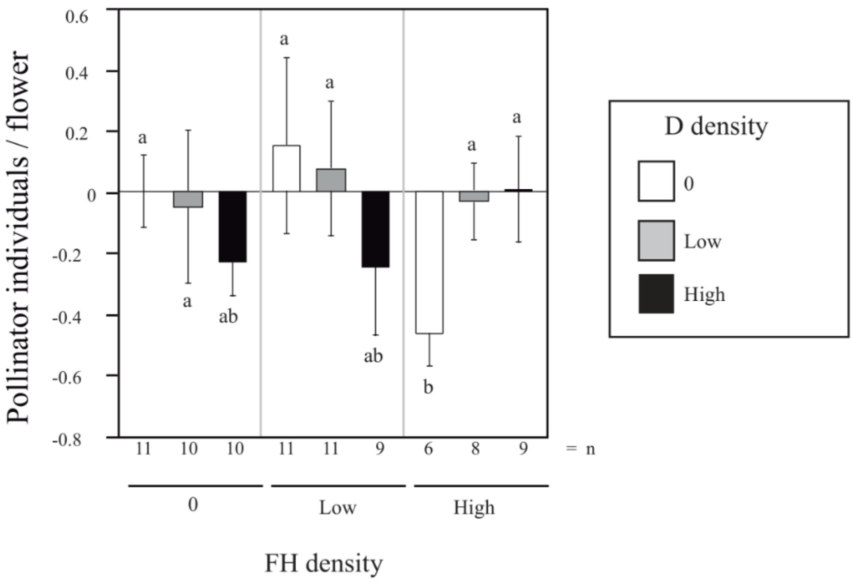


Figure GDA1. Detritivore (D) and floral herbivore (FH) density effects on pollinator individuals per flower. Shown values (mean ± SE) are relative to the focal treatment (mean D_0FH_0 = 0) and are weighted by plant identity to correct overdispersion. Letters correspond to Fischer Least Square Differences.

Glossary of terminology

Term	Definition	Reference
Complexification	incremental elaboration of solutions through adding new structure	(Stanley and Miikkulainen 2004)
Selection	relationship between a trait and fitness	(Lande and Arnold 1983)
Phenology	timing of events in the life of an organism	(Lieth 1974)
Ontogeny	developmental history of an organism within its own lifetime	(Gould 1977)
Phenotype	the observable characteristics or traits of an organism	(revisited by Dawkins 1978)
Plasticity	the ability of an organism to change its phenotype in response to different environments	
Trade-off	when one trait cannot increase without decrease in another, or vice versa	
Pleiotrophy	when one gene influences two or more seemingly unrelated phenotypic traits	
Functional trait	any trait affecting, directly or indirectly, individual performance and fitness of species	(Violle et al. 2007)
Pleitropic cost of defense	pleiotropic fitness costs when the stress factor is reduced	(Fritz and Simms 1992)
Allocation cost	when traits are directly limited by competition for resources	(sensu Pilson 2000)
Ecological cost	when coexpression of traits may be penalized depending on the environment	(sensu Pilson 2000)

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Mi gratitud para todos los que me habéis transmitido vuestro cariño por un paraje tan singular como el Barranco de Espartal, y vuestra preocupación por que se conserve tan natural como sea posible. Un afectuoso recuerdo también para Paco y Pepe del (extinto) bar Los Álamos, nuestro oasis de repostaje cuando el sol apretaba.

Una de las experiencias más enriquecedoras de estos años ha sido colaborar con y ser acogido por Caroline Müller en su grupo de Ecología Química de la Universidad de Bielefeld. Allí me encontré a una gran persona siempre dispuesta a aportar su logística y su valiosa visión a nuestra investigación. Fue una pasada la acogida que me brindaron todos y el ambiente tan colaborativo y estimulante para el desarrollo científico que me topé. Mención aparte merece Karin Djendouci por su santa paciencia no exenta de buen rollo y de chapurreo angloalemán al intruirme en las técnicas de laboratorio empleadas. Dankeschön an alle!

