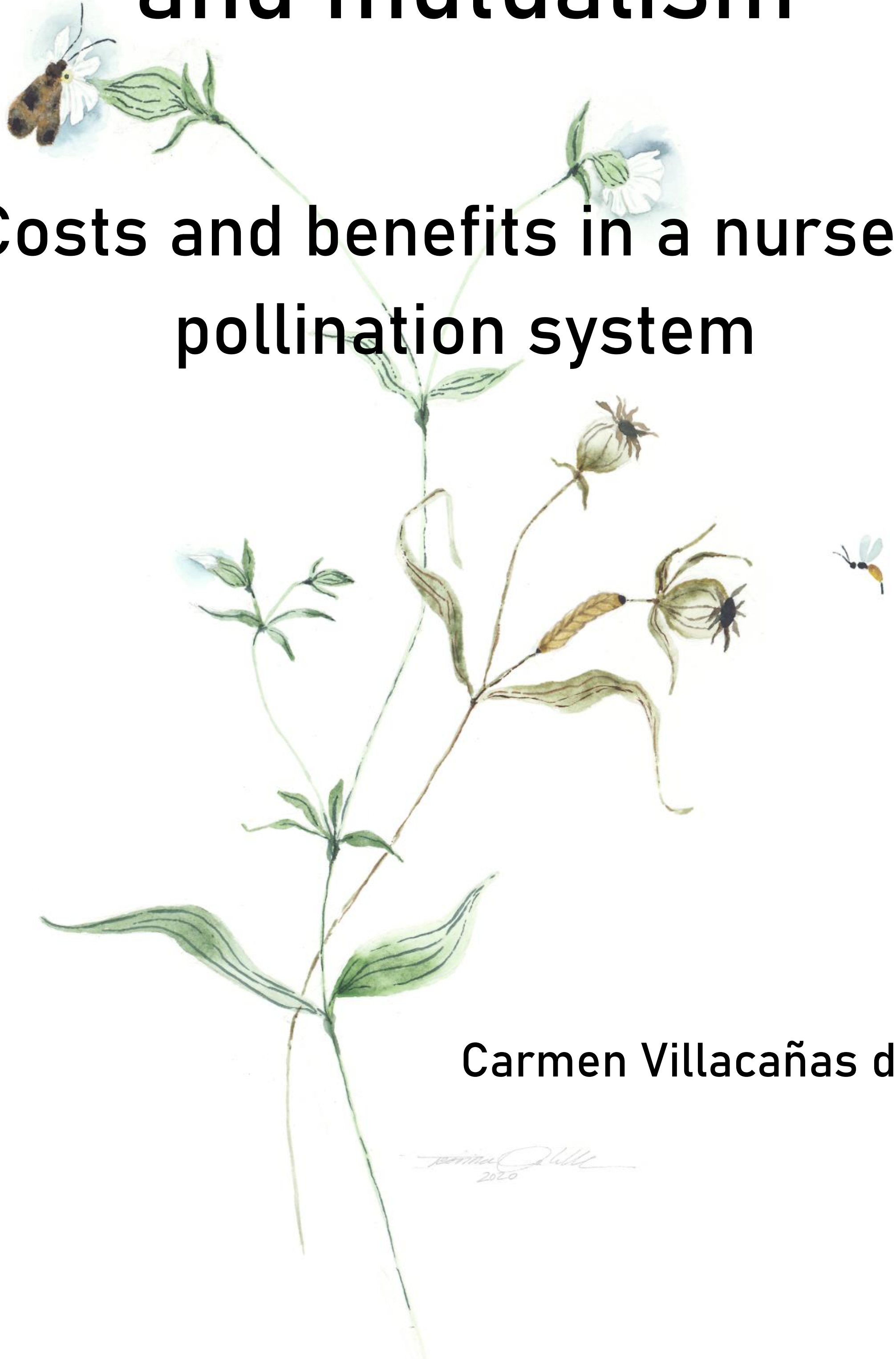
Between antagonism and mutualism



Costs and benefits in a nursery pollination system

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TABLE OF CONTENTS

SUMMARY

CHAPTER 1:

CHAPTER 2:

CHAPTER 3:

CHAPTER 4:

ANNEX

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

SUPPORTING INFORMATION

LIST OF TABLES

CHAPTER 2

CHAPTER 3

SUMMARY

SUMMARY

A hundred years after Darwin predicted the existence of a specific hawkmoth that could drink the nectar and pollinate the Malagasy star orchid, coevolution was defined as “an evolutionary change in a trait of the individuals in one population in response to a trait of the individuals of a second population, followed by an evolutionary response by the second population to the change in the first”. Since then, the demonstration that adaptations in both partner species are a result of reciprocal selection became a requirement to associate a certain pattern to coevolution. These relationships between species are dynamic and continually evolve as natural selection reshapes them. In this way, natural selection and coevolution have led to different types of interactions between species, depending on the effects on fitness of the partners involved. When the net effect is negative to one or both partners the relationship is an antagonism. Antagonisms are diverse and include predation and parasitism, among others. In commensalistic interactions, one species benefits and the other remains unaffected, while in mutualistic interactions both species benefit. Mutualisms vary greatly in the degree of obligacy and specificity, even between mutualistic partners.

The study of mutualisms has gained popularity among ecologists, and they are now recognised as providing essential ecosystem services. Several ecological and evolutionary patterns that are shared between diverse forms of mutualisms have been identified. First of all, one of the partners performs a service that benefits its associate, and in turn it receives a reward. However, services provided and rewards produced can also be costly. Hence, natural selection will favour traits that minimise such costs without interfering with the mutualism itself. As long as the benefits outweigh the costs, the association will last. The second pattern observed is conditionality: a change in the outcome of the interaction as a result of variations in the given local biotic or abiotic conditions. Such variation can change the benefit and cost ratio, shifting the interaction along a continuum from mutualism towards antagonism, and vice versa. Conditionality can occur when the services or rewards provided by partners change with age, size, or developmental stage, as well as with environmental factors such as resource availability, presence of competitors and natural enemies, or population densities. Lastly, evidence suggests that partners may be able to reinforce

mutualistic actions of their associates to reduce defection of the mutualism and decrease the probability that their partner will become an exploiter or a cheater.

Plant-pollinator interactions are some of the most well studied examples of coevolution and mutualism. Plants will experiment natural selection in those traits that will allow them to be located more easily by their partners, such as colourful and scented flowers. In exchange for pollination services, plants provide a nutritious reward, usually nectar or pollen. A specific type of pollination interactions are nursery pollination systems. In these interactions the pollinator also lays eggs in or on the host plant, which becomes a breeding site for its offspring. As the offspring develop and feed from reproductive structures of its host plant, very often seeds, the costs associated may be high. For the interaction to remain mutualistic the benefits must balance out the costs of the interaction, otherwise it would be evolutionarily unstable and eventually turn into a parasitism.

Nursery pollination systems vary in the degree of specialisation. Highly specialised, coevolved and obligate mutualisms include the interactions between *Ficus* plants-fig wasps, and *Yucca* plants-yucca moths. Other interactions such as the partnership between *Greya* moth and its host plant show conditionality, with the interaction shifting from mutualistic to commensalistic depending on the abundance of copollinators. Interactions between plants of the Caryophyllaceae family and *Hadena* moths range from antagonisms to facultative mutualisms. One of the interactions belonging to this complex is formed by host plant *Silene latifolia* and its pollinator/herbivore partner *Hadena bicruris,* which is often referred to as parasitic due to the extent of seed predation, and even as a “primitive” stage of nursery pollination mutualisms. Being a facultative mutualism with high conditionality, together with the high fitness costs, makes the *S. latifolia-H. bicruris* interaction a perfect model system to study the early stages of evolution in nursery pollination mutualisms.

The aim of this thesis is to explore under which ecological circumstances interactions may behave as a facultative mutualism, creating the possibility for them to evolve into more specialised interactions. To be able to evaluate the nature of the interaction and understand what are the causes leading to conditional outcomes, it is necessary to know the costs and benefits in the system. The positive contributions of male and female adult moths to seed production in *S. latifolia* plants were quantified through the examination of moth pollinating behaviour and efficiency with observational assays. This was followed by field data collections and the analysis of pollination, infestation, and parasitism rates by a natural enemy in order to investigate conditionality under field conditions. A theoretical model was

applied to determine interaction outcomes and the possible role of copollinators. The results show that *H. bicruris* male pollination benefits are essential for the stability of the system, but pollination, infestation and parasitism rates highly varied between populations. The amount of copollination was a good predictor for the outcome of the system, being clear that third parties associated to the *S.latifolia-H. bicruris* system play an important role modifying the outcome of the interaction.

Following the field investigation, the potential role of a natural enemy of *H. bicruris*, the ectoparasitoid *Bracon variator*, in modifying the interaction outcome between the plant and the herbivore was investigated. In particular, I examined whether the effect of the predator acted as a stabilising mechanism, reducing the level of seed consumption and therefore the costs caused to the plant, and how this in turn influenced individual plant fitness. With a series of laboratory and greenhouse experiments germination, survival, and flower production were measured as proxies for individual plant fitness. The results show that seed dormancy increased significantly in seeds from plants infested with *H. bicruris*, which could act as a short-term strategy to reduce costs in the interaction. *Silene latifolia* plants also had a higher seed output in the presence of *B. variator*, suggesting that *B. variator* may act as a regulator in the system. However, plant survival and flower production also decreased with higher seed densities. Therefore, an increase in seed output may be less beneficial for plant fitness than estimated from the number of seeds alone. There is a need to discuss whether taking simple proxies of fitness such as seed output is sufficient to determine the net effect of multitrophic interactions.

To conclude, the causes leading to conditionality in the *S. latifolia-H. bicruris* system are linked to male moth availability and abundance of copollinators, as well as plant population size. Moreover, the presence or absence of parasitoids can deeply impact conditionality in the S*. latifolia-H. bicruris* interaction, although the overall effects for plant fitness are not so clear. Further research needs to be carried out to properly assess the impact of the pollinator/herbivore and parasitoid partners on host plant population dynamics through exclusion experiments and modelling approaches at the tritrophic population level. On the other hand, natural selection should favour traits that limit predation costs inflicted by the pollinator/herbivore to avoid over-exploitation. In line with this idea, inducing seed dormancy and even investing in high amounts of seed production to compensate for the feeding of its pollinator/herbivore partner, could be possible mechanisms that have been selected for in *S. latifolia* plants to lower the costs of the interaction.

ZUSAMMENFASSUNG

Darwin sagte einst basierend auf der Sternorchidee die Existenz eines zu seiner Zeit unbekannte Schwärmers voraus, der sich von dem Nektar der Sternorchidee ernähren könnte und für diese als Bestäuber fungiert. Erst hundert Jahre danach wurde Koevolution definiert als „Eine Entwicklung eines Merkmals von Individuen einer Population angepasst auf ein Merkmal von Individuen einer anderen Population. Dies führt wiederum zu wechselseitig auf einander abgestimmten Anpassungen der Merkmale in den beiden Populationen.“ Um ein Anpassungsmuster von zwei Partnerarten der Koevolution zuzuschreiben, muss gezeigt werden, dass diese Entwicklungen ein Ergebnis reziproker Selektion sind. Diese interspezifischen Interaktionen sind dynamisch und unterlaufen stetigen Veränderungen aufgrund natürlicher Selektion. Auf diese Weise haben, abhängig von dem Effekt auf die Fitness der einzelnen Arten, sowohl natürliche Selektion als auch Koevolution zu unterschiedlichen Typen von interspezifischen Interaktionen geführt. Ist der Nettoeffekt negativ für die Fitness der einen Art, so wird die Beziehung zwischen den beiden als Antagonismus bezeichnet. Unter Anderem gehören Prädation und Parasitismus zu den Antagonismen. Während unter Mutualismus beide Arten profitieren, hat bei Kommensalismus lediglich eine Art einen Vorteil von der Interaktion mit der anderen. Bei mutualistisch miteinander lebenden Arten ist das Verhältnis zwischen den beteiligten Arten nicht unbedingt spezifisch und somit sind die Arten auch nicht zwingend aneinander gebunden in ihrer Existenz.

Mutualismus ist ein beliebtes Studienthema vieler Ökologen und inzwischen anerkannt als wichtiger Bestandteil von Ökosystemdienstleistungen. Es konnten bereits ökologische und evolutionäre Muster identifiziert werden, die in diverse Formen des Mutualismus vorkommen. Unter anderem gehört dazu ein Belohnungssystem bei dem eine der beiden Partnerarten von dem Service der anderen profitiert und diese dafür belohnt. Dienstleistungen zwischen Arten können allerdings auch große Kosten für die interagierenden Arten mit sich führen. Aufgrund natürlicher Selektion entwickeln sich eher Merkmale oder Verhaltensweisen, die so wenig Kosten wie möglich für die beteiligten Arten mit sich führen ohne die Beziehung negativ zu beeinflussen. Ist der positive Effekt größer ist

als der negative wird der Mutualismus der Arten weiterhin bestehen bleiben. Des Weiteren kann der Effekt den eine mutualistische Beziehung für zwei Arten hat durch abiotische und biotische Faktoren beeinflusst werden. Eine solche Variation in der Umgebung kann das Kosten-Nutzen-Verhältnis einer vormals mutualistische Interaktion kontinuierlich zu antagonistischen und andersrum verändern. Diese Abhängigkeit oder Konditionalität kann vorkommen, wenn eine Dienstleistung oder Belohnung von einer der Partnerarten durch Alter, Größe und Entwicklungsstadium beeinflusst wird. Andere Faktoren könnten Ressourcenverfügbarkeit, Anwesenheit von Prädatoren und natürlichen Feinden so wie Populationsdichte sein. Arten in einer mutualistischen Beziehung können durch gegenseitige Beeinflussung das Risiko, dass die Partnerart abtrünnig wird, bzw. dass die eine Art die andere ausnutzt minimieren.

Zu den sicherlich am besten untersuchten Beispielen für Koevolution und Mutualismus gehören die Pflanzen-Bestäuber-Interkationen. Die Pflanzenmerkmale, die es Bestäubern leicht machen die Pflanzen zu lokalisieren wie z.B. farbenprächtige und duftende Blüten, werden aufgrund von natürlicher Selektion bevorzugt ausgebildet. Als Gegenleistung für die Bestäubung bieten die Pflanzen Nektar oder Pollen als Nährstoffe für ihre Bestäuber. In einem sehr spezifischen System dieser Bestäuber-Interaktionen profitieren die Bestäuber nicht nur von Nektar und Pollen, sondern legen außerdem ihre Eier auf die Pflanzen und nutzen diese somit auch als Aufzuchtstation für ihre Nachkommen. Diese ernähren sich oftmals von den Samen der Pflanzen. Damit diese Form der Pflanzen-Bestäuber-Interaktion nicht zum Parasitismus umschlägt, muss der Nutzen für die Pflanzen die recht hohen Kosten ausgleichen.

Diese Art von Interaktion variiert stark in der Spezialisierung. *Ficus* Pflanzen–Feigenwespen sind genau so wie *Yucca* Pflanzen–Yucca Falter sehr stark aufeinander spezialisiert. Diese mutualistischen Beziehung sind koevolutionär entstanden und obligatorisch. Die Beziehung zwischen dem *Greya* Falter und seiner Wirtspflanze hingegen kann zwischen Kommensalismus und Mutualismus schwanken. Sie ist nicht obligatorisch mutualistisch und hängt mit der Anwesenheit weiterer Bestäuber zusammen. Interaktionen zwischen Caryophyllaceae Pflanzen und *Hadena* Faltern reichen von Antagonismen bis hin zu fakultativem Mutualismus. Die Beziehung zwischen *Silene latifolia* und *Hadena bicruris* wird aufgrund des Ausmaßes an Samenfraß oft als parasitär oder als primitive Form des Aufzucht-Bestäuber Mutualismus bezeichnet.

Ziel dieser Arbeit ist es zu untersuchen unter welchen ökologischen Umständen Interaktionen sich wie fakultativer Mutualismus verhalten und somit die Möglichkeit schaffen sich in eine spezialisierte Beziehung zu entwickeln. Es ist notwendig Kosten und Nutzen in einem solchen System zu kennen, um zu verstehen was die Auslöser für Konditionalität ist. Um den positiven Einfluss von männlichen und weiblichen Faltern auf die Samenproduktion von *S. latifolia* auszuwerten wurden Bestäubungsverhalten und -effektivität beobachtet. Im Rahmen von Feldversuchen wurde Bestäubung, Befall und Parasitismus durch einen natürlichen Feind analysiert, um Konditionalität im Freien zu untersuchen. Um Interaktionsergebnisse und die mögliche Rolle eines Kobestäubers zu ermitteln, wurde ein theoretisches Modell angewendet. Während Bestäubung, Befall und Parasitismus stark zwischen den Populationen variierten, zeigte sich, dass die Bestäubung durch *H. bicruris* Männchen ein essentieller Faktor für die Stabilität des Systems ist. Die Menge an Kobestäubung war ein guter Anhaltspunkt. Es wurde deutlich, dass weitere Arten in der Beziehung *S. latifolia-H. bicruris* eine wichtige Rolle spielen und diese nachhaltig beeinflussen können. Auf dem Feldversuch basierend wurde der mögliche Effekt des natürlichen Feindes *Bracon variator* von *H. bicruris* auf die Interaktion zwischen Pflanze und Falter untersucht. Genau genommen wurde geprüft, ob der Räuber in diesem System einen stabilisierenden Effekt hat, indem er den Samenfraß und somit die Kosten für die Pflanze reduziert und wie dies die Fitness der Pflanze beeinflusst. In einer Reihe von Labor- und Feldexperimenten wurden Frucht- und Blütenbildung sowie die Überlebensrate stellvertretend für die Fitness einer Pflanze betrachtet. Saatruhe stieg signifikant bei Pflanzen mit *H. bicruris* Befall an. Dies könnte eine kurzfristige Strategie sein, um den Samenfraß und so die Kosten der Interaktion zu reduzieren. Da in Anwesenheit von *B. variator* die Pflanzen mehr Samen produzierten, könnte *B. variator* als Regulator in dem untersuchten System fungieren. Allerdings nahmen Überlebensrate und Blütenproduktion mit größerer Samendichte der Pflanzen ab. Ein Anstieg in der Samenproduktion könnte also einen negativeren Effekt auf die Fitness der Pflanzen haben als nur die Anzahl der Samen. Es sollte hinterfragt werden, ob Werte wie die Samenproduktion stellvertretend für die Fitness einer Pflanze ausreichen, um eine Kosten-Nutzen-Effekt für multitrophische Interaktionen zu bestimmen zu können.

Zusammenfassend kann man sagen, dass Konditionalität im *S. latifolia-H. bicruris* System mit dem Vorkommen von männlichen Faltern, der Abundanz von Kobestäubern und der Populationsgröße der Pflanzen zusammen hängt. An- oder Abwesenheit von

Parasitoiden kann das System ebenfalls stark beeinflussen, allerdings ist nicht eindeutig was dies für die Pflanzenfitness bedeutet. Fortführende Ausschlussexperimente und Modellansätze für das tritrophische Populationslevel sind notwendig, um die Auswirkungen von Bestäubern/Herbivoren und Parasitoiden auf die Wirtspflanzen-Populationsdynamik bestimmen zu können. Allerdings ist es zu erwarten, dass durch natürliche Selektion Merkmale, die Prädation durch Bestäuber/Herbivore limitieren bevorzugt werden, um eine Ausbeutung zu vermeiden. Aus dieser Überlegung heraus, könnte man argumentieren, dass induzierte Samenruhe und erhöhte Samenproduktion, um den Samenfraß durch Bestäuber/Herbivoren zu kompensieren bereits selektierte Mechanismen bei *S. latifolia* Pflanzen sind, um die Kosten der mutualistischen Beziehung zu verringern.

RESUMEN

Cien años después de que Darwin predijera la existencia de una polilla específica que pudiera beber el néctar y polinizar la orquídea conocida como estrella de Belén, se definió la coevolución como "un cambio evolutivo en un rasgo de los individuos en una población en respuesta a un rasgo de los individuos de una segunda población, seguido de una respuesta evolutiva de la segunda población al cambio en la primera”. Desde entonces, la demostración de que las adaptaciones en especies asociadas son el resultado de una selección recíproca se convirtió en requisito fundamental para vincular un patrón concreto a la coevolución. Estas relaciones entre especies son dinámicas y evolucionan continuamente a medida que la selección natural las moldea. De esta manera, la selección natural y la coevolución han dado lugar a diferentes tipos de interacciones especializadas entre especies, dependiendo de los efectos sobre la eficacia biológica de los organismos involucrados. Cuando el efecto neto es negativo para uno o ambos socios, la relación es un antagonismo. Los antagonismos son diversos e incluyen depredación y parasitismo, entre otros. En las interacciones comensalistas, una especie se beneficia y la otra no se ve afectada, mientras que en las interacciones mutualistas ambas especies se benefician. Las interacciones mutualistas varían mucho en el grado de obligación y especificidad, incluso entre socios mutualistas.

El estudio de los mutualismos ha ganado popularidad entre los ecologistas, y en la actualidad son reconocidos como proveedores de servicios ecosistémicos esenciales. Se han identificado varios patrones ecológicos y evolutivos que se comparten entre diversas formas de mutualismos. En primer lugar, uno de los socios realiza un servicio que beneficia a su asociado y éste, a su vez, recibe una recompensa. Sin embargo, los servicios prestados y las recompensas producidas también pueden ser costosas. Por lo tanto, la selección natural favorecerá los rasgos que minimicen dichos costes sin interferir con el mutualismo en sí. Mientras los beneficios superen los costes, la asociación perdurará. El segundo patrón observado es la condicionalidad: un cambio en el resultado de la interacción como resultado de variaciones en las condiciones bióticas o abióticas locales. Dicha variación puede cambiar la relación entre beneficios y costes, desplazando la interacción a lo largo de un continuo desde el mutualismo hacia el antagonismo, y viceversa. La condicionalidad

puede ocurrir cuando los servicios o recompensas proporcionados por las especies asociadas cambian con la edad, el tamaño o la etapa de desarrollo de los individuos, así como por factores ambientales como la disponibilidad de recursos, la presencia de competidores y enemigos naturales o la densidad poblacional. Por último, ciertas evidencias sugieren que las especies asociadas pueden reforzar las acciones mutualistas de sus socios para reducir la deserción del mutualismo y disminuir la probabilidad de que su compañero se convierta en explotador o "tramposo".

Las interacciones planta-polinizador son uno de los ejemplos mejor estudiados de coevolución y mutualismo. Las plantas experimentarán la selección natural en aquellos rasgos que les permitirá ser localizadas más fácilmente por sus especies asociadas, como flores coloridas y olorosas. A cambio de los servicios de polinización, las plantas proporcionan una recompensa de carácter nutritivio, generalmente néctar o polen. Un tipo específico de interacciónes planta-polinizador son los sistemas de polinización "guardería". En estas interacciones el insecto polinizador además pone huevos en o sobre la planta hospedadora, que se convierte así en lugar de cría para su descendencia. Debido a que la descendencia se desarrolla y se alimenta de las estructuras reproductivas de su planta hospedadora, que muy a menudo son las semillas, los costes asociados pueden ser muy altos para la planta. Para que la interacción siga siendo mutualista, los beneficios deben equilibrar los costes de la interacción, de lo contrario sería evolutivamente inestable y eventualmente se convertiría en un parasitismo.

Los sistemas de polinización "guardería" varían en el grado de especialización. Los mutualismos altamente especializados, coevolucionados y obligados, incluyen las interacciones entre las plantas del género *Ficus* y sus avispas polinizadoras, o las yucas y sus polillas polinizadoras. Otras interacciones, como la asociación entre las polillas del género *Greya* y las plantas *Litophragma*, muestran condicionalidad, y la interacción cambia de mutualista a comensalista dependiendo de la abundancia de copolinizadores. Las interacciones entre plantas de la familia Caryophyllaceae y las polillas del género *Hadena* abarcan desde antagonismos hasta mutualismos facultativos. Una de las interacciones que pertenece a este grupo está formada por la planta hospedadora *Silene latifolia* y su socio polinizador/herbívoro *Hadena bicruris*. La interacción a menudo es descrita como un parasitismo debido al alto grado de depredación de las semillas por parte de las larvas de *H. bicruris*, e incluso como una etapa "primitiva" de la polinización "guardería". El hecho de ser un mutualismo facultativo con alta condicionalidad, sumado a los altos costes de eficacia biológica, hacen de la interacción entre *S. latifolia-H. bicruris* un sistema modelo perfecto para estudiar las primeras etapas de evolución en los mutualismos de polinización "guardería".

El objetivo de esta tesis es explorar bajo qué circunstancias ecológicas las interacciones pueden comportarse como un mutualismo facultativo, creando la posibilidad de que evolucionen hacia interacciones más especializadas. Para poder evaluar la naturaleza de la interacción y comprender cuáles son las causas que conducen a resultados condicionales, es necesario conocer los costes y beneficios del sistema. La contribución positiva de las polillas macho y hembra adultas a la producción de semillas en plantas *S. latifolia* se cuantificó mediante ensayos de observación comportamental de la polinización y su eficiencia. A continuación se recolectaron datos de poblaciones naturales y se analizaron las tasas de polinización, infestación por larvas de *H. bicruris*, y parasitismo por un enemigo natural, para investigar la condicionalidad en condiciones de campo. Se aplicó un modelo teórico para determinar los resultados de la interacción y el posible papel de los copolinizadores. Los resultados muestran que los beneficios de la polinización por machos *H. bicruris* son esenciales para la estabilidad del sistema, pero las tasas de polinización, infestación y parasitismo varían mucho entre las poblaciones. La cantidad de copolinización resultó ser un buen predictor del resultado del sistema, quedando claro que terceras especies asociadas al sistema *S. latifolia-H. bicruris* juegan un papel fundamental modificando el resultado de la interacción.

Tras la investigación de campo, se exploró el papel potencial de un enemigo natural de *H. bicruris*, la avispa ectoparasitoide *Bracon variator*, en la modificación del resultado de interacción entre la planta y su polinizador/herbívoro asociado. En particular, se examinó si el efecto del ectoparasitoide actuó como un mecanismo estabilizador, reduciendo el nivel de depredación de semillas y, por lo tanto, los costes causados a la planta. Además se analizó cómo esto influyó a su vez en la eficacia biológica individual de la planta hospedadora. Con una serie de experimentos de laboratorio e invernadero, se midieron la germinación, supervivencia y producción floral como indicadores de la eficacia biológica de las plantas. Los resultados muestran que la dormición de las semillas aumentó significativamente en aquellas procedentes de plantas infestadas por *H. bicruris*, lo que podría actuar como una estrategia a corto plazo para reducir los costes en la interacción. Las plantas de S*. latifolia* también tuvieron una mayor producción de semillas en presencia de *B. variator*, lo que sugiere que *B. variator* puede actuar como regulador en el sistema.

Sin embargo, la supervivencia de las plantas y la producción floral también disminuyeron con mayores densidades de semillas. Por lo tanto, un aumento en la producción de semillas puede ser menos beneficioso para la eficacia biológica de la planta que lo estimado tan sólo a partir del número de semillas. Así pues, es necesario debatir si es suficiente tomar medidas simples de eficacia biológica, como el *output* de semillas, para determinar el efecto neto en interacciones multitróficas.

Para concluir, las causas que conducen a la condicionalidad en el sistema *S. latifolia-H. bicruris* están relacionadas con la disponibilidad de machos adultos y la abundancia de copolinizadores, así como con el tamaño poblacional de las plantas. Además, la presencia o ausencia de parasitoides puede alterar profundamente la condicionalidad de la interacción de *S. latifolia-H. bicruris*, aunque los efectos para la eficacia biológica de la planta no están tan claros. Se necesita llevar a cabo más estudios para evaluar adecuadamente el impacto de los socios polinizador/herbívoro y parasitoides en las dinámicas poblacionales de la planta hospedadora a través de experimentos de exclusión y modelos para poblaciones tritróficas. Por otro lado, se espera que la selección natural favorezca los rasgos que limitan los costes de depredación infligidos por el polinizador/herbívoro para evitar la sobreexplotación. Asimismo, y en línea con esta idea, inducir la dormición en semillas e incluso invertir en grandes cantidades la producción de semillas para compensar la alimentación de su socio polinizador/herbívoro, serían posibles mecanismos que han sido seleccionados en las plantas de *S. latifolia* para reducir los costes de la interacción y evitar así la sobre-explotación por parte de *H. bicruris*.

2020

CHAPTER 1

General introduction

Carmen Villacañas de Castro

"Thus it would appear that there has been a race in gaining length between the nectary of the *Angræcum* and the proboscis of certain moths; but the *Angræcum* has triumphed, for it flourishes and abounds in the forests of Madagascar, and still troubles each moth to insert its proboscis as far as possible in order to drain the last drop of nectar."

—Charles Darwin. *Fertilisation of Orchids*[1]

1. COEVOLUTION

When Darwin predicted in his book on orchid pollination (Darwin 1862) the existence of a hawkmoth capable of feeding from and pollinating the Malagasy Star of Bethlehem orchid (*Angraecum sesquipedale,* often referred to as Darwin's orchid), he was proposing the first mechanistic model of a coevolutionary process (Johnson and Anderson 2010). Although he did not use the word "coevolution", he described how the orchid and its pollinator could have interacted in such a way that they reciprocally influenced each other's evolution, driven by natural selection. Darwin (1862) suggested that a spur longer than the hawkmoth proboscis would enhance pollination and benefit reproductive success in the plant, as the hawkmoth would have been forced to press its head against the reproductive parts of the flower to reach all the nectar. This in turn would also favour a selection towards a longer proboscis in hawkmoths, and therefore lead to the above mentioned "race in length" (Figure 1) (Micheneau et al. 2009, Johnson and Anderson 2010). For several decades later, naturalists and biologists would study insect-flower interactions where "reciprocal evolution" could have taken place (Proctor et al. 1996, Micheneau et al. 2009).

However, it was not until the 1960s that the first scientific articles focusing explicitly on coevolution were published. Ehrlich and Raven (1964) suggested how the diversification of butterflies and their host plant species might have been a result of coevolution in both groups, establishing a framework to study the evolution of interactions (Thompson 1982). A couple

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[1] From Darwin, C. R. 1862. On the various contrivances by which British and foreign orchids are fertilised by insects, and on the good effects of intercrossing. London: John Murray. 202-203. Sourced from http://darwin-online.org.uk, accessed April 2020.

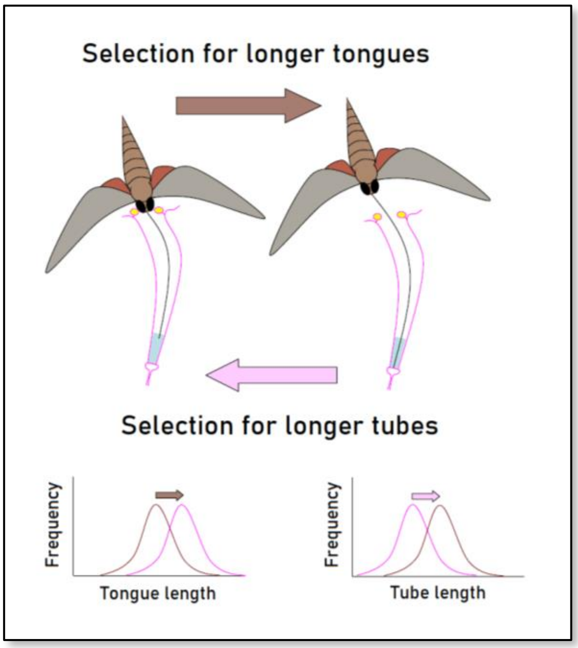

Fig. 1 Darwin's mechanistic model for the coevolution of *Angraecum sesquipedale* and its pollinator (modified from Johnson and Anderson 2010).

years later Janzen (1966) showed coevolution for the first time between swollen-thorn acacias and their ant inhabitants with a series of natural history observations and community experiments. The same author, in response of the broad and imprecise use of the term among researchers, wrote the first formal definition of coevolution in 1980, which he defined as "an evolutionary change in a trait of the individuals in one population in response to a trait of the individuals of a second population, followed by an evolutionary response by the second population to the change in the first" (Janzen 1980). From then on, the study of coevolution focused on finding general patterns among particular kinds of interactions (Connell 1980, Price 1980, Thompson 1982). The demonstration that adaptations in both partner species were a result of reciprocal selection, became a requirement to associate a certain pattern to coevolution (Brockhurst and Koskella 2013). Therefore, not all interacting species have necessarily coevolved; a bird has not necessarily coevolved with the worm that it preys on, just like a bee has not necessarily coevolved with all the different flowers it pollinates (Janzen 1980).

Since Janzen's (1980) definition of coevolution, different types have been recognised. Specific coevolution, which acts between pairs of species, is the historical view, but not the only possibility. In diffuse coevolution, coevolution is also reciprocal, yet it is acting in a community context where selection pressures are generated not only by the two-species interaction, but by a group of species (Janzen 1980, Schoonhoven et al. 2005a). A third view on coevolution would be that developed by Thompson (1994) in his Geographic Mosaic Theory, which unites the ecological and evolutionary processes shaping interactions. Putting emphasis on the geographical context of coevolution, and assuming there is genetic variation within species, it argues that pairs or groups of interacting species may exhibit distinct patterns of selected traits across different geographic landscapes (Schoonhoven et al. 2005a, Bronstein 2009). Hence, and interaction between the same pair of species could have very different outcomes in different landscapes (Thompson 1999).

Nevertheless, there are still multiple open questions as to how exactly coevolution shapes species interactions and natural communities (Thompson 1999, Occhipinti 2013). Just like individual species do not occur in nature in complete isolation, strongly interacting species and their associations do not evolve in isolation either, but within a complex community and environmental context, where other species may play an important role in modifying the interaction (Thompson 1999, Bronstein et al. 2003, Gomulkiewicz et al. 2003, Strauss et al. 2005). Consequently, these relationships are dynamic and continually evolve as natural selection reshapes them (Bronstein 2009).

2. TYPES OF INTERSPECIFIC INTERACTIONS

Natural selection and coevolution have favoured a great diversity of lifestyles which lead to different types of specialised interactions between species, depending on the negative, neutral or positive effects on fitness (i.e. the ability of an organism to survive and produce offspring (Ridley 2003)) of the partners involved. The net effect depends on the benefits and costs that each partner brings to the relationship. While the precise costs can vary greatly depending on the specific characteristics of the interaction, the benefits obtained typically include: nutrition (provision of limiting nutrients to the partner), protection (from biotic or abiotic conditions, including defence and resistance to enemies), transport (phoresy to more suitable environments or by dispersal of gametes or propagules) or a combination of these (Boucher et al. 1982, Bronstein 2015).

A common way of categorising interspecific interactions is using the “interaction grid” (see Figure 2) first developed by Haskell (1947) to study human social behaviour, and later adapted to the field of Ecology by Odum (1953) (Bronstein 2015). When the net effect of the interaction is negative to one or both partners of the interaction the relationship is antagonistic; commensalistic when one species benefits and the other remains unaffected; and mutualistic if both species benefit from the interaction (Boucher et al. 1982). A fourth possible outcome in interspecific interactions would be neutralism: the relationship between two species in which the fitness of neither is affected by the association (Odum 1953). However, it has been questioned whether it should be considered a type of ecological interaction (Arthur and Mitchell 1989) given it is almost impossible to prove. Nonetheless,

Species 1 \ Species 2	+	–	0
+	Mutualism		
–	Predation/ Parasitism	Competition	
0	Commensalism	Amensalism	Neutralism

Fig. 2 The interaction grid. The net effect (positive, negative or neutral) of each partner species determines the outcome of the interaction (modified from Bronstein 2015).

later in this thesis it will be discussed how this traditional classification of interactions do not have such clear divisions, as it ignores the variation in the net effects (Thompson 1988, Bronstein 1994).

Antagonisms are diverse and include four types of interactions: predation and parasitism (-/+), competition (-/-), and amensalism (-/0) (Bronstein 1994). Predation is when an individual of one species, the predator, kills and consumes an individual of a different species, its prey, consuming many different prey individuals in a lifetime (Lafferty and Kuris 2002). Typical predator-prey interactions include carnivorous ones such as between lions and gazelles, but also seed eating ants (Figure 3a) or caterpillars that eat through the roots of young plants, killing them (Townsend et al. 2003). On the other hand, parasitism is when an individual of a species lives throughout a major period of its life in or on an individual of a different species, its host, obtaining nutrients from the host and causing a decrease in host fitness (Thompson 1982, Combes 2001). Some examples include parasitic fungi of plants, or mites which feed on blood of poultry (Figure 3b). In most cases, parasites do no kill their host in the short term, and usually will only have one or few hosts in their lifetime, forming intimate associations, exploiting it not only for nutrition, but as a habitat (Combes 2001). Therefore, in the case the host would die before the parasite reaches the life stage where it can leave its host, the parasite would also die (Thompson 1982). However, some of these categories are simplified here for clarification purposes and can be further divided into several other trophic strategies (see Lafferty and Kuris 2002). In the case of competition, no species benefits from the interaction, as species compete for the access to limiting resources (i.e. resources that are a requisite for population growth), usually by capturing resources faster than their competitors (exploitation competition), or physically interacting with one another in an aggressive manner (interference competition) (Townsend et al. 2003). A classic example of competition is the interaction between two species of freshwater diatoms, *Asterionella formosa* (Figure 3c) and *Synedra ulna*, studied by Tilman et al. (1981). Both species need silicate to grow. When cultured on their own, they are both able to establish stable populations, maintaining their limiting resource at a constant but low concentration. However, *S. ulna* exploits silicate more efficiently, keeping silicate concentration at a lower level than *A. formosa*. As a result, when both species were grown together, *S. ulna* kept silicate levels at a concentration too low for *A. formosa* to survive, driving the species to

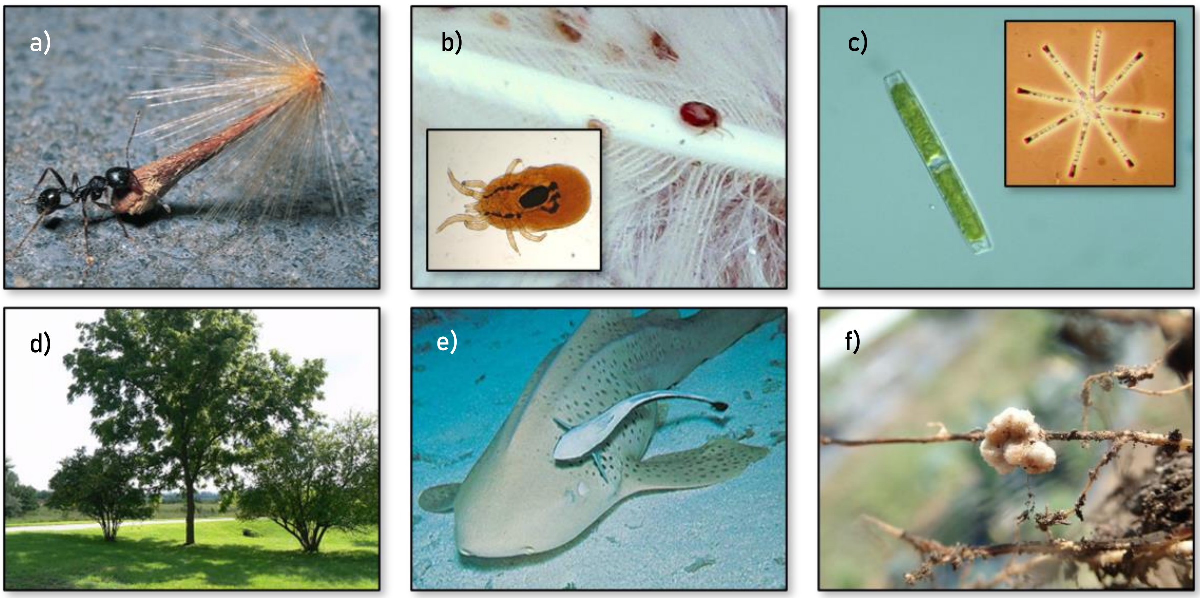

Fig. 3 Depiction of different examples of interspecific interactions. a) *Messor structor* carrying a seed of a sycamore tree. Credit: Bernhard Seifert. b) Mite on a chicken feather. Credit: Kathy Shea Mormino. Left below: Close up of a red mite *Dermanyssus gallinae.* Credit: Luis Fernández García. c) Cell body of diatom *Synedra ulna*. Credit: Yuuji Tsukii. Top right: *Asterionella formosa*. Public domain. d) Black walnut tree, *Juglans nigra*. Public domain. e) A remora fish (*Echeneis naucrates*) and its host, a zebra shark (*Stegostoma fasciatum*). Credit: Douglas Faulkner. f) *Rhizobium* nodules on a root of a faba bean (*Vicia faba*). Credit: Whitney Cranshaw.

extinction (Tilman et al. 1981). Competitive interactions tend to evolve towards avoidance, coexistence or exclusion (Townsend et al. 2003, Thompson 2019), sometimes having very asymmetrical effects. In one extreme of this asymmetry, we find amensalism, where an individual of one species harms another individual from a different species without obtaining any benefit from the interaction (Odum 1953). An example is the black walnut tree (*Juglans nigra*) (Figure 3d), which excretes juglone (a chemical toxic to other species of plants) from roots and other tissues into the environment, inhibiting the growth of any nearby plants (Rietveld 1983).

Commensalism (0/+) is an interaction in which one individual of a species obtains a benefit, while its partner of a different species obtains no benefit and is inflicted no cost, remaining unaffected (Townsend et al. 2003). One of the best known examples of commensals are remora fish (Echeneidae) (Figure 3e), which swim adhered to the bodies of sharks or other large marine animals, feeding on food leftovers of their host and free-riding (Marshall 1965).

In mutualistic relationships (+/+) (Box 1) individuals of two different species depend on and benefit from their interaction with a net benefit in fitness (Boucher et al. 1982). These types of interactions can be found across many different taxa and in very diverse forms (Boucher et al. 1982, Boucher 1985). Classical examples include nitrogen-fixing *Rhizobium* bacteria (Figure 3f) in the roots of leguminous host plants (Burns and Hardy 1975), or pollinating insects and flowering plants such as Darwin's orchid and its pollinating hawkmoth. Mutualistic interactions can be further divided into facultative and obligate. Obligate mutualisms are those in which one or both partners depend entirely on the other for survival and/or reproduction, while facultative mutualisms are those where there is a benefit in fitness, but survival or reproduction is not dependent on the interaction (Holland and Bronstein 2008). Another way of classifying these interactions is on their specificity: in species-specific mutualisms, only a single partner species can provide benefits to its mutualistic partner, while in generalised mutualisms usually a group of species is able to provide benefit (Holland and Bronstein 2008). Individual systems can vary greatly in the degree of obligacy and specificity, even between mutualistic partners (Bronstein 2009). The characteristics of these systems are the result of coevolutionary processes of which the first steps are usually unknown (Westerbergh and Westerbergh 2001, Kephart et al. 2006).

3. ECOLOGICAL AND EVOLUTIONARY PATTERNS IN MUTUALISMS

In the recent decades, the interest of ecologists in the study of mutualisms has grown significantly and nowadays they are recognised as providing essential ecosystem services and being fundamental in the shaping of communities (Bronstein 2009, Thompson 2009). From the extensive network of beneficial mycorrhizae that allow plants to efficiently extract nutrients from the soil, to corals depending on the photosynthetic algae that inhabit them, or organisms in human gut systems that once were thought to cause microbial diseases and are now known to have crucial roles in human well-being (Bäckhed et al. 2005, Dethlefsen et al. 2007), mutualistic interactions are crucial directly or indirectly for almost every species on Earth (Boucher et al. 1982, Bronstein et al. 2006). Generally, mutualisms are considered to have evolved from ancestral antagonistic interactions (Thompson 1982). However, although our understanding of how mutualistic interactions arise, evolve, and are maintained is very far from complete (Thompson 1999, Bronstein 2009), recently researchers have identified ecological and evolutionary patterns that are shared between diverse forms of mutualisms (Bronstein 1994, 2009, Jones et al. 2012).

3.1 Mutualisms as consumer-resource interactions

Traditionally, mutualisms had been viewed as unconditionally beneficial, even altruistic, interactions between two different species (Boucher et al. 1982, Bronstein 1994, Stanton 2003). In the past decades, with a greater study of the costs associated with such interactions, the perception has shifted towards a consumer-resource perspective (Bronstein 1994, Holland et al. 2005, Bronstein 2009). In most associations, one of the partners performs a service that benefits its associate, and in turn it receives a reward (Cushman and Beattie 1991, Holland et al. 2005, Holland and DeAngelis 2010). The service provided usually involves a behaviour (such as flower pollination, dispersal of seeds, or protection from natural enemies) and the corresponding rewards tend to be nutritional (nectar from the flowers, fruits containing the dispersed seeds, and sugar-rich secretions from protected insects or plants) (Jones et al. 2012). However, it is now apparent that natural selection has also acted in mutualistic interactions in the context of their costs and not only the benefits (Bronstein 2009). On the one hand, the rewards provided to the mutualistic partners can be costly to produce. Pyke (1991) provided examples in which the production of nectar could use up to 37% of the energy resources of a plant and showed how experimental removal of nectar (mimicking nectar feeding in pollinators) reduced the seed set in plants as a result from

Box 1. Mutualism and symbiosis

The word symbiosis comes from the Greek *symbíōsis* which means "living together, companionship" (Symbiosis, n.d.). Although often confused, the terms mutualism and symbiosis are not synonyms. Mutualism refers to any interaction in which the benefits exceed the costs for each partner, and therefore the outcome is mutually beneficial. This definition is independent of the intensity, specificity or history of the interaction (Bronstein 1994). On the other hand, a symbiosis is defined as an interaction in which two species exist in an intimate physical association during most or all of their lifetime and are physiologically dependent on each other (Bronstein 2009). In this case, the definition is independent of the outcome of the interaction: symbiosis can be positive, negative or neutral for each of the partners (Bronstein 1994, 2009). Therefore, mutualisms may or may not be symbiotic, and symbiosis may or may not be mutualistic.

In symbioses it is common that one of the partners, called symbiont, inhabits within the body of the other partner, its host (Holland and Bronstein 2008). An example of a mutualistic symbiosis are lichens, which are complex organisms consisting of an alga or cyanobacterium (called the "photobiont") living among the filaments of a fungus (called the "mycobiont") (Begon et al. 2006). The algae or cyanobacterium will provide carbohydrates through photosynthesis and/or fixed nitrogen, while the fungus provides a physical structure to grow upon, as well as moisture and nutrients from the environment (Begon et al. 2006). Symbiotic mutualists tend to have simple life cycles: unlike parasites they do not need an alternation between hosts and vectors are not involved (Townsend et al. 2003).

increased nectar production. Therefore, it is expected that partners are selected to minimise the cost, producing the lowest amount of reward which will still allow them to obtain a service from their associates, independent of the costs they might themselves inflict on their partners (Bronstein 1994). When providing a service, a collateral cost might also be inflicted, and traits that reduce this collateral cost without interfering with the mutualism itself can also be selected for (Bronstein 2009). An example illustrating this was provided by Willmer et al. (2009) who studied ant-tended plants. These plants have extrafloral nectaries from which ants feed in exchange for protection against herbivory. The authors showed that flowers of some ant-tended species contain volatile repellents that keep the ant guards away, to prevent them from deterring pollinators with their aggressive behaviour. Clearly, mutualistic interactions are not altruistic but quite costly, and the association will hold as long as the benefits outweigh the costs (Bronstein 1994).

3.2 Conditional outcomes

When the different types of interactions were explained, it was mentioned that the outcomes of such interactions did not always have clear boundaries. This is because, as we have seen, the benefits and costs can vary in magnitude along evolutionary time, but are also able to do so across different spatial (i.e. environmental) and time scales (Bronstein 2009, Holland and DeAngelis 2009). If the cost and benefit ratio changes, the outcome of the association will also change. The above presented classification fails to capture this dynamism (Bronstein 2009). Hence, interactions should be seen as potentially occupying dynamic positions along a continuum ranging from positive (mutualistic) to negative (antagonistic) outcomes (Bronstein 1994, Holland and DeAngelis 2009, Jones et al. 2012). A better representation of the dynamism of interactions is the “interaction compass” (Figure 4), which depicts the continuous transitions from one outcome to the other (Bronstein 2001b, Holland and DeAngelis 2009, Bronstein 2015). Depending of the environmental context and the ecological circumstances, the interactions will shift along such continuum from mutualism, towards commensalism or antagonism, and vice versa (Bronstein 1994, Bronstein et al. 2003, Holland and Bronstein 2008, Leung and Poulin 2008, Holland and DeAngelis 2009).

Thompson (1988) argued that “just as variation in traits in populations is the raw material for the evolution of species, variation in outcome is the raw material for the evolution of interactions”. Conditionality is the characteristic of interactions with context dependent variations in their outcomes (Cushman and Beattie 1991, Bronstein 1994), and although the elements involved are diverse, several life-history factors are capable to predict to some extent these variable outcomes in mutualisms (Thompson 1988, Bronstein 1994). In the first place, some of the services or rewards provided by partners might be age, size or stage dependent (Thompson 1988, Bronstein 1994). Going back to ant-tended plant species, we see that young saplings are unable to produce sufficient rewards to attract enough ants needed to avoid herbivory, while larger, older trees attract more ants (O'Dowd 1979). In such cases, rather than averaging out a net outcome within the population, it is more relevant to study

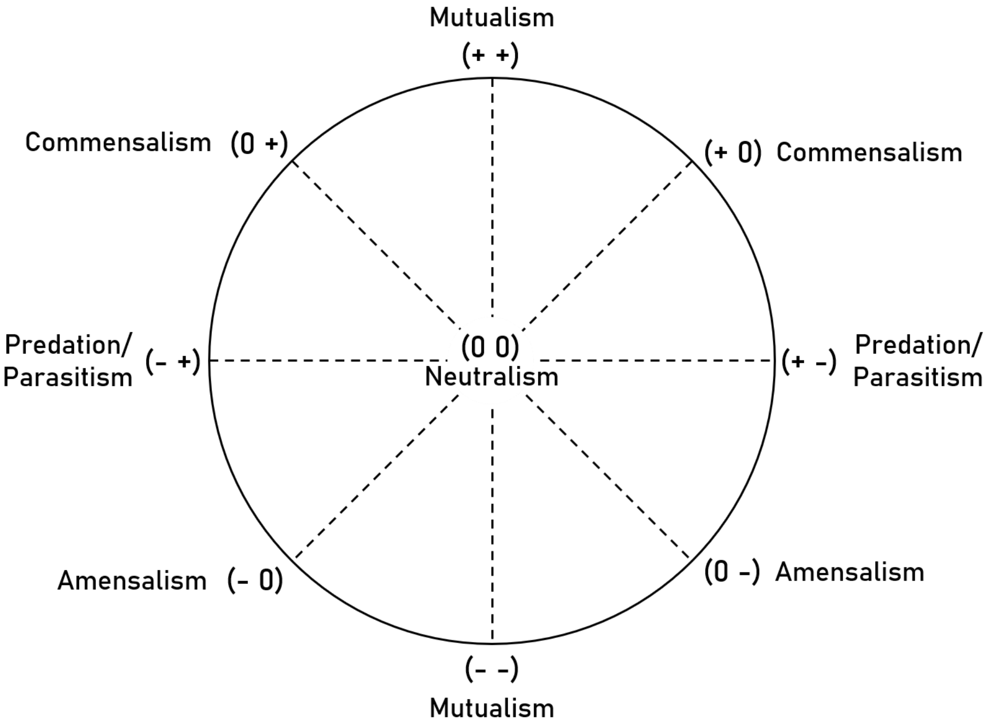

Fig. 4 The interaction compass. The first positive (+), negative (-), or neutral (0) sign represents the effect of species 1 on species 2, while the second sign represents the effect of species 2 on species 1. Moving along the radius increases the magnitude of the interaction, but does not change the direction of the net effect. Moving around the circumference shifts the direction of the net effect. The diagram illustrates the possible outcomes of interspecific interactions and how changes in the signs of one or both species results in a continuum of transitions among the different types of interaction outcomes (modified from Bronstein 2015).

the distributed outcomes of the interaction to better understand its ecology and evolution (Thompson 1988, Bronstein 1994).

Secondly, the outcomes of interactions can also vary depending on the environmental context in which they take place, as they can be influenced by abiotic and biotic factors. Resource availability can limit the production of rewards as well as the need for a service (Thompson 1988, Bronstein 1994). For example, mycorrhizas are generally mutualistic in nutrient-poor soils providing plants with phosphorous and other micronutrients in exchange for carbohydrates, but in nutrient-rich soils they can become parasitic, reducing the growth rate of the host plant (Johnson et al. 1997). The type and density of the species present in the environment can also alter the outcome, including abundance of competitors or natural enemies such as parasitoids (Bronstein 1994). Finally, the abundance of the partners themselves can also influence the outcome, as this will change the per-capita benefits and costs. Per-capita benefits tend to increase with population size of mutualist partners, but will decrease with large population sizes (Thompson 1988, Cushman and Beattie 1991), while the cost associated with the production of rewards increases excessively with large populations (Bronstein 1994). In the past decades, several studies have developed models exploring the population dynamics resulting from mutualistic interactions and density-dependent outcomes (Holland et al. 2002, Bronstein et al. 2003, Morris et al. 2003, Holland and DeAngelis 2009, Morris et al. 2010).

3.3 Exploiters and cheaters

Natural selection will favour individuals who maximise the benefits received while minimising their own costs. Conflicts of interest with the potential to destabilise the mutualism will therefore emerge (Bronstein 2001a). For example, if one of the partners evolves into a better exploiter, so that it is less costly to exploit its associate than to cooperate with it, the mutualism should break down with time (Bronstein 2001a). This is often referred to as "defection from mutualism" (Bronstein 2001a).

In addition, it is also common that mutualist partners interact with third parties that, without providing any service, will take advantage of the rewards produced for the mutualist partners (Bronstein 2001a, Douglas 2008, Jones et al. 2009). This type of exploitation is sometimes referred to as cheating, and examples include nectar robbers —insects that feed on nectar but do not pollinate the flowers— or *Rhizobium* bacteria which cease to fix nitrogen in in the root nodules of their soybean host plants (Kiers et al. 2003).

However, when these conflicts of interest emerge, evidence suggests partners are able to reduce the negative effects of exploitation, or discourage it, reinforcing mutualistic actions of their associates (Herre et al. 1999, Bronstein 2001a, Douglas 2008). A first mechanism that would act even before the mutualism has been established is partner choice, and it refers to the assessment of the quality of potential partners, and the subsequent selection of the most suitable one (Bull and Rice 1991, Bronstein 2001a, Orona-Tamayo and Heil 2013). In such cases, potential partners would signal their own quality to each other and consequently accept or reject the association (Bull and Rice 1991, Archetti et al. 2011).

Another way to protect rewards from exploiters are mechanical barriers that restrict the access to the rewards (Orona-Tamayo and Heil 2013). The length of the nectary spur in Darwin's orchid would be a good example for such mechanism, as only its specialised partner, a hawkmoth with the same proboscis length, is able to reach the nectar and carry out pollination (Darwin 1862). Nectar robbers are important exploiters that feed on nectar by biting holes in the flowers and therefore avoiding pollination of the plant (Irwin et al. 2010). Several studies have shown that certain morphological features in flowers could protect them against nectar robbers (Irwin et al. 2004). For example, plants with thicker calyces or corollas could make it mechanically more difficult for nectar robbers to access the nectar, while tightly packed flowers could restrict access to nectar spurs or corolla tubes (Irwin et al. 2004).

The third possible mechanism of control is partner fidelity feedback. In a mutualistic association, both partners interact repeatedly over time, and their actions towards each other will reflect on their own success (Bronstein 2001a). If we assume that one partner will provide lesser quality rewards if it has a lower fitness, because its partner has failed to reciprocate, it is logical to think that eventually the cheater will also have a reduced fitness as a consequence (Orona-Tamayo and Heil 2013). In this case, natural selection would favour mutualists over cheaters. A further mechanism would be applying sanctions to exploiters, usually through a decrease in reward allocation (Orona-Tamayo and Heil 2013). For example, a study by Kiers et al. (2003) showed that soybeans are able to apply sanctions to rhizobia that turn parasitic and stop fixing nitrogen by interrupting the allocations of assimilates to the cheating nodules. In such situation one of the partners is controlling the mutualistic partner and reinforcing “good” behaviours (Douglas 2008, Jones et al. 2012).

Finally, a last mechanism proposed as a stabiliser of mutualisms is partner screening. According to Archetti et al. (2011), the potential partner would be able to assess the costs and benefits of the association and to compare them to their own quality as mutualists by “screening” themselves. In this way, only adapted partners would gain enough benefits to be willing to pay the costs of the interaction. However, such mechanism could only take place when the benefit of being a mutualist is higher than the benefit obtained from being an exploiter (Heil 2013). Both Archetti et al. (2011) and later Heil (2013) suggested that several ant-*Acacia* interactions might function under such mechanism of partner screening.

Nevertheless, the stabilising mechanisms of mutualisms sometimes fail, and mutualistic interactions can revert to antagonism if cheating is an evolutionary stable strategy achieved by one of the partners (Bronstein 2001a) or if cheaters evolve through a host shift. A study by Pellmyr et al. (1996a) provided phylogenetic evidence for the reversal of obligate mutualisms within the *Yucca* plant-yucca moth complex, where certain cheater species inflict high costs to the plant by ovipositing into already fertilised flowers and young fruits, and therefore do not pollinate the plant because they lack the necessary structures to transfer pollen.

4. PLANT-POLLINATOR INTERACTIONS

Some of the most well studied examples of coevolution and mutualism are those between insects and the plants which depend on them for pollination (Bronstein et al. 2006). Pollination, the transfer of pollen from male structures (anthers) to female structures (stigmas) to allow fertilization of ovules and formation of seeds, is carried out by insects in two thirds of flowering plants (angiosperms) (Schoonhoven et al. 2005b). In plant-pollinator interactions, one partner is sedentary while the other is mobile. Plants, being sedentary, will experiment natural selection in those traits that will allow them to be located more easily by their partners, such as colourful and scented flowers which appeal to the visual and olfactory senses of their pollinating partners (Bronstein et al. 2006). Typical pollinating insects include bees, wasps, flies, moths, butterflies or beetles. In exchange for their service, plants provide a nutritious reward to maintain the interest of their mutualistic partners while the services are still required (Bronstein et al. 2006). This reward is usually in the form of nectar or pollen; other less common rewards include oils, resins and shelter (Sun and Rychtář 2015).

Like any other mutualism, plant-pollinator interactions are not exempt of costs, and therefore, of the inherent conflicts of interest resulting from maximising the benefits received while minimising their own costs (Schoonhoven et al. 2005b, Santamaría and Rodríguez-Gironés 2015). From the perspective of the plant, the perfect partner would carry as many pollen grains as possible from the anthers of other conspecific plants to their stigmas and vice versa, in a rapid manner and would exclusively search for flowers of its own species (Schoonhoven et al. 2005b). Therefore, selection would favour the production of just enough nectar to be attractive to pollinators, but not enough to satiate them quickly, as this would mean a cease in their services (Schoonhoven et al. 2005b). On the other hand, from the perspective of the insect, optimal foraging theory (Charnov 1976) suggests pollinators would feed as much as possible while minimising energy and time costs to increase efficiency, which means that flowers with larger amounts of nectar should be preferred, even if it involves visiting flowers of several species in a single foraging trip (Schoonhoven et al. 2005b). Being the mobile partners of the mutualism, insect pollinators have the ability to compare between visited plants, and therefore natural selection will act on the capacity of attraction and rewards of plants (Bronstein et al. 2006).

The constantly changing costs and benefits of plant-pollinator interactions may be important to understand different degrees of interdependence and why some are more specialised than others (Mitchell et al. 2009). Generally, if a plant is visited by several pollinators providing comparable benefits and inflicting comparable costs, there is no reason for the plant to specialise in a particular pollinator or group. Still, if one or a group of floral visitors prove to be more efficient pollinators, selection should favour specialisation and those traits which attract them (Mitchell et al. 2009). Floral traits such as flower colour, shape or orientation, and nectar volume are known to influence pollinator attraction and fidelity (Johnson and Dafni 1998, Schemske and Bradshaw 1999, Fenster et al. 2004, Brothers and Atwell 2014). Fenster et al. (2004) demonstrated that indeed floral traits in plants have evolved as a result of selection pressures exerted by insect pollinators. This can result in the occurrence of so called "pollination syndromes" (Figure 5), which are a series of floral traits, including rewards, used for the attraction of a specific functional group of pollinators, which have appeared through adaptations between plants and their specialised pollinator groups (Waser et al. 1996, Fenster et al. 2004).

The opposite relation, meaning the evolution of pollinator traits as a response to selection pressures exerted by plants, and how interactions with particular plant species affect pollinator fitness, is not so well studied (Bronstein et al. 2006). Yet, there are some clear examples of evolved characteristics which aid pollinators in providing better services or extracting rewards. Such is the case with active pollination, where "specific morphological structures and behaviour components exist in the pollinator for the purpose of picking up and transporting pollen, and depositing it on stigmas" (Pellmyr 1997). Active pollination has been documented in few specialised plant – pollinator interactions: the *Ficus* plants and fig wasps system (Janzen 1979), *Yucca* plants and yucca moths (Pellmyr et al. 1996b), senita cacti and senita moths (Fleming and Holland 1998) and more recently in the *Glochidion* trees and *Epicephala* moth system (Kato et al. 2003). All the mentioned specialised interactions are obligate, and belong to a particular type of pollination mutualisms called nursery pollination systems.

Nevertheless, even though specialisation does occur in certain pollination systems in nature, a majority of plant-pollinator interactions seem to be generalised: most plant species are pollinated by floral visitors from very different taxa rarely depending on a single species, and most pollinators also visit multiple plant species (Waser et al. 1996, Bronstein et al. 2006).

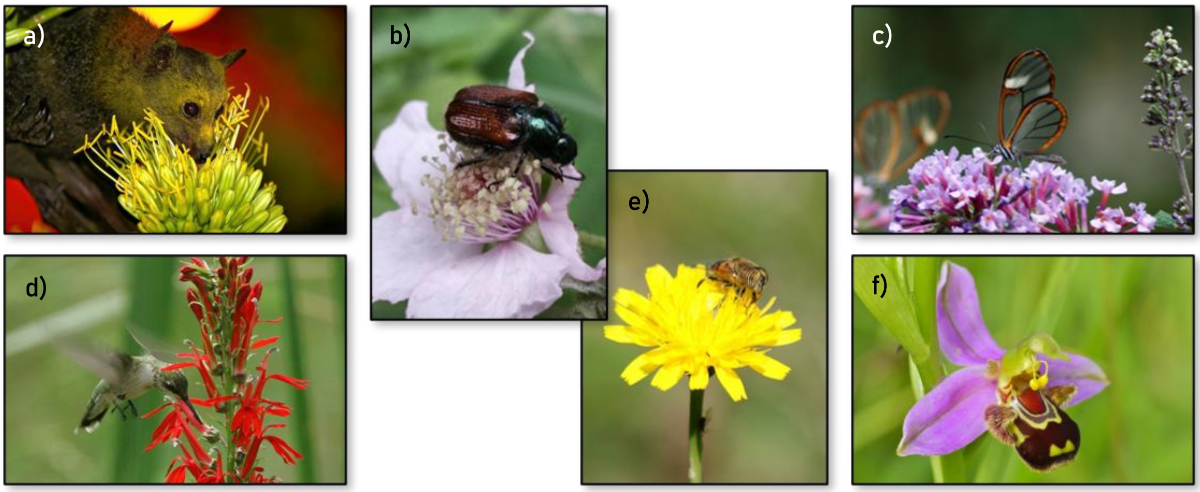

Fig. 5 A variety of pollination syndromes. a) Black flying fox (*Pteropus alecto*) feeding on plant nectar. Credit: Andrew Mercer. Bat-pollinated flowers are usually large, either bell-shaped or long and tubular, light coloured, musty scented and are opened at night (Fleming et al. 2009). b) Scarab beetle on a bramble flower. Credit: Carmen Villacañas de Castro. Flowers pollinated by beetles tend to be large and green or dull coloured, with strong scents and usually have a dish shape with readily accessible pollen (Gullan and Cranston 2005). c) *Greta oto* butterfly feeding on a *Buddleia* sp. Credit: Carmen Villacañas de Castro. Butterfly pollinated flowers tend to be very visible and colourful, often large or forming inflorescences, sweetly scented and tubular, allowing the butterfly to drink nectar with their long proboscis (Gullan and Cranston 2005). d) Ruby-throated hummingbird at a cardinal flower. Credit: public domain. Flowers specialised on hummingbird pollination tend to be bright coloured (usually red, pink, orange, or purple), long and tubular with a pendulous shape, often odorless, and with high amounts of sweet nectar (Fenster et al. 2004). e) Syrphid fly collecting nectar and/or pollen from a yellow hawkweed flower. Credit: Carmen Villacañas de Castro. Fly-pollinated flowers are usually more plain than other but in some specific cases can have fetid smells mimicking that of decaying animals or faeces (Gullan and Cranston 2005). f) *Ophrys apifera* orchid attracts male *Eucera* sp. bees by mimicking the scent and resemblance of the female bees (Fenster and Martén-Rodríguez 2007). Credit: Bernard Dupont.

5. NURSERY POLLINATION SYSTEMS

A particular type of pollination interactions, defined as "nursery pollination systems" (*sensu* Dufay and Anstett 2003), have been receiving quite some attention in the past years (Westerbergh 2004, Kephart et al. 2006, Reynolds et al. 2012, Labouche and Bernasconi 2013, Prieto-Benítez et al. 2017). In these interactions the insect acts as a pollinator, but it also lays eggs in or on the plant, which therefore is providing the pollinator with a breeding site for its offspring, as well as some kind of nutritious reward for itself (Holland and Fleming 1999). Breeding its own pollinators might be a mechanism for plants to specialise on a particular flower visitor, but the costs associated may be high (Pettersson 1991, Fleming and Holland 1998, Després 2003), as the offspring will develop and feed from the reproductive structures of the plant, which very often will be the seeds (Dufaÿ and Anstett 2003, Kephart et al. 2006). Therefore, the insects are pollinators and herbivores of the host plant simultaneously. For the plants, the benefits obtained from the pollination services, and the costs of predation can both be easily quantified in terms of seeds (Bronstein 2001a). For the insects, fitness benefits will be measured in terms of offspring, and costs will be measured in time and energy spent flying between flowers and pollinating. Still, for the interaction to remain mutualistic the benefits must balance out the costs of the interaction, otherwise the interaction would be evolutionarily unstable and eventually turn into a parasitic one (Bronstein 1994, Pellmyr et al. 1996a, Kawakita et al. 2015).