Que un fuera de serie como Peter Hambäck me acogiera en la Universidad de Estocolmo también ha sido una de esas sonrisas del destino. Allí me encontré con una manera de combinar el trabajo científico y las relaciones sociales fascinante, y con un Peter dispuesto a discutir cualquier interacción biótica en la pizarra. *Nature is complex*. Tack!

En la Universidad de Granada he podido conocer gente maravillosa. El desarrollo de esta tesis no hubiera sido lo mismo sin la amistad, el compañerismo y los momentos de distensión que nos hemos dado entre la chavalería de Zoología, Ecología, Genética y Botánica. Casi prefería no dar nombres para no dejarme a ninguno, pero no puedo dejar de acordarme de Gianluca, Alba, Indra, Óscar, Senda, Elena, Hugo, Fran, Pedro, Dani, Bea, Marco, Anas, Lucía, Carolina, Paquillo, Rubén, Javi, Moha y demás. Gracias por todos esos momentos, y por los que puedan venir. Qué decir de Manolo Tierno, siempre dispuesto a animar y echar una mano, igual que Rafa Rubio. Y del resto de compañeros del Departamento que me habéis dado un espaldarazo en más de un momento.

Siguiendo con las personas que marcan a uno, no puedo olvidarme de los compañeros del máster. Tanta buena gente y tantas conversaciones sobre el impulso que debe darse a la agroecología acaban determinando la vocación de uno. Haberme cruzado en el camino con científicos natos como Juan Antonio López Ráez y María José Pozo también deja huella.

Tengo la gran suerte de ser parte ahora del Laboratorio de Entomología Agrícola del IFAPA de Málaga. Me he topado con grupo de personas irrepetible, que me demostráis día a día que hacer buena ciencia no está reñido con disfrutar cada paso. Seguimos!

Los colegas de aquí y de allá. Gracias por hacerme sentir en casa en Granada, y hacerme sentir que vuelvo a casa cuando voy a Bilbo. Eskerrik asko, espero tener más tiempo a partir de ahora y retomar muchos planes.

Mis padres. Tantos veranos junto a la marisma, tantos libros de naturaleza y tanto ir al monte desde pequeño tienen sus consecuencias. Insuflarme tanta moral, y tanto esfuerzo por vuestra parte apoyándonos en la crianza y el cuidado de Nahia ha sido vital. Ez ditut eskertzeko hitzak topatzen. Igual por parte de mi hermana, la mejor de las hermanas que se pueden tener, siempre ahí. Y por extensión Jon. Todos vosotros, y el resto de la familia, sois un ejemplo de trabajo y perseverancia para mí. Los que estáis, y los que se fueron pero siguen presentes. Eutsi goiari!

La familia de Almería que me ha hecho sentir como uno más desde el inicio y me ha apoyado mucho, no puedo dejar de teneros presente en estos momentos, mil gracias!

Mi pequeña Nahia, que ha ido creciendo a la par que esta tesis. Cuya mirada y sonrisa es capaz de desvanecer todos los males. Eres mi llama. Te he robado tantas horas, espero darte lo mejor de mí a partir de ahora.

Y por último mi Sara. Si a alguien tengo que agradecer y dedicar esta tesis es a tí. Qué inmensa suerte tengo de que estés a mi lado. Sin tu sacrificio, tus consejos y tu amor esta tesis nunca hubiera llegado a puerto. También eres el arquetipo de investigador en el que intento convertirme. Ahora tengo que darte de vuelta mucho, y ser el compañero de viaje que mereces. Me hacéis muy feliz, os quiero!

P.D.: Gracias a tí también, si se me había pasado. Gracias a todos los que habéis obviado preguntarme cuándo iba a depositar la tesis. Ahora a sonreír más.

"Yo soy yo y mi *Moricandia*"

Posiblemente adaptado. Anónimo, o no.