Nursery pollination systems and their conflicts of interest were reviewed by Dufaÿ and Anstett (2003), describing a total of 13 documented cases of interactions, with diverse degrees of specialisation. Since then other systems have been discovered (see Kato et al. 2003, Kawakita and Kato 2004, Song et al. 2014, Nunes et al. 2018). Highly specialised, co-evolved and obligate mutualisms include the interactions between *Ficus* plants and fig wasps (Janzen 1979) and *Yucca* plants and yucca moths (Pellmyr et al. 1996b), which are considered "perfect" mutualisms (Dufaÿ and Anstett 2003). Other interactions in this group are not specialised to that degree, such as the partnership between *Greya* moth and its host plant, *Lithophragma parviflorum*, which lacks active pollination and where copollinators are present (Thompson and Pellmyr 1992). In years or populations were copollinators are responsible for a large portion of the seed set, the pollination benefits from *Greya* moths are masked, and therefore the interaction becomes commensalistic (Thompson and Pellmyr 1992). On the other hand, interactions between host plants from the Caryophyllaceae family and pollinators/herbivores from the *Hadena* genus range from antagonisms to facultative mutualisms (Kephart et al. 2006, Prieto-Benítez et al. 2017).

As we have seen, the outcome of pairwise interactions highly depend on the environmental context in which they take place. This is also the case for nursery pollination systems, where the cost and benefits of both partners can be affected by the presence or absence of third parties such as copollinators, exploiters, or natural enemies of the pollinator/herbivore (Pellmyr 1989, Thompson and Pellmyr 1992, Holland and Fleming 2002, Bronstein et al. 2003, Gomulkiewicz et al. 2003, Harrison 2014). Therefore, such interactions could also potentially change positions along the continuum ranging from mutualistic to antagonistic outcomes (Bronstein 1994, Thompson and Cunningham 2002, Dufaÿ and Anstett 2003, Westerbergh 2004, Bronstein et al. 2006, Thompson and Fernandez 2006). Moreover, certain life history traits could predict when obligate mutualisms may develop between a plant and its pollinator (Addicott et al. 1990, Waser et al. 1996, Holland and Fleming 1999). Long-lived plants with several reproductive episodes are likely to specialise on an efficient pollinator if its population dynamics are predictable, while a pollinator may specialise on its host plant when there is a synchronization of the flowering phenology and the life cycle of the pollinator, and when its generation time is not longer than the flowering season of the host plant (Addicott et al. 1990, Waser et al. 1996).

5.1 The fig-fig wasp interaction: a perfect mutualism

Fig trees (*Ficus* spp) are pollinated by wasps of the Agaonidae family (Hymenoptera, Chalcidoidea). Due to their exceptional coevolution and specialisation, the life cycles of both species are tightly connected. Janzen (1979) described the mutualistic interaction in detail: fig flowers are enclosed inside a fleshy type of inflorescence called "syconium" (Figure 6). At first, a female wasp will enter the syconium through a small opening called "ostiole", a process in which she usually loses her wings. The female wasp will actively pollinate the flowers by spreading pollen she picked up from her first host fig on the stigmas of the female flowers. She will also oviposit eggs in some of the female flowers of the syconium, inside the ovule, before dying. Ovules that received eggs develop into a gall and, as the eggs hatch, larvae will feed on the developing seeds, predating upon approximately 50% of the seeds (Janzen 1979). In monoecious figs, each female flower inside the syconium will either produce

Fig. 6 Adult *Seres rotundus* fig wasps emerging from the syconium of a larged-leaved rock fig, *Ficus abutilifolia.* Credit: Alan Manson.

seeds or a pollinator wasp (Anstett et al. 1996). Dioecious figs are even more specialised, and female trees will have seed producing syconia, while male trees will produce pollen and pollinators (Harrison 2014). Once the wingless male wasps emerge from their galls, they mate with female wasps through a small hole in the galls, and then exit the syconium creating a tunnel, dying short time after (Harrison 2014). At this point, winged females emerge from their galls and pack pollen from the male flowers, exiting the syconium through the tunnel previously dug by the males, ready to look for another fig tree to pollinate (Janzen 1979). The syconia quickly mature into fig fruits, which will be eaten by vertebrate frugivores (Harrison 2014). By looking at the life cycles it is clear that both organisms are fully dependent on each other for their reproduction, which might be the reason for their success (Harrison 2014).

The fig-fig wasp, like the yucca-yucca moths mutualisms, differ from other nursery pollination systems because they form very specific pairs of interactions where copollinators are absent and where pollination is carried out actively (Holland and Fleming 1999). From the 900 species of *Ficus* trees many have their own species of agonid fig wasp pollinator (Janzen 1979). Given that the only pollen vectors of fig trees are the wasps born within each syconium, the host plants are selected to both produce seeds and rear their pollinators (Anstett et al. 1996). The interaction is evolutionarily stable, producing enough seeds to sustain both the host plant and the pollinator (Westerbergh and Westerbergh 2001).

6. THE *Silene-Hadena* SYSTEM

From the known nursery pollination interactions, Kephart et al. (2006) and later Prieto-Benítez et al. (2017) reviewed a complex formed by plants of the Caryophyllaceae family and moths from the *Hadena* genus. They found a total of 21 different *Hadena* species which predated upon flowers and seed capsules of 70 caryophyllaceous host plants during the larval stage, interactions with very different outcomes from mutualisms to antagonisms. One of the interactions belonging to this complex is formed by host plant *Silene latifolia* and its pollinator/herbivore partner *Hadena bicruris.* A facultative mutualism, and even considered a "primitive" stage of nursery pollination mutualisms compared with the highly specialised fig-fig wasp mutualism (Dufaÿ and Anstett 2003, Giménez-Benavides et al. 2007, Bernasconi et al. 2009), it is often referred to as parasitic due to the extent of seed predation (Giménez-Benavides et al. 2007, Reynolds et al. 2012, Brothers and Atwell 2014, Kula et al. 2014, Prieto-Benítez et al. 2017). This system has been studied in depth in the past decades to research different biological questions (Bernasconi et al. 2009): from the evolution of sex chromosomes (Kejnovsky and Vyskot 2010), biotic interactions (Elzinga et al. 2003, Biere and Honders 2006, Jolivet and Bernasconi 2006, Elzinga et al. 2007a, Elzinga et al. 2007b, Cozzolino et al. 2015, Litto et al. 2015, Stucchi et al. 2019), and how they are affected by human activity (Elzinga et al. 2005, Geestman 2013), to biological invasions (Wolfe 2002, Blair and Wolfe 2004) or disease evolution and ecology (Antonovics et al. 2002). Nevertheless, despite of the extensive knowledge of the system, there are still interesting unresolved questions in the ecology and evolution of this interaction (Bernasconi et al. 2009), which will be crucial to answer and, thus, will be the main focus of this thesis.

6.1 *Silene latifolia*: the host plant

The white campion, *Silene latifolia* POIR. (= *S. alba* [MILL.] Krause, *Melandrium album* [MILL.] Garcke) is a short-lived perennial weed of the family Caryophyllaceae, native to Europe and the Mediterranean region (Baker 1947). Normally found in open disturbed habitats such as field margins, roadsides, or grazing fields (Baker 1947), it tends to exist in natural metapopulations, which can lead to drastic changes in population size or even extinctions (Richards et al. 2003). It is dioecious with male (Figure 7a) and female (Figure 7b) plants and therefore an obligate out-crossing species, fully depending on pollinator visitation for successful reproduction. Field populations of *S. latifolia* usually have a female biased sex ratio (Purrington 1993). *Silene latifolia* emerges in early spring, blooms from May to October and overwinters as a rosette (Jolivet and Bernasconi 2007). The flowers have white petals which open at dusk, emitting a lilac fragrance (Dötterl et al. 2005), and close up again in the late morning, although in cool cloudy days they may remain open (Kephart et al. 2006). All of these traits are typical for plants with a nocturnal pollination syndrome (Baker and Hurd 1968), which suggests there has been some specialisation, at least to a functional group of pollinators. Both female and male flowers produce nectar as a reward for pollinators, with a production peak in the evening hours (Barthelmess et al. 2006). It is pollinated mainly by the noctuid moth *Hadena bicruris*, its specialist pollinator/herbivore (Brantjes 1976b, Jürgens et al. 1996, Elzinga et al. 2005, Anderson et al. 2015). However, there is a variety of less efficient nocturnal and diurnal copollinators visiting the flowers (Jürgens et al. 1996, Young 2002, Barthelmess et al. 2006, Anderson et al. 2015), and therefore the interaction with *H. bicruris* remains facultative. Female flowers have a long corolla tube, and once they have been pollinated, they wilt (Barthelmess et al. 2006) and cease scent emission and nectar production within the next hours (Dötterl et al. 2005, Muhlemann et al. 2006). After pollination, a fruit is formed, also called a seed capsule, in which typically several hundreds of seeds will develop (Jolivet and Bernasconi 2007). Five to eight weeks after pollination, dehiscent fruits scatter the mature seeds which will be dispersed by gravity. Seeds can remain within the capsules, which then will be buried after the plants stems decay (Purrington and Schmitt 1995). Consequently, it has a short dispersal range, with relatively large groups of sibling seeds coexisting in close proximity within the seed bank (Purrington and Schmitt 1995, Barluenga et al. 2011).

Although dioecy is widespread in plants, this characteristic makes *Silene* plants quite unique within the group of nursery pollination systems. In all other systems consisting of dioecious plants reviewed by Dufaÿ and Anstett (2003), the larvae attacks tissues with male sexual function. In these cases, the cost to the plant is low, as the pollen has already been dispersed, and the reproductive episode is over (Dufaÿ and Anstett 2003). However, in the case of *Silene*, the sexual function of the tissues eaten by the larvae of the pollinator is the female one, consisting usually of fruit and seeds, thereby imposing high costs to the plant (Dufaÿ and Anstett 2003). These high costs and sex-specific fitness consequences due to the attack upon the female plants, makes *Silene* the perfect model plant to study the early stages of evolution in nursery pollination mutualisms (Westerbergh 2004).

6.2 *Hadena bicruris*: the pollinator/herbivore

Adult lychnis moths, *Hadena bicruris* HUFN. (Lepidoptera; Noctuidae) (Figure 7c) have a wingspan of 30-40mm and are good colonisers, with dispersal ranges of more than 2km per generation (Elzinga et al. 2005). The only sign of sexual dimorphism is the presence of the ovipositor in adult female moths (Magalhaes and Bernasconi 2014). *Hadena bicruris* moths have a dual role in their partnership with *S. latifolia*. On the one hand they are the main pollinator of *S. latifolia* plants in its native range, but this species is also their most important seed predator (Brantjes 1976b, Elzinga et al. 2005, Kephart et al. 2006). Male and female adults are nocturnal and fly from male to female *S. latifolia* plants, drinking the nectar reward and passively pollinating the flowers between the months of May and October (Elzinga et al. 2005, Burkhardt et al. 2009). Both female and male moths are efficient pollinators (Labouche and Bernasconi 2010). Female moths oviposit usually a single egg inside or on female flowers, and "mark" (with a volatile oviposition deterrent) the flowers that have already been parasitised to avoid superparasitism (Brantjes 1976a, b, Roitberg and Prokopy 1987). Female moths are able to discriminate male and smut infected flowers and avoid laying any eggs on them (Page et al. 2014). Typically, female moths can lay up to 40 eggs per night (Elzinga et al.

2005) and oviposition decision are taken during nectaring and probing of the flowers (Page et al. 2014). After egg eclosion, the first instar larva enters the fruit by eating off a tiny hole in the fruit wall, and starts feeding on the developing seeds inside the seed capsule (primary seed capsule) (Elzinga et al. 2005), where it is protected from natural enemies such as parasitoid wasps (Brantjes 1976b). On rare occasions, females will lay more than one egg per flower, but due to larval competition and cannibalism in the early instars, fruits will hold a single larva (Brantjes 1976a). The same entrance hole is gradually enlarged and used to discharge the faeces from the capsule (Elzinga et al. 2007b). Once it has consumed all developing seeds in the primary seed capsule the larva, which is usually in its fourth instar, leaves to find secondary capsules to continue feeding, destroying two to five other capsules on the same plant (Brantjes 1976a, Elzinga et al. 2005). At this stage, the larva might be too

Box 2. Parasitoids

A special type of parasite, a parasitoid is an insect whose offspring feeds and develops within (endoparasitoids) or on the surface (ectoparasitoid) of the bodies of other arthropods, eventually killing the host (Thompson 2019). Female parasitoids deposit one (solitary parasitoid) or several eggs (gregarious parasitoid) through their ovipositor in the eggs, larvae or pupae of their hosts, providing a single host to each clutch. Before oviposition, parasitoids first immobilise their host by stinging it with the ovipositor and injecting paralysing venom (Godfray 1994). In most cases, ectoparasitoids are also idiobiont, and the venom prevents any further development of the host (Askew & Shaw 1986). On the other hand, endoparasitoids tend to be koinobionts, and only partially paralyze their host, which eventually recovers and continues its development until the parasitoid offspring reaches maturity (Askew & Shaw 1986). This means that koinobiont parasitoids develop in more hostile environments as they have to fight the immune systems of the host, so they are often specialised and have short host ranges (Lopez-Vaamonde et al. 2005; Desneux et al. 2012). Ectoparasitoids, on the contrary, tend to be generalists and have wider host ranges (Askew & Shaw 1986). Although most parasitoids are wasps or flies, there are also parasitoid species from other taxa such as beetles, moths or lacewings (Thompson 2019).

large to fit completely inside secondary capsules, and part of its body might be exposed to natural enemies (Figure 7d) such as parasitoids (Biere et al. 2002) (Box 2). Around 3-4 weeks after egg eclosion, the larva will pupate either hidden inside a capsule (personal observation) or by slightly burying in the soil (Elzinga et al. 2007b). In field conditions, adults will hatch approximately three weeks after pupation or they will enter diapause to overwinter (Elzinga et al. 2003).

Hadena bicruris is widely spread in *S. latifolia* populations in Western Europe, where it has a prevalence of over 90%, although at varying densities (Elzinga et al. 2005). With a 25-70% fruit destruction (Biere and Honders 1996, Wolfe 2002, Elzinga et al. 2005), the degree of damage varies greatly and so does its impact on plant fitness. This facultative interaction is usually referred to as antagonistic, with *H. bicruris* parasitising *S. latifolia* (Brantjes 1976b). However, the degree to which it may be antagonistic may vary from year to year, depending on the ratio between pollination benefits and predation costs. This gives us a unique opportunity to study different ecological contexts in which the same partners of an interaction produce opposite results, and therefore better understand or even predict under which ecological circumstances these interactions may became mutualistic and persist in time (Dufaÿ and Anstett 2003, Kephart et al. 2006, Bernasconi et al. 2009, Kula et al. 2014).

6.3 A third player: *Bracon variator*

In a model developed by Bronstein et al. (2003), natural enemies had the potential to stabilise mutualisms by altering population sizes of their partners, while other studies such as Nunes et al. (2018) have described parasitoids mediating in the outcome of interactions by changing the cost/benefit ratio of the partnership. As already mentioned, at a certain stage in its development the larva of *H. bicruris* might be exposed to natural enemies. Indeed, several parasitoid species have been found to attack *H. bicruris* in the field. The parasitoid complex associated to the *S. latifolia-H. bicruris* system was described in depth by Elzinga et al. (2007b). The parasitoid species with a higher incidence parasitising *H. bicruris* are *Microplitis tristis* (Hymenoptera; Braconidae) and *Eurylabus tristis* (Hymenoptera; Ichneumonidae) (Elzinga et al. 2007b). *Microplitis tristis* and *E. tristis* are gregarious and solitary koinobiont endoparasitoids, respectively, and most frequently attack *H. bicruris* larvae in their L4 and L5 instars. Thus, the seed predating larva is killed only after substantial damage has been inflicted to the plant (Elzinga et al. 2003, Elzinga et al. 2007b). In addition, *E. tristis* offspring hatches only after the host has pupated, having therefore an even smaller impact on the reduction of herbivory by *H. bicruris* (Elzinga et al. 2007b). On the other hand, parasitoid wasp

Bracon variator NEES (Hymenoptera; Braconidae) (Figure 7e) has also been found to attack *H. bicruris* larva, although at much lower frequencies in the field (Elzinga et al. 2007b). Being an idiobiont ectoparasitoid, *B. variator* oviposits on the tegument of the host, immobilising it by injecting a paralysing venom with its ovipositor. This also prevents any further development of the host larva (Askew and Shaw 1986). Once they hatch, the wasp larvae feed on the body fluids of the host (Figure 7f). This parasitoid usually attacks hosts in their their L3-L5 instars, slightly sooner than *M. tristis* and *E. tristis*. Most importantly, by arresting host development, parasitoid attack prevents any further seed consumption by the herbivore (Elzinga et al. 2007b). This means, that *B. variator* has the potential to cause a decrease in predation of *S. latifolia* seeds by parasitising *H. bicruris* larvae, limiting its population size and possibly reducing the costs of the interaction for the plant.

7. SIGNS OF SPECIALISATION IN THE *S. latifolia-H. bicruris* INTERACTION

The interaction between *S. latifolia* and *H. bicruris* is under constant debate as to whether it consists of a facultative mutualism or a parasitism. Dufaÿ and Anstett (2003) even stated that the interaction had the lowest degree of specialisation among the nursery pollination systems. Although not to the degree of the fig-fig wasp “perfect” mutualism, subsequent studies have found evidence for potential specialisation between the host plant and its most efficient pollinator/herbivore, which I will explore below.

7.1 Distribution and Habitat

The most obvious requirement for specialisation to occur between species is a similar distribution pattern and habitat preference, as otherwise the species would not coexist. In theory, the distribution of a parasite should not exceed that of its host, while mutualists should have matching geographic distributions (Bopp and Gottsberger 2004). In this case, the host plant can be found in the whole area of distribution of *H. bicruris,* although the distribution range of *S. latifolia* exceeds that of the pollinator/herbivore (Bopp and Gottsberger 2004), as it can be found as an invasive plant species in North America, where *H. bicruris* is absent (Wolfe 2002). Both species also share habitat preferences for warm and dry conditions, and avoid higher elevations (Bopp and Gottsberger 2004).

Fig. 7 The *Silene latifolia–Hadena bicruris–Bracon variator* multitrophic system. a) Male *S. latifolia* flower. Credit: Rosa-Maria Rinkl. b) Female *S. latifolia* flower. Credit: public domain. c) Adult *H. bicruris* moth. Credit: Patrick Clement. d) Larva of *H. bicruris* predating on a *S. latifolia* secondary seed capsule. e) A female ectoparasitoid wasp *B. variator* (notice the prominent ovipositor) foraging on a *S. latifolia* seed capsule. f) A larva of ectoparasitoid *B. variator* feeding off a parasilised *H. bicruris* larva. Photos d)-f) were kindly provided by Jeroen Voogd.

7.2 Phenology

Studies have shown that pollinators can act as a selective force on phenology and floral display through their positive impact on seed set (Elzinga et al. 2007a, Sandring and Ågren 2009, Chen et al. 2017), as plants —especially obligate out-crossers— need to be fertilised. In contrast, pre-dispersal seed predation could also act as a selective force in host plants, shifting their phenology away from peak herbivore activity to avoid high predation costs (Biere and Honders 1996, Pilson 2000, Kolb et al. 2007). However, differentiating between the effects of correlated traits is a hard task. In this particular case, *H. bicruris* has a dual role as an efficient pollinator and a seed herbivore, being possible that both factors impose selection on flowering phenology, and that the end result is a compromise between the different conflicting selection pressures (Pilson 2000, Elzinga et al. 2007a, Ehrlén and Münzbergová 2009). On the one hand, Bopp and Gottsberger (2004) studied the oviposition phenology of *H. bicruris* and compared it to the flowering phenology of *S. latifolia*, and their research showed a remarkable coincidence between both broods of *H. bicruris* (as a bivoltine species it produces two generations per year) and both flowering peaks of *S. latifolia* (Biere and Honders 1996, Elzinga et al. 2005). Alternatively, asynchrony with host plant phenology has a detrimental impact on herbivore fitness, and selection can act to link the life cycles of herbivores with phenological events of their host plants (Biere and Honders 1996). The fruits of *S. latifolia* develop while the young *H. bicruris* larva is inside its primary capsule. During the first instars, the larva can only feed on tender developing seeds, and only after the larva has reached the fourth instar and leaves the primary capsule, the seeds ripen (Bopp and Gottsberger 2004). It is apparent that the life cycles and phenology of both species are in synchrony. However, we cannot know in which direction selection took place, whether *S. latifolia* adjusted flowering time and seed maturation to the period of activity and development of *H. bicruris* larvae as future pollinators, or if it was vice versa (Bopp and Gottsberger 2004).

7.3 Pollination syndrome and floral traits

Previously in this chapter, it was discussed how selection can favour certain traits in plants to attract its most efficient pollinator (Mitchell et al. 2009), resulting in so called pollination syndromes. *Silene latifolia* shows a typical nocturnal pollination syndrome, with tall, erect flowering stems with large, white scented flowers that open at dusk (Baker and Hurd 1968, Dötterl et al. 2005), all traits expected to attract *H. bicruris* moths (Kephart et al. 2006). A recent study by Brothers and Atwell (2014) showed that indeed *H. bicruris* visitation was influenced by flower height and size, as well as number of flowers, a pattern consistent with the expectation that *S. latifolia* has evolved traits attractive to moths. Jürgens et al. (1996) found a correlation between mean proboscis length in *H. bicruris* moths and the calyx length in *S. latifolia* flowers, suggesting that it was a strong indication of adaptation of the host species to its most efficient pollinator.

Floral scent is considered one of the traits to have evolved and diversified in response to preferences of visiting pollinators, as nocturnally pollinated plants tend to rely on strong floral scents for attraction (Jürgens et al. 2002, Fenster et al. 2004, Dötterl et al. 2006). In specialised interactions, flower scents serve as a mechanism of communication between host plants and pollinating insects (Dötterl et al. 2005, Dötterl et al. 2006). As seen above, the pollination syndrome of *S. latifolia* reveals a certain degree of specialisation to maximise nocturnal moth pollination (Kephart et al. 2006). To expand on this question, Jürgens et al. (2002) analysed the chemical profile of *S. latifolia* floral scent, and a few years later Dötterl et al. (2006) studied which of those chemical compounds elicited an antennal and behavioural response in *H. bicruris* moths. The latter study concluded that there was an electrophysiological and behavioural adaption of *H. bicruris* to its most important host plant, and that lilac aldehydes (compounds not often found in floral scents) were used as key volatile markers for *S. latifolia* location. Moreover, these specific key compounds make up approximately 80% of the floral scent of the host plant (Muhlemann et al. 2006). *Silene latifolia* plants stop emitting scent almost completely after 24 hours of being pollinated (Dötterl et al. 2005, Muhlemann et al. 2006), and the same key compounds contribute the most to such decrease in scent, while the absolute amounts of the majority of other compounds did not change after pollination (Muhlemann et al. 2006). Muhlemann et al. (2006) interpreted that the drastic reduction in key volatile compounds used for locating *S. latifolia* plants was a mechanism to avoid further parasitism by the pollinator/herbivore *H. bicruris*, while directing the moths to remaining unpollinated flowers and this way maximise overall reproductive success.

7.4 Defences against over-exploitation

In nursery pollination systems, minimising predation costs is essential for a mutualistic outcome, as otherwise the interaction would be evolutionarily unstable (Bull and Rice 1991, Bronstein 1994, Pellmyr et al. 1996a, Kawakita et al. 2015). This should lead to the evolution in plants of strategies to prevent over-exploitation from their pollinator/herbivore partners. Recent studies suggest that plants from the Caryophyllaceae family have evolved phenological, chemical, morphological or physiological adaptations to limit over-exploitation

(Prieto-Benítez et al. 2017). However, to reduce interaction costs without reducing the benefits from pollination services offered by the adult moths is a complicated task. Some of the known strategies include changes in flowering phenology and floral scents (briefly mentioned above), but there are also other known defence mechanisms in the *S. latifolia-H. bicruris* interaction.

Fig. 8 *Silene latifolia* infested seed capsule about to be abscised from the plant. Credit: Public domain.

One well studied mechanism is selective fruit abortion (Figure 8), which has been previously documented in the yucca-yucca moth nursery pollination system when egg loads are high or flowers have received low quality pollen (Huth and Pellmyr 2000). Jolivet and Bernasconi (2006) tested whether the rate of selective fruit abortion varied in response to experimental predation, and found a significant six-fold increase. By abscising primary infested fruits (thus, primary seed capsules with young larva), *S. latifolia* may potentially reduce seed predation of secondary capsules in the same plant, and free the resources intended to develop the infested fruits, reallocating them into flower production (Wright and Meagher 2003, Jolivet and Bernasconi 2006). However, to be effective as a defence, abortion of infested fruits needs to lower larval survival or decrease reproductive success in the adult moths, regulating their population density, otherwise it would not reduce the costs of predation (Shapiro and Addicott 2004). Burkhardt et al. (2009) demonstrated that indeed selective fruit abortion had a negative effect in *H. bicruris* larval fitness, reducing mass and anticipating emergence from the primary capsule due to lack of resources, implying higher risks of predation and parasitism.

A defence mechanism acting prior to selective fruit abortion could further help reduce costs and save resources. Labouche and Bernasconi (2013) thought that oviposition site within the flower could affect success of fruit attack and predation costs. To test this idea, they carried out experiments to study whether female floral traits could predict oviposition site, and whether variations in egg positions could affect plant and moth fitness. They found novel evidence that a higher length of the corolla tube affected the ability of moths to insert the abdomen in the flower, forcing them to oviposit on the petal instead of directly touching the ovary. This constraint reduced the risk of successful fruit predation and therefore directly decreased the costs of the interaction (Labouche and Bernasconi 2013). As reported in other systems, eggs placed on the petal had higher mortality, and larva emerging from eggs inside the flower had a 150% higher chance to become established (Holland and Fleming 1999, Shapiro and Addicott 2003, Labouche and Bernasconi 2013). However, both egg positions implied costs for the plant, as larvae that hatched from eggs oviposited inside the flower were smaller, and so were those fruit capsules (probably due to a reduced resource allocation in infested fruits). Therefore, a trade off might exist for moths, to either cope with low larval fitness or maximise success of attack (Labouche and Bernasconi 2013). Finally, because pollination services in this species is independent to oviposition behaviour (Labouche and Bernasconi 2010), there is no trade off with pollination services offered by the adult moths (Labouche and Bernasconi 2013).

9. AIMS AND THESIS OUTLINE

In summary, even though there are evident signs of a certain degree of specialisation between the partners, the interaction between host plant *S. latifolia* and pollinator/herbivore *H. bicruris* represents an early stage of nursery pollination mutualisms, with clear conditionality. The ecological context, particularly third parties such as copollinators or natural enemies, may play an important role in determining the outcome of the interaction. Therefore, it constitutes an excellent study system to explore under which ecological circumstances interactions may behave as a facultative mutualism and perhaps evolve into more specialised interactions.

In nursery pollination systems, it is easy to quantify the costs and benefits from the perspective of the host plant: benefits will be obtained as pollination services and number of seeds produced, while the costs can be quantified as predation or number of seeds eaten. However, while the costs and benefits of the pollinator/herbivore are harder to quantify being a mobile partner, the outcome of the interaction for the pollinator/herbivore is expected to be positive. The work here presented has therefore focused on the outcome of the *S. latifolia*–*H. bicruris* interaction from the perspective of the plant. The experimental work carried out for this thesis can be divided into two main sets, which I have written as separate chapters. Each of the chapters is a compilation of experiments, which derive logically one from the other.

In CHAPTER 2, I investigate the conditional outcomes of the *S. latifolia-H. bicruris* system in the field. To be able to evaluate the nature of the interaction and understand what are the causes leading to such conditional outcomes, it is necessary to know the costs and benefits in the system. There is still a lack of knowledge in relation to the benefits provided by adult *H. bicruris* moths on plant reproduction. The present thesis tackles this issue by directly quantifying the positive contributions of male and female adult moths to seed production in *S. latifolia* plants. Moth pollinating behaviour and pollination efficiency are examined through observational assays. This is followed by field data collections and the application of a theoretical model to determine interaction outcomes in each field and the possible role of copollinators.

In CHAPTER 3 I study the potential role of a natural enemy, the ectoparasitoid *B. variator*, in modifying the interaction outcome between the plant and the herbivore along the continuum ranging from mutualism to antagonism. In particular, I examine whether the effect of the predator acts as a stabilising mechanism, reducing the level of seed consumption and therefore the costs caused to the plant by *H. bicruris* through seed predation. This is followed by an investigation on the possible consequences in individual plant fitness, with a series of laboratory and greenhouse experiments were germination, survival, and flower production are measured as proxies for individual plant fitness.

Finally, in the General Discussion (CHAPTER 4) I wrap up the thesis and address the research carried out as a whole and any insights gained from this project. I summarise my findings, discussing them in regard to the different ecological and evolutionary concepts explained in the General Introduction, as well as relating them to other studies.

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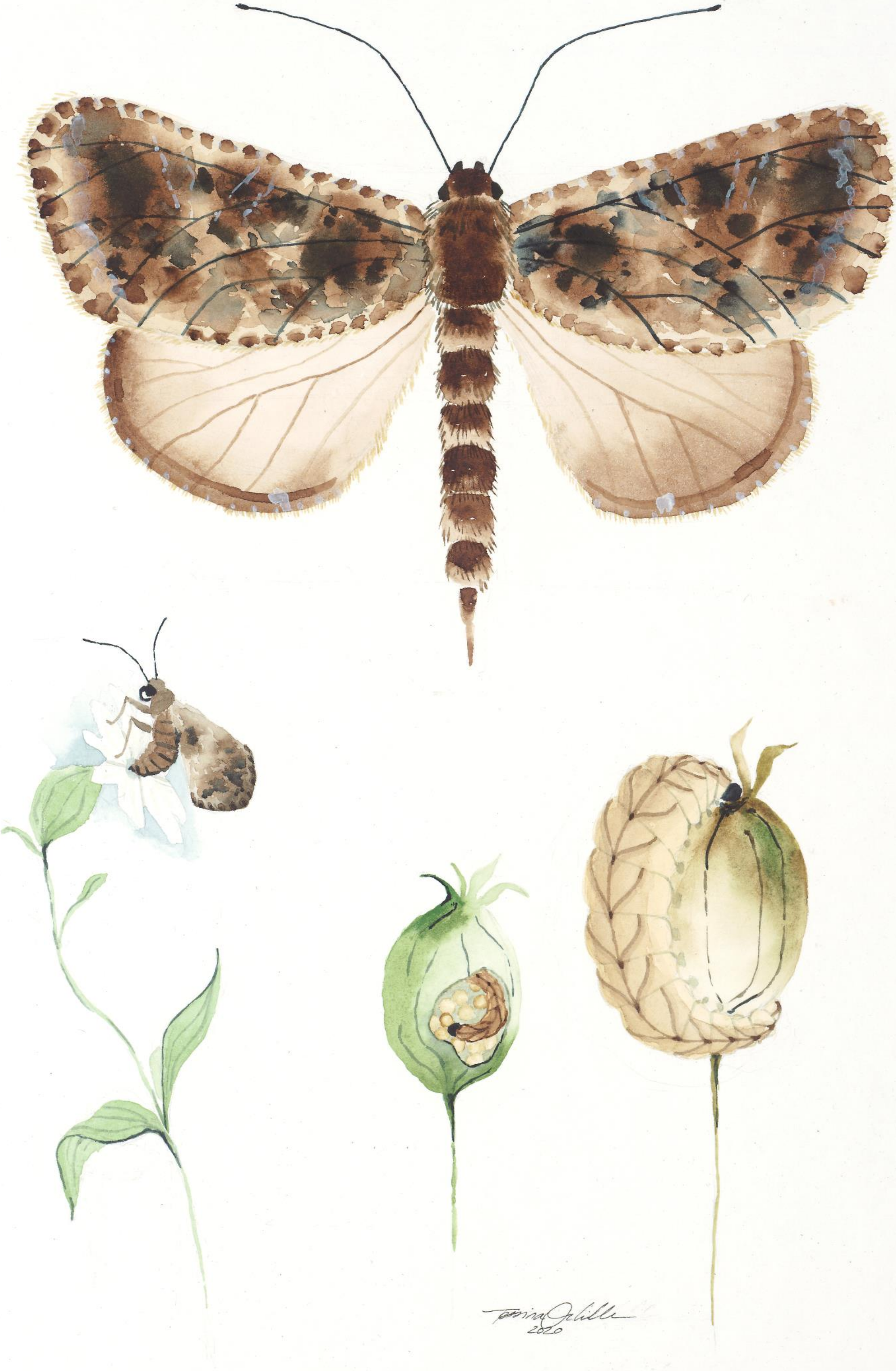

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CHAPTER 2

Cost/benefit ratio of a nursery pollination system in natural populations: a model application

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ABSTRACT

Nursery pollination systems are a type of mutualistic interaction in which a pollinating insect also uses its host plant to reproduce. While the host plant partner depends on the associated insect for pollination, the insect depends on the reproductive structures of the host plant for larval development. The outcome of these interactions depends on the balance between benefits and costs, and can vary according to the ecological context or the third parties present. Less specialised systems, such as the interaction between the plant *Silene latifolia* and its pollinator/herbivore *Hadena bicruris,* may fluctuate along a gradient between mutualism and antagonism. To better understand the origin of such conditionality, we need to know the costs and benefits in the system. Therefore, we first quantified the positive contribution of male and female adult moths to seed production in *S. latifolia* plants. Moth pollinating behaviour and pollination efficiency were examined through observational assays. This was followed by field data collections from five different populations and the analysis of pollination and infestation rates, as well as parasitism rate by parasitoid wasp *Bracon variator*. Finally, we applied a theoretical model to determine interaction outcomes in each field, as well as the possible role of copollinators. Our results clearly showed that *H.bicruris* male pollination benefits are essential for the stability of the system. Pollination, infestation and parasitism rates highly varied between fields, although some of the factors causing such variability remained unclear. The amount of copollination turned to be a good predictor for the outcome of the system, as only those fields with low copollinator presence behaved mutualistically. Overall, it is clear that third parties associated to the *S.latifolia-H. bicruris* system —specially copollinators and natural enemies— play an important role modifying the outcome of the interaction.

KEYWORDS: *Silene-Hadena* system, nursery pollination, cost/benefit ratio, mutualism.

1. INTRODUCTION

Nursery pollination systems (*sensu* Dufaÿ and Anstett 2003) are a particular type of mutualistic interaction in which a pollinating insect also uses its host plant to reproduce. The insect (normally a moth, fly, or wasp) will pollinate the flowers and at the same time lay eggs in or on the plant, meaning that the offspring will develop and feed on the fruits, seeds, or other tissues of the same plant. This could imply high costs for the plant, as it is pollinated at the expense of its own reproductive structures or even offspring (Dufaÿ and Anstett 2003, Kephart et al. 2006). However, in a mutualistic interaction the costs and benefits ratio between the pollinator/herbivore and the host plant must have a positive net outcome, otherwise the mutualism would evolve into a parasitic interaction (Bronstein 1994, Pellmyr et al. 1996, Kawakita et al. 2015).

Dufaÿ and Anstett (2003) reviewed nursery pollination systems and described 13 documented cases, and since then other interactions have been discovered (see Kawakita and Kato 2004, Song et al. 2014, Nunes et al. 2018). The nature of the interactions within this group ranges from obligate mutualisms to antagonisms. Characteristics of obligate mutualistic systems include active pollination of the host plant and an absence of copollinators in the system. Some well-known examples include the *Ficus* trees and fig wasps, *Yucca* plants and yucca moths and senita cacti and senita moths (Anstett et al. 1996, Pellmyr et al. 1996, Holland and Fleming 1999, Dufaÿ and Anstett 2003). Here, the level of specialisation and coevolution between the interacting partners is such that the system remains very stable over time, facilitating the evaluation of costs and benefits for each partner. Nonetheless, there are other less sophisticated systems where third parties (such as copollinators, exploiters, predators, or parasites) are present and may play an important role modifying the interaction and its cost/benefit ratio (Holland and Fleming 2002, Bronstein et al. 2003, Gomulkiewicz et al. 2003, Schatz et al. 2006, Harrison 2014). Such systems are less stable and may shift positions in a gradient between antagonism and mutualism, depending on the specific context in which they take place, even within short periods of time (Thompson and Cunningham 2002, Westerbergh 2004, Kephart et al. 2006).

Kephart et al. (2006) and more recently Prieto-Benítez et al. (2017) reviewed the complex formed by plants of the Caryophyllaceae family and moths of the *Hadena* genus. A total of 21 different *Hadena* species were found to predate upon flowers and seed capsules of 70 caryophyllaceous plant hosts during the larval stage, with interactions ranging from antagonisms to facultative mutualisms. One such interaction is between the host plant *Silene latifolia* and its pollinator/seed predator partner *Hadena bicruris,* which is facultative and even considered to be a basic state of nursery pollination (Dufaÿ and Anstett 2003, Bernasconi et al. 2009), but is often being referred to as parasitic due to the extent of seed predation (Giménez-Benavides et al. 2007, Reynolds et al. 2012, Kula et al. 2014, Prieto-Benítez et al.

2017). In the *S. latifolia–H. bicruris* system there are copollinators present but the moth *H. bicruris* itself lacks active pollination, meaning there is an absence of any specific morphological structures and behaviours to ensure the pollination process (Pellmyr 1997). This lower degree of specialisation makes the system less robust, and therefore likely to shift along the gradient spatially and over short periods of time (Bronstein 1994, Thompson and Cunningham 2002, Westerbergh 2004, Kephart et al. 2006). This gives us a unique opportunity to study different ecological contexts in which the same partners of an interaction produce opposite results, and therefore better understand or even predict under which ecological circumstances these interactions may became mutualistic and persist over time (Dufaÿ and Anstett 2003, Kephart et al. 2006, Bernasconi et al. 2009, Kula et al. 2014).

In the past decades, several models have been developed to shed some light into how mutualisms could persist over time and coevolve. Vandermeer and Boucher (1978) were the first to study population dynamics of mutualisms by adapting the Lotka-Volterra competition equations with positive interaction coefficients. The model of Bronstein et al. (2003) studied the ecological and evolutionary persistence of mutualisms in the presence of antagonist species, similar to Jones et al. (2009) who developed predictions for coevolved mutualist-exploiter communities, while Gomulkiewicz et al. (2003) included the effect of a third mutualistic species in the interaction. However, all of these models assumed that the species partners of the interaction always behaved as either facultative or obligate mutualists. As already mentioned above, the *S. latifolia-H. bicruris* interaction varies temporally and spatially along a gradient between antagonism and a facultative mutualism, so a prior step is necessary to analyse the nature of this interaction in natural conditions.

Westerbergh and Westerbergh (2001) developed a theoretical model describing the parameters that affect the fitness of both partners in a specialised host plant and pollinator/herbivore interaction. They identified two key parameters: seed production and seed consumption, with the following relationship for mutualistic outcomes:

$$\text{Seed production by the seed predator} \;>\; \text{seed consumption by the seed predator}$$

$$h\,P_1\,(1 - P_2)\,F_2 + h'P_1'\,(1 - P_2)\,F_1 \;>\; y\,F_2\,(1 - x^n)$$

This equation states that when seed consumption costs outweigh the benefits, the interaction is truly antagonistic, while if the benefit of pollination (thus, seed production) is larger than the costs, the system will behave that year as a facultative mutualism (Westerbergh and Westerbergh 2001; see Table 1 for description of parameters). If this situation persists over time —for example, if it consists of an isolated population where copollinators are absent or

Table 1 Description of the parameters from the Westerbergh and Westerbergh (2001) model.

	Parameter description
F_1	proportion of flowers without eggs
F_2	proportion of flowers infested with eggs
h	number of seeds produced per infested and attacked capsule
h'	number of seeds produced per intact capsule
n	number of eggs per flower laid by a single female seed predator
P_1	proportion of infested flowers pollinated by the seed predator
P_1'	proportion of intact flowers pollinated by the seed predator
P_2	proportion of flowers pollinated by other flower visitors
x	probability that an egg and a larva will die at an early stage
y	number of seeds eaten per capsule

rare— then the system will be on the evolutionary pathway towards mutualism, as beneficial coevolution becomes possible (Westerbergh 2004).

One very important parameter in this model is P_2, which refers to the contribution of copollinators to the seed set, as the *S. latifolia*-*H. bicruris* system has not yet excluded them. In a situation where copollinators are responsible for a large portion of the seed set, the benefits obtained by *S. latifolia* plants from *H. bicruris* pollinators will be comparatively low (Dufaÿ and Anstett 2003, Westerbergh 2004, Reynolds et al. 2012): the damage of seed predation will outweigh the benefits of additional pollination, resulting in an antagonistic interaction between both partners. On the other hand, if only low levels of pollination are provided by insects other than *H. bicruris*, the plant would indeed benefit from the interaction, despite the seed eating.

In certain nursery pollination systems oviposition has a crucial role in ensuring pollination, such as in the yucca-yucca moth interaction (Pellmyr 1997), and in some of the interactions from the fig-fig wasp complex (Jousselin and Kjellberg 2001). In these systems, where males do not provide pollination services but feed on seeds as larvae, breeding males is a cost to the plant (Després 2003). Nevertheless, it has also been observed, although less frequently, that male insects can have an important role, i.e. in the globeflower and globeflower fly interaction, where both sexes passively pollinate the plants (Després 2003). According to Labouche and Bernasconi (2010), pollination efficiency in male and female *H. bicruris* moths is equal, with an average of 45% of the ovules being fertilised after the first visit, and 80% of visits resulting in fruit initiation. Consequently, something which should be considered is that the model developed by Westerbergh and Westerbergh (2001) does not take into account that male *H. bicruris* moths also pollinate the flowers without causing any costs to the plant. The positive contribution of males through pollination could partly compensate for the costs caused through seed predation of the larvae and therefore the seed production to seed predation ratio (benefit/cost ratio) could be affected (Labouche and Bernasconi 2010). Therefore, it is important to investigate the role of males in providing pollination services to better understand how these systems are maintained.

Although much is known about the costly effects of *H. bicruris* through predation, there is still a lack of knowledge in relation to the benefits provided by adult *H. bicruris* moths on plant reproduction (Reynolds et al. 2012). In this paper, we specifically address two research questions: 1. What is the positive contribution of male *Hadena bicruris* moths to seed production in *Silene latifolia* plants? And 2. What is the nature of the *Silene latifolia-Hadena bicruris* system in the field? We investigated female and male moth pollinating behaviour and pollination efficiency through observational assays and carried out field collections of different populations, applying the Westerbergh and Westerbergh (2001) model to assess the nature of the interaction.

2. MATERIALS AND METHODS

2.1 The model system: the *Silene latifolia-Hadena bicruris-Bracon variator* interaction

The host plant: The white campion *Silene latifolia* (Caryophyllaceae, Figure 1a) is a short-lived, dioecious, perennial weed which usually forms metapopulations in open disturbed habitats such as roadsides and field margins (Elzinga et al. 2003, Elzinga et al. 2007a, Elzinga et al. 2007b). Within the nursery pollination systems, plants from the *Silene* group are quite unique. In all other systems consisting of dioecious plants reviewed by Dufaÿ and Anstett (2003), the larvae attacks the tissues with male sexual function, therefore having low or no cost to the plant as the reproductive episode is over once the pollen has been

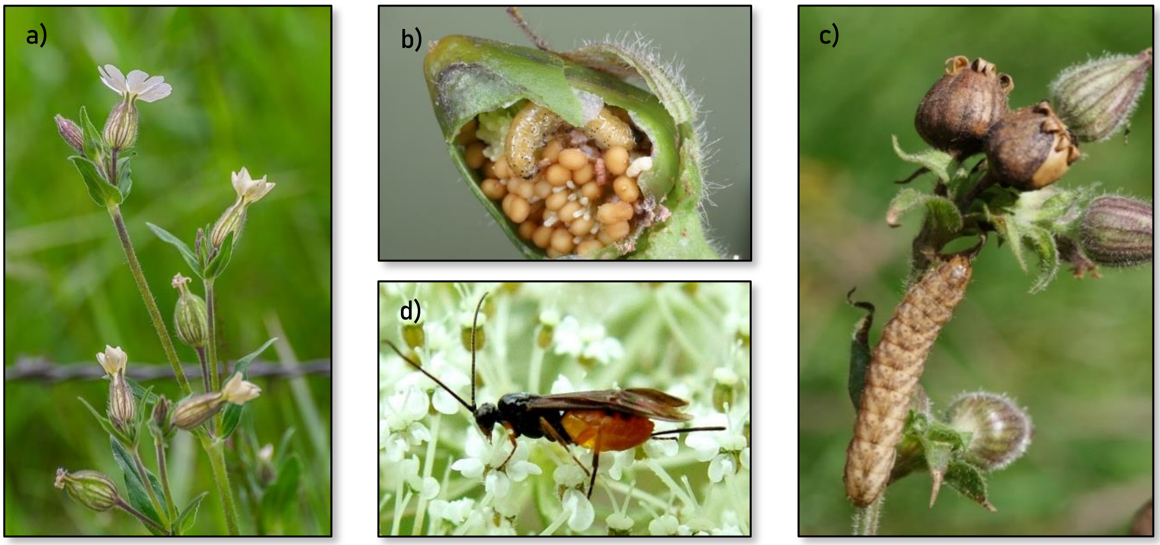

Fig. 1 The model system: *Silene latifolia–Hadena bicruris–Bracon variator* interaction. a) *Silene latifolia* female plant (notice the enlarged ovaries of the two pollinated flowers). Credit: Krzysztof Golik. b) *Hadena bicruris* young larva feeding on the developing seeds within the primary capsule (manually opened for illustration purposes). Image provided by Jeroen Voogd. c) *H. bicruris* late instar larva searching for a secondary capsule to feed on. Image provided by Jeroen Voogd. d) *Bracon cf. variator* female (*det.* Donald Quicke) drinking nectar from undetermined flowers. Credit: Marion Friedrich.

dispersed (Dufaÿ and Anstett 2003). However, in the case of *Silene* plants, pollinator larvae feed on the fruit and seeds, thereby imposing high costs to the plant (Dufaÿ and Anstett 2003).

The pollinator/herbivore: Adult lychnis moths, *Hadena bicruris* (Lepidoptera; Noctuidae), are the main pollinator of *S. latifolia* plants, but this species is also their most important seed predator (Brantjes 1976a, Elzinga et al. 2005, Kephart et al. 2006). Adult moths are active after dusk and feed on nectar from male and female plants, passively pollinating the flowers. Female moths also oviposit on female flowers, usually laying a single egg (Brantjes 1976a, b), and use cues to avoid superparasitism by leaving a volatile oviposition deterrent indicating that the flower has already been parasitised by a conspecific (Brantjes 1976a, b, Roitberg and Prokopy 1987). Once the larva hatches from the egg, it feeds on the developing seeds from inside the fruit capsule where it has enclosed (the primary capsule, Figure 1b) (Elzinga et al. 2005). After consuming all the seeds from the primary capsule, the larva, already at a late developmental stage, moves to secondary capsules in order to continue feeding (Figure 1c), destroying up to five other capsules on the same plant (Brantjes 1976a, Elzinga et al. 2005). The density of *H. bicruris* in natural populations varies greatly, but it has been found in over 90% of *S. latifolia* plant populations in Western Europe (Elzinga et al. 2005), with an average of 50% of fruit destruction (Biere and Honders 1996, Elzinga et al. 2005). These high rates of fruit and seed destruction are supposed to have a major impact on plant fitness, although the degree of damage also varies significantly between periods (Biere and Honders 1996). For this reason, the interaction is usually described as an antagonistic one, with *H. bicruris* parasitising *S. latifolia* (Brantjes 1976b); however the degree to which it may be antagonistic may vary from year to year.

The natural enemy: The parasitoid wasp *Bracon variator* (Figure 1d) (Hymenoptera; Braconidae) is a generalist species which has been found to attack *H. bicruris*, although it occurs at low incidence in the field (Elzinga et al. 2007b). As an idiobiont ectoparasitoid, the adult female immobilises the host larva by injecting a paralysing venom, which also prevents any further development, before ovipositing on the surface of the host (Askew and Shaw 1986). Most commonly, the female will parasitise host larva in the L3-L5 instars, preventing any further seed consumption by the herbivore (Elzinga et al. 2007b). Therefore, *B. variator* can cause a decrease in predation of *S. latifolia* seeds by parasitising *H. bicruris* larvae, potentially reducing the costs of the interaction for the plant. However, the Westerbergh and Westerbergh (2001) model does not take mortality by parasitoids into account, as already considerable damage has been sustained to the seed capsule at the stage at which parasitism might occur.

2.2 Part 1: Male moth contribution

To evaluate the male moth contribution to seed set through pollination, we carried out behavioural observation assays with *H. bicruris* adult moths and analysed two main components. On the one hand we analysed the number of flower visits per bout in male and female moths, and secondly, we analysed the pollination efficiency of female and male moths, measured as the percentage of fertilised ovules per fruit capsule. Based on literature, we hypothesised that male and female moths would show similar visitation rates and pollination efficiencies.

2.2.1 Rearing of individuals

S. latifolia seeds were collected from natural field populations in the municipality of Ottersberg (53.1102, 9.1512; Lower Saxony), close to Bremen (Germany). From these seeds, plants were reared in the greenhouse facilities of the experimental garden of the University of Bremen over the summer of 2018. Seeds were sowed in large seedling trays (8 x 12 cells; ø 6 cm) and seedlings were transplanted to 6 cm ø pots at the six-leaf stage. Plants were transplanted to their final pots (11 x 11 x 10 cm) as soon as the basal rosette was formed. Plants were watered as needed and temperature and light were dependent on environmental conditions. We divided plants into groups: experimental plants and plants used for the rearing of insects. Male and female plants from the experimental group were isolated in 1 m^3 net tents (Nature®) as soon as they started blooming, to avoid any previous contact with pollinators.

The greenhouse plants used for rearing had access to pollinators, which allowed us to collect *H. bicruris* larva regularly to establish a laboratory population. All collected individuals were kept inside climate cabinets at 23°C with a 16L:8D light regime. Newly hatched and young instar *H. bicruris* larvae were left inside their primary seed capsules to feed from the tender developing seeds to increase survival rate. Late instar larvae were fed with an artificial diet, prepared according to Elzinga et al. (2002), which was refreshed on alternate days. In order to avoid cannibalism, which is common in first instar larvae (Brantjes 1976b), larvae were kept separately in small plastic vials, and moved to a population box as soon as they pupated. This population box was checked daily for emerging adults, and newly hatched *H. bicruris* adults were sexed and moved to population boxes to mate (34.5 x 22 x 30 cm) and were fed a honey-water solution. These boxes were sorted by age to ensure equal conditions in our observation assays.

2.2.2 Observation assays

The observation assays were carried out in a field adjacent to the greenhouse facilities of the experimental garden of the University of Bremen during the summer of 2018. A closed insect net (ø 2.8 m) was set up in the field, and the interior was divided into a grid with unit size of 40 cm x 40 cm, with a total of 30 units numbered in order from bottom to top and left to right as a reference for position. Each of these units contained a single potted *S. latifolia* male or female plant with a single open flower each. This was achieved by removing all opened flowers except one. Male and female plants were selected randomly from the group of experimental plants and given a unique ID to identify them. Plants were positioned in the grid in alternate order for sex, and we ensured an equal distance of 40 cm between plants (see Figure 2). The observations were performed approximately 30 minutes after sunset, corresponding to the activity time of *H. bicruris* moths. For each observation, a single three-day old moth, previously starved for 12 hours, was added to the closed insect net. A headlamp

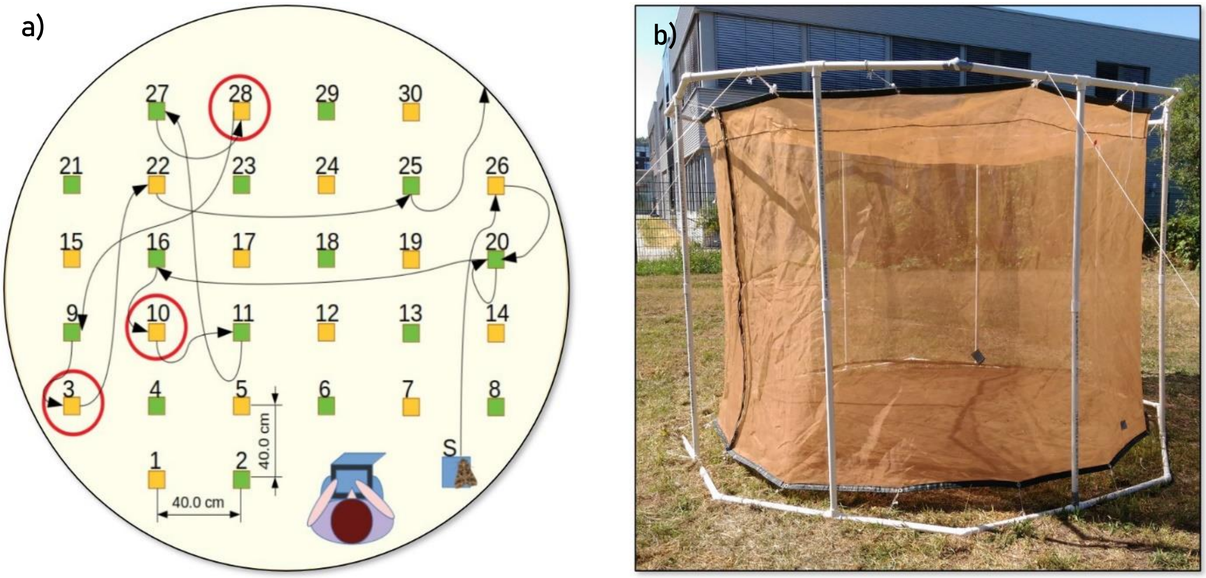

Fig. 2 Experimental set up for the observation assays. a) Top view of the experimental set-up. The big enclosing circle represents the insect net, orange squares represent female plants, while green squares are male plants. The numbers are positions within the grid. The blue square labelled "S" indicates the starting point (i.e. from where the moth was released). The arrows indicate the visitation sequence on flowers (for illustration purposes, we show the observed sequence for replicate n° 29, female moth #16). Note that original trajectories are not represented (e.g. in between two plant landings the moths could have landed on the net). The red circles show female flowers that were visited immediately after a male flower and successively dissected for seed/ovules counts. b) Photo of the insect net used to carry out the observations. Credit: Daniele Buono.

with red light was used for proper visualisation of the insects (Labouche and Bernasconi 2010). Moth behaviour was recorded using licensed software "The Observer® XT" version 7.0 (Noldus Information Technology 2007), and classified in five types: landing, feeding, ovipositing, flying, and resting (for definitions of each behaviour see Table 2). For each replicate, the number and sequence of flowers visited, duration and type of behaviour observed were recorded, with a total of 44 replicates (20 replicates from female moths, and 24 replicates from male moths). The replicate was considered finished once the moth had not visited any flower for 20 minutes. Plants were reused in consecutive trials if they were not visited during a replicate. If visited, the flower was immediately removed to prevent a physiological reaction to pollination (Wright and Meagher 2003), and the plant was used again when a new flower opened.

To evaluate pollination efficiency of female and male moths we counted fertilised and unfertilised ovules in *S. latifolia* capsules developed after pollination. Directly after every trial, when available, we selected three female flowers that had been visited by the moths immediately after feeding on a male flower. Any eggs found were removed from the flowers, and the plants were isolated. After 10 days, the developing fruit was removed from the plant and dissected. Developing seeds and unfertilised ovules were counted under a stereo microscope with the help of a counting chamber with 100 squares and a mechanical tally counter.

2.2.3 Statistical analysis

All statistical analyses were performed using "R" version 3.5.3 (R Core Team 2019) and the interface "RStudio" (RStudio Team 2016). To analyse the number of flower visits by male and female moths we used Generalized Estimating Equation (GEE, Hardin and Hilbe 2002) models, function "geeglm" from the package "geepack" (Yan 2002, Yan and Fine 2004, Højsgaard et al. 2006). By using GEE with "id = moth" and correlation structure "exchangeable" we corrected for data correlation in repeated measurements, i.e., each moth had two entries in the dataset, one for each plant sex. We used poisson error distribution (for count data) with a log link function. To analyse the proportion of fertilised ovules in fruits pollinated by female and male moths we also used a GEE Model, this time using a binomial error distribution with logit link function and "id = plant", as some of the fruits dissected belonged to the same plant, although collected from different trials. The "ggplot2" package (Wickman 2016) was used to create the bar graphs in Figure 4 and Figure 5, and the "emmeans" package (Lenth 2019) was used to calculate the confidence intervals represented in Figure 4 and Figure 5.

Table 2 Description of the behaviours recorded in the observation assays

	Behaviour description
landing	moth lands on a flower
feeding	moth inserts proboscis into flower calyx
ovipositing	moth insert abdomen into flower calyx
flying	moth flies inside the experimental area
resting	moth lands and rests on a surface that is not a flower

2.3 Part 2: Cost/benefit ratio in the field

2.3.1 Collection of data

In order to determine if the system is behaving as an antagonism or facultative mutualism in the field, we needed to estimate all the parameters from the model by Westerbergh (2004). We sampled five different natural field populations (see map of the area in Figure 3) in the municipality of Ottersberg (53.1102, 9.1512; Lower Saxony), close to Bremen (Germany). We collected all seed capsules found in each of the fields and noted the following data: number of female and male plants in the population, and for each plant the number of open (unpollinated) flowers, number of pollinated capsules and status of the capsule (infested with *H. bicruris* or intact). Capsules were kept individually in plastic cups to avoid any loss of seeds and were given a unique code for identification.

Once in the lab, capsules were dissected to double check the status. Infested capsules were further categorised depending on the stage of the insect present: egg, larva, dead larva, pupa, dead pupa, parasitoid. and empty (if it had signs of infestation such as exuvia or faeces but no insect was present). We used this data to create life tables and obtain mortality values (parameter x in the model), pollination rates, infestation rates by *H. bicruris* and parasitism rates by the parasitoid for each field population. Finally, the number of seeds per capsule was counted (only for populations "Ebbensiek3", "Kampe" and "Ottersberg"), which allowed us to calculate parameters h and h' from the model. Parameter y will be estimated as the equivalent to $h'-h$. We assumed that P_1 (proportion of infested flowers pollinated by *H. bicruris*) is equal to 1, as female moths will passively pollinate the flower while ovipositing. Parameter P_1' refers to the proportion of intact flowers pollinated by *H. bicruris*. This was calculated following the same method as Westerbergh (2004), using the formula $(F_{2(1)} / F_{2(2)}) \times (F_{1(2)} / F_{1(1)})$. Our initial event was the field sampling, from which we calculated $F_{1(1)}$ and $F_{2(1)}$. We took our observation assays as our second event (taking into account female and male moth contributions), and used the data obtained to calculate $F_{1(2)}$ and $F_{2(2)}$. The proportion of flowers pollinated by other flower visitors was taken as $P_2 = 1-P_1'$. Once all parameters were calculated, the model was applied to each field site and the outcome of the interaction, whether antagonistic or facultative mutualistic, was compared between sites.

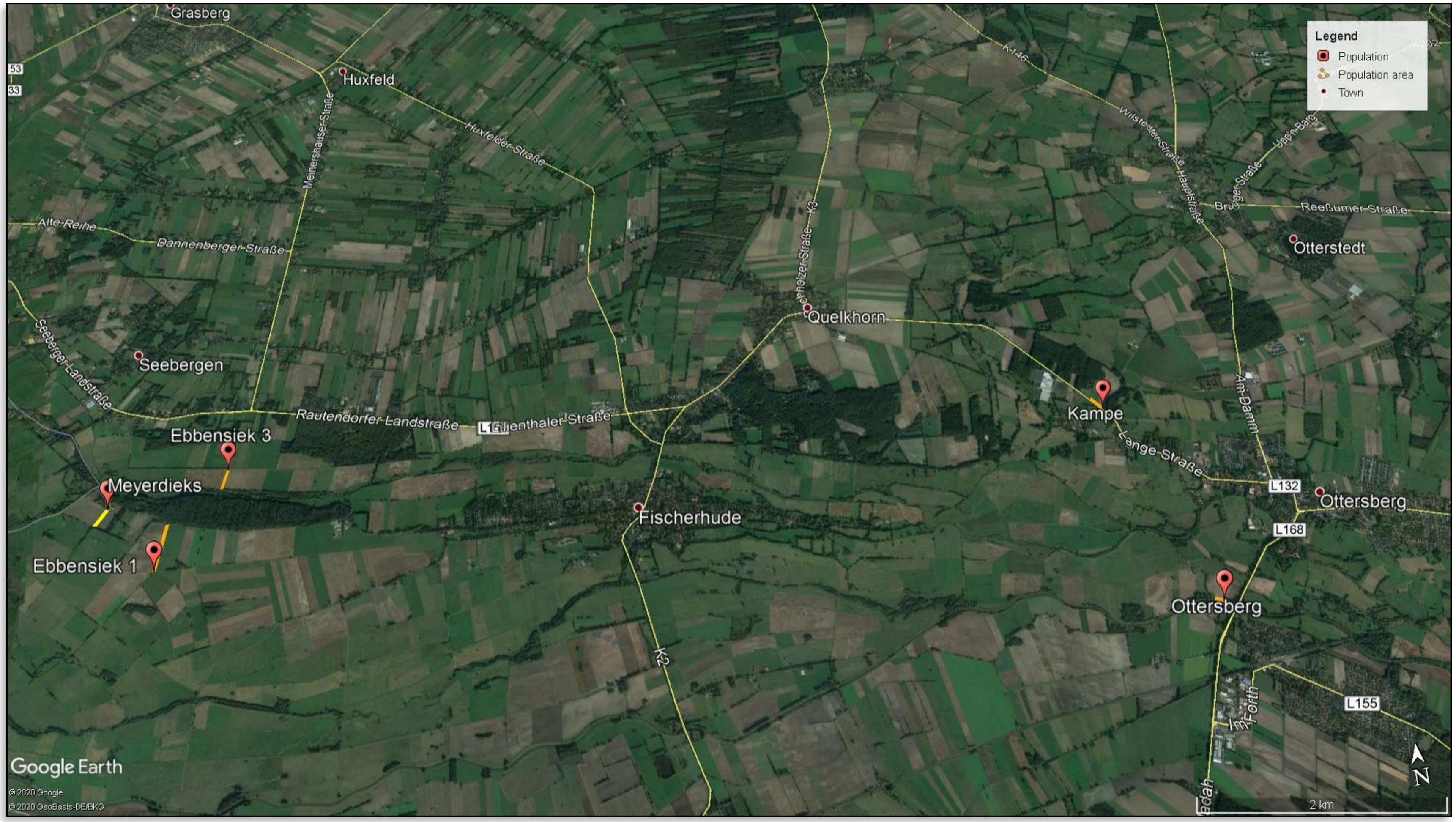

Fig. 3 Map of the sampling area with the five different populations (red markers: Ebbensiek1, Ebbensiek3, Meyerdieks, Kampe, and Ottersberg). The coloured lines within each site represents the extension of the population. Modified from Google Earth.

2.3.2 Statistical analysis

Generalized linear models (GLM, Nelder and Wedderburn 1972) were applied to analyse pollination, infestation, and parasitism rates between field populations using the “car” package (Fox and Weisberg 2011) and a binomial error distribution with logit link function. GLMs were also used to analyse the number of seeds per capsule as a function of field population and status (infested or intact), this time using a poisson error distribution with correction for overdispersed data and a log link function. We performed Bonferroni-adjusted pairwise comparisons for the two-way interaction terms using the “lsmeans” package (Lenth 2016). Additionally, for all three rates, we used paired samples *t*-tests to compare group means with a “Benjamini-Hochberg” correction to control for the false discovery rate. To analyse whether pollination, infestation, and parasitism rates were dependent on the number of female plants in the *S. latifolia* population, we used the Generalized Estimating Equation (GEE, Hardin and Hilbe 2002) models, and the “geeglm” function from the “geepack” package (Yan 2002, Yan and Fine 2004, Højsgaard et al. 2006). By using GEE with “id = field” and correlation structure “exchangeable”, we corrected for data correlation in repeated measurements, and a binomial error distribution with a logit link function was used. Finally, the “ggplot2” package (Wickman 2016) was used to create the bar graphs in Figure 6, Figure 7, Figure 9 and Figure 10.

3. RESULTS

3.1 Part 1: Male moth contribution

The analysis of the total male and female *S. latifolia* flower visits by female and male moths showed no significant effects of plant sex ($X^2_{df=1;\ n=44}$ = 0.901; *p*-value = 0.34), moth sex ($X^2_{df=1;\ n=44}$ = 0.925; *p*-value = 0.34), and their interaction ($X^2_{df=1;\ n=44}$ = 0.354; *p*-value = 0.55; Figure 4; GEE poisson distribution with log-link function, id = moth, corstr = exchangeable). Male and female moths show the same visitation rates, showing no apparent preference for either plant sex. In addition, there was no significant difference in the proportion of fertilised ovules in fruits pollinated by female (63.90%) and male moths (67.20%) (Figure 5; $X^2_{df=1;\ n=95}$ = 0.246; *p*-value = 0.62; GEE binomial distribution with logit-link function, id = plant, corstr = exchangeable).

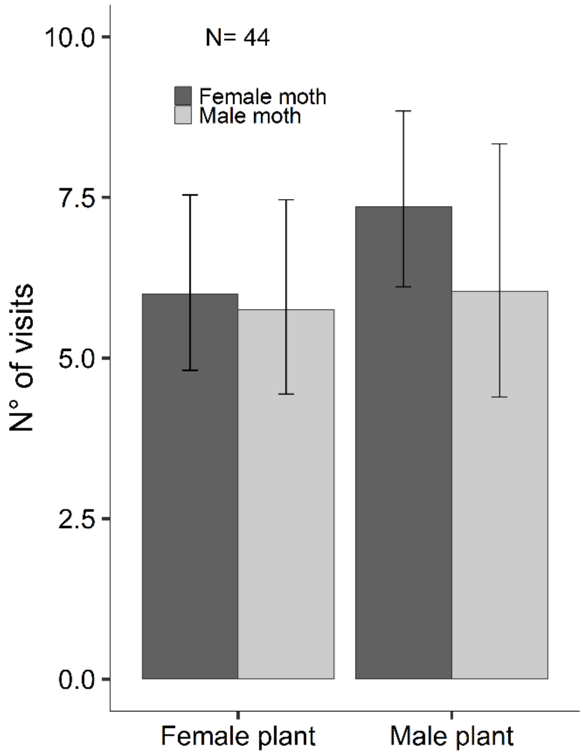

Fig. 4 Number of visits to male and female *Silene latifolia* flowers by female and male *Hadena bicruris* moths. There was no significant effects of plant sex ($X^2_{df=1;\ n=44}$ = 0.901; *p*-value = 0.34), moth sex ($X^2_{df=1;\ n=44}$ = 0.925; *p*-value = 0.34), or their interaction ($X^2_{df=1;\ n=44}$ = 0.354; *p*-value = 0.55). The bars represent model estimates and confidence intervals. GEE poisson distribution with log-link function, id = moth, corstr = exchangeable.

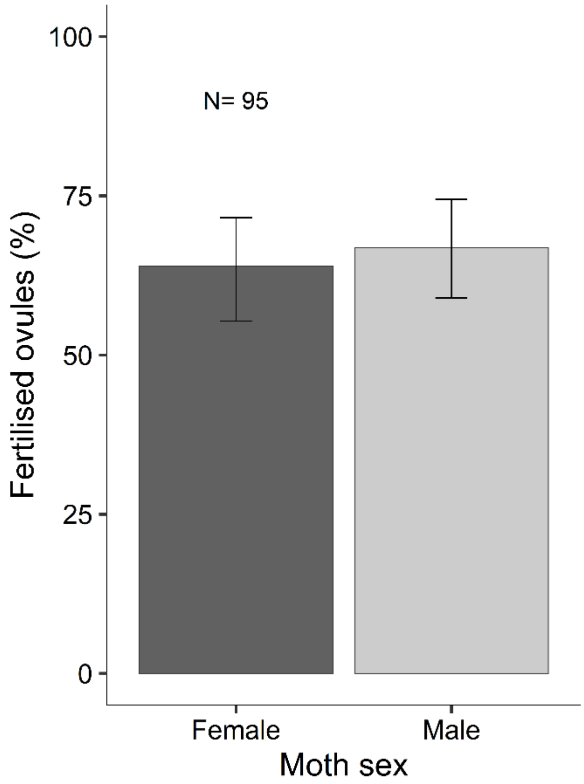

Fig. 5 Percentage of ovules fertilised by *Hadena bicruris* male and female moths. Moth sex was not significant ($X^2_{df=1;\ n=95}$ = 0.246; *p*-value = 0.62). The bars represent model estimates and confidence intervals. GEE binomial distribution with logit-link function, id = plant, corstr = exchangeable.

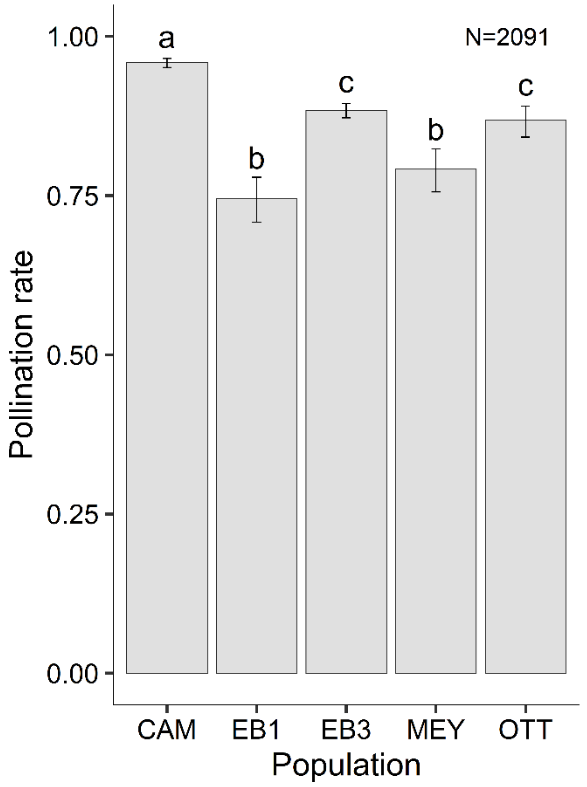

Fig. 6 Pollination rates of *Silene latifolia* female flowers, with significant differences between fields ($X^2_{df=a;\ n=2091}$ = 85.97; *p*-value < 2.20e-16). The bars represent model estimates and confidence intervals. GLM binomial distribution with logit-link function.

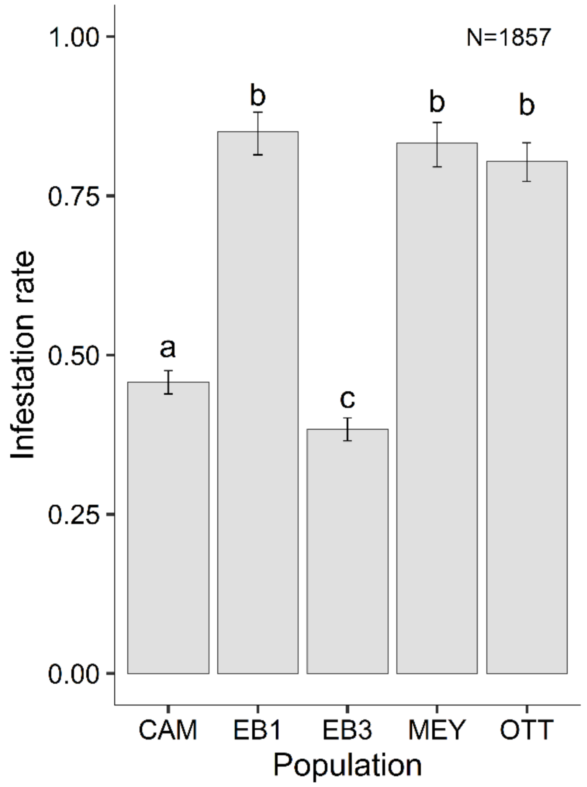

Fig. 7 Infestation rates of *Silene latifolia* female flowers by *Hadena bicruris* larva, with significant differences among populations ($X^2_{df=4;\ n=1857}$ = 229.59; *p*-value < 2.20e-16). The bars represent model estimates and confidence intervals. GLM binomial distribution with logit-link function.

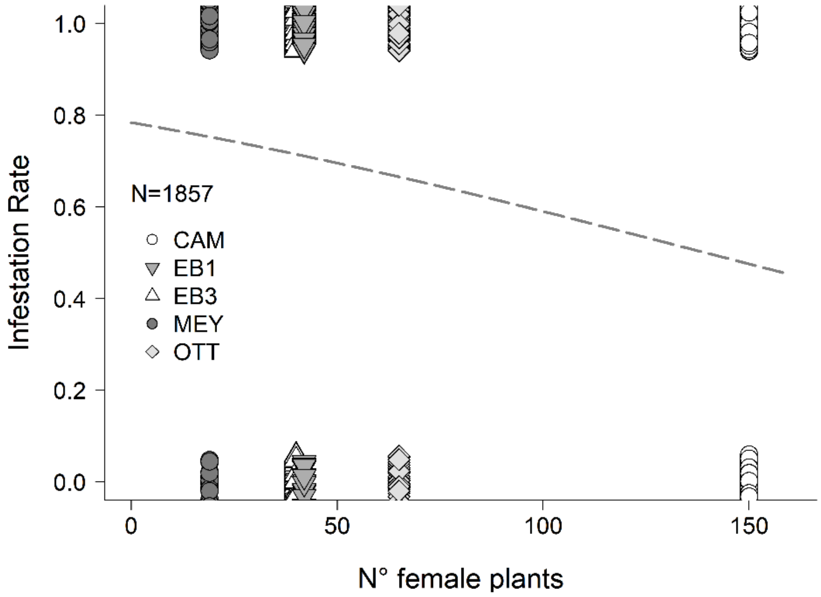

Fig. 8 Infestation rates by *Hadena bicruris* as a function of the number of female *Silene latifolia* plants in the population, represented by the best fitting line ($X^2_{df=1;\ n=1857} = 4.26$; *p*-value = 0.039). GEE binomial distribution with logit-link function, id = field, corstr = exchangeable.

3.2 Part 2: Cost/benefit ratio in the field

3.2.1 Differences in population rates

The field populations varied greatly in their pollination, infestation and parasitism rates. All populations had high pollination rates, with significant differences between fields ($X^2_{df=a;\ n=2091} = 85.97$; *p*-value < 2.20e-16; GLM binomial distribution with logit-link function, see Figure 6). The paired samples *t*-tests (Table 3) showed that the population "Kampe" had significantly the highest rate with over 95% of all flowers pollinated, followed by "Ebbensiek3" with 88% and "Ottersberg" with 87%, which were not significantly different from each other. Lastly, populations "Meyerdieks" with 79%, and "Ebbensiek1" with 75% had the lowest levels of pollination, yet were not significantly different from each other. Pollination rates were not dependent on the number of female *S. latifolia* plants in the population ($X^2_{df=1;\ n=2091} = 0.36$; *p*-value = 0.55; GEE binomial distribution with logit-link function, id = field, corstr = exchangeable).

Infestation rates varied significantly between field populations (Figure 7; $X^2_{df=4;\ n=1857} = 229.59$; *p*-value < 2.20e-16; GLM binomial distribution with logit-link function). The pattern this time was different, with "Ebbensiek1" (85%), "Meyerdieks" (83%) and "Ottersberg" (80%) having significantly higher infestation rates, followed by "Kampe" (46%), and "Ebbensiek3" (38%) with the lowest infestation rate (Table 4). Moreover, infestation rates decreased significantly with a higher number of female *S. latifolia* plants in the population (Figure 8; $X^2_{df=1;\ n=1857} = 4.26$; *p*-value = 0.039; GEE binomial distribution with logit-link function, id = field, corstr = exchangeable).

Parasitism rates by the parasitoid were also significantly different between populations (Figure 9; $X^2_{df=4;\ n=1857} = 108.53$; *p*-value < 2.20e-16; GLM binomial distribution with logit-link function). While in populations "Ebbensiek1", "Ebbensiek3" and "Meyerdieks" there was an absence of parasitoids, with close to 0% of the *H. bicruris* larvae being parasitised by *B. variator*, "Kampe" had a significant increase to a 6% parasitism rate, with "Ottersberg" having again the highest rate with almost 25% of the larvae being parasitised (Table 5). Parasitism rates were not dependent on the number of female *S. latifolia* plants in the population ($X^2_{df=1;\ n=1857} = 0.94$; *p*-value = 0.33; GEE binomial distribution with logit-link function, id = field, corstr = exchangeable).

Finally, the number of seeds per capsules showed significant effects of field population ($F_{[2,1195]} = 153.37$; *p*-value < 2.20e-16), status ($F_{[1,1195]} = 519.01$; *p*-value < 2.20e-16), and their interaction ($F_{[2,1195]} = 35.11$; *p*-value = 2.61e-14; Figure 10; GLM poisson distribution with log-link function and correction for overdispersion). In all field populations intact capsules had a significantly higher number of seeds than infested capsules (Table 6). Population "Ottersberg" had an overall higher seed production than "Ebbensiek3" and "Kampe". However, both intact and infested capsules in "Ebbensiek" and "Kampe" did not produce a significantly different number of seeds (Table 6).

3.2.2 Model parameters and outcome

The data used and obtained in the calculation of the model is summarised in Tables 7-11. In Table 7 we can see the data obtained in our behavioural assays. Female moths visited 124 female flowers in total, and oviposited in 86 of them. While we cannot be sure that all the 124 flowers were pollinated (especially female flowers visited before any male flower), we can safely assume that all 86 flowers in which we found eggs were pollinated. This gives us an oviposition/pollination to visitation ratio of 0.69. As the results show that female and male moths have the same pollination efficiency, this allowed us to use the ratio to calculate how many of the female flowers visited by male moths were pollinated. With this data we calculated parameters $F_{1\,(2)}$ = proportion of unpollinated flowers and $F_{2\,(2)}$ = proportion of pollinated flowers.

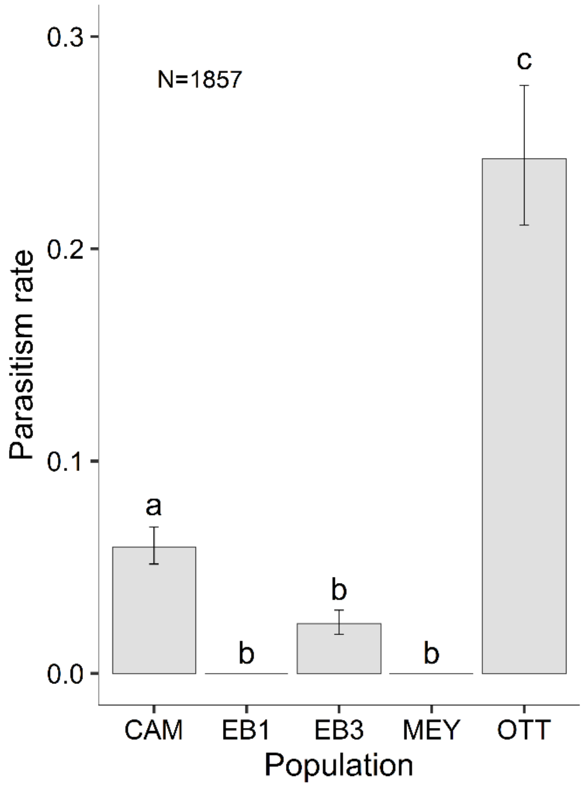

Fig. 9 Parasitism rates of *Hadena bicruris* larvae by the parasitoid wasp *Bracon variator* with significant differences between populations ($X^2_{df=4; n=1857}$ = 108.53; *p*-value < 2.20e-16). The bars represent model estimates and confidence intervals. GLM binomial distribution with logit-link function.

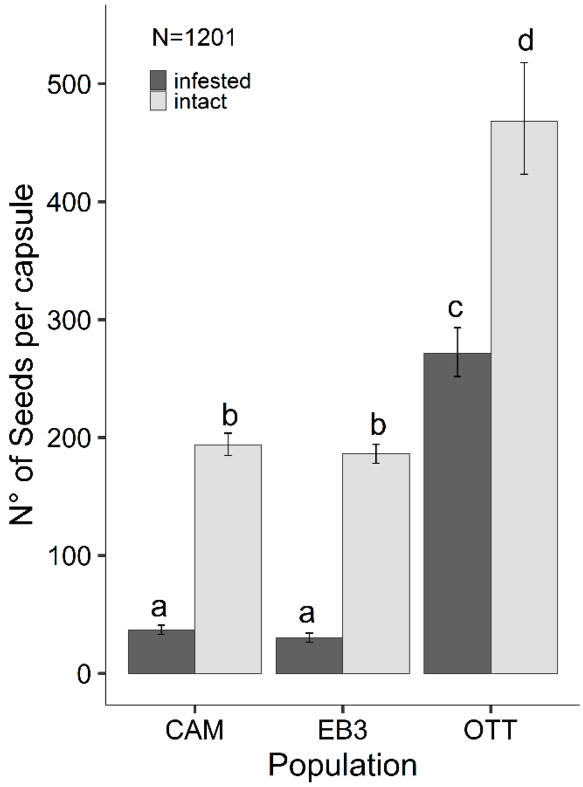

Fig. 10 Number of *Silene latifolia* seeds per infested and intact capsules in different field populations. Field, ($F_{[2,1195]}$ = 153.37; *p*-value < 2.20e-16), status (i.e. infested or intact) ($F_{[1,1195]}$ = 519.01; *p*-value < 2.20e-16), and their interaction ($F_{[2,1195]}$ = 35.11; *p*-value = 2.61e-14) all had significant effects on the number of seeds per capsule. The bars represent model estimates and confidence intervals. GLM poisson distribution with log-link function and correction for overdispersion.

Table 3 Paired samples *t*-tests for differences in pollination rates between all fields, with a “Benjamini–Hochberg” adjustment method. Field population names are coded: CAM = Kampe, EB1 = Ebbensiek1, EB3 = Ebbensiek3, MEY = Meyerdieks, OTT = Ottersberg. Significant differences between pairs are shown in bold (*$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Populations	CAM	EB3	EB1	MEY
EB3	1.80e-06 ***	-	-	-
EB1	1.90e-14 ***	6.60e-07 ***	-	-
MEY	6.00e-09 ***	0.0011 **	0.20	-
OTT	0.00027 ***	0.51	0.00027 ***	0.026 *

Table 4 Paired samples *t*-tests for differences in infestation rates between all fields, with a “Benjamini–Hochberg” adjustment method. Field population names are coded: CAM = Kampe, EB1 = Ebbensiek1, EB3 = Ebbensiek3, MEY = Meyerdieks, OTT = Ottersberg. Significant differences between pairs are shown in bold (*$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Populations	CAM	EB3	EB1	MEY
EB3	0.0038 **	-	-	-
EB1	3.80e-16 ***	<2.00e-16 ***	-	-
MEY	6.00e-15 ***	<2.00e-16 ***	0.78	-
OTT	<2.00e-16 ***	<2.00e-16 ***	0.52	0.68

Table 5 Paired samples *t*-tests for differences in parasitism rates by the parasitoid between all fields, with a "Benjamini-Hochberg" adjustment method. Field population names are coded: CAM = Kampe, EB1 = Ebbensiek1, EB3 = Ebbensiek3, MEY = Meyerdieks, OTT = Ottersberg. Significant differences between pairs are shown in bold (**p* < 0.05; ** *p* < 0.01; *** *p* < 0.001).

Populations	CAM	EB3	EB1	MEY
EB3	0.0033 **	-	-	-
EB1	0.0098 **	0.32	-	-
MEY	0.0098 **	0.32	1.00	-
OTT	<2.00e-16 ***	<2.00e-16 ***	<2.00e-16 ***	<2.00e-16 ***

Table 6 Bonferroni-adjusted pairwise comparisons for the number of seeds per capsule with the interaction term field*status. Field population names are coded: CAM = Kampe, EB3 = Ebbensiek3, OTT = Ottersberg. Significant differences between pairs are shown in bold (**p* < 0.05; ** *p* < 0.01; *** *p* < 0.001).

Populations		CAM		EB3		OTT	
Status		intact	infested	intact	infested	intact	infested
OTT	infested	-	-	-	-	-	-
	intact	-	-	-	-	-	0.0003***
EB3	infested	-	-	-	-	< 0.0001***	< 0.0001***
	intact	-	-	-	< 0.0001***	< 0.0001***	0.0002***
CAM	infested	-	-	< 0.0001***	1.00	< 0.0001***	< 0.0001***
	intact	-	< 0.0001***	1.00	< 0.0001***	< 0.0001***	0.0030**

Table 7 Direct observations of *Hadena bicruris* activity behaviour. Number of flowers pollinated, not pollinated and total number of visits by female and male moths. $F_{1(2)}$ = proportion of unpollinated flowers. $F_{2(2)}$ = proportion of pollinated flowers.

	pollinated	unpollinated	total visits	$F_{1(2)}$	$F_{2(2)}$
female moths	86	38	124	0.145	0.328
male moths	96 (*1)	42	138	0.160	0.366
Total	182	80	262	0.304	0.695

(*1) Calculated using the oviposition/pollination to visitation rate ratio of females: 138 * 0.69

Table 8 Data collected from field populations. Number of flowers/capsules infested with *Hadena bicruris,* intact flowers/capsules, and the total flowers/capsules found in the population. Field population names are coded: CAM = Kampe, EB1 = Ebbensiek1, EB3 = Ebbensiek3, MEY = Meyerdieks, OTT = Ottersberg. $F_{1\ (1)}$ = proportion of not infested flowers/capsules. $F_{2(1)}$ = proportion of infested flowers/capsules.

	infested	intact	Total	$F_{1(1)}$	$F_{2(1)}$
CAM	337	432	769	0.562	0.438
EB1	97	56	153	0.366	0.634
EB3	277	541	818	0.661	0.339
MEY	95	49	144	0.340	0.660
OTT	136	59	195	0.303	0.697

Table 9 Data collected from field populations. Number of *Hadena bicruris* larvae that died at a young stage and number of infested flowers/capsules found in the population. Field population names are coded: CAM = Kampe, EB1 = Ebbensiek1, EB3 = Ebbensiek3, MEY = Meyerdieks, OTT = Ottersberg. x = probability of the larva of dying at a young stage.

	larva died young	total infested	x
CAM	80	337	0.212
EB1	13	97	0.134
EB3	42	277	0.152
MEY	20	95	0.211
OTT	26	136	0.191

Table 10 Model parameters calculated for the different field populations. Field population names are coded: CAM = Kampe, EB1 = Ebbensiek1, EB3 = Ebbensiek3, MEY = Meyerdieks, OTT = Ottersberg.

	CAM	EB1	EB3	MEY	OTT
P_1	1.00	1.00	1.00	1.00	1.00
P_1'	0.343	0.761	0.225	0.852	1.00 (*1)
P_2	0.657	0.239	0.775	0.148	0.00 (*2)
h	36.45	62.00	29.61	62.00	271.76
h'	193.90	200.00	186.11	200.00	468.21
y	157.45	138.00	156.50	138.00	196.45
n	1.00	1.00	1.00	1.00	1.00

(*1) Rounded to fulfil de model assumptions. Original value: 1.013

(*2) Rounded to fulfil de model assumptions. Original value: -0.013

In Table 8 we present the data obtained from the field collections in terms of numbers of infested flowers/capsules (the sum of the open flowers with eggs and infested capsules), intact flowers/capsules (the sum of the open flowers without signs of oviposition and not infested capsules), and the total flowers/capsules of the population (the sum of all open flowers and capsules). This data allowed us to calculate parameters $F_{1(1)}$ = proportion of not infested flowers/capsules and $F_{2(1)}$ = proportion of infested flowers/capsules.

The calculation of parameter x is shown in Table 9. We had data on how many of the capsules found showed signs of infestation (specifically small entry holes and faeces) but no insect was present, and we considered this to be due to early mortality. However, when capsules were already found mature and open, we decided to be conservative, and consider all capsules with 0-10 seeds as a survival, as the larva could have already consumed most of the seeds in the capsule and then left. This value was then divided between the total number of infested capsules and the result was parameter x.

Finally, in Table 10 we show the remaining parameters used for the calculation of the model. The population "Ottersberg" had a P_1' value slightly higher than 1.00, which by definition (proportion of intact flowers pollinated by the seed predator) should not be possible, so it was rounded down. We took the average number of seeds in intact (h') and infested (h) capsules (see Figure 10), and number of seeds eaten ($y = h'-h$) from populations "Kampe", "Ebbensiek3" and "Ottersberg", and used it as a value for the same parameters for populations "Ebbensiek1" and "Meyerdieks", as we had no seed count data from these two populations. Lastly, parameter n (number of eggs per flower laid by a female moth) was estimated to be $n = 1$ in accordance to what was observed in our behavioural assays (personal observation), and taking into account that larval competition and cannibalism in the first instar larvae is very common in this species (Brantjes 1976b), which avoids the presence of more than one larva per capsule.

With all the model parameters ready we could finally calculate the outcome for each population (Table 11). The benefit/cost ratio (B/C ratio) was calculated by dividing the seed production term of the equation by the seed predation term. When the B/C < 1, the interaction is behaving antagonistically. If the B/C > 1, the interaction is behaving as a facultative mutualism for the plant. According to the model by Westerbergh and Westerbergh (2001) populations "Kampe" (0.33), "Ebbensiek1" (0.95), and "Ebbensiek3" (0.18) suffered from higher costs (seed predation) than benefits (seed production) obtained through the interaction with *H. bicruris*, and therefore the interaction in these fields should be considered antagonistic. On the other hand, populations "Ottersberg" (3.04) and "Meyerdieks" (1.14) are behaving as facultative mutualisms with an overall positive benefit/cost ratio. However, population “Ottersberg” showed much higher seed production and seed predation values.

4. DISCUSSION

The first aim of this study was to determine the contribution of male *H. bicruris* moths to seed production in *S. latifolia* plants. To this end, we carried out observational assays with both female and male moths and compared their visitation rates and pollination efficiency. Our results support our first hypothesis, as male and female *H. bicruris* moths show equivalent visitation rates and pollination efficiencies, with an average of 65% of the available ovules being fertilised. This is in line to what was reported by Labouche and Bernasconi (2010), although our results indicate higher pollination efficiency values. It is evident that the role of male pollinators in nursery pollination systems is more valuable than initially suspected, and male pollination services should be further studied to better understand how these systems are maintained.

Table 11 Model outcome calculated for the different field populations. The left side of the equation represents seed production (pollination) by *Hadena bicruris,* the right side represents seed predation by *H. bicruris* larvae. B/C ratio refers to the benefit/cost ratio, and overall outcome of the interaction. If production is higher than predation, the interaction is a facultative mutualism (B/C > 1). If seed production is lower than seed consumption, the interaction is considered an antagonism (B/C < 1). Field population names are coded: CAM = Kampe, EB1 = Ebbensiek1, EB3 = Ebbensiek3, MEY = Meyerdieks, OTT = Ottersberg.

	$h P_1 (1 - P_2) F_{2(1)} + h' P_1' (1 - P_2) F_{1(1)}$		$y F_{2(1)} (1 - x^n)$	B/C ratio
CAM	18.28480	<	54.37164	0.33
EB1	72.36345	<	75.76651	0.95
EB3	8.49136	<	44.94037	0.18
MEY	84.28376	>	71.83188	1.14
OTT	337.47665	>	110.84213	3.04

According to (Brantjes 1976b) moths can distinguish between flower sexes, and yet in our study the number of male and female flowers visited by adult *H. bicruris* moths was statistically equivalent. On the one hand, male flowers are known to produce less nectar but with a higher concentration of sugars (Shykoff 1997), making it a higher quality resource. On the other hand, female flowers are essential for reproduction, and survival of offspring decreases significantly when the eggs are laid outside the floral tube (Labouche and Bernasconi 2013). Therefore, if moth adults would show preference or specialise on one flower sex, it would potentially compromise pollen transfer, decrease pollination efficiency, and limit larval survival (Labouche and Bernasconi 2010). This is consistent with the fact that there are no sex-specific cues in the floral scent emitted by *S. latifolia* flowers, as shown by (Dötterl et al. 2005).

The effects of various measures of local abundance in pollination and reproductive success in plant populations was reviewed by Kunin (1997a). While the negative effect of low local population density (defined as interindividual spacing) on pollination and subsequent seed set was almost unanimous, the results for plant population size (the number of individuals within it) effects were not so clear. Kunin (1997b) concluded himself short time later that the size of a population would most likely not influence pollination. Our results are in line with these studies as pollination rates were not influenced by the number of female plants present in the population. Although we cannot explain the significant differences in pollination rates between the field populations, it is likely that these have to do with some other variable not analysed in this study (i.e. temporal differences in pollinator availability). However, the high pollination rates in all fields could be explained by the strong dispersal capacity and good patch locating abilities of *H. bicruris* moths, which are able to colonise fields which are more than 2 km away from the nearest source population (Elzinga 2005, Elzinga et al. 2007a).

In contrast to the previously discussed pollination rates, population infestation rates did decrease significantly with a higher number of females in the population. Elzinga et al. (2005) found a similar pattern in his study and concluded that oviposition and proportion of infested plants, increased in small and low-density populations, while the proportion of infested seed capsules was in addition affected by isolation (distance to the nearest population). There are several mechanisms that could explain this pattern. Firstly, if we look at optimal foraging theory (Charnov 1976) it is likely that females allocate more time in smaller patches, visiting and ovipositing in relatively more flowers, while in larger populations there is a "saturation effect" as female moths become egg limited (they are able to produce about 40 eggs during a single night (Brantjes 1976b, Elzinga et al. 2002)) and incapable of ovipositing in all female flowers found in the population (Elzinga et al. 2005, Biere and Honders 2006). This mechanism could also explain our observations. Secondly, predation of capsules could be higher in smaller populations because this implies fewer resources to feed on, and therefore larva would be forced to consume more fruits per host plant (Kula et al. 2014). Unfortunately, in our case, we cannot elucidate whether this applies to our results, as we only identified capsules from individual plants in populations "Kampe" and "Ebbensiek", which is insufficient to carry out a proper analysis.

A third mechanism that could explain a decrease in herbivory in larger populations is a higher expected incidence of natural enemies, such as parasitoids, resulting in lower larval survival rates and therefore a decrease in predation, as suggested in various previous studies (Jeffries and Lawton 1984, Roland 1993, Elzinga et al. 2005, Elzinga et al. 2007a, Kula et al. 2014). As already mentioned, the parasitoid *B. variator* is an idiobiont species, which means that female wasps will arrest host development before parasitising its host (Askew and Shaw 1986). This also stops any further herbivory of *S. latifolia* seed capsules and could potentially reduce the costs of the interaction for the plant. However, this would remain inconclusive in our study, as higher levels of infestation were not always accompanied by a lower parasitism rate in our fields, and parasitoid presence was not significantly related to population size. Looking at population "Kampe", which has the largest population size, we see a parasitism rate of 6% (consistent with *B. variator* incidence results in Elzinga et al. 2005, Elzinga 2007a, Elzinga 2007b) and lower infestation rates. In the case of populations "Ebbenisek1", "Ebbensiek3" and "Meyerdieks", with the smallest population sizes, parasitoid incidence was very low to null. Yet, while populations "Ebbenisek1" and "Meyerdieks" had very high infestation rates, "Ebbensiek3" showed the lowest infestation rates, moving away from the pattern. On the other hand, population "Ottersberg" shows the opposite tendency: having the highest parasitism rate (25%), it also shows very high infestation rates. The reason for the absence and very low incidence of parasitoids in populations "Ebbenisek1", "Ebbensiek3" and "Meyerdieks" is not clear. Although parasitoids tend to disperse less well than pollinators, *B. variator* is a generalist species and therefore less susceptible to possible isolation of *S. latifolia* patches, as other suitable hosts would be available in the surrounding environment (Elzinga et al. 2007a). This might also help explain why parasitism rates in our study were not affected by *S. latifolia* population size. Therefore, it is clear that there are other factors influencing both infestation and parasitism rates in the fields, which unfortunately we cannot account for with this study (i.e. habitat fragmentation and heterogeneity, or use of pesticides in the crop fields adjacent to the populations).

Regarding the number of seeds per capsule, there are some patterns worth commenting. First of all, we see a much higher overall seed production in field "Ottersberg" compared to fields "Kampe" and "Ebbensiek3". We believe this can be explained by differences in pollinator behaviour. As mentioned previously, *S. latifolia* has not excluded copollinators from the system, and several studies have shown that *Silene* plants are frequently visited by diurnal and nocturnal insects (Jürgens 2004, Kephart et al. 2006, Giménez-Benavides et al. 2007, Reynolds et al. 2009, Prieto-Benitez et al. 2016). Even though *H. bicruris* is still the most efficient pollinator (Jürgens et al. 1996, Bopp and Gottsberger 2004), in a context where there is a low availability of *H. bicruris,* copollinators can offer valuable pollination services at the expense of producing a smaller seed set (Young 2002, Scopece et al. 2018). Looking at the estimates obtained for the proportion of flowers that have been pollinated by other insects (P_2, see Table 10) we see that populations "Kampe" and "Ebbensiek3" have the highest values (P_2 > 65%). On the contrary, population "Ottersberg" seemed to be exclusively pollinated by *H. bicruris* moths, with an estimated P_1' of 100%. This could therefore explain the lower seed set produced in populations "Kampe" and "Ebbensiek3", and a twofold increase in the number of seeds produced in intact capsules from population "Ottersberg". In addition, a lower contribution of *H. bicruris* to the total pollination services would also help explain lower infestation rates in populations "Kampe" and "Ebbensiek3". The differences in number of seeds produced in infested capsules, could be explained by a combination of factors, including a higher initial (pre-predation) yield as mentioned above and a relatively high early mortality rate (approx. 20%, see Table 9). Moreover, high parasitism rates by *B. variator* in population "Ottersberg" (almost 25%) could also have a positive impact in seed set in infested capsules by reducing larval seed predation. Recent studies have shown that parasitoids are able to mediate the outcome of nursery pollination interactions by reducing the costs of the partnership between the host plant and its pollinator/herbivore and helping in the stabilisation of the system (Harrison 2014, Nunes et al. 2018, Stucchi et al. 2019). However, whether the action of parasitoid wasps is enough to enable the *S. latiolia-H. bicruris* interaction to shift from parasitic to a facultative mutualistic interaction is something which needs further examination.

In total we analysed and parameterised five field populations, with a large variation in model outcomes. According to our results, only two of these fields ("Meyerdieks" and "Ottersberg") behaved as a facultative mutualism, while the other three ("Kampe", "Ebbensiek1" and "Ebbensiek3") lay in the antagonistic side of the spectrum. In this sense, the amount of copollination (P_2) in the system is a good predictor for the outcome of the system. In fields where copollinators are responsible for a large portion of the seed set such as in "Kampe" and "Ebbensiek3", the benefits obtained by *S. latifolia* plants from *H. bicruris* pollinators are low, and the costs in terms of seed predation outweigh them, resulting in an antagonistic interaction between the partners (Dufaÿ and Anstett 2003, Westerbergh 2004, Reynolds et al. 2012). Therefore, it is surprising that population "Ebbensiek1", which has low estimated levels of copollination (24%) has an antagonistic outcome. Given such copollination values, and the above described differences in seed set between fields, we believe that the mean values we took for the number of seeds in intact (h') and infested (h) capsules for populations "Ebbensiek1" and "Meyerdieks" (see Table 10) were underestimated, and this as a consequence underestimated the benefits obtained through *H. bicruris* pollination.

5. CONCLUSION

The *S. latifolia*-*H. bicruris* interaction has a lower degree of specialisation than other nursery pollination systems, which makes it less robust and stable. As we have shown, field ecological conditions vary greatly between populations. Our study gives some insight into how different ecological circumstances can impact the outcome of the population and shift the balance along the antagonism-mutualism gradient (Bronstein 1994, Thompson and Cunningham 2002, Westerbergh 2004, Kephart et al. 2006). It is clear that associations between organisms never evolve in isolation, but within a complex environmental context, where third parties —such as copollinators or natural enemies— may play an important role modifying the interaction. To continue improving our understanding on how these types of interactions became mutualistic, it is necessary to consider the biotic factors, and especially the role of parasitoids as stabilising actors.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA ACCESABILITY

The data reported in this article will be publicly available upon acceptance in a data repository.

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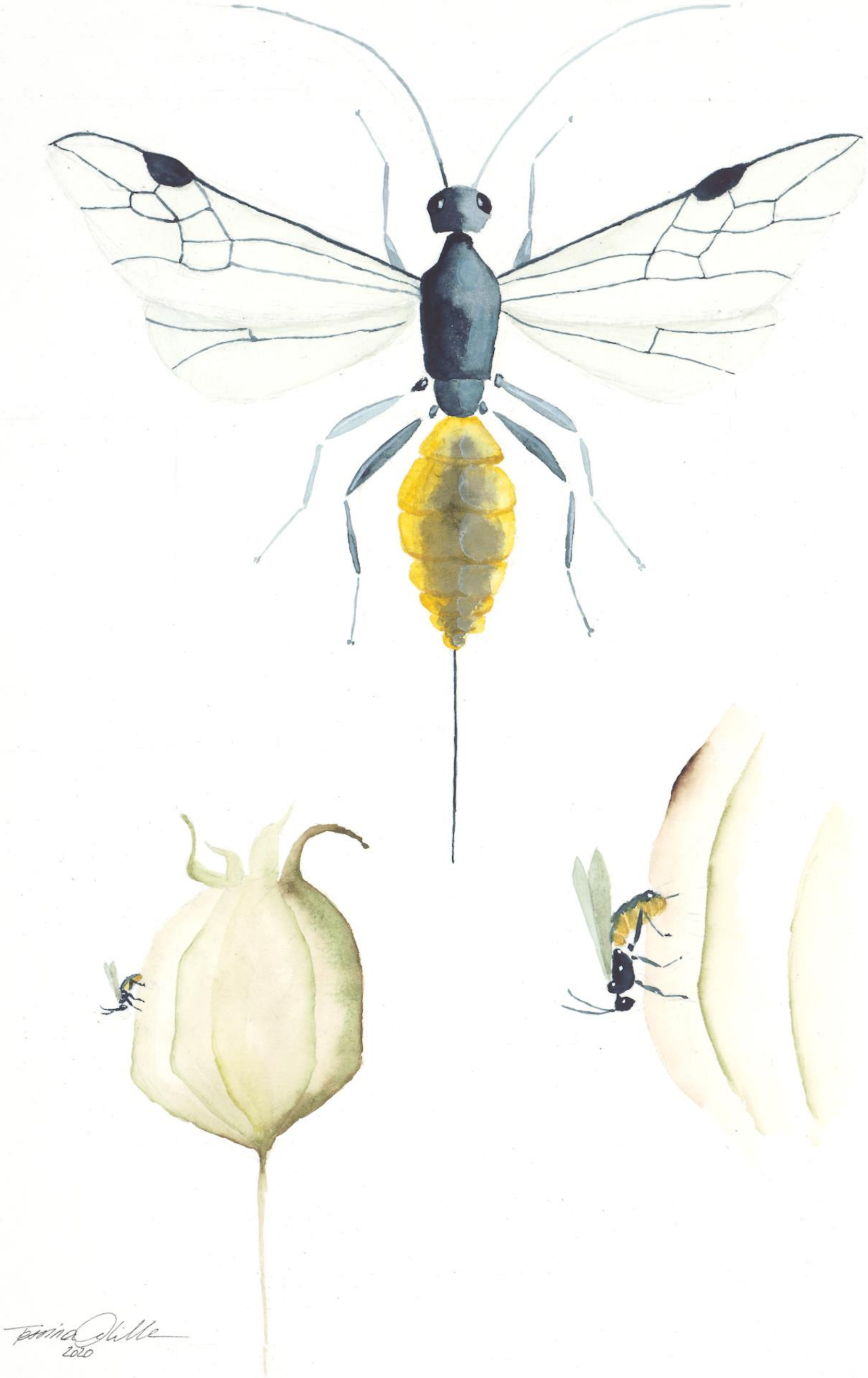

Tamina Hille
2020

CHAPTER 3

Friend or foe?
A parasitic wasp shifts the cost/benefit ratio in a nursery pollination system impacting plant fitness.

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*The format has been adapted to fit the general style of this thesis.

ABSTRACT

Nursery pollination systems are species interactions where pollinators also act as fruit/seed herbivores of the plant partner. While the plants depend on associated insects for pollination, the insects depend on the plants' reproductive structures for larval development. The outcome of these interactions is thus placed on a gradient between mutualism and antagonism. Less specialised interactions may fluctuate along this gradient with the ecological context, where natural enemies can play an important role. We studied whether a natural enemy may impact the level of seed consumption of a nursery pollinator and how this in turn may influence individual plant fitness. We used the plant *Silene latifolia*, its herbivore *Hadena bicruris,* and its ectoparasitoid *Bracon variator* as a model plant-herbivore-natural enemy system. We investigated seed output, germination, survival, and flower production as proxies for individual plant fitness. We show that *B. variator* decreases the level of seed consumption by *H. bicruris* larvae which in turn increased seed output in *S. latifolia* plants, suggesting that parasitism by *B. variator* may act as a regulator in the system. However, our results also show that plant survival and flower production decrease with higher seed densities, and therefore an increase in seed output may be less beneficial for plant fitness than estimated from seed output alone. Our study should add another layer to the complex discussion of whether parasitoids contribute to plant fitness, as we show that taking simple proxies such as seed output is insufficient to determine the net effect of multitrophic interactions.

KEYWORDS: cost/benefit ratio*,* host-parasitoid interaction*,* plant fitness, *Silene-Hadena-Bracon* system.

1. INTRODUCTION

Mutualistic interactions are fascinating examples of coevolution, in which individuals of different taxa depend on and provide benefits to each other (Boucher et al. 1982). Even though scientists have been studying mutualism for many decades (Micheneau et al. 2009), recent research makes it ever more evident that our understanding of the general interactions driving these processes is far from complete. For example, organisms that once were thought to cause microbial diseases are now known to act as mutualists in our gut systems, having crucial roles in our well-being (Bäckhed et al. 2005, Dethlefsen et al. 2007). In another case studied by Kawakita et al. (2015) the authors suggest that the presence of a third-party partner (a braconid wasp) could explain the reversal of mutualism to parasitism in leaf-flower moths. A whole new world has opened up focusing on mutualism (e.g., see Dubilier et al. 2008) and there is a need to understand how these systems evolve, transform, and are maintained.

A particular type of mutualism, nursery pollination systems (*sensu* Dufay and Anstett 2003), have been receiving quite some attention in the past years (Westerbergh 2004, Kephart et al. 2006, Reynolds et al. 2012, Labouche and Bernasconi 2013, Prieto-Benítez et al. 2017). The partners in these interactions are a host plant and an insect (normally a moth, fly, or wasp) that acts as a pollinator but at the same time lays eggs in or on the plant. The offspring of the insect will then develop and feed from the reproductive structures of the plant, possibly implying high fitness costs for the plant (Dufaÿ and Anstett 2003, Kephart et al. 2006). However, there must be a balance between the costs and benefits for both partners to achieve a positive net outcome, otherwise the mutualism would eventually turn into a parasitic interaction (Bronstein 1994, Pellmyr et al. 1996a, Kawakita et al. 2015).

Dufaÿ and Anstett (2003) reviewed nursery pollination systems and described a total of 13 documented cases, although since then other systems have been discovered (see Kawakita and Kato 2004, Song et al. 2014, Nunes et al. 2018). Within these systems there are some which are obligate mutualisms, such as the interaction between *Ficus* trees and fig wasps, *Yucca* and yucca moths, or senita cacti and senita moths (Anstett et al. 1996, Pellmyr et al. 1996b, Holland and Fleming 1999, Dufaÿ and Anstett 2003). The insects here actively pollinate their host plant with morphological structures that increase pollen transfer and copollinators are absent from the system. This high specialisation makes the system more stable and prone to a positive cost/benefit ratio. Other interactions such as the partnership between Greya moth and its host plant, *Lithophragma parviflorum*, are not specialised to that degree, lacking active pollination and having copollinators present (Thompson and Pellmyr 1992). In the same vein, the interaction between the host plant *Silene latifolia* and its pollinator/seed predator partner *Hadena bicruris* is also facultative and even considered a basic state of nursery pollination (Dufaÿ and Anstett 2003, Bernasconi et al. 2009), often being referred to as parasitic due to the extent of seed predation (Giménez-Benavides et al. 2007, Reynolds et al. 2012, Kula et al. 2014, Prieto-Benítez et al. 2017). This interaction, however, is just one pair belonging to a complex formed by plants from the Caryophyllaceae family and moths from the *Hadena* genus. Kephart et al. (2006) and later Prieto-Benítez et al. (2017) reviewed this system and found a total of 21 different *Hadena* species which predated upon flowers and seed capsules of 70 caryophyllaceous plant hosts during the larval stage, interactions ranging from antagonisms to facultative mutualisms.

The cost/benefit analysis of these interactions is never simple, as associations between organisms do not evolve in isolation, but rather within a complex ecological context, where third parties —such as copollinators, exploiters, predators, or parasites— may play an important role modifying the plant-insect interaction (Holland and Fleming 2002, Bronstein et al. 2003, Gomulkiewicz et al. 2003, Schatz et al. 2006, Harrison 2014, Scopece et al. 2018). This means that the net outcome of the interaction may change from a mutualism to an antagonism or commensalism in a reversible fashion depending on the specific environment in which the interaction occurs (Pellmyr 1989, Thompson and Pellmyr 1992, Bronstein 1994, Pellmyr et al. 1996b, Westerbergh and Westerbergh 2001, Thompson and Cunningham 2002, Dufaÿ and Anstett 2003, Westerbergh 2004, Bronstein et al. 2006, Thompson and Fernandez 2006). Taking this into account, these interactions should not be described as being either fully antagonistic or fully mutualistic, but be placed somewhere along a gradient between antagonism and mutualism (Bronstein 1994). Nevertheless, until recent, known cases were often described in the extreme categories rather than along a continuum (Janzen 1979, Pellmyr 1989, Pettersson 1991a, b, Thompson and Pellmyr 1992, Anstett et al. 1996). To further our understanding, it is thus important to elucidate under which ecological circumstances these systems may shift along the aforementioned gradient. From the plants' perspective, any factor that enhances the plants' reproductive success, such as interactions with natural enemies of the seed predators that would interfere with herbivore consumption, could be a first step in that shift towards a mutualism. As mentioned above, the *S. latifolia*–*H. bicruris* system is not a specialised mutual system: copollinators are present in the system and *Hadena* lacks active pollination, meaning there is an absence of any specific morphological structures and behaviours to assure the pollination process (Pellmyr 1997). This lower degree of specialisation makes the system less robust, and therefore likely to shift along the gradient over short periods of time (Bronstein 1994, Thompson and Cunningham 2002, Westerbergh 2004, Kephart et al. 2006). This shift is dependent on the specific context in which the system occurs, and the third parties involved, such as copollinators, parasitoids, or other natural enemies. The question therefore arises: how exactly may these third parties influence the outcome of the interaction?

To answer this question, Bronstein et al. (2003) developed general models to explain how antagonists (such as predators and parasites of the pollinators) could affect population dynamics and evolution of the mutualist partners. Some of the outcomes suggested antagonist species could alter population sizes of the mutualists in such a way that they could stabilise the dynamics of the interaction. In an empirical case study, though not of a nursery pollination system, van Loon et al. (2000) found that parasitisation of the herbivore *Pieris rapae* significantly reduced seed loss of its host plant *Arabidopsis thaliana*, suggesting that parasitism of herbivores potentially increased plant fitness. In that line, a study by Nunes et al. (2018) described a new nursery pollination system formed by a weevil and its orchid host plant, in which parasitoid wasps mediated the outcome of the interaction by killing the weevil larvae and therefore changing the cost/benefit ratio of the partnership. Moreover, very recently Stucchi et al. (2019) developed a population dynamics model demonstrating how the *Silene-Hadena* system might be more stable in the presence of parasitoids. Therefore, parasitic wasps of the pollinator *H. bicruris* have the potential to substantially alter the balance of the pollinator/predator and host plant interaction (Holland and Fleming 2002, Schatz et al. 2006, Harrison 2014).

On the other hand, *S. latifolia* produces large numbers of seeds per capsule (several hundred, Brantjes 1976b, Young 2002, Jolivet and Bernasconi 2007), and therefore it is unlikely that the plant is seed limited. As mentioned, *S. latifolia* depends on moth pollination and has gravity seed dispersal, therefore it will have a short dispersal range (Barluenga et al. 2011). According to a study by Peroni and Armstrong (2001), where they estimated seed density and dispersion by monitoring seedling emergence from soil cores, *S. latifolia* seeds follow a clumped dispersion pattern, with very high estimated densities per m^2. It is known that certain plant species can have negative density dependent recruitment, meaning that seedling survival decreases with local conspecific seed density (Sheffer et al. 2013, Jansen et al. 2014). Yoda et al. (1963) identified self-thinning as one of the main effects of intraspecific competition in plants. Waser et al. (2010) concluded that the probability of survival until adulthood and the total number of flowers produced were density dependent. Whereas many studies use fecundity as a direct measure for fitness, this relationship might not always be so straight forward. This was very well shown by Campbell et al. (2017) in an experiment with *Ipomopsis aggregata* plants, where offspring recruitment and reproduction were higher for seeds from low-fecundity parents due to density-dependent effects. Therefore, in an intraspecific competition scenario for *S. latifolia* plants, *H. bicruris* larvae might be less detrimental as expected to the plant's reproductive success and fitness, as it could be reducing part of this intraspecific competition by predating on a portion of the seeds.

In this paper, we specifically address two research questions: can a natural enemy impact the level of seed consumption by the seed predator, and if so, what are the consequences at the level of individual plant fitness. We used the *S. latifolia–H. bicruris* interaction as a model system, and the ectoparasitoid *Bracon variator* as a natural enemy. We investigated seed output, germination, survival to adulthood, and lifetime flower production as proxies for individual plant fitness with a series of laboratory and greenhouse experiments.

2. MATERIALS AND METHODS

2.1 The model system: the *Silene latifolia–Hadena bicruris–Bracon variator* interaction

The host plant: The White Campion *Silene latifolia* (Caryophyllaceae) is a short-lived perennial weed that exists in natural metapopulations and is normally found in open disturbed habitats such as field margins, roadsides, or grazing fields (Elzinga et al. 2003, Elzinga et al. 2007a, Elzinga et al. 2007b). Plants of the *Silene* group are dioecious and although dioecy is widespread in plants, this characteristic makes *Silene* plants quite unique within the nursery pollination systems, as they are the only dioecious plants in which the sexual function of the tissues eaten by the larvae of the pollinator is the female one (Dufaÿ and Anstett 2003). In all other systems consisting of dioecious plants reviewed by Dufaÿ and Anstett (2003), the larvae attacked the tissues with male sexual function. When pollinator larvae develop at the expense of the male structures, there is low or no cost to the plant, as its pollen has already been dispersed and the reproductive episode is over (Dufaÿ and Anstett 2003). However, in the case of *Silene* plants, pollinator larvae will feed on the fruit and seeds, thereby imposing high costs to the plant (Dufaÿ and Anstett 2003). These high costs and sex-specific fitness consequences due to the attack upon the female plants, makes *Silene* the perfect model plant to study the early stages of evolution in nursery pollination mutualisms (Westerbergh 2004).

The pollinator/herbivore: Adult Lychnis Moths, *Hadena bicruris* (Lepidoptera; Noctuidae), are the main pollinator of *S. latifolia* plants, but this species is also their most important seed predator (Brantjes 1976b, Elzinga et al. 2005, Kephart et al. 2006). Both males and females are active at night and fly from male to female plants, feeding on nectar and passively pollinating the flowers. Female moths also oviposit a single egg on female flowers and use cues to avoid superparasitism by leaving a volatile oviposition deterrent which indicates the flower has already been parasitised by a conspecific (Brantjes 1976a, b, Roitberg and Prokopy 1987). After egg eclosion, the young larva feeds on the developing seeds inside the seed capsule where it had hatched (Elzinga et al. 2005). Once it has grown to a late developmental stage and has consumed all of the seeds in the primary capsule the larva moves to secondary capsules for feeding (Figure 1), destroying up to five other capsules on the same plant (Brantjes 1976a, Elzinga et al. 2005). *Hadena bicruris* is widely spread in *S. latifolia* populations, where it was found in over 90% of plant populations in Western Europe, although at varying densities (Elzinga et al. 2005). The degree of seed capsule destruction varies greatly, with an average of 50% of all fruits being destroyed (Biere and Honders 1996, Elzinga et al. 2005); thus, periods with high seed destruction have a major impact on plant fitness (Biere and Honders 1996). The interaction is usually described as an antagonistic one, with *H. bicruris* parasitising *S. latifolia* (Brantjes 1976b); however the degree to which it may be antagonistic may vary from year to year.

Fig. 1 *Hadena bicruris* larva at a late developmental stage feeding on a *Silene latifolia* secondary seed capsule. Credit: Carmen Villacañas de Castro.

The natural enemy: The parasitoid wasp *Bracon variator* (Hymenoptera; Braconidae) has been found to attack *H. bicruris*, although it occurs at low incidence in the field (Elzinga et al. 2007b). As an idiobiont ectoparasitoid, it oviposits on the surface of the host, immobilising it by injecting a paralysing venom which also prevents any further development of the host (Askew and Shaw 1986). This species attacks hosts in their L3-L5 instars, and by arresting host development prevents further seed consumption by the herbivore (Elzinga et al. 2007b). Therefore, *B. variator* can cause a decrease in predation of *S. latifolia* seeds by parasitising *H. bicruris* larvae, potentially reducing the costs of the interaction for the plant.

2.2 Experiment 1: the role of *B. variator* as a natural enemy

2.2.1 Rearing of individuals

S. latifolia plants were reared in the cold-frame greenhouse facilities of the experimental garden of the University of Bremen over the summers of 2017 and 2018 from seeds collected from natural field populations in the municipality of Ottersberg (53.1102, 9.1512; Lower Saxony), close to Bremen. We used large seedling trays (8 x 12 cells; ø6 cm) for sowed seeds, and transplanted the seedlings to 6 cm ø pots at the six-leaf stage. Once the basal rosette was formed plants were transplanted to their final pots (11 x 11 x 10 cm). Temperature and light were not controlled for in the cold-frame and were dependent on environmental conditions. Plants were watered as needed. Plants were divided into two groups: experimental plants and plants for the rearing of insects. To make sure that experimental plants and flowers had no previous contact with pollinators, once they started blooming both male and female plants were kept in 1 m^3 net tents (Nature®). Laboratory populations of *H. bicruris* and *B. variator* were established in both years from individuals collected from the field at the beginning of the season and replenished with individuals found in the greenhouse plants used for rearing. All collected individuals were kept inside climate cabinets at 23°C with a 16L:8D light regime. Parasitoid clutches collected in the field were kept separately in small plastic vials until all offspring hatched. Adult parasitoids were kept in population boxes where they could mate and were fed on drops of honey and water. Newly hatched and young instar *H. bicruris* larvae were fed freshly pollinated *S. latifolia* capsules with tender developing seeds to increase survival rate, while late instar larvae were fed with an artificial diet, prepared according to Elzinga et al. (2002), which was refreshed on alternate days. All larvae were kept separately in small plastic vials to avoid cannibalism and once pupated they were moved to a pupation box, which was checked daily for emerging adults. Newly hatched *H. bicruris* adults were sexed and moved to population boxes (34.5 x 22 x 30 cm), sorted by age and fed a honey-water solution.

2.2.2 Reduction of seed predation by parasitoid attack

We measured the impact of the parasitoid wasp *B. variator* on larval seed predation and hypothesised that the parasitisation of *H. bicruris* larvae by *B. variator* would reduce seed loss in *S. latifolia* plants.

The experiment to test seed production of plants (a) "without herbivore attack", (b) "with herbivore attack", and (c) "with herbivore attack plus parasitoids" was carried out under controlled laboratory conditions by employing collapsible insect rearing cages (60 x 60 x 90 cm, Aerarium®). Each cage contained a single female *S. latifolia* plant with a minimum of six open flowers, and a male *S. latifolia* plant with a minimum of 10 open flowers to ensure sufficient pollen for pollination. All plants used belonged to the group of experimental plants reared in the cold-frame greenhouse. A 4-6 days old mated female, previously starved for 24 hr, was added to the cage in the evening and left to feed and pollinate the flowers overnight. The next morning the moths were removed. Each replicate consisted of three treatments: (a) "control" treatment (negative control): After pollination by the female moth, all eggs were removed from the plant to avoid infestation by *H. bicruris*; (b) "herbivore" treatment: *S. latifolia* plants pollinated and parasitised by a single *H. bicruris* larva which fed undisturbed (to ensure this all visible eggs except one visibly fertilised egg that was haphazardly chosen were removed from the plant, and in case this method failed and more than one larva was detected later the replicate was discarded and repeated); and (c) "herbivore + parasitoid" treatment: *S. latifolia* plants pollinated and parasitised by a single *H. bicruris* larva; once the larva emerged from the primary capsule to move to secondary capsules, a mated and experienced *B. variator* female was released inside the cage until parasitism of the larva. The end of each replicate was marked as the moment when the larva from the herbivore treatment had either pupated or been paralysed by the parasitoid. At this point all capsules, damaged or undamaged, were counted, gathered, and stored individually and per plant. All seeds were later counted and data on the number of seeds per capsules and per plant was collected. Each capsule, and all its seeds (without plant tissue) were also weighed on a precision scale (Quintix®, Sartorius Lab Instruments) to collect data on average seed weight per capsule.

2.2.3 Early germination

Rapid germination is a quality of many ruderal plants and especially of those growing near arable fields (Grime 1977). It benefits plants as they can start using resources to outcompete other individuals, therefore timing and speed of germination can be crucial for a successful seedling establishment (Gioria and Pyšek 2017). In order to test if there is any qualitative change in the seeds due to attack by *H. bicruris*, we carried out an early germination test, with the seeds obtained from the previous experiment. We hypothesised that seeds coming from unattacked plants would have a higher quality and therefore earlier germination, while seeds from damaged capsules would have a lower quality than those coming from undamaged capsules. The set-up included three different treatments: (a) seeds from pollinated plants without herbivore attack, (b) seeds from undamaged capsules from plants with herbivore attack (with and without parasitoid), and (c) seeds from damaged capsules from plants with herbivore attack (with and without parasitoid). We used large seedling trays (8 x 12 cells; ø6 cm) and sowed each seed in one separate cell, randomly allocating the treatments. Seeds were watered every day and kept inside the greenhouse facilities of the experimental garden of the University of Bremen. After 7 days, the trays were checked for germination (recorded as 0 if the seed had not germinated or 1 if the seed had germinated). In our experience, *S. latifolia* in greenhouse conditions would typically start germinating in 3-5 days, and therefore we consider a week enough time to see differences in the early germination of seeds from different treatments, as proxy for seed quality.

2.2.4 Statistical analyses

All statistical analyses were performed using “R” (Version 3.5.3) for statistical computing (R Core Team 2019) and the interface “RStudio” (RStudio Team 2016). Seed output per capsule was analysed taking into account the treatment of the plant (“control”, “herbivore” and “herbivore + parasitoid” treatments) and whether the capsules were damaged (attacked by *Hadena*) or not. The combination of both factors gives five categories: (a) “control” (always undamaged as there is no presence of larvae), (b) “herbivore + undamaged” (capsules produced by a plant in the respective treatment that were not attacked by the larva of *H. bicruris*), (c) “herbivore + damaged” (capsules attacked by the larva *H. bicruris*), (d) “herbivore + parasitoid + undamaged” (capsules produced by a plant in the respective treatment that escaped larval attack from *H. bicruris*) and (e) “herbivore + parasitoid + damaged” (capsules attacked by the larva *H. bicruris*, which eventually was parasitised by *B. variator*). To analyse seed output per capsule and mean seed weight, we used Generalised Estimating Equation (GEE, Hardin and Hilbe 2002) models, function “geeglm” from the package “geepack” (Yan 2002, Yan and Fine 2004, Højsgaard et al. 2006). By using GEE with “id = plant” and correlation structure “exchangeable” (for seed output) and “ar1” (for mean seed weight), we corrected for data correlation in repeated measurements, i.e., the measurement of several capsules per plant. We used poisson error distribution (for count data) with a log-link function. In addition, we performed contrast tests following the close test principal (Bretz et al. 2010) with the function “esticon” from the package “doBy” (Højsgaard and Halekoh 2018). Generalised linear models (GLM, Nelder and Wedderburn 1972) were applied to analyse total seed output per plant using the package “car” (Fox and Weisberg 2011) and a poisson error distribution with correction for overdispersed data and a log-link function. To analyse the proportion of undamaged capsules in different treatments and early germination rate, we also used GLMs, this time using binomial error distributions with logit link function. In either case there was a need to correct for multiplicity as treatment consisted only of three groups. Therefore contrast tests were applied between pairs of categories using the package “contrast” (Kuhn et al. 2016). Finally, package “ggplot2” (Wickman 2016) was used to create the bar graphs in Figures 2-4 and Figure S1. Package “emmeans” (Lenth 2019) was used to calculate the confidence intervals represented in Figure 2 and Figure S2.

2.3 Experiment 2: consequences for plant fitness

We measured the impact of initial seed density on the number of germinating seeds, seedling to adult plant recruitment and flower anthesis in adult female and male *S. latifolia* plants (measured as the total number of open flowers in a lifetime) as a proxy for individual plant fitness. We hypothesised that at higher densities, germination, survival, and flower production would decrease due to intraspecific competition.

We created artificial seed densities by extrapolating the estimated mean of 535.29 seedlings/m^2 (Peroni and Armstrong 2001) to the dimension of our pots (11 x 11 x 10 cm), which gave us a density of 6.48 seedlings per pot. Taking this information into account and looking at our results from the previous experiment of total seed production, we decided to create seed densities of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 40, 80, and 150 seeds per pot. All seeds used were randomly selected from a pool of seeds collected at the field sites described in the methods section. This was repeated for five simultaneous replicates. The experiment was set up in the cold-frame greenhouse facilities of the experimental garden of the University of Bremen, where temperature and light were not controlled for but rather dependent on environmental conditions. Pots were watered as needed. After 10 days, the number of seedlings (germinated seeds) was counted.

Plants were reared until the adult stage. Stems were given individual codes, as at high densities it was hard to distinguish between plants, and their sex was recorded. Plants were checked every day to collect data on the number of new open flowers produced per stem. After being counted, open flowers were picked from the plant. This was done until all stems died. At this point the experiment was concluded by removing plants from their pots to identify the origin of the stems, and surviving plants from each pot were counted to obtain data for seedling to adult plant recruitment.

2.3.1 Statistical analyses

All statistical analyses were performed using "R" (Version 3.5.3) for statistical computing (R Core Team 2019) and the interface "RStudio" (RStudio Team 2016). Graphical analysis of the proportion of surviving plants as a function of density suggested the responses were density dependent so we fitted nonlinear regression models (Bates and Watts 1988) with the function "nls" (nonlinear least squares) in the native "stats" package, and we used the "nlme" (Pinheiro et al. 2018) package to check the residuals. Models for germination and survival probability were fitted following a logarithmic equation ($y = a + b*\log(x)$) where x is the initial seed density and a and b are the regression coefficients. The response variable total flower anthesis per plant was log-transformed to achieve a normal distribution, and it was analysed with an additive Linear Mixed Model (LMM, Zuur et al. 2009) as a function of density, sex and number of stems as a random factor, using package "lme4" (Bates et al. 2015), and package "Mumln" (Barton 2019) to obtain an R^2 value for the same model.

3. RESULTS

3.1 Seed output

We found significant differences in the seed output of capsules from different categories (Figure 2; $X^2_{df=4;\ n=126}$ = 122.67; *p*-value = 2.20e-16; GEE poisson distribution with log-link function, id = plant, corstr = exchangeable). The contrast test between all pairs of categories (Table 1) showed that undamaged capsules from the three treatments ("control", "herbivore + undamaged", and "herbivore + parasitoid + undamaged") do not significantly differ in their seed outputs. However, the seed output of undamaged capsules was significantly different to the seed output of damaged capsules from both treatments ("herbivore + damaged" and "herbivore + parasitoid + damaged" respectively), the latter two also being significantly different from each other. While capsules from the herbivore treatment that were attacked by the larvae barely produced a seed output of a few surviving seeds, capsules attacked by *H. bicruris* and later parasitised by *B. variator* had a significantly higher seed output yet lower than the seed output from undamaged seed capsules (Figure 2). Moreover, the "herbivore + parasitoid" treatment produced a significantly higher proportion of undamaged capsules compared to the "herbivore treatment" ($X^2_{df=1;\ n=28} = 19.245$; p-value = 1.10e-05; see Figure S1). On the other hand, mean weight of seeds in *S. latifolia* capsules under different treatments was not significantly different ($X^2_{df=4;\ n=85} = 3.76$; p-value = 0.44; see Figure S2).

Seed output per plant was analysed counting the total number of seeds contained in all capsules from each plant. This was done for all three treatments ("control", "herbivore" "herbivore + parasitoid"). Treatment has a significant effect on the total seed output per plant (Figure 3; $F_{[2,33]} = 21.51$; p-value = 1.48e-06; McFadden's R^2 = 57,43%; GLM Poisson distribution with log-link function). The "control" treatment had a significantly higher (p-value = 0.029) total seed output per plant compared with the "herbivore + parasitoid" treatment, which in turn had a significantly higher (p-value = 0.0013) total seed output than the "herbivore" treatment with the lowest total seed output per plant. The same pattern was observed when we analysed the total number of seeds as a function of treatment ($F_{[2,32]} = 24.82$; p-value = 3.1e-07) and total number of capsules produced per plant ($F_{[1,32]} = 8.46$; p-value = 0.0066) with an additive model (McFadden's R^2 = 67,60%; see Figure S3).

Table 1 Contrast tests following the close test principal (Bretz et al. 2010) for seed output per capsule between all pairs of categories.

p-values	Herbivore + parasitoid + undamaged	Herbivore + parasitoid + damaged	Herbivore + undamaged	Herbivore +damaged
Herbivore + parasitoid + damaged	4.63e-05 ***	-	-	-
Herbivore + undamaged	n.s	0.0001 ***	-	-
Herbivore + damaged	1.11e-16 ***	0.0006 ***	<0.0001 ***	-
Control	n.s	1.36e-05 ***	n.s	2.22e-16 ***

Significant differences between pairs are shown in bold (*$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

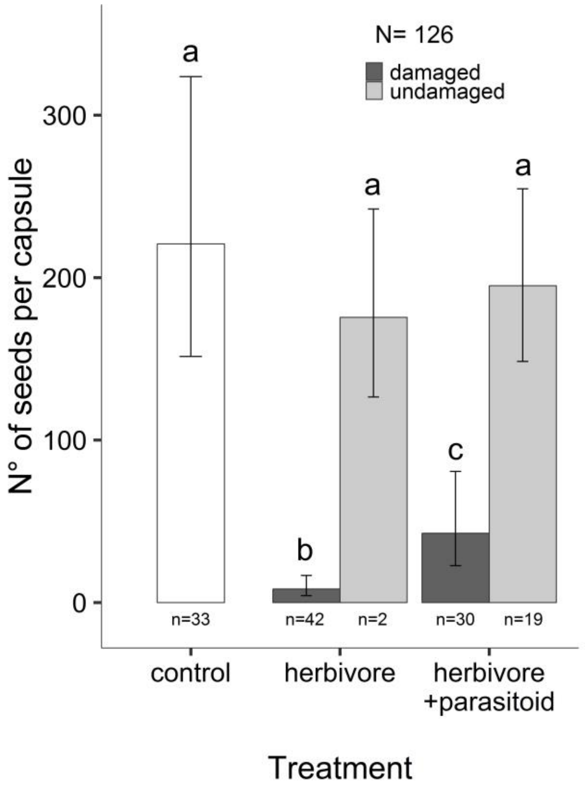

Fig. 2 Seed output per capsule in *Silene latifolia* plants under different treatments: "control" (negative control, no presence of the larvae and hence no predation), "herbivore" (larva is present and allowed to feed freely on seed capsules) and "herbivore + parasitoid" treatment (the predating larva is attacked by the parasitoid *Bracon variator*). Undamaged capsules (light grey filled bars) escaped predation by the larva of *Hadena bicruris*, while damaged capsules (dark grey filled bars) were predated upon by the larva *H. bicruris.* The bars represent model estimates and confidence intervals. (GEE Model with a poisson error distribution and log-link function, id = plant, corstr = exchangeable; $X^2_{df=4;\ n=126}$ = 122.67; *p*-value = 2.20e-16)

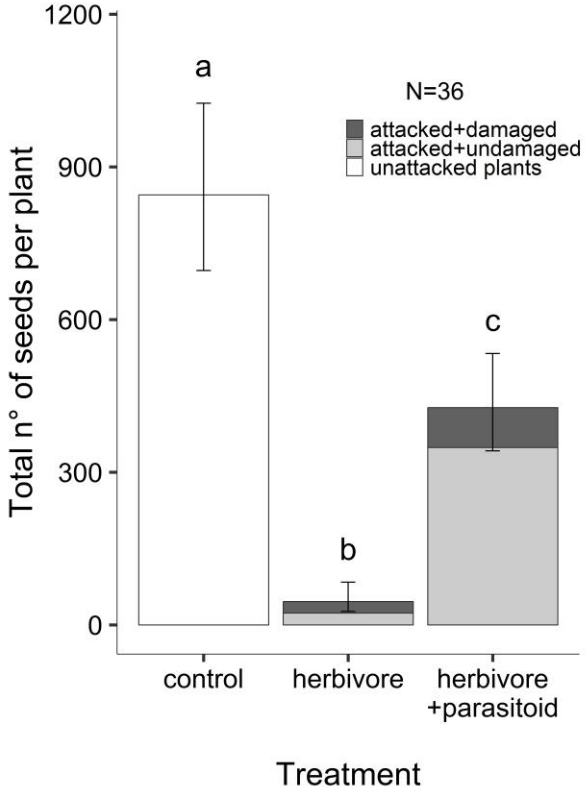

Fig. 3 Total seed output per plant for *Silene latifolia* plants under different treatments: "control" (no presence of the larvae and hence no predation), "herbivore" (larva is present and allowed to feed freely on the plant) and "herbivore + parasitoid" (the predating larva is attacked by the parasitoid *Bracon variator*). Undamaged capsules (light grey filled bars) escaped predation by the larva of *Hadena bicruris*, while damaged capsules (dark grey filled bars) were predated upon by the larva *H. bicruris.* The bars represent model estimates and confidence intervals. (GLM with a poisson error distribution with a correction for overdispersion and log-link function; $F_{[2,33]}$ = 21.51; *p*-value = 1.48e-06; McFadden's R^2 = 57,43%)

3.2 Early germination

Overall, we found significant differences in early germination between seeds coming from different treatments (Figure 4; $X^2_{df=2;\ n=288}$ = 57.20; *p*-value = 3.80e-13; GLM binomial distribution with logit-link function). Seeds from capsules from pollinated plants without herbivore attack and seeds from damaged capsules from plants with herbivore attack both had high early germination (84.4% and 75%, respectively), and the contrast test showed no significant difference between these two treatments (*p*-value = 0.11), which was against our initial expectations. Seeds from undamaged capsules from plants with herbivore attack had a low early germination of 35.4%, and the contrast test confirmed this result was significantly lower compared with the previous treatments (*p*-value < 0.0001). However, the variance explained through our model is very low (McFadden's R^2 = 15.33%) and therefore it is very likely that there are other processes involved that we are not aware of.

3.3 Density-dependent effects

We found that the germination probability did not follow a density-dependent response (Figure 5a; coeff.a = 0.55, *SE* = 0.057, t-value = 9.60, *p*-value = 2.84e-14; coeff.b = 0.013, *SE* =

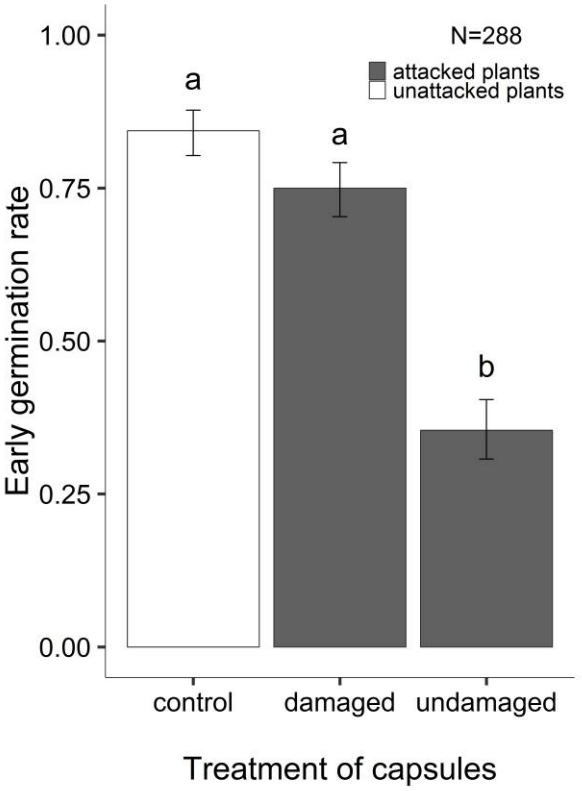

Fig. 4 Early germination for *Silene latifolia* seeds under different treatments: "control" (seeds from capsules from pollinated plants without herbivore attack), "damaged" (seeds from damaged capsules from plants with herbivore attack) and "undamaged" (seeds from undamaged capsules from plants with herbivore attack). The bars represent model estimates and confidence intervals. (GLM with a binomial error distribution and logit-link function; $X^2_{df=2;\ n=288}$ = 57.20; *p*-value = 3.80e-13; McFadden's R^2 = 15.33%).

0.022, t-value = 0.58, *p*-value = 0.57; NLS Model). Yet, survival probability did follow a density-dependent logarithmic response to initial seed density (Figure 5b; coeff.a = 0.93, *SE* = 0.044, t-value = 21.14, *p*-value < 2e-16; coeff.b = -0.13, *SE* = 0.017, t-value = -7.71, *p*-value = 3.25e-10; NLS Model). As initial seed density increases, there is a strong decrease in the proportion of plants that survive to adulthood. In addition, the analysis of the total flower anthesis per plant showed significant effects of both initial seed density ($X^2_{df=1;\ n=151}$ = 30.10; *p*-value = 4.20e-08) and sex ($X^2_{df=1;\ n=151}$ = 35.50; *p*-value = 2.60e-09; Figure 6; LMM with number of stems as random term; conditional R^2 = 48.18%). We can see a strong negative effect of initial seed density on the total number of flowers produced starting at low densities, with an overall lower production of flowers in female plants (y_1 = exp(-0.38*x + 4.57)) than male plants (y_2 = exp(-0.38*x + 5.38)) (model estimates have been backtransformed to calculate the best fitting lines for the original data, with a poisson distributed response variable).

4. DISCUSSION

This study aims to determine whether the parasitoid *B. variator* has an impact on the level of seed consumption by the seed predator *H. bicruris,* and thus, whether it modifies the interaction between the plant and herbivore along the mutualism-antagonism gradient. To this end we tested how parasitoid action translates into possible consequences in individual plant fitness. At first, our results support our first hypothesis, suggesting that *B. variator* can indeed decrease the level of seed predation by the larvae of *H. bicruris,* therefore reducing the costs of the interaction for the host plant partner *S. latifolia*. However, to which extent this increase in seed output is translated into an increase in plant fitness is something that needs to be discussed.

Under our experimental conditions, the parasitism of *H. bicruris* by *B. variator* resulted in an increase in seed output in *S. latifolia* at both the capsule and plant level. The fact that there are no significant differences between the seed outputs of undamaged capsules suggests that infested and uninfested plants allocate their resources per capsule equally. We conclude that the increase in seed output shown by damaged capsules and plants where the natural enemy was present is a direct result of the parasitism by the parasitoid *B. variator* and not due to a differential allocation of resources in plants assigned to different treatments.

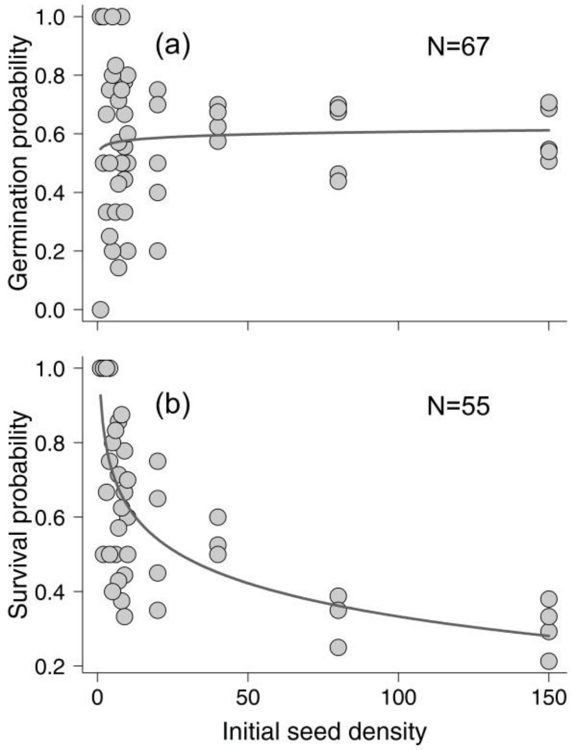

Fig. 5 Germination (a) and survival (b) probability of *Silene latifolia* plants as a function of initial seed density, represented by the best fitting lines (NLS Models, fitted to a logarithmic equation ($y = a + b*\log(x)$), starting values: a = 0.1, b = 0.1; (a) coeff.a = 0.55, SE = 0.057, t-value = 9.60, p-value = 2.84e-14; coeff.b = 0.013, SE = 0.022, t-value = 0.58, p-value = 0.57; r^2 = 0.48%; (b) coeff.a = 0.93, SE = 0.044, t-value = 21.14, p-value < 2e-16; coeff.b = -0.13, SE = 0.017, t-value = -7.71, p-value = 3.25e-10; r^2 = 52.87%).

Nursery pollination systems require a balance between the costs and the benefits of the interaction for both partners to achieve a positive outcome, which means that there must be mechanisms which prevent over-exploitation by either mutualistic partner or which ensure survival of future generations. These mechanisms are varied, ranging from selective abortion of infested fruits, cannibalism, or changes in phenology, to the presence of third parties (Brantjes 1976a, Wright and Meagher 2003, Holland and DeAngelis 2006, Burkhardt et al. 2012, Reynolds et al. 2012, Stucchi et al. 2019), or seed dispersal in space and time (e.g., seed dormancy) (Fenner and Thompson 2005). Seed dormancy, defined as failure of an intact viable seed to complete immediate germination under favourable conditions (Bewley 1997), may lead to variation in seed dispersal in time. The function of dormancy is crucial as it prevents germination when the probability of survival of the seedling is low (Fenner and Thompson 2005). The general mechanisms of seed dormancy are well studied and understood (see reviews by Finch-Savage and Leubner-Metzger 2006, Bentsink and Koorneef 2008, Nonogaki 2014), while the same cannot be said about the specific mechanisms by which parental plants can alter the dormancy state of seeds. Baskin and Baskin (1998) reviewed the effects of parental plants on seed dormancy, in cases where parent plant detects a particular stimulus and responds to it by altering the level of dormancy (Fenner and

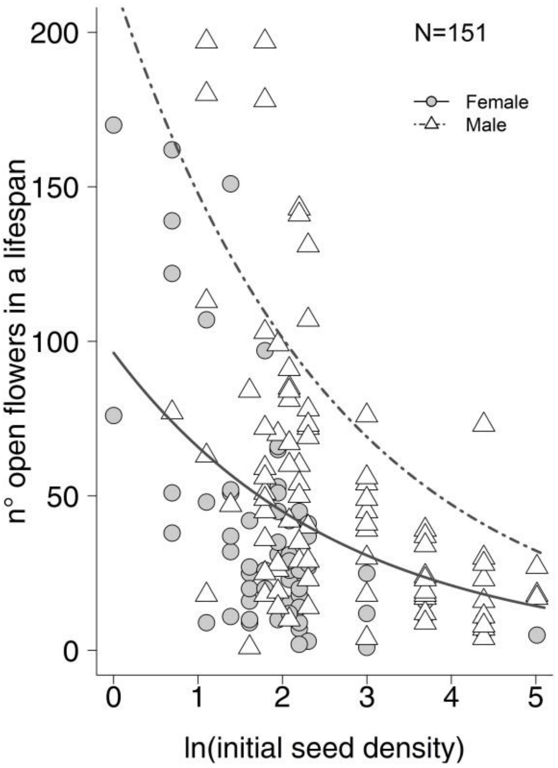

Fig. 6 Total flower anthesis per plant in *Silene latifolia* as a function of initial seed density ($X^2_{df=1; n=151}$ = 30.10; *p*-value = 4.20e-08) and sex ($X^2_{df=1; n=151}$ = 35.50; *p*-value = 2.60e-09), represented by the best fitting lines (LMM with number of stems as random term; model estimates have been backtransformed to fit the original data; females: y_1= exp(-0.38*x + 4.57); males: y_2= exp(-0.38*x + 5.38); conditional R^2 = 48.18%).

Thompson 2005). Although the mechanisms are not well understood, a recent study by Singh et al. (2017) on *Arabidopsis thaliana* showed that herbivory pressure suffered by the maternal plant can result in the loss of dormancy in its offspring, a process regulated by phytohormones.

Our germination rate results suggest that there might be some maternal effect occurring. It is possible that the presence of the predator can act as a trigger to increase seed dormancy in seeds from capsules that escaped herbivore attack on infested plants. The germination rates in seeds from attacked plants that did suffer predation by the herbivore vary between damaged and undamaged capsules. These differences in germination rates are not due to seed size differences, as average seed weight was not significantly different between treatments of capsules (see Figure S2). It is possible that attacked plants, as a response to the high herbivore pressure they were suffering in their damaged capsules, increase dormancy levels to seeds from undamaged capsules. Although this contradicts the results from Singh et al. (2017), in our view it could serve as a possible means to escape from herbivory and increase survival probability. Uninfested plants that did not suffer predation by *H. bicruris* had very high early germination, which is in line with this idea. On the other hand, enhancing seed dormancy in damaged capsules which are currently infested with the herbivore might be a waste of resources, given that without parasitoid attack most of these seeds will be consumed by the herbivore as shown by our results in Figure 2.

Previous works have explored the role of third parties in balancing the costs and benefits in nursery pollination systems. Elzinga et al. (2003) studied parasitism of *H. bicruris* larvae by the koinobiont endoparasitoid *Microplitis tristis* and its effect on larval feeding behaviour. Parasitism resulted in lower food consumption of the herbivore and the authors suggested this could positively impact *S. latifolia* populations, although it was only tested on larvae feeding on artificial diet. Various other studies suggest that high rates of parasitism of *H. bicruris* in the field could decrease the seed damage caused by the larvae, and in such cases the benefits obtained through the adult moth pollinators might counteract the costs of seed predation by the offspring (Biere et al. 2002). Later Elzinga et al. (2005) and Elzinga et al. (2007b) dismissed this idea as in the field the highest incidence rates corresponded to koinobiont parasitoid species (such as *M. tristis*), which do not arrest host growth or seed predation post parasitism. However, in this study we have a different scenario, as *B. variator* is an idiobiont ectoparasitoid commonly found in the field populations we sampled, which does indeed prevent its host larva from developing and feeding any further, and also by following a clear quantitative approach. Crabb and Pellmyr (2006) showed how a braconid parasitoid wasp could affect seed predation of yucca moth offspring, increasing the production of yucca seeds and reducing the costs of pollination. The already mentioned study by Nunes et al. (2018) showed that parasitoids could rescue part of the fruits of the orchid host plant *Dichaea cogniauxiana* from predation by the weevil larvae, changing the cost/benefit ratio of the host plant and pollinator/herbivore interaction to a positive one. As previously mentioned, the *S. latifolia*-*H. bicruris* system has been referred to as an antagonistic interaction in the literature due to the extent of seed predation caused by *H. bicruris* larvae, which often impose larger costs than those benefits granted through pollination by adult individuals. In line with these studies, our results show that parasitism by *B. variator* could act as a regulator in the *S. latifolia*-*H. bicruris* system, reducing the costs imposed by larval feeding and controlling pollinator/seed predator populations, therefore possibly acting as a stabilising mechanism of the interaction across evolutionary time.

However, our density-dependent experiments show that increasing seed density can lead to a negative impact in adult plant survival and fitness. The probability to germinate was not density dependent and likely more affected by other unknown factors, like dormancy triggered by *Hadena* predation. On the other hand, in our experimental setting, survival probability rapidly decreased at high densities. Although these densities might only be achieved in the field under ideal conditions, it is likely that *S. latifolia* has a negative density dependent recruitment. The same effect was observed for total flower anthesis per plant, with a strong decrease in flower production at high densities. This is in line with studies by Lara-Romero et al. (2016) on *Silene ciliata,* in which they report self-thinning in recruits and a lower adult reproductive performance at higher conspecific density, and also agrees with the already mentioned study by Campbell et al. (2017). This means that even when plants survive, they might suffer the effect of higher densities throughout their lifetime, achieving a lower fitness. Inevitably, this leads us to question what benefit the increase in seed output seen in plants due to parasitism by parasitoid *B. variator* provides to individual plant fitness. Clearly a higher seed output likely means higher seed density in the soil, and the possible negative effects of competition that may accrue from it. At some conditions *B. variator* might indirectly be causing an increase in density-related intraspecific competition and therefore diminishing its positive impact on plant fitness, whereas *H. bicruris* could actually be decreasing intraspecific competition by feeding on *S. latifolia* seeds, increasing the chances of the remaining seeds to be successful. In this scenario, the interaction between *S. latifolia* and *H. bicruris* should not be viewed as antagonistic, but rather much more specialised than it has been considered until now, as it would mean that the host plant invests in high amounts of seed production to compensate for the feeding of its mutualistic partner, and therefore lowers the costs of their interaction.

The parasitism of *Hadena* by *B. variator* might not only affect the seed availability for the next plant generation, it might also impact the pollination level at the next generation, given it reduces the moth populations considerably and given other pollinators would not compensate for this. This would be an intriguing line of thought best addressed with a modelling approach at the tritrophic population level, but this is beyond the scope of this paper.

Ever since it was demonstrated three decades ago that plants emitted volatile compounds as a response to several forms of herbivory attack (Dicke and Sabelis 1988, Dicke et al. 1990, Turlings et al. 1990, van Loon et al. 2000, Steidle et al. 2005) or even oviposition (Hilker and Meiners 2006), there has been a standing discussion whether plants “crying for help” to attract the natural enemies of their herbivores may actually be beneficial for plant fitness (Dicke and Baldwin 2010, Kessler and Heil 2011, Heil 2014). Only a few studies have provided evidence for a net increase in plant fitness as a direct result of natural enemies attacking their herbivores (van Loon et al. 2000, Hoballah and Turlings 2001, Schuman et al. 2012, Gols et al. 2015, Cuny et al. 2018). More interestingly, recent studies have also shown that in certain cases, natural enemies may confer a negative effect on plant fitness (Smallegange et al. 2008, Xi et al. 2015). Smallegange et al. (2008) studied the effect of a koinobiont endoparasitoid on plant fitness and found out that unparasitised caterpillars and caterpillars with high load of parasitoid larvae consumed more flowers than caterpillars with single parasitoid broods, and as a result there was a decrease in seed production. Xi et al. (2015) concluded that parasitism by a koinobiont endoparasitoid increased seed damage caused by the seed predating larvae of a species of tephritid flies. Our study should add another layer to the complex discussion of whether parasitoids contribute or not to plant fitness, as we have shown that taking only seed output into account is not enough to determine the net effect of this relationship. Other factors should be taken into consideration to properly examine the indirect effects of parasitoids on plant fitness.

5. CONCLUSION

The *S. latifolia-H. bicruris* interaction is usually described as parasitic, however, most studies have only focused on the net outcome of the interaction at the seed production level. Our research offers new insight into the role of parasitoids in the *S. latifolia-H. bicruris* nursery pollination system. The presence of a braconid ectoparasitoid wasp can increase seed output of the host plant, making the system more stable. However, such an increase in seed density has a negative effect on *S. latifolia* survival and flower production, and therefore we should consider whether this increase in seed output is indeed beneficial to plant fitness. These results emphasize the need to focus on different measures of fitness when studying pollination systems and the complex relationship between natural enemies and host plants.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

CVC and TSH conceived and designed the work. CVC performed fieldwork, data collection, analysis and interpretation, and wrote the manuscript. TSH provided critical revision of the manuscript.

DATA AVAILABILITY STATEMENT

The data reported in this article are available in the Dryad data repository with https://doi.org/10.5061/dryad.rjdfn 2z75.

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CHAPTER 4

General discussion

Carmen Villacañas de Castro

> "It is not necessary … in order to establish mutualism to show that the organisms do no injury to each other. Mutualism of the kind we meet with in the vegetable kingdom involves sacrifice on the part of the host. The parasite is not there gratuitously. It is there to steal from its host the living it is hereditarily and constitutionally indisposed to make for itself. If the host gains any advantage from the relation, it can only do so by sacrificing – by giving the parasite the benefit of its labor that it may subsist."
>
> —Roscoe Pound. *Symbiosis and mutualism* [1]

One of the aims of evolutionary theory is to understand how interactions are shaped, transformed and diversified under different ecological conditions (Thompson 1988). The interaction between *Silene latifolia and Hadena bicruris* has a lower degree of specialisation than other nursery pollination systems, which makes it less stable, and more prone to conditional outcomes. Understanding how biotic factors —like the presence of copollinators or parasitoids— play a role in such conditionality will help improve our knowledge on how these mutualistic interactions evolve, transform, and are maintained (Bronstein 1994). However, to do so, it is vital to determine the costs and benefits to the partners in the system. In nursery pollination systems, from the perspective of the host plant, it is relatively easy to quantify the benefits obtained from the pollination services, and the costs of predation. While the costs and benefits of the pollinator/herbivore are harder to quantify, being a mobile partner, the outcome of the interaction for the pollinator/herbivore is expected to be positive. The work here presented has therefore focused on the outcome of the *S. latifolia*–*H. bicruris* interaction from the perspective of the plant.

In the first part of this thesis, the positive contribution of male and female adult moths as pollination services for *S. latifolia* plants was quantified by analysing visitation rates and pollination efficiency. Moreover, different field populations were sampled and parametrised. A theoretical model was applied to investigate the conditional outcomes of the *S. latifolia-H. bicruris* system in the field and to discern the possible role of copollinators in shaping such interaction outcomes. In the second part of this thesis, the potential role of a natural enemy,

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[1] From Pound, R. 1893. Symbiosis and mutualism. American Naturalist 27:59-520, p.519. Sourced from Boucher (1985), p. 15.

the ectoparasitoid *Bracon variator*, as a stabilising mechanism that reduces the seed predation costs caused by *H. bicruris* was examined. Finally, laboratory and greenhouse experiments were carried out to study the possible consequences of an increase in seed output in individual plant fitness. In the last chapter of this thesis, I will summarize and discuss my findings in regard to some of the concepts of eco-evolutionary theory presented in the introduction.

1. ECOLOGICAL PATTERNS IN THE *S. latifolia-H. bicruris* INTERACTION

Conditionality refers to a change in the outcome of an interaction between two species as a result of variations in the local biotic or abiotic conditions where it occurs (Cushman and Beattie 1991, Bronstein 1994, Holland and DeAngelis 2009). As the costs and benefits for each partner change, so will the net effect (+,0,-) of the interaction for each of them, and consequently the outcome will shift along a continuum from mutualism to antagonism (Bronstein 1994, Bronstein et al. 2003, Westerbergh 2004, Holland and Bronstein 2008, Leung and Poulin 2008, Holland and DeAngelis 2009). In this thesis a total of five field populations of *S. latifolia-H. bucruris* were parametrised and the results reflect a high conditionality in the outcomes of the interaction for the host plant, with three populations behaving antagonistically and only two out of the five fields as a facultative mutualism. The reasons for conditional outcomes are diverse and some were already explored in the introduction. Hence, I will focus on two most likely affecting the *S. latifolia-H. bucruris* interaction: population size and the presence of other interacting species.

It is known that per-capita costs and benefits of one partner might change depending on its own population size or the population size of its mutualistic partner (Thompson 1988, Holland and DeAngelis 2001). By modifying the cost and benefit ratio, interaction outcomes will also change. My findings suggest that in *S. latifolia* plants pollination benefits are not influenced by the number of female plants in a population, but infestation rates by *H. bicruris* larvae decrease significantly in larger plant populations. It is to be expected that the per-capita seed predation costs imposed by such infestation also decrease with higher plant population sizes. On the other hand, in fields with higher pollinator/herbivore abundance relative to host plants, infestation rates and consequent seed predation costs could be high enough to outweigh the pollination benefits for the plant, which can lead to a shift in the interaction from mutualism to parasitism, a pattern predicted in the model developed by

Holland and DeAngelis (2009). Other studies have suggested that abundance of pollinators relative to flowers can influence the costs of the interaction (Herre and West 1997, Holland and DeAngelis 2001, Holland et al. 2002, Aizen et al. 2014). Thus, we can conclude that population size is a cause for conditionality in the *S. latifolia*-*H. bicruris* system.

In regards to pollination behaviour, the observational assay results confirm that both female and male *H. bicruris* moths show equivalent visitation rates and pollination efficiencies, with an average fertilisation of 65% of the ovules. Therefore, it is obvious that the pollination services offered by *H. bicruris* moths are highly valuable to *S. latifolia* plants. In addition, male moths bring benefits to the interaction without imposing many costs to its host plant, as only the females lay eggs (Labouche and Bernasconi 2010). This can potentially increase the benefits provided in relation to the costs, partly compensating for the seed predation, and having an impact in the outcome of the interaction. In fact, in a study on the nursery pollination system between *Trollius europaeus* and *Chiastocheta* flies, Després (2003) showed that male flies contribute to pollination benefits, suggesting it could buffer the antagonistic effects of ovipositing females. Moreover, Stucchi et al. (2019) developed a population dynamic model for the *S. latifolia*-*H. bicruris* system, together with a natural enemy, and concluded that male moth initial population size and relative presence had a strong effect on the stability of the whole system. Therefore, ecologists should continue investigating the role of male pollinators in modifying the strength of interaction outcomes and conferring stability in other nursery pollination systems (Labouche and Bernasconi 2010).

On the other hand, the density of copollinators also seems to be decisive in the outcome of the interaction, strongly influencing conditionality (Pettersson 1991, Dufaÿ and Anstett 2003, Westerbergh 2004). Non-predating copollinators have a similar impact as male *H. bicruris* moths: they provide pollination services with almost no costs to the host plant, although they produce a lower seed set as they are less efficient (Young 2002, Scopece et al. 2018). However, in terms of the interaction between *S. latifolia* and *H. bicruris*, if a large proportion of the seed set is the result of copollination, such benefits obtained will mask those provided by *H. bicruris* moths (Dufaÿ and Anstett 2003, Westerbergh 2004, Reynolds et al. 2012), as the larvae will feed upon more seeds than those produced by the adult moths. Therefore, in an environment with a low density of copollinators and a high density of *H. bicruris* moths, the outcome of the interaction between *S. latifolia* and *H. bicruris* is likely to be mutualistic despite seed predation, while in an environment with a high density of copollinators and a low density of *H. bicruris* moths, the outcome is likely to be antagonistic. This is not the only nursery pollination system with such dynamics, as this type of conditionality is also present in the interaction between *Greya* moths and their host plant *Lithophragma* (Thompson and Pellmyr 1992, Thompson and Fernandez 2006).

Although several studies have suggested that a higher incidence of natural enemies in larger populations result in a decrease in fruit predation rates (Jeffries and Lawton 1984, Roland 1993, Elzinga et al. 2005, Elzinga et al. 2007b, Kula et al. 2014), the results from the field were inconclusive as to whether the presence of parasitoid *B. variator* could decrease infestation rates and increase seed output. I carried out laboratory experiments to follow up this question and elucidate if the action of the parasitoid had an impact on the level of seed consumption by the seed predator *H. bicruris.* My findings show that under experimental conditions *B. variator* decreases seed predation by the larvae, producing an increase in seed output. This limitation on the costs of the *S. latifolia H. bicruris* interaction could potentially help stabilise the system and regulate the pollinator/herbivore populations. Bronstein et al. (2003) suggested that adding an antagonist to a pairwise mutualism could regulate mutualist success and population sizes, avoiding an excessive population growth which would lead to extinction of both species. Subsequent studies have shown that specifically parasitoids are able to mediate the outcome of nursery pollination interactions, reducing the costs of the partnership between the host plant and its pollinator/herbivore and helping in the stabilisation of the system towards a mutualism (Harrison 2014, Nunes et al. 2018, Stucchi et al. 2019). In the same study by Stucchi et al. (2019), the dynamic population model results showed that parasitoid attack was an effective regulatory mechanism, and even a small population of parasitoids was enough to shift the system to a new stable stationary point where plant population was favoured. Therefore, once again it becomes clear that the presence or absence of third parties can deeply impact conditionality in the S*. latifolia-H. bicruris* interaction.

The effects of seed predation by *H. bicruris* on the population dynamics of *S. latifolia* is something not yet fully understood. In most cases the research has focused on whether the interaction was parasitic or mutualistic, with emphasis on simple measures of fitness such as seed production vs. seed predation. However, in systems such as this one with high conditionality and variation in pre-dispersal seed predation, both plant population dynamics and plant trait evolution will likely be influenced (Kolb et al. 2007). It is obvious that seed predation by *H. bicruris* has a negative impact on the performance of individual plants, but looking only at the proportion of seeds eaten in relation to seeds produced might not be a good measure of total fitness (Ehrlén 2003, Kolb et al. 2007). Instead, it is preferable to analyse how seed predation affects next generation population size and dynamics of the plant population (Kolb et al. 2007). A low seed output due to seed predation may not be limiting if density dependent responses compensate for seed loss, for example by increasing survival probability of seedlings due to a decrease in intraspecific competition (Kolb et al. 2007). In this line, the density-dependent experiments carried out as part of this thesis show that increasing sowing seed density can lead to a negative impact in adult plant survival and reproductive performance, typical of a negative density dependent recruitment response.

On the other hand, in North America, outside its native range, *S. latifolia* has been found to behave invasively (Wolfe 2002, Blair and Wolfe 2004). Wolfe (2002) tested the enemy-release hypothesis comparing European and North American populations of *S. latifolia,* and results indicated that any random plant was 17 times more likely to be attacked in its native range than in North America. Therefore, it appears that *S. latifolia* managed to escape its specialist seed predator, *H. bicruris*, when it was introduced in North America approximately 200 years ago (Wolfe 2002). So far, it seems that no other seed predator has so far filled this niche, which could partly explain its success as an invasive plant (Wolfe 2002). Specialised enemies are good at controlling host populations as they are the result of generations of interacting and possibly of coevolution, and therefore it would be rare that native North American enemies would occupy the niche vacated by *H. bicruris* (Wolfe 2002).

Given the complexity of the system (Figure 1) and how pairwise interactions and population dynamics can vary greatly as species are added or removed (Thompson 1988, Bronstein 1994), further research needs to be carried out to properly assess the impact of the pollinator/herbivore and parasitoid partners on host plant population dynamics through exclusion experiments and modelling approaches at the tritrophic population level.

2. COEVOLUTIONARY PATTERNS IN THE *S. latifolia-H. bicruris* INTERACTION

When studying conditional outcomes in interspecific interactions due to fluctuating partner densities, Thompson (1988) came to the conclusion that selection of hosts in certain mutualisms was dependent on the probability of encounter between the partners. Thus, when interacting with a particular partner in a lifetime becomes inevitable, selection may favour host genotypes that decrease antagonistic effects of the partner or increase mutualistic ones (Thompson 1988). Therefore, in a plant-pollinator interaction, only when pollinator visits to the host plant, and host plant availability to the pollinator are consistently high, selection for

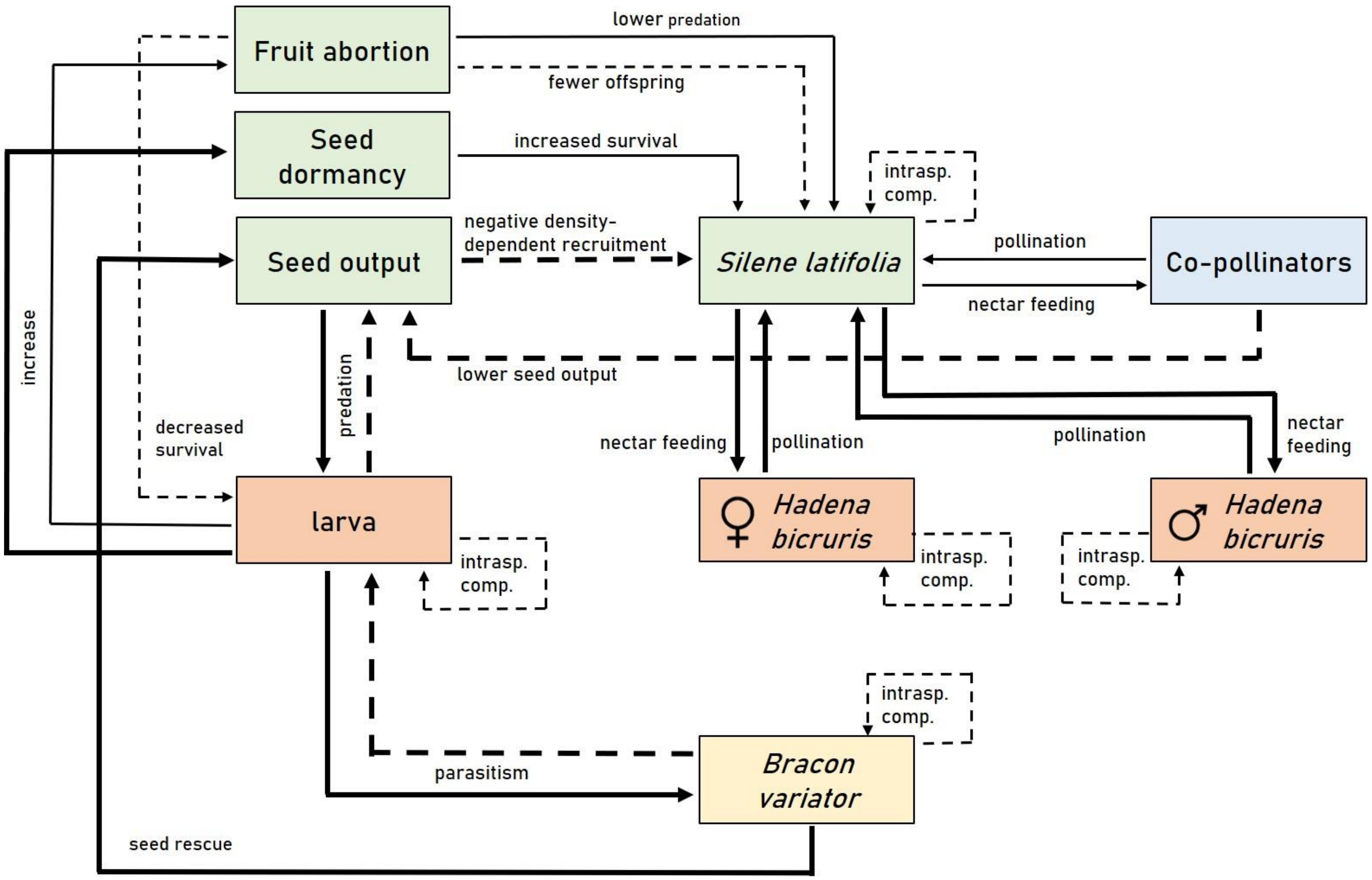

Fig. 1 Conceptual diagram of the *Silene latifolia – Hadena bicruris* system. Positive effects are represented by solid lines and negative effects by dashed lines. Bold lines represent the factors investigated in this thesis. Different colours represent different species, same colours represent the same population (Modified from Stucchi et al. 2019).

specific mutualistic coevolution in a population would be effective (Thompson 1988). In fact, some authors suggest that pollinator/herbivore partners may put more selective pressure in their host plant through their costly actions as seed predators than through their benefits as pollinators (Burkhardt et al. 2012). If the pollinator/herbivore causes any change in a specific plant trait which is directly linked to fitness, an evolutionary change will be possible (Kolb et al. 2007). Therefore, pre-dispersal seed predation can be a strong selective force in the evolution of plant traits (Kolb et al. 2007). In line with this idea, it is expected that in nursery pollination systems natural selection favours genotypes that are able to reduce the predation costs inflicted by the pollinator/herbivore and by doing so avoid over-exploitation. For example, the *Yucca-Tegeticula* moth nursery pollination system presents selective fruit abortion when the pollinator/herbivore egg loads are too high, or flowers have received low quality pollen, increasing the net benefits of fruit production and regulating pollinator/herbivore populations (Huth and Pellmyr 2000, Holland and DeAngelis 2001, Shapiro and Addicott 2004, Holland and DeAngelis 2006).

As already mentioned in the introduction there is evidence supporting the idea that host plant *S. latifolia* has evolved certain adaptations to avoid over-exploitation by *H. bicruris:* from changes in scent production (Dötterl et al. 2005, Muhlemann et al. 2006, Piesik et al. 2014) to constraining of the oviposition site (Labouche and Bernasconi 2013), including selective fruit abortion (Jolivet and Bernasconi 2006, Burkhardt et al. 2009). Nevertheless, defensive traits are usually costly, so interactions prone to conditional outcomes and in which partner densities tend to fluctuate, may select for plasticity in defence responses, with such traits only being expressed when induced by pollinator/herbivore attack (Jolivet and Bernasconi 2006). In this sense, we would expect nursery pollination systems to present inducible defences when predation rates are high. My findings suggest that seed dormancy could act as a short-term strategy to reduce costs in the interaction and ensure survival of future generations, although the mechanism through which the mother plant would induce dormancy is unknown. According to Baskin and Baskin (2014) an increase in seed predation will lead to increased seed dormancy, allowing a delay in germination until conditions are favourable for seedling survival and reproductive success. Although clearly this field needs further research, inducing seed dormancy could be a defensive mechanism that has been selected for in *S. latifolia.* In the North American populations of *S. latifolia* where there has been a release of enemy pressure, investing in costly defences is unnecessary, and therefore selection is expected to favour a shift in the allocation of those resources towards enhancing reproduction and growth (Blossey and Notzold 1995, Blair and Wolfe 2004, Wolfe et al. 2004).

Indeed, Blair and Wolfe (2004) provided evidence that *S. latifolia* plants from North American populations showed greater germination rates, a more vigorous growth, enhanced reproductive output, and a lower calyx trichome production (presumed to have a defensive function). Shortly after, Wolfe et al. (2004) corroborated that such differences in life-history traits between European and North American populations were not due to plasticity but genetically-based, and showed that *S. latifolia* plants from North America exhibited a higher susceptibility to fruit predation and attack when exposed to *H. bicruris* and other natural enemies from its native range. All of this supports the idea that *S. latifolia* has evolved adaptations to defend itself from the over-exploitation by *H. bicruris*, and highlights a higher degree of specialisation of the interaction than initially thought (Dufaÿ and Anstett 2003).

Another plant reproductive trait which can be selected for as a result of pre-dispersal seed predation is seed number. Indeed, Sakai and Harada (2007) developed a model to study the effect of predation on seed size and number. They concluded that if predation is high or unpredictable (which are both characteristics of the *S. latifolia-H. bicruris* interaction) plants will increase the number of ovules produced to ensure a minimum offspring number. Consistent with this model, a recent study by Desoto et al. (2016) suggested a plastic response to predation in *Juniperus thurifera* in populations with high and low predation rates. Spanish populations with high pre-dispersal predation rates produced multi-seeded cones with smaller seeds, while Moroccan populations with low levels of seed predation produced single-seeded cones, with a larger seed. In addition, fruits with higher number of seeds could potentially satiate pre-dispersal seed predators sooner, avoiding the loss of the total seed content and lowering the overall seed predation rate (Desoto et al. 2016). With this in mind, one can speculate whether *S. latifolia* has similarly been selected to invest in high amounts of seed production to compensate for the feeding of its pollinator/herbivore partner, and therefore lower the costs of their interaction (Kephart et al. 2006). On the one hand, it would be consistent with the fact that *S. latifolia* survival and fitness decreases at higher sowed seed densities. On the other hand, at first glance this seems to contradict the dynamic population model by Stucchi et al. (2019), which suggested that parasitoid attack of *H. bicruris* larvae and the subsequent increase in seed output (also observed in my experiments), is an effective regulatory mechanism. However, it does not need to be so, as the combined effect of the different species interacting with *S. latifolia* could lead to stable population dynamics. The *S. latifolia-H. bicruris* interaction is part of a complex ecological community where it can interact with multiple other species beyond copollinators or parasitoids. Other organisms may predate upon *S. latifolia* seeds, increasing total seed loss, which in turn would decrease density-dependent competition, allowing a higher seedling recruitment. It is possible that in field conditions, *S. latifolia* seeds that have already been dispersed by gravity are foraged by organisms such as harvester ants, which would both increase seed predation and seed dispersal. A study by Detrain and Pasteels (2000) analysed the harvesting behaviour of Mediterranean ant *Messor barbarus*, and showed that among a community of 78 seed species, seeds from the catchfly or *Silene gallica* were among those that were preferentially harvested. In the endangered herb *Silene diclinis,* endemic to Valencia (Spain), ants are significant seed predators, with clumped seed distributions resulting in severe seed predation (mean seed predation of 81%) (Montesinos et al. 2006).

3. CONCLUSION

When studying plant-insect interactions, and even more such complex ones like those involved in nursery pollination systems, it is vital to remember that taking simple, partial measures of fitness is not enough to understand the outcomes and overall influences on partner performance. It is evident that interactions between the partners involved in nursery pollination systems are highly dynamic and continue to evolve within complex ecological communities which impose selection pressures that constantly reshape them (Thompson and Pellmyr 1992, Schoonhoven et al. 2005, Thompson and Fernandez 2006). Sometimes, such selection pressures can be conflicting (Pilson 2000, Frey 2004, Elzinga et al. 2007a, Ehrlén and Münzbergová 2009). For example, in this line, it was hypothesised by Prieto-Benítez et al. (2017) that, as parasitoids attack *H. bicruris* larva when they are too large to take refuge inside secondary capsules, smaller fruit capsule sizes could be a potential trait indirectly selected for to minimise predation costs by the pollinator/herbivore. In contrast, larger *S. latifolia* flowers have higher visitation rates by *H. bicruris* moths and produce more seeds per capsule, so the bigger flower size could be selected for to maximise pollination (Biere and Honders 2006, Burkhardt et al. 2009, Burkhardt et al. 2012, Brothers and Atwell 2014). However, larger flowers are also more prone to predation by *H. bicruris* larva, contributing to selection of flower size again in the opposite direction (Biere and Honders 2006, Burkhardt et al. 2009, Burkhardt et al. 2012, Brothers and Atwell 2014). In a community context, such opposing selection pressures will limit evolutionary trajectories, but this does not exclude partners from responding to selection pressures by individual specific agents (Kolb et al. 2007).

Overall, this study has provided new insight into the causes of conditionality in the *S. latifolia-H. bicruris* system, of which male moth availability, abundance of copollinators, and plant population size are the most important. In addition, it demonstrates the impact that the presence or absence of parasitoids can have in the *S. latifolia-H. bicruris* interaction. However, the overall effects for plant and moth population dynamics are not yet fully understood, and need to be further investigated through modelling approaches. Although adding species to the study of this system clearly adds extra complexity, it would be very interesting to examine whether there are any other species in the field affecting the dynamics of the *S. latifolia-H. bicruris* system, such as harvester ants or other seed dispersers. Finally, questions have also arisen from the results presented in this thesis. While there is evidence that pre-dispersal seed predation can be a strong selective force in the evolution of plant traits, whether induction of dormancy in *S. latifolia* seeds has been selected for and the mechanisms behind it are unknown. Further germination experiments and phytohormone concentration analyses would be a step in the right direction towards understanding the mechanisms involved in such phenomenon.

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ANNEX

SUPPORTING INFORMATION

CHAPTER 3

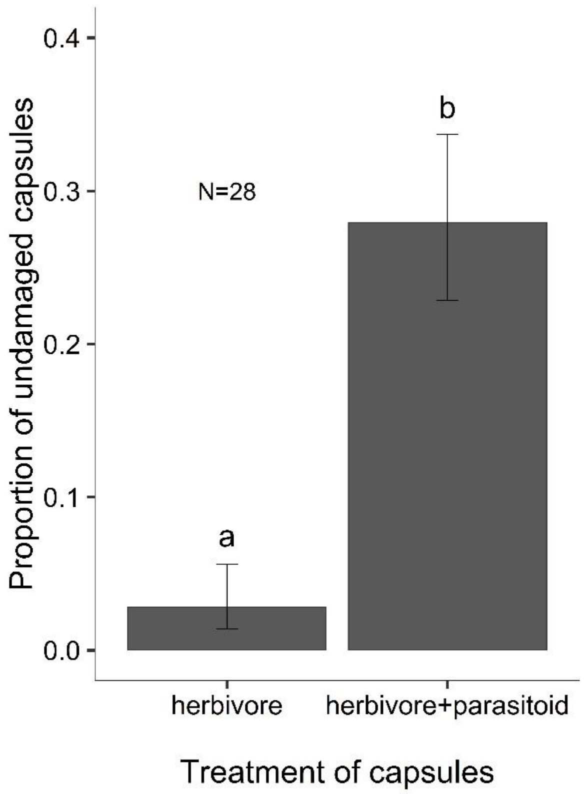

Fig. S1 Proportion of undamaged capsules for *Silene latifolia* plants as a function of treatment ($X^2_{df=1; n=28}$ = 19.245; *p*-value = 1.10e-05): "herbivore" (control, larva is present and allowed to feed freely on seed capsules) and "herbivore + parasitoid" treatment (the predating larva is attacked by the parasitoid *Bracon variator*). The bars represent model estimates and confidence intervals. (GLM with a binomial error distribution and logit-link function; McFadden's R^2 = 44,2%).

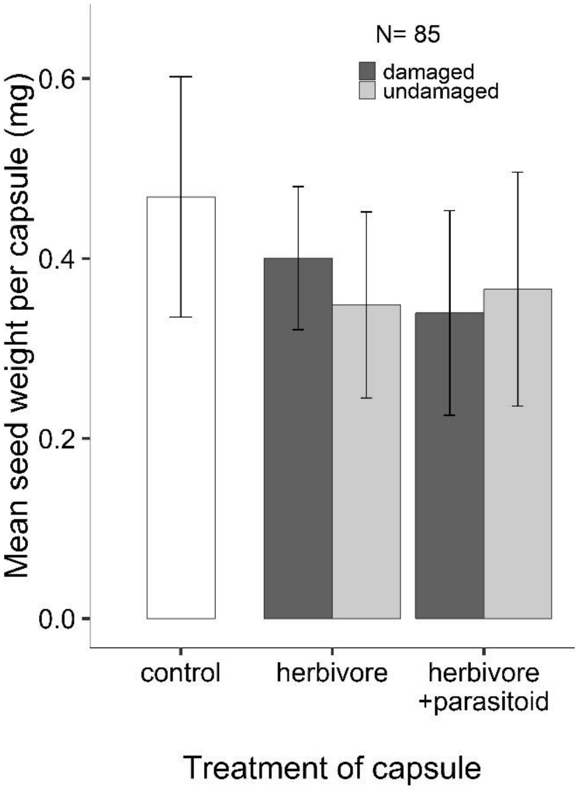

Fig. S2 Mean weight of seeds in *Silene latifolia* capsules under different treatments: "control" (negative control, no presence of the larvae and hence no predation), "herbivore" (control, larva is present and allowed to feed freely on seed capsules) and "herbivore + parasitoid" treatment (the predating larva is attacked by the parasitoid *Bracon variator*). Undamaged capsules (light grey filled bars) escaped predation by the larva of *Hadena bicruris*, while damaged capsules (dark grey filled bars) were predated upon by the larva *H. bicruris.* The bars represent model estimates and confidence intervals. (GEE Model with a gaussian error distribution, id = plant, corstr = ar1; $X^2_{df=4;\, n=85}$ = 3.76; *p*-value = 0.44).

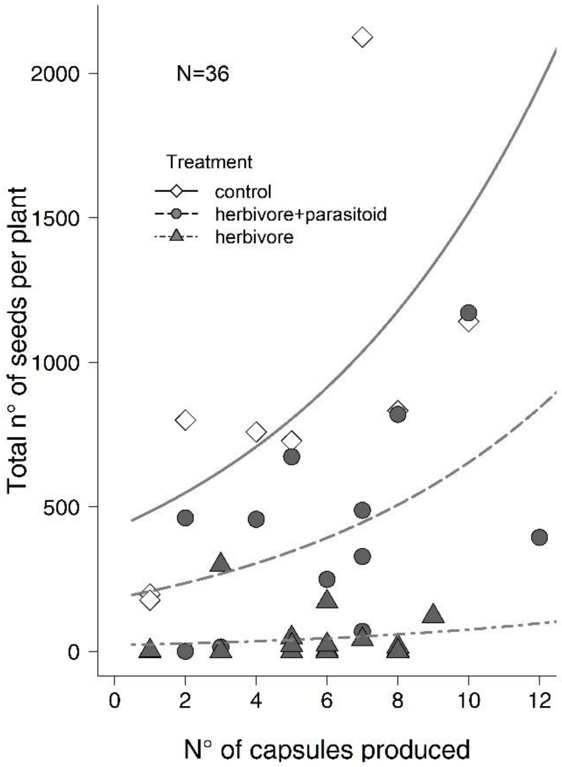

Fig. S3 Total seed output per plant for *Silene latifolia* plants as a function of treatment ($F_{[2,32]}$ = 24.82; *p*-value = 3.1e-07) and total number of capsules produced ($F_{[1,32]}$ = 8.46; *p*-value = 0.0066), represented by the best fitting lines (GLM with a poisson error distribution with a correction for over-dispersion and log-link function; McFadden's R^2 = 67,60%) The treatments are: "control" (negative control, no presence of the larvae and hence no predation), "herbivore" (control, larva is present and allowed to feed freely on the plant) and "herbivore + parasitoid" (the predating larva is attacked by the parasitoid *B. variator*).

LIST OF IMAGE SOURCES

CHAPTER 1

Cover: *Silene latifolia.* Artwork: Tessina De Lille, 2020. Watercolour on paper. A3.

Figure 1 Darwin's mechanistic model for the coevolution of *Angraecum sesquipedale* and its pollinator. Source: modified from Johnson and Anderson 2010.

Figure 2 The interaction grid. Source: modified from Bronstein 2015.

Figure 3 Depiction of different examples of interspecific interactions. Sources:

a) http://www.antbase.net/english/ants-of-germany/ebene1/messor-structor.html
b) https://the-chicken-chick.com/poultry-lice-and-mites-identification/
Left below:
https://upload.wikimedia.org/wikipedia/commons/0/02/Dermanyssus-gallinae.jpg
c) http://protist.i.hosei.ac.jp/PDB/Images/Heterokontophyta/Araphidineae/Synedra/ulna_4.html
Top right:
https://upload.wikimedia.org/wikipedia/commons/2/29/Asterionella_formosa.jpg
d) https://upload.wikimedia.org/wikipedia/commons/5/54/Black_Walnut_middle.JPG
e) https://www.britannica.com/animal/remora
f) https://www.invasive.org/browse/detail.cfm?imgnum=5573435

Figure 4 The interaction compass. Source: modified from Bronstein 2015.

Figure 5 A variety pollination syndromes. Sources:

a) https://upload.wikimedia.org/wikipedia/commons/d/d1/Black_Flying_Fox_-_AndrewMercer_IMG01870.jpg
b) Original image by the author, Carmen Villacañas de Castro.
c) Original image by the author, Carmen Villacañas de Castro.
d) https://upload.wikimedia.org/wikipedia/commons/f/fa/Ruby-throated_Hummingbird_at_Cardinal_Flower_%285278271834%29.jpg
e) Original image by the author, Carmen Villacañas de Castro.

f) https://upload.wikimedia.org/wikipedia/commons/8/8d/Bee_Orchid_%28Ophrys_apifera%29_%2814374841786%29_-_cropped.jpg

Figure 6 Adult *Seres rotundus* fig wasps emerging from the syconium of a larged-leaved rock fig, *Ficus abutilifolia*. Source: https://www.inaturalist.org/observations/35200304

Figure 7 The *Silene latifolia-Hadena bicruris-Bracon variator* multitrophic system. Sources:

a) https://upload.wikimedia.org/wikipedia/commons/6/64/Wei%C3%9Fe_Lichtnelke%2C_Bogen.jpg
b) https://commons.wikimedia.org/wiki/Category:Silene_latifolia_in_Germany#/media/File:20120621Silene_latifolia1.jpg
c) https://upload.wikimedia.org/wikipedia/commons/f/f0/73.281_BF2173_The_Lychnis%2C_Hadena_bicruris_%2814393560953%29.jpg
d) Image kindly provided by Jeroen Voogd.
e) Image kindly provided by Jeroen Voogd.
f) Image kindly provided by Jeroen Voogd.

Figure 8 *Silene latifolia* infested seed capsule about to be abscised from the plant. Source: https://commons.wikimedia.org/wiki/File:20160720Silene_latifolia2.jpg

CHAPTER 2

Cover: *Hadena bicruris.* Artwork: Tessina De Lille, 2020. Watercolour on paper. A3.

Figure 1 The model system: *Silene latifolia-Hadena bicruris-Bracon variator* interaction. Sources:

a) https://commons.wikimedia.org/wiki/File:Silene_latifolia_in_Aveyron_01.jpg
b) Image kindly provided by Jeroen Voogd.
c) Image kindly provided by Jeroen Voogd.
d) https://arthropodafotos.de/dbge.php?lang=eng&sc=0&ta=t_43_hym_apo_bra

Figure 2 Experimental set up for the observation assays. Sources:

a) Image provided by Daniele Buono.
b) Image provided by Daniele Buono.

Figure 3 Map of the sampling area with the five different populations. Source: Modified from Google Earth.

CHAPTER 3

Cover: *Bracon variator*. Artwork: Tessina De Lille, 2020. Watercolour on paper. A3.

Figure 1 *Hadena bicruris* larva at a late developmental stage feeding on a *Silene latifolia* secondary seed capsule. Source: original image by the author, Carmen Villacañas de Castro.

CHAPTER 4

Cover: System *Silene-Hadena-Bracon.* Artwork: Tessina De Lille, 2020. Watercolour on paper. A3.

Figure 1 Conceptual diagram of the *Silene latifolia-Hadena bicruris* system. Source: modified from Stucchi et al. 2019.

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Although I am not a huge fan of rollercoasters, I must say this PhD has definitely felt like riding one. There's been steep slopes that appeared to never end, tight turns that changed my direction, and a couple free-falls where everything seemed to be lost. I have felt fear, excitement, anxiety, adrenaline rushes and euphoria, sometimes all in the same evening! However, I have made it through, and I wish to thank everyone that has helped me, in one way or the other, arrive to the end safe and sound.

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DECLARATION OF CONTRIBUTION

CHAPTER 1:
Title: General introduction
Author: Carmen Villacañas de Castro.
Contribution of the candidate: 100%

CHAPTER 2:
Title: Cost/benefit ratio of a nursery pollination system in natural populations: a model application
Authors: Carmen Villacañas de Castro, Daniele Buono, Thomas S. Hoffmeister
Contribution of the candidate:

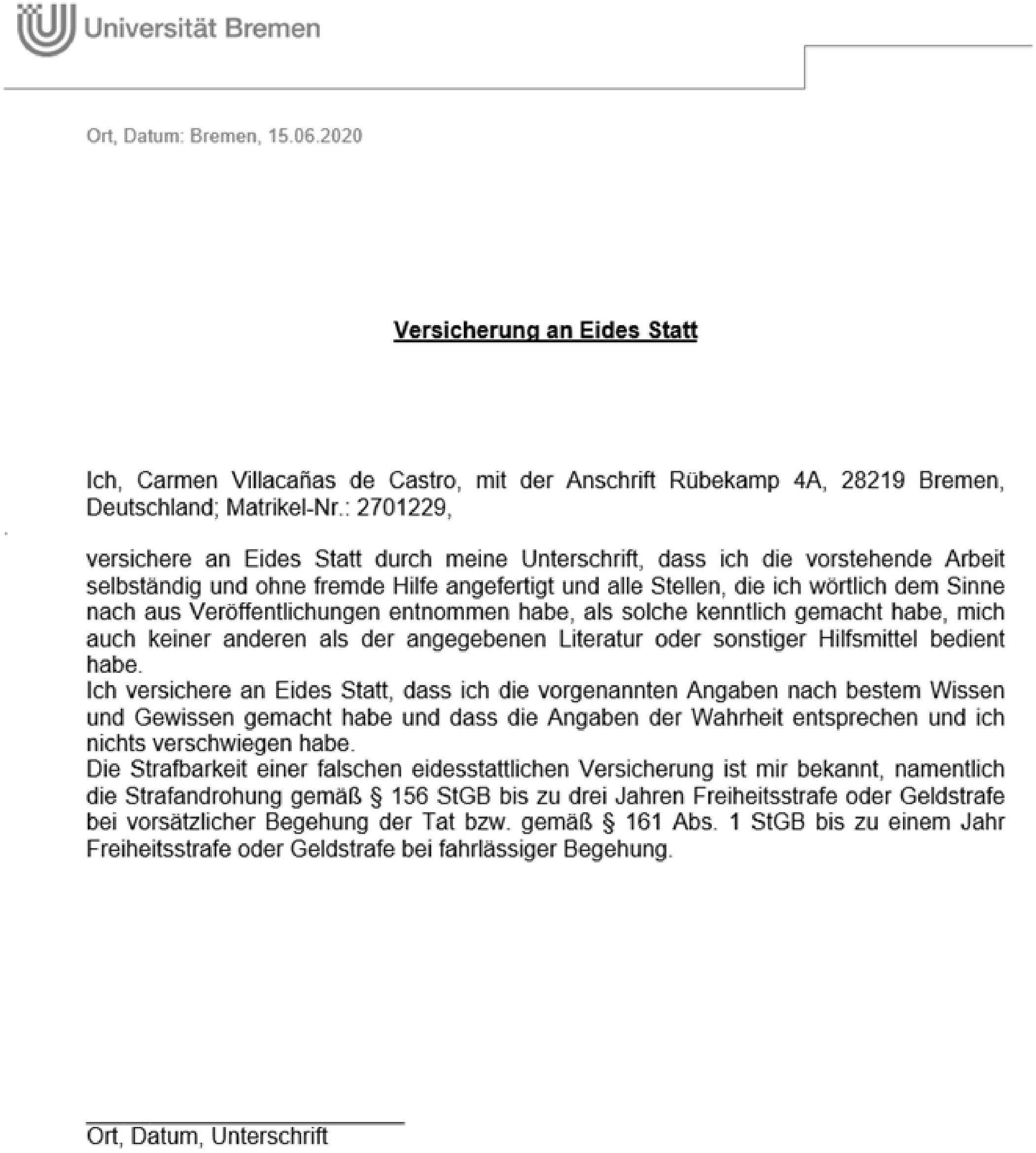
Experimental concept and design: 60%
Experimental work and acquisition of data: 65%
Data analysis and interpretation: 100%
Preparation of figures and tables: 100%
Drafting of the manuscript: 100%

CHAPTER 3:
Title: Friend or foe? A parasitic wasp shifts the cost/benefit ratio in a nursery pollination system impacting plant fitness.
Authors: Carmen Villacañas de Castro, Thomas S. Hoffmeister
Contribution of the candidate:

Experimental concept and design: 70%
Experimental work and acquisition of data: 100%
Data analysis and interpretation: 100%
Preparation of figures and tables: 100%
Drafting of the manuscript: 100%

CHAPTER 4:
Title: General discussion
Author: Carmen Villacañas de Castro.
Contribution of the candidate: 100%

AFFIRMATION IN LIEU OF OATH



Universität Bremen

Ort, Datum: Bremen, 15.06.2020

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Date, place of birth: 31.08.1987, Valencia (Spain)

SCHOOLING

Student at English School Los Olivos following British Education System, passing all the relevant stages, covering Primary and Secondary education.	1990-2005
GCSE (General Certificate of Secondary Education) through the Cambridge and Edexcel-London examining boards.	2002-2003
GCE A-Level (Advanced Level) through the Edexcel-London examining board.	2004
High School Diploma ("Título de Bachiller")	2005

UNIVERSITY EDUCATION

Degree in Biology Student at the University of Valencia	2005-2010
Academic exchange year Erasmus student at the Freie Universität-Berlin.	2008-2009
International Program MSc. Ecology Student at the Universität Bremen.	2011-2014
PhD candidate Universität Bremen, Populations- und Evolutionsökologie. Advisor: Prof. Dr. Thomas S. Hoffmeister	since 2014

RESEARCH EXPERIENCE

Projekt-Praktikum “Feeding acceptance and oviposition of the Mustard leaf beetle *Phaedon cochleariae*” 2009
Freie Universität-Berlin, Angewandte Zoologie/Ökologie der Tiere
Advisor: Prf. Dr. Monika Hilker.
(01.07.2009 – 31.08.2009)

Research project “Size-fitness relationship and clutch size decisions in ectoparasitoid females of *Bracon brevicornis*”. 2012
Universität Bremen, Populations- und Evolutionsökologie.
Advisor: Dr. Andra Thiel.
(15.08.2012 – 15.11.2012).

Master Thesis “Host choice behaviour of the parasitoid wasp *Bracon variator*”. 2013
Universität Bremen, Populations- und Evolutionsökologie
Advisor: Dr. Andra Thiel.
(22.05.2013 – 21.12.2013)

Research stay abroad 2013
Agroscope Reckenholz-Tänikon ART Research Station (Zurich, Switzerland)
As part of my Master’s degree dissertation.
Advisor: Dr. Jana Collatz.
(22.05.2013 – 30.06.2013)

Intern for the project “Natural History of Panamanian Cassidine beetles”. 2014
Smithsonian Tropical Research Institute (Panama City, Panama)
Earl S. Tupper Research and Conference Center.
Advisor: Dr. Donald Windsor.
(15.09.2014 – 15.12.2014)

Pre-doctoral Researcher for project “Between antagonism and mutualism: can a natural enemy shift the balance in a nursery pollination system?”. 2016-2019
Universität Bremen, Populations- und Evolutionsökologie.
Advisor: Prof. Dr. Thomas S. Hoffmeister
(01.10.2016 – 30.09.2019)

PUBLICATIONS

Villacañas de Castro, C., Thiel, A. (2017) Resource-Dependent Clutch Size Decisions and Size-Fitness Relationships in a Gregarious Ectoparasitoid Wasp, Bracon brevicornis. Journal of Insect Behaviour 30, 454-469. doi.org/10.1007/s10905-017-9632-2.

Villacañas de Castro, C., Hoffmeister, T.S. (2020) Friend or foe? A parasitic wasp shifts the cost/benefit ratio in a nursery pollination system impacting plant fitness. Ecol Evol. 2020;00:1–13. doi.org/10.1002/ece3.6190.

CONFERENCES AND MEETINGS

19th Graduate Meeting of the German Zoological Society, Bremen, Germany. 2014
Oral Presentation on "Clutch size decision and size-fitness relationship in females of the gregarious ectoparasitoid *Bracon brevicornis*".
C. Villacañas de Castro & A. Thiel.

4th International Entomophagous Insects Conference, Málaga, Spain. 2015
Oral presentation on "Clutch size decision and size-fitness relationship in females of the gregarious ectoparasitoid *Bracon brevicornis*".
C. Villacañas de Castro & A. Thiel.

22nd Graduate Meeting of the German Zoological Society, Plön, Germany. 2017
Oral Presentation on "Between antagonism and mutualism: how a natural enemy can shift the balance in a nursery pollination system" *(Work in progress).*
C. Villacañas de Castro & T.S. Hoffmeister.

Ecology Across Borders, BES, GfÖ, NECOV and EEF, Ghent, Belgium. 2017
Poster "Between antagonism and mutualism: can a natural enemy shift the balance in a nursery pollination system?" *(Preliminary results)*
C. Villacañas de Castro & T.S. Hoffmeister.

Entomologentagung der DGaaE, Halle, Germany. 2019
Oral Presentation on "Between antagonism and mutualism: can a natural

enemy shift the balance in a nursery pollination system?".
C. Villacañas de Castro & T.S. Hoffmeister.

112th Annual Meeting of the German Zoological Society, Jena, Germany. 2019
Oral Presentation on "Can parasitoids shift the outcome in an antagonistic nursery pollination system? An ecological detective story."
C. Villacañas de Castro & T.S. Hoffmeister.

TEACHING AND SUPERVISION

Teaching Assistant 2016-2018
Experimental Design and Data Analysis Course, MSc. Ecology
Universität Bremen, Population and Evolutionary Ecology Group.
(25.10.2016 – 25.11.2016, 24.10.2017 – 24.11.2017, 23.10.2018 – 23.11.2018)

Teaching Assistant 2017-2019
Statistik für Naturwissenschaften, BSc. Biologie,
Universität Bremen, Population and Evolutionary Ecology Group.
(SoSe 2017, SoSe 2018, SoSe 2019)

Teaching Assistant 2018
Introduction to Behavioural Ecology Seminar, MSc. Ecology
Universität Bremen, Population and Evolutionary Ecology Group.
(13.03.2018 – 16.03.2018)

Supervision of a student project 2018
Daniele Buono "Male and Female Moths Provide Comparable Pollination Services in *Hadena bicruris/Silene latifolia* Nursery System"
MSc. Ecology Research Project (01.07.2018 – 30.09.2018)

PARTICIPATION IN WORKSHOPS

Workshop "Intercultural Competence: Global Skills for University and Professional Life", by Dr. Kerstin Lange. Organised by Bremen Early Career Researcher Development Universität Bremen (29.11.2016).

Workshop “Scientific Publishing for Natural Scientists”, by Dr. Andrew Davis. Organised by Bremen Early Career Researcher Development Universität Bremen (27.04.2017-28.04.2017).

Workshop “Academic Teaching in English”, by Dr. Darren Paul Foster. Organised by Bremen Early Career Researcher Development Universität Bremen (05.10.2017-06.10.2017).

Workshop “Getting it Done: Gemeinsam die Doktorarbeit meistern” by Dr. Jan Stamm, ImpulsPlus Qualitätsnetzwerk Kompetenzentwicklung in Wissenschaft. Organised by Bremen Early Career Researcher Development Universität Bremen (26.11.2018-27.11.2018).

Workshop “Open Access to scholarly publications” by Benjamin Ahlborn, Staats- und Universitätsbibliothek Bremen (29.11.2018).

Workshop “How to get your papers published and how to referee papers”, by Mike Elliott and Tim Jennerjahn. Organised by Leibniz Centre for Tropical Marine Research (06.12.2018).

Workshop “Selbstvermarktungsstrategien für Akademiker”, Institut für Berufliche Bildung AG (24.02.2020-28.02.2020).